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Antony van der Ent
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# Agromining: Farming for Metals

## Extracting Unconventional Resources Using Plants

Springer

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# Agromining: Farming for Metals

## Extracting Unconventional Resources Using Plants

SOCIETY for GEOLOGY APPLIED to MINERAL DEPOSITS

Springer

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# Preface

Metallophytes (metal-tolerant plants) have been used for centuries to locate valuable metallic ore deposits (in fact as early as 1556 in Georgius Agricola's *De Re Metallica*). A subset of metallophytes, those plants that accumulate extraordinary amounts of metals or metalloids within their living tissues, are called hyperaccumulators. Although first reported in 1865 in the zinc-hyperaccumulating *Noccaea caerulescens*, the reporting of the exceptional blue-green latex, containing 25 wt% Ni, in the endemic New Caledonian tree *Pycnandra acuminata* by Jaffré et al. 40 years ago, has really spawned scientific interest in these unusual plants. We have come a long way since then to find innovative uses for these plants. One such emerging technology is phytomining or agromining, which involves the cultivation of hyperaccumulator plants and harvesting their biomass to obtain particular metals or metalloids. Whereas phytomining describes the process of exploiting plants to obtain valuable elements, agromining refers to the full agronomic chain in using hyperaccumulator plants as 'metal crops'. The process involves the farming of 'metal crops' on sub-economic deposits or industrial or mineral wastes to obtain valuable element(s) from their harvest biomass via the production of a 'bio-ore'. The demand for critical metals, including rare earth elements (REEs), platinum group elements (PGEs), nickel and cobalt, is more under pressure in the twenty-first century as a result of resource depletion and geopolitical factors. Agromining is expected to be transformative in the extraction of unconventional resources of these elements not accessible by traditional mining techniques.

This seminal book presents the complete chain of metal farming—'agromining'. It brings together for the first time individual contributions by active research scientists and practitioners currently engaged in fundamental and applied aspects of hyperaccumulator plants. The emergence of the great opportunities they present has taken several decades for acceptance, a fact which is discussed in detail in the introductory chapter. Further chapters address the agronomy of 'metal crops', the latest developments in the processing of bio-ores and associated products, the current state of knowledge on the global distribution and ecology of hyperaccumulator plants, biogeochemical pathways involved in the basic processes, the influence of rhizosphere microbes, as well as aspects of propagation and conservation of these unusual plants. It then summarizes the state of the art in new tools for hyperaccumulator discovery and in the understanding of their physiology and molecular biology. The opportunities for incorporating agromining into rehabilitation and mine closure strategies are presented and ecosystem service provision and life cycle analysis discussed. The possibilities for agromining nickel, cobalt, manganese, selenium, arsenic, thallium, REEs and PGEs are discussed in separate case study chapters. Finally, an overview concludes the book, looking ahead to the prospects for the development of agromining in the future.

This book is edited and authored by pioneers in the field who have been at the forefront of the development of agromining over the past three decades. The book is timely, as agromining is now on a pivotal point in its development, with rapid expansion of activities in the field worldwide. As such, the book will be of significant interest to environmental professionals in the minerals industry, government regulators and academia.

Nancy, France
March 2017

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# Contents

# The Long Road to Developing Agromining/Phytomining

Rufus L. Chaney, Alan J.M. Baker, and Jean Louis Morel

**Abstract**

The concept of phytomining is a natural extension of botanical prospecting and the study of metal biochemistry and biogeography of metal hyperaccumulator plants. Some elements may be phyto-extracted to remediate soils, but the recovered biomass would have little economic value (Cd, As, etc.) and disposal of the biomass would be a cost. A few elements may have sufficient economic value in phytomining biomass to support commercial practice (Ni, Co, Au). The development of phytomining requires (1) selection of high-biomass hyperaccumulator plant species; (2) evaluation of genetic diversity and breeding of improved strains with higher yields of the phytoextracted element; (3) development of agronomic practices to maximize economic return; and (4) development of methods to recover the phytomined element from the plant biomass. Plant species and methods for phytomining of soil Ni have been demonstrated for several species and locations (temperate and tropical climates). Production of Ni metal in an electric arc furnace smelter, and of $Ni(NH_4)_2SO_4$ using a hydrometallurgical method, have been demonstrated. Full commercial phytomining of Ni is beginning in Albania using *Alyssum murale*, and major trials in Malaysia are underway using *Phyllanthus securinegioides*. Variable prices of commodity metals add confusion to the development of commercial phytomining.

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## 1 Background

Phytoremediation of soil metals to prevent adverse effects in the environment includes phytostabilization (converting the metals to forms that are not phyto- or bio-available), phytoextraction (using plants to remove metals from the soil), and agromining/phytomining (growing plants to mine soil metals as an alternative agricultural technology). After research on metals in the environment began in the 1970s, research on each of these technologies has been intensive and productive. These technologies are only useful if they are economic compared to engineering alternatives such as removal and replacement of the tillage depth of soil, which cost \$2.5 million ha$^{-1}$ in Japan where more than 1000 ha of rice paddy soils were remediated using engineering methods (Iwamoto 1999).

Chaney (1983a) and Chaney et al. (1981a, b) introduced the concepts of phytoextraction and phytomining in papers dealing with land treatment of hazardous wastes. He had developed the concept of the soil-plant barrier to characterize the food-chain transfer of elements in contaminated or mineralized soils (Chaney 1983a, b). Food chains are protected from nearly all trace elements in contaminated soils because animals tolerate, in lifetime diets, the element concentrations that accumulate in shoots of nearly all plant species when the plants suffer phytotoxicity. For example, nearly all crop species suffer Zn phytotoxicity when shoot Zn reaches 500 μg g$^{-1}$ dry weight (DW), and the most sensitive livestock tolerate this level of diet Zn (see Chaney 1993). Crop plants suffer Ni phytotoxicity at about 100 mg kg$^{-1}$ DW, but even sensitive livestock species tolerate >100 μg g$^{-1}$ in foliar biomass. Metal hyperaccumulator biomass would be expected to cause toxicity to wildlife and livestock if only that biomass was consumed; livestock avoid most known hyperaccumulators (e.g. Cannon 1960; Chaney and Baklanov 2017). At the same time, hyperaccumulator plants (Ernst 1974, 1975; Jaffré and Schmid 1974; Brooks et al. 1977) of several potentially toxic elements had been reported in the literature. The first paper that reported a Ni hyperaccumulator, by Minguzzi and Vergnano (1948), has been repeatedly confirmed, but because analysis of trace levels of Ni was difficult in 1948, little attention was paid to this original evidence, until Jaffré, Brooks and colleagues began studying Ni hyperaccumulators.

## 2 The Need for Cleaning Up Contaminated Land in the USA

The risk-assessment research and papers that Chaney prepared focused on land application of municipal sewage sludge (now biosolids) and treated municipal wastewater effluent applied to agricultural land. In the 1970s, many biosolids were highly enriched in specific metals discharged by local industry, such that metal phytotoxicity (Zn, Cu, Ni, Co) could occur if soils became acidic. For example, in even 1980, a single application of 5 dry t ha$^{-1}$ of a typical biosolids containing 2500 μg g$^{-1}$ Zn and 25 μg g$^{-1}$ Cd would apply 12.5 kg Zn and 125 g Cd ha$^{-1}$, much more than removed in a 20 t ha$^{-1}$ crop of maize forage (0.5 kg Zn and 4 g Cd) (Table 1). Similarly, the rate of removal of Ni by crop plants is trivial compared to the potential removal with hyperaccumulator species (Table 2).

Because of recognition in the early 1970s that soil Cd could cause adverse health effects in rice farmers, many questions were raised about the accumulation of Cd and how to remove Cd from contaminated soils. The mining and smelting industries had caused Zn and Cd contamination of large areas of soils in many countries, whilst historic application of biosolids had caused excessive Cd accumulation in some cities. Public concern about hazardous wastes followed several internationally recognized cases, and regulatory agencies were starting to try to identify methods to remediate the risks of contaminated soils.

In 1980, a US-EPA scientist, Carlton Wiles, who worked on problems of hazardous waste contaminated soils, contacted Chaney about extending the USDA research on metals from biosolids to include remediation of hazardous soils. An Inter-Agency Agreement between

**Table 1** Estimated removal of Zn and Cd in crop biomass *vs*. hyperaccumulator biomass, or improved phytoextraction crop (Remediation)

| Plant species | Ni in shoot biomass | | Ash-Ni | | |
|---|---|---|---|---|---|
| Yield | | | | | |
| % of soil | | t ha$^{-1}$ | μg g$^{-1}$ | | kg ha$^{-1}$ |
| Maize (Control) | 20 | 1 | 0.02 | 0.01 | 0.002 |
| Maize (50% YD) | 10 | 100 | 1 | 0.01 | 0.2 |
| *Alyssum* in pasture | 3 | 10,000 | 30 | 0.3 | 5–10 |
| Wild *Alyssum* crop | 10 | 15,000 | 150 | 1.5 | 20–30 |
| *Alyssum*+Agric[a] | 20 | 20,000 | 400 | 4 | 20–30 |
| *Alyssum* cultivar[a,b] | 20 | 30,000 | 600 | 6 | 25–30 |

Presume soil is highly contaminated by smelter emissions, and pH is managed to achieve moderate Zn phytotoxicity (50% yield reduction) due to Zn phytotoxicity of crop species. The contaminated soil is assumed to contain 2000 μg Zn g$^{-1}$ = 4000 kg Zn (ha 15 cm)$^{-1}$ and 20 kg Cd kg$^{-1}$ = 40 kg Cd (ha 15 cm)$^{-1}$, whereas the control soil contains 50 μg g$^{-1}$ Zn [100 kg (ha 15 cm$^{-1}$)] and 0.20 μg g$^{-1}$ Cd [0.4 kg (ha 15 cm$^{-1}$)]

[a] Appropriate agronomic practices: N, P, K, S, Ca, and B fertilizers; herbicides; planted seeds

[b] Improved cultivar bred to maximize shoot Ni content (yield—concentration) at annual harvest

**Table 2** Potential of crop species maize (*Zea mays*) and Ni hypernickelophore *Alyssum murale* to phytomine Ni from soils

| | | Yield | Zn in Crop | Zn in Ash | | |
|---|---|---|---|---|---|---|
| Soil | Species | t ha$^{-1}$ | μg g$^{-1}$ | kg ha$^{-1}$ | % of soil | % |
| Control | Maize | 20 | 25 | 0.5 | 1 | 0.025 |
| Contaminated | Maize | 10 | 500 | 5 | 0.00125 | 0.5 |
| Contaminated | *Noccaea caerulescens* | 5 | 250,000 | 125 | 3.12 | 25 |
| Contaminated | Remediation | 10 | 250,000 | 250 | 6.25 | 25 |
| | | Yield | Cd in Crop | Cd in Ash | | |
| Soil | Species | t ha$^{-1}$ | μg g$^{-1}$ | kg ha$^{-1}$ | % of soil | % |
| Control | Maize | 20 | 0.2 | 0.004 | 1 | 0.005 |
| Contaminated | Maize | 10 | 5 | 0.05 | 0.025 | 0.25 |
| Contaminated | *Noccaea* ('Prayon') | 5 | 250 | 1.75 | 4.4 | 2 |
| Contaminated | *Noccaea* ('Ganges') | 5 | 2000 | 10 | 25 | 2 |
| Contaminated | Remediation | 10 | 2000 | 20 | 50 | 25 |

Assume soil contains 2500 μg Ni g$^{-1}$ which equals 10,000 kg Ni (ha 30 cm)$^{-1}$ deep. Presume soil pH is low enough to reduce maize yield by 50%, or high enough to achieve maximum Ni in *Alyssum* species

US-EPA and USDA-ARS was initiated to support a review of the literature, and then supply $1 million/year for 4 years of research and demonstrations of technologies dealing with both metals and xenobiotics. Scientists in the Biological Waste Management Laboratory, led by Dr James F Parr, worked on the literature review, preparing both a formal report for US-EPA (Chaney et al. 1981a, b), and a book (Parr et al. 1983). In the 1980s the US-EPA received reduced funding and redirected many programs. The Inter-Agency Agreement was cancelled after the preparation of the literature review. Lacking the US-EPA funding, USDA-ARS terminated that research program and redirected the research of the team.

As part of that review process, Chaney reviewed the important findings of Ernst, Baker, Brooks, Jaffré, Rascio (1977), Reeves, Wild and others about the hyperaccumulation of metals by rare plants. It was conceivable that growing high yields of hyperaccumulator plants on contaminated soils could remove enough metal to alleviate the hazardous nature of the soils at considerably lower cost than removal and replacement of the contaminated soil. The potential to remove Cd from contaminated soils (phytoextraction) seemed promising and a need had been clearly identified for the technology to remove Cd from contaminated soils to protect food chains. The phytomining concept was summarized with regard to Cd, Zn, and Ni hyperaccumulation (Chaney 1983a). Table 1 presents the potential removal of Zn and Cd by crop plant maize (*Zea mays*) grown as a forage crop, compared to removal by the hyperaccumulator *Noccaea* (*Thlaspi*) *caerulescens*. It is clear that crop plants remove so little Cd or Zn that growth for centuries would not deplete soil levels from contaminated soils, but that hyperaccumulators might remove significant amounts of Cd. Table 2 summarizes similar information about Ni phytomining by hypernickelophore species such as *Alyssum murale*. Although phytoextraction of Zn and Ni would not be rapid, with the southern France or so-called 'Ganges' ecotype of *N. caerulescens*, Cd removal might be rapid enough to achieve soil remediation goals to protect food safety by phytoextraction of soil Cd at low cost (see Simmons et al. 2015). Rice genotypes with high accumulation of Cd under aerobic soil conditions grow much better in tropical rice paddy soils than does the temperate *N. caerulescens* (Murakami et al. 2009). For soils with Cu co-contamination with Cd and Zn, corn inbreds with relatively high Cd accumulation have been demonstrated (Broadhurst and Chaney 2016). For soil with high levels of Zn, Ni, Cu, Pb and some other metals, phytostabilization is quite effective in reducing plant uptake and even bioavailability of the metal in soils ingested by wildlife and livestock (see Chaney et al. 2014; Chaney and Baklanov 2017).

In 1979, Chaney attended a Trace Element Symposium in Los Angeles, CA, which was also attended by Alan Baker. Their discussion included metal tolerance and accumulation and the unusual hyperaccumulator species, and Chaney's concept of phytoextraction (see Moskvitch 2014). Until that time, Baker, Brooks and others (Cannon 1960) had conceived of botanical prospecting for metal ores by analysis of herbarium specimens for elements (Brooks et al. 1977), and were studying the metal tolerance and biogeography of such species as *N. caerulescens*, *Alyssum*, etc. In the Proceedings of that Symposium, a classic paper by Baker reviewed metal tolerance by exclusion or accumulation (Baker 1981). Baker and Brooks (1989) summarized the concepts of hyperaccumulator species and closed with a discussion of the potential for phytoextraction/phytomining in a seminal review that spread the meme of phytoextraction widely. This concept was also promoted independently in an article in *New Scientist* by Baker et al. (1988), which received wide international interest and triggered fundamental research worldwide on hyperaccumulator plants.

## 3 The Situation and Response in Europe

In Europe, soil contamination by heavy metals, their absorption by roots, and subsequent transfer to the food chain were important issues in the 1970s (e.g. in France, research programs launched by the Ministry of Environment and the Anred, now Ademe). In the same way, as in the United States, much research was conducted on the fate of metals in agrosystems, as the use of sewage sludge and urban waste in agriculture raised great concern about the risks of soil pollution and crop contamination. In fact, during this period, some urban sludge exhibited abnormal concentrations of metals (e.g. >150 μg $g^{-1}$ Cd; Morel 1977). In addition, application rates were much higher than those currently permitted, and the probability of transfer of toxic metals to plants was very high at that time (Morel and Guckert 1984; Morel et al. 1988). Processes and mechanisms that control the uptake and transfer of metals to plants were then thoroughly studied in the 1980s and 1990s, allowing a better understanding of plant contamination, taking into account soil reactivity, root activity, and plant metabolism. For example, at the soil-root interface, the role of exudates in metal dynamics was demonstrated (Morel et al. 1986; Mench et al. 1986). The regulation for use of sludge in agriculture and improvement of the quality of urban sludge considerably reduced the risk of contamination of agrosystems by heavy metals, while ensuring the recycling of essential elements such as phosphorus (Sommellier et al. 1996). Still, large surface areas had been contaminated by heavy metals not only by wastes, but also by industrial atmospheric deposits (e.g. smelters) (Sterckeman et al. 2000). In general, soils strongly affected by human activities, in urban and industrial areas may contain elevated concentrations of metals (De Kimpe and Morel 2000).

As of a consequence, the strong economic transformations of the 1980s that impacted mining and heavy industry, the closure of activities such as coal, iron and non-ferrous metal mining, steel industry, and textiles, led to the development of numerous industrial wastelands. If these territories could be used for new economic activities, they were, nonetheless, a threat for human and environment health. Organic and inorganic contaminants were present and very commonly firmly attached to the soil matrix, thus making their elimination a difficult task. Soil remediation technologies were based on excavation and land disposal in landfills or *ex situ* treatment using biological, thermal, or physicochemical techniques. However, in spite of their efficiency, these techniques were not suitable for large-scale sites, for technical as well as economic reasons. This problem triggered the emergence of multidisciplinary structures during this period, in order to develop innovation in the treatment of large polluted sites and soils. The Gisfi (Groupement d'Intérêt Scientifique sur les Friches Industrielles) is an example of groups that combined a wide range of disciplines to cope with complex situations (www.gisfi.fr). Research activity was directed to in situ treatment, including chemical and biological, to treat persistent pollutants on very large areas such as brownfields and agricultural land contaminated with metals from atmospheric deposition. Phytoremediation was one of the options.

In the early 1990s, the phytoremediation technology proposed by Chaney (1983a) was considered as a potential alternative. However, in Europe, decontamination professionals rather designated phytoremediation a "sweet dream of plant lovers" and did not take it seriously. It is true that the knowledge about the effects of plants and their possible contribution to the cleaning of polluted soils was rather scarce. But this did not prevent some operators from promoting the technology and presenting it as a reliable solution for dealing with contaminated environments. The consequence of the lack of solid scientific foundations inevitably led to a series of failures in the 1990s that marginalized the technology for several years.

## 4 The Saga of Chelator-Enhanced Pb Phytoextraction

Much has been learned about the potential for phytoextraction to remove enough metal to achieve phytoremediation. The concept lay dormant until Ilya Raskin and colleagues at Rutgers University invited a lecture by Chaney on metal accumulation. Raskin et al. recognized that Pb-contaminated soils were a significant industrial and urban problem, and that if plants could remove soil Pb efficiently it would create a significant market. Unfortunately, Raskin et al. tested Pb uptake under conditions that kept Pb highly soluble. It was well known at that time if the nutrient solution was deficient in phosphate, that Pb was readily absorbed and translocated to plant shoots causing Pb phytotoxicity (Miller and Koeppe 1971). Raskin et al. chose to test a known Se accumulator species, *Brassica juncea,* and grew the crop with low levels of P and S, then washed the sand and added a P- and S-free nutrient solution having a high level of soluble Pb and when the plants suffered Pb phytotoxicity, harvested and analyzed the plant shoots. Some accessions of this species accumulated >1% Pb (10,000 μg $g^{-1}$) in shoot (Kumar et al. 1995). Raskin et al. obtained a patent for phytoextraction of essentially all elements (Raskin et al. 1994) and obtained investment to start commercialization. Of course, *B. juncea* grown on Pb-contaminated field soils did not accumulate much Pb, typically less than 100 mg $kg^{-1}$ DW. Their team looked at alternatives to increase Pb accumulation (P deficiency in soil, foliar P fertilization), then tested application of chelating agents such as ethylenediaminetetraacetate (EDTA) added to the soil surface when the plants had grown significant biomass but before flowering changed growth patterns. With the addition of EDTA and other chelators, they achieved high shoot Pb concentrations (Blaylock et al. 1997), but actually more metals were leached as EDTA chelates than were taken up by the plants.

Testing of reported Pb-accumulating plant species such as *Thlaspi rotundifolium*, containing very high Pb levels where it grew naturally on highly Pb-contaminated mine wastes (Reeves and Brooks 1983), found <300 μg $g^{-1}$ Pb DW when grown in field-contaminated soils using methods to prevent soil splash contamination of the plants. Even a locally adapted strain of *Ambrosia artemisiifolia* L. (ragweed), which was found to accumulate more Pb than other species occurring on Pb-contaminated land controlled by the DuPont Corp., only accumulated about 300 mg $kg^{-1}$ DW (Huang and Cunningham 1996). It is now recognized that the very high Pb levels in the field-collected *Thlaspi rotundifolium* was likely due to soil particle contamination of this very small plant (e.g. Faucon et al. 2007). In the field testing by Phytotech, in addition to the effect of EDTA addition and growth of *B. juncea*, tillage caused dispersal and dilution of Pb concentration in the surface soil hot spots that most required remediation. It should be noted that their process achieved the environmental standard for the site, which was a Pb concentration in the upper 2.5 cm of soil, even if the induced phytoextraction technology was unacceptable in the environment.

As Chaney et al. (2002) reported, the cost of adding EDTA made this an unacceptable technology; leaching of Pb-EDTA and other metal chelates to groundwater was also unacceptable in the environment. As a result, US and EU governments prohibit the application of EDTA and other chelating agents to induce phytoextraction. In an estimate reported in Chaney et al. (2014), using the lower cost of bulk-purchased EDTA, the addition of 10 mmol of EDTA $kg^{-1}$ contaminated soil would cost more than US$23,500 $ha^{-1}$ per year. Thus, induced phytoextraction was neither economic nor acceptable in the environment. Field tests showed the extent of metal chelate leaching. These problems were reviewed in detail by Chaney et al. (2014).

Raskin et al. (1994) had obtained a patent for Phytoremediation of Metals that they described as covering all metals and all plant species. Because the Chaney et al. team was working to develop a patentable technology for Cd or Ni phytoextraction, we contacted our patent advisers (University of Maryland and USDA), and immediately prepared a patent application. Fortunately, Raskin et al. stressed that non-crop metal accumulator plant species were so small and difficult to grow that they were not useful for phytoextraction, which left us the possibility of patenting Ni phytoextraction/phytomining. We were also advised that obtaining economic support to develop the full commercial technology would likely be impossible without a patent. Hence, the team worked with patent advisers and eventually obtained patents for a method of phytomining nickel, cobalt, and other metals from soil (e.g. Chaney et al. 1998). Phytotech Inc. licensed the Raskin et al. patent, but ultimately went bankrupt when it became evident that chelator-induced Pb phytoextraction would not be permitted in the USA.

## 5 Developing the First Phytomining Trials in the USA

In about 1990, Alan Baker, Scott Angle, Yin-Ming Li, and Rufus Chaney began collaborating to test potential phytoextraction technologies starting with Cd and Zn, but quietly also starting a study of potential Ni phytomining. Although much research has been conducted to understand better how plants hyperaccumulate Cd, and large land areas have become contaminated with Cd and cause excessive food-chain transfer of Cd, governments have not ordered remediation of the large land areas where Cd phytoextraction would be applicable. Without legislation to mandate the remediation of soil Cd from polluted soils, no commercialization has occurred. Both Cd phytoextraction using hyperaccumulator plant species, and use of high biomass energy species (Ruttens et al. 2011) or rice genotypes (Murakami et al. 2009) have been demonstrated, but the lack of government impetus to remediate polluted areas prevents progress in use of these technologies. The production of energy crops such as willow or poplar trees, which may also accumulate appreciable amounts of Cd and Zn, might be cost effective, if the energy value alone would provide profitable agricultural use of the contaminated soils, as phytoextraction is achieved very slow with these species. Cd and Zn would need to be effectively recovered from incinerator stack emissions in order to make products of the combustion safe in the environment.

The Chaney et al. team worked with artist Mel Chin to establish a field test of Cd, Zn, and Pb phytoextraction by *N. caerulescens* and several crop species from a landfill in St. Paul, Minnesota. In the process of seeking a grant to pay for the art piece (entitled ‘Revival Field’), Chin’s proposal was initially rejected by the U.S. National Endowment for the Arts. This caused a large response from the art community and eventually a half-page article on Revival Field was published in *Science* (Anonymous 1990). This article spread the word about the potential value of phytoextraction to the scientific community and jump-started phytomining research in the USA.

Nickel phytomining seemed to offer greater economic opportunity than did that for cadmium. Chaney had obtained seed of *Dicoma niccolifera* Wild from the renowned botanist Hiram Wild in Zimbabwe (Wild 1970), but it was not a hypernickelophore on testing at Beltsville. Excessive soil Cd presents a human and environmental health risk, because Cd is readily translocated to plant shoots. Baker and colleagues in the UK had begun basic research and field phytoextraction trials with *N. caerulescens* (e.g. Baker et al. 1994) and provided seed of the Cd-accumulating Prayon population of *N. caerulescens* from Belgium to many researchers to promote study.

Ernst (1996, 2000) raised several questions about the practicality of phytoextraction. He had published many papers on native-metal hyperaccumulators and reported the shoot small size of *N. caerulescens* and the low density of plants in contaminated sites. He reasoned that the harvest of standing biomass at contaminated sites with hyperaccumulator plants would remove so little metal mass that no benefit could result. We look at this information and stress that agronomic methods to maximize metal amounts in annual harvests and improved cultivars of hyperaccumulators are required for successful phytoextraction, and especially for phytomining.

In spite of all the ensuing ‘hype’ (Ernst 2000), it was soon recognized that nickel phytomining could be profitable if developed. So, Chaney and his US colleagues quietly began research with seeds of several *Alyssum* species supplied by Baker from accessions collected in Mediterranean Europe and Eurasia. Independently, they obtained seeds of cold-tolerant *A. murale* collected from serpentine soils in the southern Bulgarian mountains. As they conducted research on Ni phytomining, they contacted potential sources of funding to develop Ni phytomining technology. Separate work with INCO, Ltd., Ontario, Canada, had addressed risk assessment for soil Ni phytotoxicity and methods to alleviate the toxicity (reported in Kukier and Chaney 2000, 2001, 2004; Siebielec et al. 2007; Chaney et al. 2003). Angle and Chaney visited several Ni industry firms to present our developing Ni phytomining technology and seek funding.

Separately, U.S. Bureau of Mines scientists undertook testing of *Streptanthus polygaloides* Gray (Nicks and Chambers 1995, 1998; Brooks et al. 1998) on a serpentine soil in California. This species was reported to be able to accumulate 1% Ni in dried leaves (Reeves et al. 1981). However, the trials with *Streptanthus* were of limited success because of the growth pattern of this species. Although leaves can accumulate greater than 1% Ni, leaves represent only a small portion of the shoot biomass at harvest, which has to occur at flowering before the leaves fall to the soil. Their field studies were reported in Nicks and Chambers (1995, 1998) and Brooks et al. (1998), and was the subject of a news note in *Discover Magazine* (Anonymous 1994), the paragraph from which was read by Jay Nelkin (of Viridian LLC) who contacted Nicks and Chambers who referred Nelkin to Chaney and Angle about the commercialization of Ni phytomining. We described concepts and data about Ni phytomining in the USA and solicited funding for a Cooperative Research and Development Agreement (CRADA), which would allow the CRADA commercial co-operator to obtain licenses to any patents of the technology and germplasm developed during the CRADA. After prolonged discussion of what might be possible, a meeting in Beltsville (attended by the Nelkin family, Chaney, Angle, Li, our managers and Alan Baker) at which the extraordinary Ni accumulation in leaves of hyperaccumulators was demonstrated by showing the reaction of hyperaccumulator leaves to dimethylglyoxime-impregnated filter paper (resulting in an instant purple colour-response). Negotiations occurred over several years during which time a US patent for Ni phytomining using Ni hyperaccumulators was granted (Chaney et al. 1998). With the patent issued, commercial interest greatly increased. The CRADA started in June, 1998, and led to our development of commercial Ni phytomining technology (see Angle et al. 2001; Li et al. 2003b; Chaney et al. 2007).

## 6 Advancing the Development of Phytomining

Brooks extended the evaluation of potential phytomining by estimating the value of hyperaccumulator biomass for elements with known hyperaccumulator plants (Brooks et al. 1998, 1999). Table 3 is based on his paper with some additional adjustments shown for current metal prices. The price of metals varies somewhat widely with global economic conditions, thus making it difficult to decide on developments in phytomining. Several approaches to modelling or improving phytoextraction and phytomining have been reviewed by researchers. Robinson et al. (2003, 2009) developed a computer program to estimate annual metal removals, and reviewed the phyto-management of trace elements in soils. One greatly unexpected outcome from the basic research by the CRADA team was the recognition that higher Ni accumulation by *Alyssum* species as soil pH was raised (Li et al. 2003a; Kukier et al. 2004) was opposite the effect of soil pH on the solubility and extractability of soil Ni. Several estimates of potential phytomining of Ni from different soils were premised on DTPA-extractable or ammonium acetate-extractable Ni,

**Table 3** Example first reported hyperaccumulator plant species, their estimated biomass and element concentrations together with the value of the biomass metals

| Element | Species | Concentration μg g$^{-1}$ | Biomass t ha$^{-1}$ | Reference |
|---|---|---|---|---|
| Au | *Brassica juncea* | 57 | 5 | Anderson et al. (2005) |
| Cd | *Noccaea caerulescens* ‘Ganges’ | 3000 | 4 | Reeves et al. (2001) |
| Co | *Haumaniastrum robertii* | 10,200 | 4 | Brooks et al. (1977) |
| Cu | *Haumaniastrum katangense* | 8356 | 5 | Brooks et al. (1977) |
| Pb | *Thlaspi rotundifolium* | 8200 | 4 | Reeves and Brooks (1983) |
| Mn | *Macadamia neurophylla* | 55,000 | 30 | Jaffré (1979) |
| Ni | *Alyssum bertolonii* | 13,400 | 9 | Minguzzi and Vergnano (1948) |
|  | *Berkheya coddii* | 17,000 | 18 | Morrey et al. (1992) |
| Se | *Astragalus pattersoni* | 6000 | 5 | Cannon (1960) |
| Tl | *Iberis intermedia* | 3070 | 8 | Leblanc et al. (1999) |
| U | *Atriplex confertifolia* | 100 | 10 |  |
| Zn | *Noccaea caerulescens* | 10,000 |  |  |

Reinterpretation of some species changes the potential value of Cu and Co hyperaccumulators both of which increase as pH declines, opposite the reaction of *Alyssum* species. In the case of Cd and Zn, phytoextraction using *N. caerulescens*, this species accumulates more Cd and Zn at lower pH where Cd and Zn have much higher solubilities (Wang et al. 2006).

Chaney et al. (2017) evaluated the role of convection of a soil solution containing Ni to *Alyssum* roots vs. the diffusion of Ni from soil solid phases. For a Brockman gravelly loam soil from Oregon with 4700 μg $g^{-1}$ Ni, the Ni in soil saturation extracts was only 0.047 mg $L^{-1}$ or 0.8 μM. Assuming the plants used 250 mL of soil solution to produce 1 g of shoot dry matter, and the shoot dry matter contained 15,000 μg $g^{-1}$ Ni, the soil solution would have needed to contain 60 mg $L^{-1}$ or 1.02 mM Ni to provide the Ni by convection. Hence, convection could account for only 0.8/1010 or 0.078% of *Alyssum* absorbed soil Ni. Many reviews have since been published, and valuable up-to-date reviews are included in the present book. In particular, readers are referred to chapter "Agronomy of 'Metal Crops' Used in Agromining" by Nkrumah et al. on agronomic management of Ni phytomining to attain commercial phytomining of Ni.

During the 1990s, the Morel et al. team studied two orientations for phytoremediation in Europe: phytodegradation of organic pollutants with the accelerated degradation of petroleum hydrocarbons in the rhizosphere (Chaîneau et al. 1995, 2000), and phytoextraction to eliminate heavy metals and metalloids from polluted soils. Our encounter with Alan Baker in 1993 initiated our research with the hyperaccumulator *N. caerulescens* (Prayon, Belgium; Baker et al. 1997), and helped to demonstrate the potential of the species to extract metals from the soil (Zn) and from different matrices, including wastes (Schwartz 1997). It is with this species that we demonstrated the proliferation of roots in contact with hotspots of metallic pollution (Zn and Cd; Schwartz et al. 1999). Beyond the Prayon population, reputed to accumulate only Zn, we conducted a survey in France, which led to the discovery of a number of *T. caerulescens* populations able to hyperaccumulate Cd (Reeves et al. 2001) and the initiation of research work on Cd phytoextraction (Schwartz et al. 2001a, b, 2003, 2006; Perronnet et al. 2003; Sterckeman et al. 2004; Saison et al. 2004; Sirguey et al. 2006). In parallel, thanks to collaborations established in the late 1980s with colleagues in Albania, we conducted in 1996 a survey of the flora of ultramafic environments of the Balkans. The survey made it possible to establish a list of species growing on these environments, which represent more than 10% of the Albanian territory, and to identify Ni hyperaccumulators, in particular *A. murale*, widespread in this geographic region (Shallari et al. 1998). Later, other plants originating from the Balkans were also added to the list (e.g. *Leptoplax emarginata, Bornumuellera tymphaea*; Chardot et al. 2005; Bani et al. 2009, 2010, 2013).

The success of phytoextraction, hence phytomining, depends closely on soil properties, in particular metal bioavailability (Gérard et al. 2001; Morel 2012), i.e. the capacity of the solid phase to supply the soil solution with metal ions where they can be absorbed by roots (Morel 1997). This property has generated a lot of work since the eighteenth century among agronomists willing to measure the size of the available nutrient reserve, e.g. P. Much progress has been made with the use of isotopic techniques, with the measurement of L- and E-values (Larsen 1952) that give an exact quantification of the pools of available nutrients (e.g. Fardeau 1981). The availability (lability) of trace elements such as Cd, Hg, Ni and Zn was later assessed using these techniques (Fardeau et al. 1979; Morel 1985; Echevarria et al. 1998; Sinaj et al. 1999; Gérard et al. 2001). The extraordinary ability of hyperaccumulators to absorb soil metals had led to the belief that these plants would be capable of extracting metal ions from the soils that were not able to be extracted by other 'normal' plants. However, with isotopic techniques we demonstrated that, in fact, hyperaccumulators and 'normal' plants absorb metals from the same available (labile) pool of metals (Echevarria et al. 1998; Shallari et al. 2001; Gérard et al. 2001). Hyperaccumulator plants just exhibit an amazing ability to deplete this pool. For example, a single crop of *N. caerulescens* was shown to take up more than 20% of the available soil Cd (Gérard et al. 2001) and even 40% of soil Cd if the soil were acidified to maximize Cd phytoextraction (Wang et al. 2006). This finding proves also that hyperaccumulators are excellent agents to reduce the risks associated with soil-to-plant transfer of metals; the co-culture of a hyperaccumulator and an edible plant on contaminated agricultural land being a practical application of this phenomenon (Wu et al. 2007; Jiang et al. 2010, 2015).

Cutting and harvesting hyperaccumulators breaks the natural cycle of metal in the soil-plant-litter system. The cutting is aimed either to remove the metal contained in the aboveground biomass or to recycle the metal if it has a sufficient economic value. Data accumulated over the years have demonstrated that phytoextraction/phytomining was a relevant option. However, there was a need for tests to be run at large scale in order to prove the feasibility of this approach. As a continuation of the work carried out on *A. murale* (Shallari 1997), we initiated a series of multiple year field trials to establish the real potential of the species to extract Ni from ultramafic soils. The thesis conducted by A. Bani (2009) showed that under extensive conditions it was possible to harvest up to 120 kg of Ni $ha^{-1}$ per year, making it feasible to implement the technology on a wider scale (Bani et al. 2007, 2010). Hence, complementary to the Oregon studies (Li et al. 2003b), we proved the economic feasibility of the production of biomass containing metals of industrial interest. A subsequent phase of optimization of the agronomic part of the chain allowed the refining of conditions necessary for fertilization and herbicide treatment (Bani et al. 2007, 2009, 2015a, b), the selection of the best individuals, the improvement of substrate fertility, the use of chemical agents (Wu et al. 2006) the decrease of toxicity, and the increase of availability of metals of interest (e.g. Rees et al. 2015, 2016), taking into account the whole cycle of processes (Rodriguez et al. 2016).

In natural environments, hyperaccumulating plants contribute to a change in the chemical status of metals. Metal ions are extracted from the soil available compartment, transferred to the aerial parts, and then deposited on the ground as litter during senescence. Morel et al. demonstrated that metals are much more available when present in litter than in the soil (e.g. Cd; Perronnet et al. 2000). Consequently, the plant contributes to increase size of the available metal pool of the surface of the soil, a pool where it preferentially picks up metal ions during its life. Therefore, in natural environments, hyperaccumulators thrive on a restricted metal pool that is permanently renewed by litter and root deposits. The role of rhizosphere microorganism was also investigated (Aboudrar et al. 2007, 2013; Chardot et al. 2013) with applications in the inoculation of PGPR to enhance extraction of metals (Durand et al. 2016). Recent work using fractionation of stable isotopes in the soil-plant-system that show fractionation during the various processes taking place in hyperaccumulators have brought new insights into the mechanisms of uptake, translocation, sequestration, and secondary redistribution into the plant of Cd and Ni (Montarges-Pelletier et al. 2008; Tang et al. 2012a, 2016; Deng et al. 2014, 2016; Estrade et al. 2015). The collaboration with groups in Guangzhou (PR China) contributed to increasing our knowledge about the physiology of various hyperaccumulators, including *P. divaricata* and *S. alfredii* (Ying et al. 2010; Du et al. 2011; Tang et al. 2013), and to propose strategies to design cropping systems suitable to obtain value from contaminated environments (Tang et al. 2012b). This dynamic gave birth in 2015 to an international joint laboratory, named ECOLAND, standing for Ecosystem Services Provided by Contaminated Land, with a strong focus on the use of phytoremediation technologies to get value from polluted territories.

The chain, however, would have been incomplete without the second part, i.e. metal recovery, which was crucial. The recovery of the metal contained in the biomass had grown interest since the 2000s with a focal point on Ni leading to several Ni upgrading routes, metals, salts, or catalysts (Li et al. 2003b; Barbaroux et al. 2009, 2011, 2012; Chaney et al. 2007; Losfeld et al.

2012; Zhang et al. 2014; Vaughan et al. 2017). The 'ups and downs' of phytomining in the United States, described above, had probably a negative effect in the development of the whole chain. However, in France, two start-ups were created, by Stratoz (2013) and Econick (2016), to market metal compounds derived from harvested hyperaccumulator biomass.

## 7 Outlook

Recently, we have introduced the term 'agromining' as it reflects the entire chain of processes of the production of metals of economic value from cultivation of plants on metalliferous environments (Morel 2013; van der Ent et al. 2015). Agromining is similar in concept and complexity to the chain of processes that are required to produce cash crops. The word also stresses the need for multi-disciplinary studies. Indeed, agromining would not have emerged without a dynamic that favoured the gathering of a large set of disciplines. Multi-disciplinarity can be illustrated by projects such as the LORVER project aimed at the production of biomass for industrial use on polluted sites and even on polluted matrices (e.g. industrial wastes). It is also one of the reasons for the success of the Laboratory of Excellence 'Ressources21' aimed at developing the green mining of strategic metals, and for supporting research on agromining. Hence, agromining is no longer an idea, it is already a chain of processes that is being implemented at field scale thanks to research and demonstration projects funded by the National Agency for Research (e.g. Agromine, ANR, France) and the EU (e.g. Life), wherein international teams combine efforts to make agromining feasible under different substrates and climatic conditions.

Tropical regions have substantial unrealized opportunities for Ni agromining operations. Recent advances by the team of van der Ent in Australia have revealed that the extensive ultramafic outcrops in these regions have suitable characteristics that include high Ni phytoavailability and good soil physical properties, required for profitable agromining. At the moment, some ultramafic areas in these regions are not readily accessible, whereas others have challenging topography, and the rocky nature of other substrates limit usage. Nonetheless, large expanses of ultramafic substrates are available for consideration. Attempts by other researchers to capitalize on this huge expanse of substrates by employing the widely used Mediterranean-climate hyperaccumulator *Alyssum* species, did not yield useful outcomes (van der Ent et al. 2013). In view of this, van der Ent and colleagues have embarked on extensive field surveys and systematic herbarium screening that have led to the discovery of more than 50 new hyperaccumulator plant species in Sabah (Malaysia) and Halmahera (Indonesia) (van der Ent et al. unpublished data). Potential 'metal crops' are being selected from these hyperaccumulator species for agronomic trials to ascertain growth performance, nutrient requirements, and Ni yield. Pioneering studies in Sabah are currently underway and consist of a detailed, large, randomized block growth trial using *Phyllanthus securinegioides* and *Rinorea bengalensis* undertaken over 12 months, and a 1.5-ha field using the same species. The pot trial is aimed at testing, under controlled conditions, the effects of N, P, K, Ca and S fertilization, pH adjustment, and organic matter amendments, which will ultimately be critical for field-scale agronomic systems. The field trial is aimed at demonstrating the feasibility of commercial-scale Ni agromining in tropical regions. Early results from the pot trial suggest that a Ni yield of 200–300 kg $ha^{-1}$ can be achieved under appropriate agronomic systems—the highest so far achieved with agromining, which is indicative of the hitherto untapped metal resources in tropical regions. It is envisaged that economic tropical Ni agromining could replace marginal agriculture on poor ultramafic soils, serving as an income source for local communities in Malaysia, Indonesia and the Philippines to farm

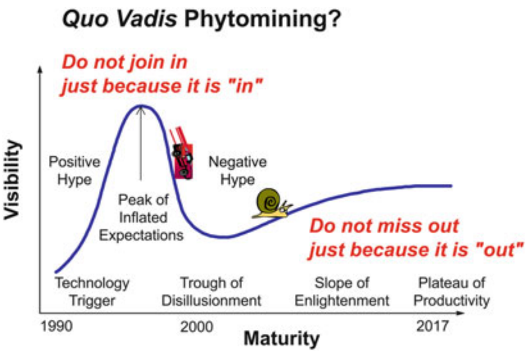

**Fig. 1** The phytomining life cycle of research and development. Source: Alan J.M. Baker

for metals. Other benefits likely to emerge include improvement of these substrates for future use such as productive agriculture and agroforestry. The agromining technology could also be an integral part of strip mining operations, during the initial project phase, and then as part of the rehabilitation strategy. Efforts are underway to explore and secure more potential sites for implementation of tropical Ni agromining; the success of the first field trial will be critical in providing baseline information. Unknown is whether improved agronomic management, breeding improved cultivars of hypernickelophores, or development of transgenic high-yielding crop plant species with hyperaccumulator ability will be the effective direction for progress in phytomining. Transgenic *Brassica juncea* accumulated more Se than the wild type, but still far lower than the amount accumulated by *Astragalus* species (Pilon-Smits and Pilon 2002). Consideration of the public acceptance of crop plants made into trace-element hyperaccumulators suggests, however, that public acceptance would be difficult (Angle and Linacre 2005).

The saga of the 'long and winding road' for the development of phytomining and now agromining continues even in the face of the current low world price for base metals. The story has been well told in a *New Scientist* article by Moskvitch (2014). It is also summarized in the stylized time-line illustrated in Fig. 1. We are now at a point where, at least for nickel, the prospect for commercialization of Ni products through agromining represents a real economic and socially desirable prospect. The future also clearly holds great opportunities for similar approaches for other metals and metalloids of industrial and commercial interest. The following chapters document our present state of knowledge on agromining/phytomining and their applications.

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# Agronomy of ‘Metal Crops’ Used in Agromining

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**Abstract**

Agromining involves growing selected hyperaccumulator plant species (‘metal crops’) on low-grade ore bodies or mineralized (ultramafic) soils, or anthropogenic metal rich materials (e.g. contaminated soils, mine spoils, industrial sludge), prior to biomass harvesting and incineration to recover valuable metals or salts. This chapter begins with an introduction that explains the concepts of phytomining and agromining. We then acknowledge the role of agronomy in enhancing metal yield of ‘metal crops,’ with emphasis on Ni. Highlighted in the selection of sites section is the issue of potential agromining substrates, and the role of metal phytoavailability in economic agromining. We present criteria for selecting potential ‘metal crops’ and possible regions where these species are most suited for successful agromining operations. We then discuss thoroughly the soil and plant management practices that have been proposed to increase biomass and metal yield of ‘metal crops.’ Also reported is progress of the tropical agronomic trials. Finally, we provide a conclusion and present an outlook on the agronomy of ‘metal crops’ used in agromining.

## 1 Introduction

Chaney (1983) was the first to propose the use of metal hyperaccumulator plant species for soil remediation, and introduced the concept of phytomining (a technology that uses such plants to accumulate soil Ni into the plant shoots where they can be harvested and used as an alternative

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ore for Ni). Nearly three decades later, Morel (2013, 2015) proposed the term ‘agromining’ to broaden the scope of the technology to include the entire soil-plant-ore agrosystem (Fig. 1). Agromining involves cultivation of selected hyperaccumulator plant species (‘metal crops’) on low-grade ore bodies or mineralized (ultramafic) soils, or anthropogenic metal-rich materials (e.g. contaminated soils, mine spoils, industrial sludge), prior to harvesting and incineration of the biomass to recover target metals or salts (Barbaroux et al. 2012; van der Ent et al. 2015a). The target elements may include As, Se, Cd, Mn, Ni, Tl and Zn, as well as rare earth elements, but most research has focused on the development of Ni agromining. Large-scale demonstration of agromining of Ni with *Alyssum murale* has been undertaken in the USA (Fig. 2) and Albania (Fig. 3).

Appropriate agronomic practices, based on insights from laboratory (Fig. 4) and field tests, have been proposed to maximize yields of the

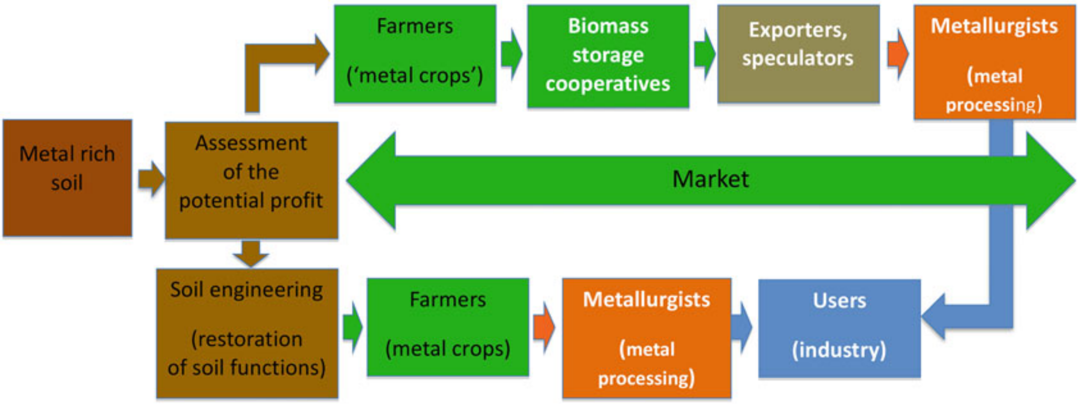

**Fig. 1** The concept of agromining (Morel 2015)

**Fig. 2** Field of flowering *Alyssum murale* (*left*) and *Alyssum corsicum* (*right*) near Cave Junction, Oregon, USA. Soil was derived from ultramafic parent materials and is rich in Ni

**Fig. 3** Large-scale demonstration of agromining of Ni with *Alyssum murale* growing on ultramafic substrate in the Balkans (Albania)

**Fig. 4** Large pot experiment with *Alyssum* species conducted at Beltsville, USA

selected 'metal crop' (Li et al. 2003a; Bani et al. 2007; Nkrumah et al. 2016). Here we discuss the agronomy of 'metal crops' that are more important for developing efficient agromining with special emphasis on Ni as a realistic example. Because most of these agronomic systems have been tested in temperate regions mainly for *A. murale* and *A. corsicum*, we report also on the progress of the agronomic trials in Sabah, Malaysia using two tropical species (*Phyllanthus* cf. *securinegoides* and *Rinorea bengalensis*) to determine whether the trends in temperate regions could be confirmed in a wet tropical environment.

## 2 Selection of Sites for Cultivating 'Metal Crops' Used in Agromining

The cultivation of 'metal crops' could be undertaken on large, metal-rich surface areas. For Ni: cultivation is feasible on naturally occurring ultramafic areas, or smelter contaminated sites (van der Ent et al. 2015a). Figure 5 shows a typical agromining site in a large ultramafic area in Sabah that has been prepared for cultivation. Ultramafic soils develop from the weathering of ultramafic bedrock (Brooks 1987), and are usually enriched in Mg, Fe, Mn, Cr, Ni, and Co, whereas the concentrations of Ca, N, P, K, Mo and B are typically low (Baker and Brooks 1989; Proctor and Woodell 1975).

High Ni phytoavailability is important for economic Ni agromining (Massoura et al. 2004). Echevarria et al. (1998, 2006) and Shallari et al. (2001) have shown that both Ni hyperaccumulator plants and 'normal' plants absorb Ni from the same soil-hosted labile Ni pools, but the former has very efficient root absorption rate (Centofanti et al. 2012; Deng et al. 2014). Metals in the hyperaccumulator biomass exhibit a higher phytoavailability than in the soil from which they derive, as demonstrated for Cd in the hyperaccumulator *Noccaea caerulescens* (Perronnet et al. 2000). Recent isotopic fractionation studies have revealed the contribution of the leaf litter of hyperaccumulator plants to phytoavailable Ni in local ultramafic substrates (Estrade et al. 2015), but the Ni is rapidly adsorbed on the Fe and Mn oxides within the soil (Zhang et al. 2007). We stress that although the biomass of the Ni-rich shoot will be removed by harvest during agromining operations, the next crop will obtain enough Ni required for sustainable operations from the remaining pools of Ni. Recently, Chaney et al. (unpublished) demonstrate the role of subsoil metal on the overall metal yield of a 'metal crop' (Fig. 6). Our previous study suggests that Ni agromining could be sustainable for 15–50 years before the phytoavailable Ni is depleted (Nkrumah et al. 2016), and long-term experiments are required to provide evidence. Because there is currently no chemical extraction method that can accurately predict Ni uptake by hyperaccumulator plants as shown in Fig. 7, future studies should report Ni phytoavailability assays to predict Ni yield in 'metal crops' as already established for 'normal' plants using DTPA extraction (e.g. Echevarria et al. 1998). The suggested cycle of Ni in a hyperaccumulator cropping system is shown in Fig. 8.

Soil physical properties play major role during the cultivation of 'metal crops' for agromining operations. Good soil drainage promotes the growth of 'metal crops', whereas poorly drained soils are detrimental to biomass production and Ni yield of 'metal crops' especially in wet climate. Chaney et al. (2007a) addressed the issue of poor drainage soils in the US field plots by employing ridge tilling and surface drainage (Fig. 9); tilling ensures maximum root penetration. For economic Ni agromining, the sites should have good soil water-holding capacity because soil moisture enhances biomass production and Ni yield of hyperaccumulator species, as has been demonstrated for *A. murale* and *Berkheya coddii* (Angle et al. 2003).

## 3 Selection and Breeding of 'Metal Crops' for Nickel

The main characteristics of Ni hyperaccumulator plants that are considered for agromining include high biomass yield and high shoot Ni concentrations (Chaney et al. 2007a). These species need to be fast-growing. In addition 'metal crops' with $> 1\%$ Ni in shoots are excellent candidates for economic agromining operations (Chaney et al. 2007a, b; van der Ent et al. 2013). Table 1 lists the 'metal crops' that are currently

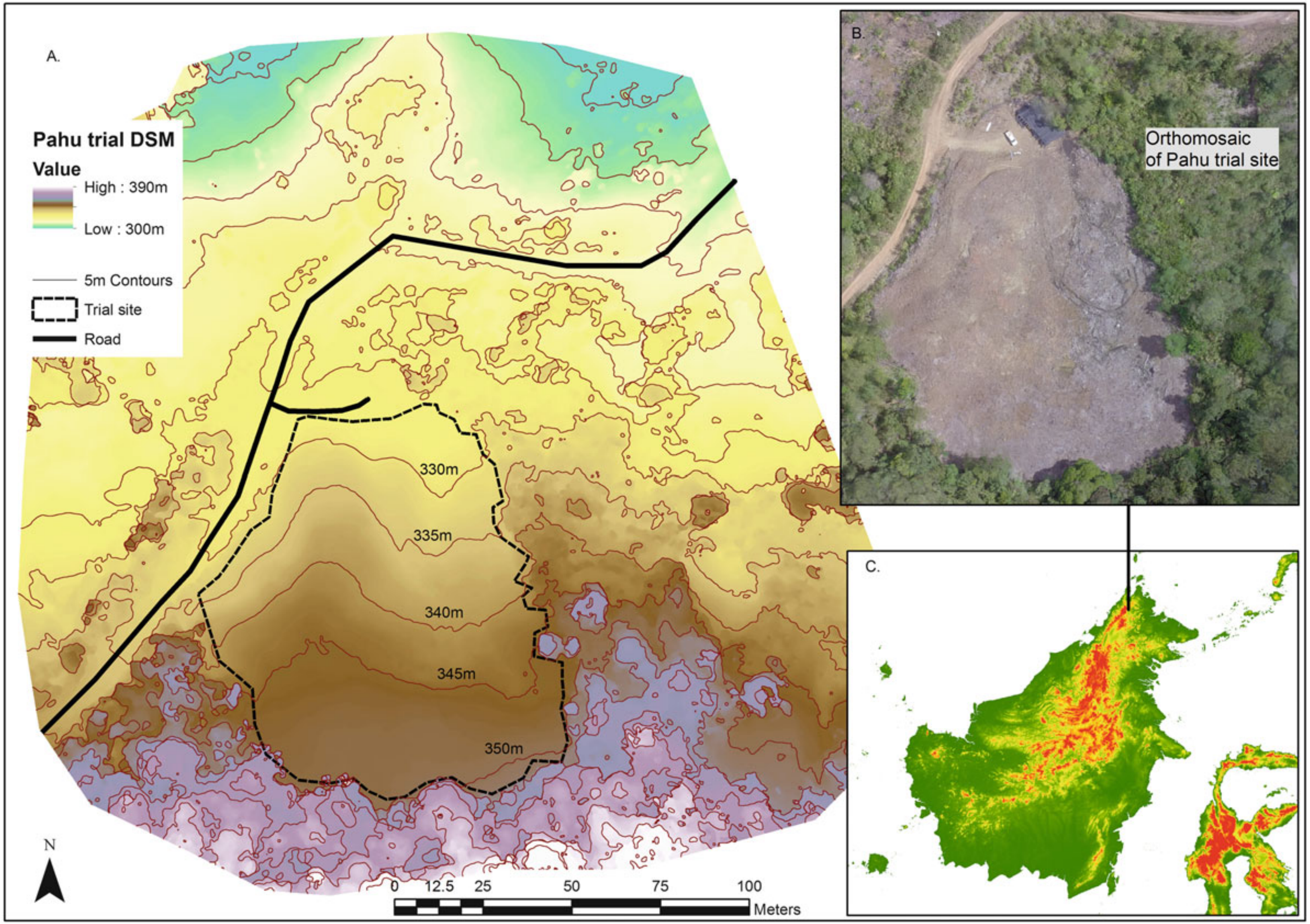

**Fig. 5** Large-scale field site for tropical Ni agromining demonstration in Sabah, Malaysia

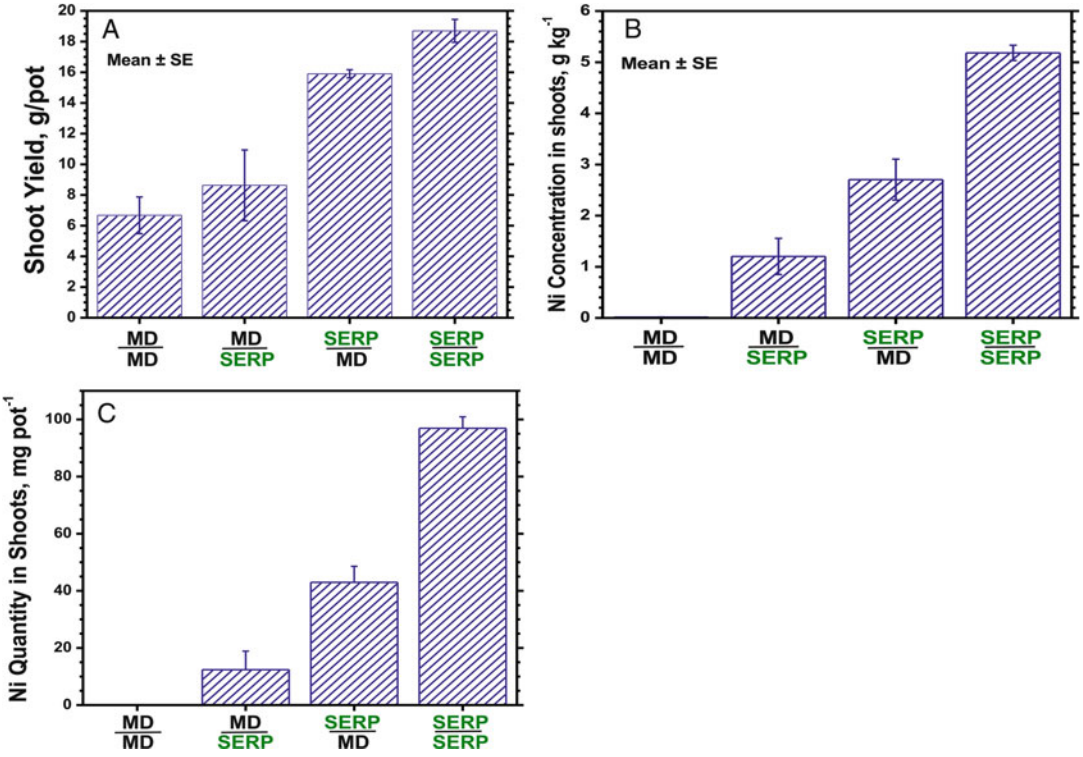

**Fig. 6** Effect of normal soil from Maryland, USA (Christiana fine sandy loam) or ultramafic soil from Oregon, USA (Brockman variant cobbly loam) used at topsoil or subsoil on: (**a**) growth, (**b**) Ni concentration, and (**c**) Ni content in shoots of *Alyssum corsicum* (Chaney, Paul and Baklanov, unpublished)

known to have great potential for agromining. Local plant species are recommended because of their adaptation to local climatic conditions (Baker 1999; Bani et al. 2007). The propagation of suitable candidates need to be relatively easy to ensure mass production, and can include direct seeding, transplantation, or by using cuttings (Angle et al. 2001; Brooks et al. 1998). Hyperaccumulators show large natural variation for trace metal accumulation as shown for *Noccaea caerulescens* (Schwartz et al. 2006; Gonneau et al. 2014). In view of the large natural variation, plant breeding techniques need to be employed to obtain improved cultivars. Up-to-date, only one study (Li et al. 2003b) has used this approach via recurrent selection. The authors observed a significant increase in shoot Ni concentration and overall Ni yield of *A. murale* and *A. corsicum* germplasm (Fig. 10). Clearly plant breeding techniques can increase the profitability of agromining operations, and research should be expanded to include other potential 'metal crops.'

## 4 Inorganic Fertilization to Increase Biomass Production

### 4.1 NPK Fertilizers

Inorganic fertilization plays a major role in maximizing the growth and metal yield of 'metal crops' (Álvarez-López et al. 2016; Bani et al. 2007, 2015a; Li et al. 2003a). The hyperaccumulator species cultivated on ultramafic soils have significant response to increasing concentrations of soil N, P, and K, despite the very poor fertility of their native habitats (e.g. Bani et al. 2015a). Specifically, the aboveground biomass production of Ni hyperaccumulator plants as well as overall Ni

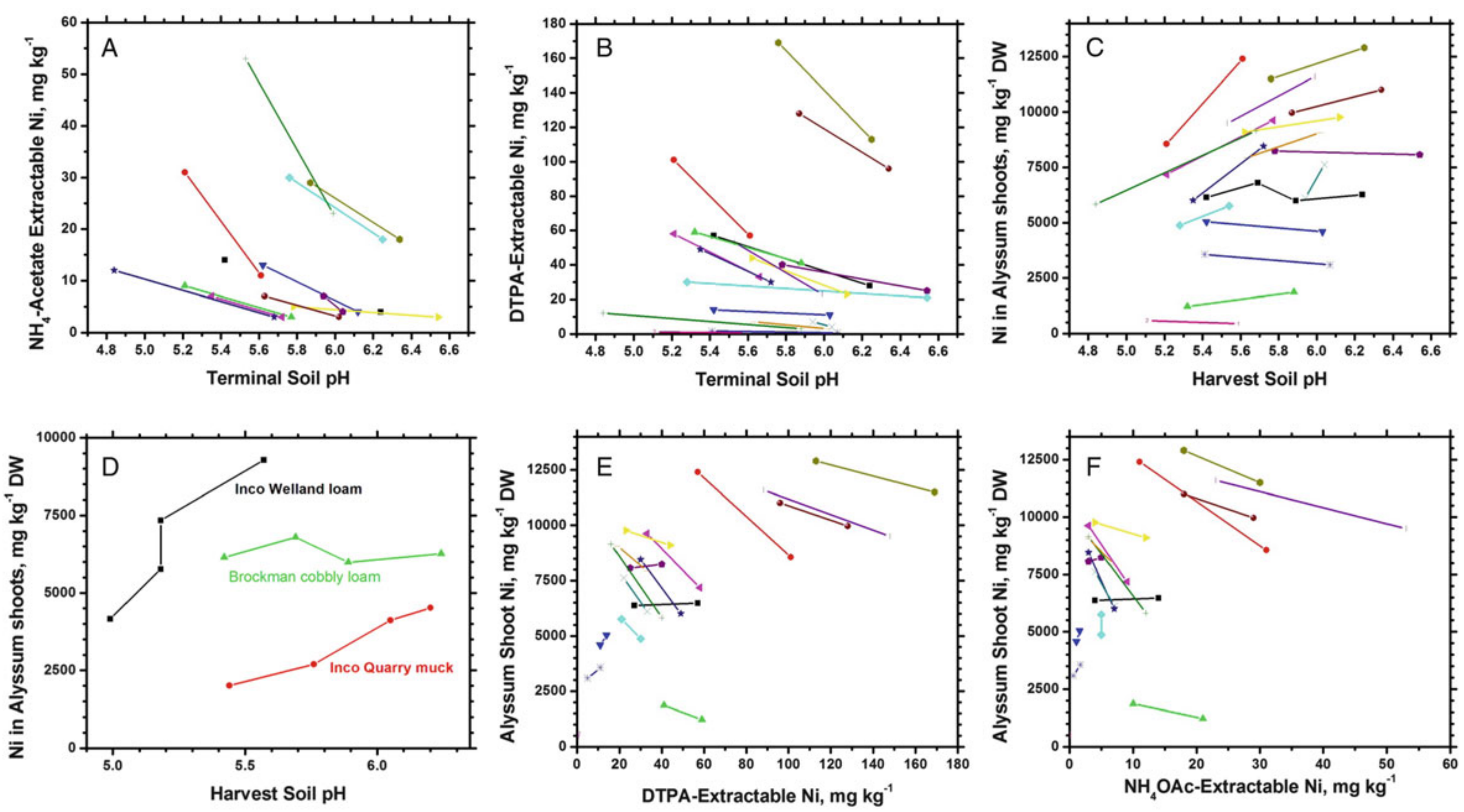

**Fig. 7** The effect of adjusted pH on (**a**) 1.0 M $NH_4$-acetate-extractable; (**b**) DTPA-extractable Ni; and (**c**) Ni accumulation in shoots of *Alyssum* species grown for 120 days in 17 ultramafic soils from Oregon and Maryland, USA. (**d**) Effect of pH on Ni accumulation in shoots of *Alyssum* species grown for 120 days on two soils collected near a Ni refinery at Port Colborne, Ontario (Quarry muck; Welland loam; organic and mineral soils respectively), and the Brockman cobbly loam. (**e**) The effect of adjusted pH (control treatment 2, and acidified treatment 6, which have been described in Table 3) on 1.0 M $NH_4$ acetate-extractable and, (**f**) DTPA-extractable Ni in relation to Ni phytoextraction from 17 ultramafic soils from Oregon and Maryland, USA. Soil pH was adjusted by addition of $HNO_3$, followed by leaching of soluble ions, and fertilization for the growth of *Alyssum* species to test the effect of soil pH on Ni accumulation

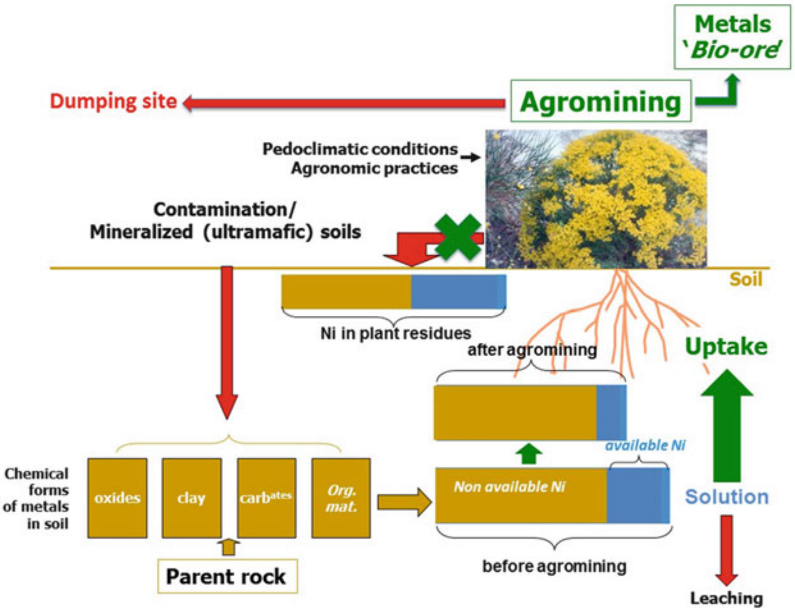

**Fig. 8** Influence of hyperaccumulators and agromining on metal cycle (after Morel 2012)

**Fig. 9** *Alyssum murale* phytomining Ni from: (**a**) poorly drained, and (**b**) better drained muck field at Port Colborne, Ontario, Ni-refinery contaminated soils

**Table 1** Nickel hyperaccumulator species with over 1% Ni ('hypernickelophores') which have high potential for application as 'metal crops' in Ni phytomining operations (adapted from Nkrumah et al. 2016)

| *Species* | Potential application area | Native distribution | Height (m) | Cropping system | Shoot Ni (%) | References |
|---|---|---|---|---|---|---|
| *Alyssum* spp. | Mediterranean and Eurasian Region | S & SE Europe, Turkey, Armenia, Iraq, Syria | 0.5–1 | Perennial herb | 1–2.5 | Reeves et al. (1983) |
| *Leptoplax* spp. | Mediterranean and Eurasian Region | Greece | 1–1.5 | Perennial herb | 1–3.5 | Reeves et al. (1980) |
| *Bornmuellera* spp. | Mediterranean and Eurasian Region | Greece, Albania, Turkey | 0.3–0.5 | Perennial herb | 1–3 | Reeves et al. (1983) |
| *Buxus* spp. | Tropical Central America | Cuba | 0.3–12 | Ligneous shrub | 1–2.5 | Reeves et al. (1996) |
| *Leucocroton* spp. | Tropical Central America | Cuba | 1–3.3 | Ligneous shrub | 1–2.7 | Reeves et al. (1996) |
| *Phyllanthus* spp. | Tropical Asia-Pacific Region | Southeast Asia and Central America | 1–6 | Ligneous shrub | 2–6 | Baker et al. (1992), van der Ent et al. (2015b) |
| *Rinorea bengalensis* | Tropical Asia-Pacific Region | Southeast Asia | 5–20 | Ligneous shrub | 1–2.7 | Brooks and Wither (1977) |
| *Berkheya coddii* | Southern Africa | South Africa, Zimbabwe | 1–2 | Perennial herb | 1.1 | Morrey et al. (1989) |
| *Pearsonia metallifera* | Southern Africa | Zimbabwe | 0.35–1.5 | Perennial herb | 1.4 | Wild (1974) |

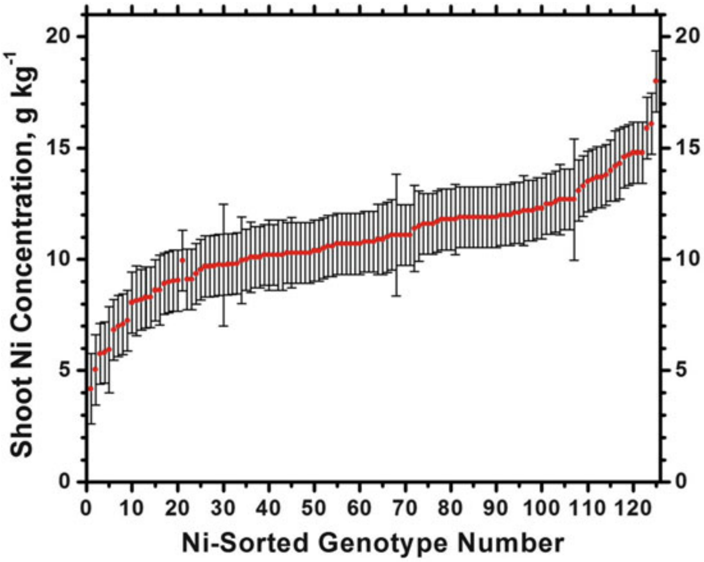

**Fig. 10** Variation in shoot Ni concentration among *Alyssum murale* genotypes grown to (mid-flowering) harvest stage on an Oregon Brockman variant ultramafic soil with 5500 μg g$^{-1}$ Ni (Adapted with permission from Li et al. 2003b)

yield greatly improve upon fertilizer additions in trials using ultramafic soils (Table 2). The effect of P fertilization is particularly remarkable. The overall effect of inorganic fertilization is more pronounced in unfertilized soils (Table 3), whilst negligible response can be observed in previously fertilized soils (Bennett et al. 1998; Robinson et al. 1997). Split fertilizer application

**Table 2** Outcomes of Ni phytomining agronomic trials showing that N+P+K fertilization and organic matter additions significantly increase the biomass of Ni hyperaccumulator plants, with the former causing negligible dilution in shoot Ni content whereas the latter may reduce it

| Agronomic practices | Species | Field and/or pot trials | Locations | Substrates | Effects on Ni yield | References |
|---|---|---|---|---|---|---|
| N+P+K fertilization | *Alyssum bertolonii*, *Berkheya coddii*, *Streptanthus polygaloides*, *Alyssum murale*, *Alyssum serpyllifolium* ssp. *Lusitanicum*, *A. serpyllifolium* ssp. *malacitanum*, *Noccaea goesingense* | Field trial, Pot trials | Tuscany (Italy); New Zealand; Pogradec (East of Albania); Spain | Ultramafic soil, 3:1 mixture of bark: crushed serpentine rock | Increases Ni yield by increasing biomass production while causing negligible dilution in shoot Ni | Álvarez-López et al. (2016), Bani et al. (2015a), Bennett et al. (1998) |
| Sulphur addition | *Berkheya coddii* | Pot trials | Palmerston North, New Zealand | 1:1 ultramafic soil:pumice soil mixture | Increases Ni yield by increasing Ni phytoavailability | Robinson et al. (1999) |
| Soil pH adjustment | *Alyssum murale*, *Alyssum corsicum* | Pot trials | Ontario, Canada | Ni refinery contaminated soil (Welland loam and Quarry muck), Ultramafic soil | Increases Ni yield while decreasing Ni extractability | Kukier et al. (2004), Li et al. (2003b) |
| Different substrates on the basis of soil Ni phytoavailability | *Leptoplax emarginata*, *Bornmuellera tymphaea*, *Thlaspi caerulescens*, *Alyssum murale* | Pot trials | France | Ultramafic soil, agricultural Calcaric Cambisol, acid agricultural soil (Haplic Luvisol) | High Ni phytoavailability increases Ni yield | Chardot et al. (2005) |
| Bacterial inoculation | *Alyssum murale*, *Noccaea tymphaea*, *Bornmuellera tymphaea* | Pot trials | Oregon, USA; France | Ultramafic soil | Increases Ni yield by increasing Ni uptake and biomass production | Abou-Shanab et al. (2006), Durand et al. (2015) |
| Plant growth regulators | *Alyssum corsicum*, *Alyssum malacitanum*, *Alyssum murale*, *Noccaea goesingense* | Pot trials | Spain | Ultramafic soil | Positive effects on biomass, but the effects on Ni yield is not clear | Cabello-Conejo et al. (2014) |

(continued)

**Table 2** (continued)

| Agronomic practices | Species | Field and/or pot trials | Locations | Substrates | Effects on Ni yield | References |
|---|---|---|---|---|---|---|
| Weed control | *Alyssum murale* | Field trial | Pogradec, East of Albania | Ultramafic soil | Enhances Ni yield by reducing competition for essential nutrients and water between the 'metal crop' and weeds | Bani et al. (2015a) |
| Plant density | *Alyssum murale* | Field trial | Pojskë and Domosdovë, Albania | Ultramafic soil | Optimum plant density increases Ni yield | Bani et al. (2015b) |
| Organic matter additions | *Alyssum serpyllifolium*, *Alyssum murale*, *Alyssum bertolonii*, *Noccaea goesingense* | Pot trials | Spain; USA | Ultramafic soil | No significant effect on Ni yield | Álvarez-López et al. (2016), Broadhurst and Chaney (2016) |

Plant growth regulators increase the biomass of Ni hyperaccumulator plants, but the effect on Ni yield is not clear. In addition, soil pH adjustment, S addition, N fertilization and bacterial inoculation increase uptake and accumulation of Ni shoots in Ni hyperaccumulator species (adapted from Nkrumah et al. 2016)

**Table 3** Effect of amending Brockman cobbly loam ultramafic soil (fine, magnesic, mesic Vertic Haploxerepts) from an unmanaged pasture field in Josephine County, Oregon, USA with phosphate (kg ha$^{-1}$ P), pH adjusting, or Ca fertilizer ($CaSO_4 \cdot H_2O$, t ha$^{-1}$) treatments on terminal soil pH, mean yield and macronutrient composition of shoots of two *Alyssum* species (*A. murale* and *A. corsicum*) grown for 120 days (GM designates geometric mean)

| Treatments | | Final pH | GM-yield | GM-P | Mg | Ca | K |
|---|---|---|---|---|---|---|---|
| | | | g pot$^{-1}$ | g kg$^{-1}$ | | | |
| 1 | None | 6.56 a‡ | 4.1 c | 1.04 e | 4.06 d | 17.5 ab | 9.1 d |
| Phosphate | | | | | | | |
| 3 | 0 P | 5.82 e | 1.6 d | 0.61 f | 6.47 a | 17.5 ab | 10.0 cd |
| 2 | 100 P | 6.24 b | 24.5 a | 2.16 cd | 6.20 bc | 17.1 ab | 16.5 b |
| 4 | 250 P | 6.14 bcd | 23.2 ab | 3.00 b | 6.46 bc | 19.8 a | 19.9 a |
| 5 | 500 P | 6.16 bc | 26.5 a | 3.59 a | 6.40 bc | 18.2 ab | 19.8 a |
| pH | | | | | | | |
| 6 | Lo pH | 5.42 g | 27.4 a | 2.03 d | 4.92 cd | 16.7 ab | 18.4 ab |
| 7 | MLo pH | 5.69 f | 26.2 a | 2.12 d | 6.42 bc | 18.5 ab | 17.0 b |
| 8 | MHi pH | 5.89 e | 27.0 a | 2.07 d | 5.31 bcd | 16.2 ab | 18.4 ab |
| 2 | As is pH | 6.24 b | 24.5 a | 2.16 cd | 6.20 bc | 17.1 ab | 16.5 b |
| Ca:Mg | | | | | | | |
| 9 | 0.0 Ca | 6.10 cd | 19.3 b | 2.43 c | 5.66 bc | 14.8 b | 12.4 c |
| 2 | 1.0 Ca | 6.24 b | 24.5 a | 2.16 cd | 6.20 bc | 17.1 ab | 16.5 b |
| 10 | 2.5 Ca | 6.04 cd | 25.2 a | 2.10 d | 6.74 b | 18.4 ab | 17.7 ab |
| 11 | 5.0 Ca | 6.03 d | 24.2 a | 1.94 d | 6.26 bc | 16.2 ab | 17.2 ab |

For single variable treatments, all other nutrients were applied as in treatment 2 (100 kg ha$^{-1}$ P; 1.0 t $CaSO_4 \cdot 2H_2O$ ha$^{-1}$). Bray-1 extractable P was 0.49, 11.1, 49.9 and 100 μg g$^{-1}$ soil for the 0, 100, 250 and 500 kg ha$^{-1}$ P treatments (applied as $Ca(H_2PO_4)_2 \cdot 2H_2O$); all except treatment 1 received 200 kg ha$^{-1}$ N as $NH_4NO_3$. The experimental design, set-up and conditions have been described by Li et al. (2003b) in which the data from the Port Colborne soils were reported similar to the serpentine soil treatments

‡Means followed by the same letter are not significantly different ($P < 0.05$ level) according to the Duncan-Waller K-ratio t-test

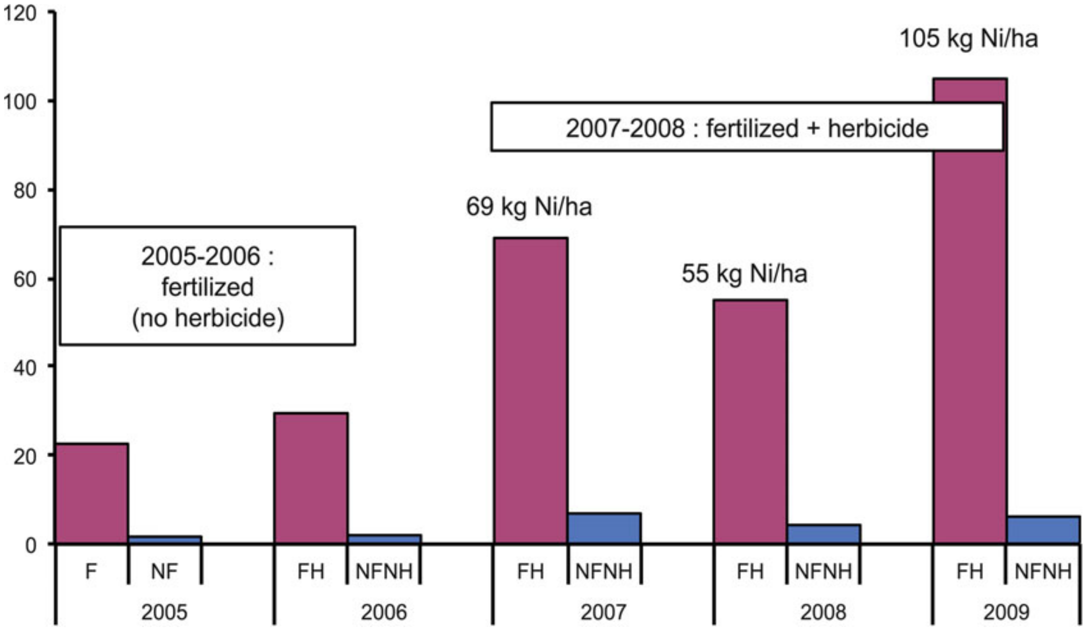

**Fig. 11** Agromining of Ni in ultramafic areas of Albania using *A. murale*—plant response to fertilization and weed control. *F* Fertilized, *NF* Not fertilized, *H* Herbicide, *NH* No herbicide (adapted from Bani et al. 2015a, b)

can be employed to ensure sustainable growth of 'metal crops' and minimize environmental risks especially for N addition (Li et al. 2003a). In ultramafic Vertisols in Albania, Bani et al. (2015b) have demonstrated a 10-fold increase in *A. murale* yield by employing inorganic fertilization. This is indicative of the usefulness of standard inorganic fertilization in sustainable agromining operations (Fig. 11).

## 4.2 Calcium and Sulphur Application

Ultramafic soils usually have very low Ca. Notwithstanding, *Alyssum* hyperaccumulator species native to these soils are able to accumulate Ca, even without fertilization. Several studies have reported exceptional concentrations of Ca in leaves of *A. murale*, predominantly in the form of $CaCO_3$ nodules on the surface of the trichomes (Broadhurst et al. 2004a, b, 2009; Tappero et al. 2007). Calcium accumulation may increase Ni tolerance in hyperaccumulator plants (Chaney et al. 2008), but a negative (Bani et al. 2009) or positive correlation (van der Ent and Mulligan 2015) exists in the foliar concentrations of Ca and Ni in some 'metal crop' species. *Alyssum* species take up more Ca than Mg, which may partially explain its role in minimizing Mg and Ni toxicity (Bani et al. 2014). Calcium supply may not be very critical in agromining operations because high shoot Ca concentration is not useful during Ni recovery process. Nonetheless, some substrates that are very deficient in Ca may require Ca fertilization to promote normal plant growth. Moreover, significant phytoavailable Ca is depleted during biomass harvest, and Ca addition may be required to sustain agromining operations (Bani et al. 2015a, this volume; Chaney et al. 2007a, 2008). The application of Ca may also increase soil pH, which has been observed to increase Ni uptake in *Alyssum* species in ultramafic soils (Kukier et al. 2004).

Sulphur additions may play significant role in agromining operations. Plant species belonging to the Brassicaceae family accumulate exceptional S concentrations, mostly for metabolic purposes (Booth et al. 1995). Studies suggest that S may play critical role in Ni tolerance in

*Noccaea* Ni hyperaccumulator plants, including *N. goesingense*, *N. oxyceras* and *N. rosulare* (Freeman et al. 2004; Na and Salt 2011). Broadhurst et al. (2004b, 2009) showed that $SO_4^{2-}$ provides counter-ion for the high concentrations of Ni that are stored in the vacuoles *A. murale* and *A. corsicum* to ensure charge balance. Research is still needed to explore: (i) the role of phytoavailable S and Ca in Ni uptake during agromining operation, (ii) the mechanisms that may be involved in the accumulation and sequestration of Ca and S in Ni hyperaccumulator plants, (iii) the effect of shoot Ca and S concentrations during the Ni recovery process, and (iv) the uptake and accumulation of Ni in relation to other trace elements such as Fe, Co, Cr, Cu, Mn, and Zn.

Ultramafic soils are usually deficient in micronutrients especially Mo and B (Walker 1948, 2001). It may be important to consider low levels of Mo and B fertilizers in previously unfertilized ultramafic soils to meet normal plant growth requirements. It is likely foliar application may be more effective, but this needs to be tested.

## 4.3 Soil pH Adjustments to Optimize Ni Uptake

Soil pH strongly influences the uptake of Ni from ultramafic soils by Ni hyperaccumulator plants (Chaney et al. 2000, 2007b; Kukier et al. 2004). In ultramafic soils, the effect is unusual (Table 4; Fig. 7c), unlike in smelter-contaminated soils (Fig. 7d) (Chaney et al. 2007a). The shoot Ni concentration of *A. murale* and *A. corsicum* increases in a pH range of 5 to 7 or higher in smelter-contaminated sites. The case in ultramafic soils is different; highest shoot Ni is observed near pH of 6.5 (Table 4; Fig. 7c) (Chaney et al. 2007b). Soil pH has varying effects on extractability of soil Ni as well as uptake by *Alyssum* species (Fig. 7e, f). Hence, it is challenging to attempt to predict Ni in shoot of Ni hyperaccumulator species based on extractable Ni data. Low values of $R^2$ (Fig. 12) were observed when Ni accumulation from ultramafic topsoils was regressed onto total soil Ni or DTPA-extractable Ni. Future studies need to develop robust predictive tools for Ni uptake in hyperaccumulator plants. The optimum soil pH

**Table 4** Effect on terminal soil pH, mean yield and microelement composition of shoots of *Alyssum* species grown for 120 days (GM designates geometric mean) under conditions noted in Table 3

| | | | GM-yield | GM-Ni | GM-Co | GM-Mn | GM-Zn | GM-Fe | Cu |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | | Final pH | g pot$^{-1}$ | µg g$^{-1}$ | | | | | |
| 1 | None | 6.56 a[‡] | 4.1 c | 14,740 a | 34.3 c | 56.5 e | 63.4 bc | 154. b | 3.0 cd |
| Phosphate treatments | | | | | | | | | |
| 3 | 0 P | 5.82 e | 1.6 d | 6250 cd | 19.4 ef | 62.3 cde | 118. a | 273. a | 2.8 d |
| 2 | 100 P | 6.24 b | 24.5 a | 6270 cd | 19.9 ef | 60.9 cde | 59.9 bc | 112. cd | 3.6 bc |
| 4 | 250 P | 6.14 bcd | 23.2 ab | 6810 bc | 22.6 def | 65.2 cde | 60.2 bc | 104. d | 4.2 ab |
| 5 | 500 P | 6.16 bc | 26.5 a | 5690 d | 18.1 f | 67.2 cde | 55.1 cd | 92. d | 4.0 ab |
| pH treatments | | | | | | | | | |
| 6 | Lo pH | 5.42 g | 27.4 a | 6150 cd | 224 a | 462. a | 63.1 bc | 144. bc | 4.4 ab |
| 7 | MLo pH | 5.69 f | 26.2 a | 6800 bc | 50.4 b | 132. b | 68.7 b | 117. bcd | 4.6 a |
| 8 | MHi pH | 5.89 e | 27.0 a | 5990 cd | 28.8 cd | 73.1 cd | 58.2 bcd | 96. d | 3.6 bc |
| 2 | As is pH | 6.24 b | 24.5 a | 6270 cd | 19.9 ef | 60.9 cde | 59.9 bc | 112. cd | 3.6 bc |
| Ca:Mg treatments | | | | | | | | | |
| 9 | 0.0 Ca | 6.10 cd | 19.3 b | 7860 b | 21.1 ef | 55.6 e | 49.4 d | 87. d | 3.1 cd |
| 2 | 1.0 Ca | 6.24 b | 24.5 a | 6270 cd | 19.9 ef | 60.9 cde | 59.9 bc | 112. cd | 3.6 bc |
| 10 | 2.5 Ca | 6.04 cd | 25.2 a | 6050 cd | 18.4 ef | 58.2 de | 59.6 bc | 87. d | 3.8 bc |
| 11 | 5.0 Ca | 6.03 d | 24.2 a | 5630 d | 24.4 de | 78.5 c | 63.3 bc | 93. d | 3.6 bc |

[‡] Means followed by the same letter are not significantly different ($P < 0.05$ level) according to the Duncan-Waller K-ratio t-test

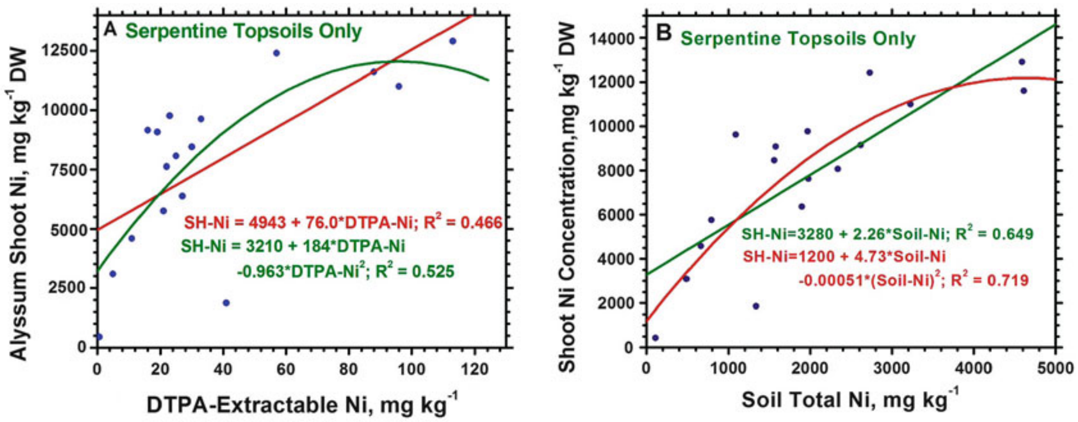

**Fig. 12** (**a**) Prediction equations for shoot Ni concentration vs. soil total Ni; (**b**) DTPA-Extractable Ni for *Alyssum* species grown for 120 days on 17 ultramafic soils

for effective agromining needs to be tested for each location and species.

### 4.4 Soil Organic Matter Additions and Ni Accumulation

Broadhurst and Chaney (2016) utilized a standard, mature, dairy manure compost product from USDA Beltsville to investigate the effect of organic matter amendments on growth and metal yield of *A. murale*. The authors did not observe any significant positive effect on the biomass and yield of the 'metal crop'. However, within the inorganic fertilized ultramafic soil, the *Alyssum* species grew very well, which further underscores the usefulness of inorganic fertilization in agromining operations. Similarly, Álvarez-López et al. (2016) showed that compost addition reduces extractable Ni and shoot Ni concentration, but the overall shoot Ni yield increases because of enhanced biomass. Compost can also change the rhizosphere environment of agromining sites. For instance it can exert adverse impact on serpentine-endemic rhizobacteria, which is important for Ni uptake (Broadhurst and Chaney 2016). However, growing hyperaccumulators on industrial waste material or mine spoils requires improvement of soil fertility, even soil construction. A construction of soil profile is based on the use of organic amendments derived from urban or industrial activities, avoiding clean topsoil (Séré et al. 2008; Chaney and Mahoney 2014). Furthermore, biochar has been proved to considerably increase growth conditions for hyperaccumulators and subsequent accumulation of Ni in plant biomass (Rue et al. in prep.). With appropriate soil conditioning, agromining could be conducted successfully on initially extreme substrates considering their metal contents. We stress that if a compost source is required, field/pot trial of every species/accession needs to be undertaken before application.

## 5 Plant Management Practices

### 5.1 Weed Control and Co-cropping

Agromining employs weed control to minimize competition for vital nutrients and water between the weeds and the 'metal crops' (Bani et al. 2015a; Chaney et al. 2007a). Co-cropping may not play any significant role in Ni agromining. Broadhurst and Chaney (2016) have shown in an experimental study that co-cropping neither increases yield nor Ni uptake in hyperaccumulator species. The authors co-cropped *A. murale* and ryegrass (*Lolium perenne*) and found that ryegrass interfered with both shoot and root systems of the hyperaccumulator plants, and that this interference has a negative effect on overall Ni yield of *A. murale*. It had been suggested that phytosiderophores secreted by roots of grasses to obtain soil Fe could solubilize Ni and improve phytoextraction of soil Ni. Jiang et al. (2015) co-cropped a N-fixing plant, *Lupinus albus*, and *A. murale* in natural ultramafic soils and observed negligible improvement in overall Ni yield in *A. murale*. Therefore, monoculture of 'metal crops' may be sufficient to produce maximum metal yield when adequate inorganic fertilization is supplied. However, research is still needed to develop profitable agromining systems that take into account the principles of agroecology, with adequate plant associations, e.g. hyperaccumulators and legumes. See Nkrumah et al. (2016) for detailed information on other plant management practices (including plant density optimization, use of plant growth regulators, rhizobacteria and mycorrhiza) that are considered during agromining operations.

## 5.2 Harvest Method/Schedule

The management of propagation and harvest will necessarily be dependent upon climate and the species being used for phytomining. If seeds are readily available, and remain viable for some time, then sowing seeds will allow the establishment of phytomining fields. If seeds are easy to work with, 'metal crop' management could follow the patterns used for crop plants. But if seeds are in short supply or it is difficult to establish a stand by sowing in the field, and if the plant is perennial, coppicing can be used to make annual harvests and cuttings can be rooted for transplanting to the field. Alternatively, seedlings raised under careful management in potting media during the dry season could be another possibility. So many factors influence these management choices that it is clear that planting and harvest management will be species-specific and location-specific.

**Fig. 13** (**a**) Mechanical and (**b**) manual modes of harvesting *Alyssum murale*, and subsequent baling operations (**c**, **d**)

For *Alyssum* and several other 'hypernickelophores' in southern Europe, the Mediterranean climate may drive operations. Seedlings could be established in an ultramafic soil-based potting medium during the dry summer using irrigation water, and the seedlings transplanted to the field in the desired density when rainfall returns in the autumn. This could be performed by the farmer using local resources and would appreciably increase the Ni yield of the first year. During flowering at the beginning of the rain-free summer, the crop can be cut and dried in the field to make collection of the dried shoots (a hay) economic (Fig. 13). In tropical areas, harvest might be scheduled during periods of low rainfall.

In the case of the tropical 'hypernickelophores', multiple patterns of management appear possible with different species. With woody species, some accumulate appreciable Ni in their trunk over years, such that harvesting the trees for Ni could yield effective phytomining (e.g. *R. bengalensis*). For most species evaluated to date, the Ni concentration in leaf biomass is much more than in the stem or whole biomass, such that for an annual harvest collecting foliage before leaf fall will be most effective for phytomining. This plan appears to be possible for *Phyllanthus* species, but multi-year biomass collection has not yet been tested in the field. Collection of leaves from the soil surface could be conducted mechanically several times during the year if leaf fall is not seasonal. This approach to foliar biomass harvest has not been evaluated for phytomining or for other species, because 'normal' crops are not harvested in this fashion (e.g. tea and tobacco are harvested by hand).

## 6 Pioneering Studies on the Agronomy of Tropical 'Metal Crops'

Most agromining trials have been occurring in temperate regions mainly for *A. murale* and *A. corsicum* (Li et al. 2003a, b; Bani et al. 2015a). To date, the agronomy of tropical 'metal crops' has not been tested. We present the progress of the agronomic trials undertaken in Sabah (Malaysia) to determine whether the trends observed in temperate regions could be confirmed in a wet tropical environment. The species that are currently being used include *R. bengalensis* and *P.* aff. *securinegoides*; the ultramafic substrates include saprolite, limonite, and young serpentinite soils. The trial is undertaken in two phases: (i) extensive pot trials under controlled conditions to test the fertilization and agronomic systems (Fig. 14), and (ii) a large-scale field trial (Fig. 5) to incorporate the preliminary results from the pot trials. Data are

**Fig. 14** Pot trials that are undertaken under controlled conditions to test the agronomic systems of two species of tropical 'metal crops'; *Phyllanthus* cf. *securinegioides* (small leaf blades) and *Rinorea bengalensis* (large leaves)

being collected on the rhizosphere environment and the foliar tissues periodically prior to treatment application (N, P, K, Ca and S fertilization, pH adjustment, and organic matter amendments). We also monitor the plants' growth rates and physiological parameters. This is the first study to: (i) develop the agronomy of potential tropical 'metal crops,' (ii) determine whether the trends in agronomic trials in temperate regions could be confirmed in a wet tropical environment, and (iii) demonstrate the feasibility of commercial-scale Ni agromining in tropical regions.

## 7 Summary

A range of agronomic systems is available to increase the biomass and metal yield of 'metal crops' to be used in sustainable agromining. We have demonstrated that:

- Because hyperaccumulators show large natural variation for trace metal accumulation, the collection of diverse germplasm, followed by uniform evaluation of yield and metal accumulation, and then normal breeding techniques to improve the 'metal crop' is clearly a key step in developing agromining.
- High soil metal phytoavailability will always be a desired property of soils intended for commercial agromining. Subsoil metal concentration increases shoot yield, shoot Ni concentration, and Ni quantity in shoots of Ni 'metal crops.'
- Standard inorganic fertilization is more essential for efficient agromining operations with *Alyssum* Ni hyperaccumulator species than organic soil amendments. However, the extreme conditions of some substrates may require the application of amendments to improve local soil conditions necessary for normal plants growth.
- Co-cropping may have pronounced positive effects on Cd 'metal crops' and may be also useful in Ni agromining.
- The management of propagation and harvest will necessarily be dependent upon the species being used for agromining and climate of the production site.
- The feasibility of agromining for Ni is demonstrated by laboratory and field experiments, whereas the demonstration of its applicability to other strategic elements (e.g. Co, Mn, rare earths) is underway, and should borrow the same general approach.
- Future developments of agromining should match the goals and constraints of agroecology, i.e. optimize the functioning of the agro-ecosystem and preserve or even improve a large range of ecosystem services (e.g. biodiversity, water quality).

Because most agromining trials have usually been performed in temperate regions, and then mainly on *A. murale* and *A. corsicum*, the ongoing pioneering study in Sabah, Malaysia, using tropical species will be critical to determining whether the trends in temperate regions can be confirmed in a wet tropical environment.

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# Processing of Bio-ore to Products

Marie-Odile Simonnot, James Vaughan, and Baptiste Laubie

**Abstract**

Hyperaccumulator plants may contain valuable metals at concentrations comparable to those of conventional metal ore and can be significantly upgraded by incineration. There is an incentive to recover these metals as products to partially counter-balance the cost of disposing the contaminated biomass from contaminated soils, mine tailings, and processing wastes. Metal recovery is included in the agromining chain, which has been developed over the past two decades for Ni and Au. More than 450 Ni-hyperaccumulator species are currently known and some grow quickly providing a high farming yield. Nickel recovery involves an extraction step, ashing and/or leaching of the dry biomass, followed by a refining step using pyro- or hydrometallurgy. The final products are ferronickel, Ni metal, Ni salts or Ni catalysts, all being widely used in various industrial sectors and in everyday life. Gold can be recovered from mine tailings using a number of plant species, typically aided by a timed addition of an Au-chelating extractant to the soil. Dry biomass is ashed and smelted. This approach enables the treatment of resources that could not be effectively processed using conventional methods. In addition to nickel and gold, the recovery of other metals or elements (e.g. Cd, Zn, Mn, REEs) has been investigated. Further effort is required to improve process efficiency and to discover new options tailored to the unique characteristics of hyperaccumulator plant biomass.

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## 1 Introduction

Soil remediation was the first area of focus for the application of hyperaccumulator plant (HA) technology (Robinson et al. 1997a, b). Whilst this topic has been extensively investigated in terms of extracting metals from soil into the plant tissue, the fate of contaminated biomass has seldom been considered. However, the topic of hyperaccumulator biomass processing is becoming increasingly important, because large-scale application of this soil remediation technology (Chaney et al. 1998) would give rise to vast amounts of contaminated biomass that would have to be treated or disposed (Sas-Nowosielska et al. 2004; Keller et al. 2005). The pioneering work of Chaney and co-workers (Li et al. 2003) has shown how Ni could be commercially recovered from the biomass of *Alyssum murale* L., introducing the concept of phytomining that has recently been broadened to agromining (Morel 2013; van der Ent et al. 2015).

Metal recovery from HA biomass is based on the principles of extractive metallurgy (Hayes 2003; Crundwell et al. 2011). Pyrometallurgy is the high-temperature chemical processing of metals, including unit operations such as calcination, roasting, smelting, and converting. In contrast, hydrometallurgy is the aqueous chemical processing of metals that is carried out at relatively low temperatures and that includes leaching, solution-phase upgrading, and purification followed by product recovery. Both of these techniques are used to enable the production of metals, alloys, concentrates, salts, and intermediate compounds. The processes typically require chemical and energy input and become more challenging and expensive to run with increasing complexity and an ever-decreasing grade of the metal-bearing feedstock.

In the past, metals were considered abundant and easily extractable. In recent decades, the demand for raw materials has sharply increased, because of the economic growth of emerging countries and global demographic pressure. Many elements, including nickel and cobalt, have become strategic metals or even critical raw materials. In this context and also with the aim of creating a circular economy, metal extraction from secondary sources is becoming increasingly important. Therefore, the development of new technologies and strategies to recycle metals has become a major economic, geopolitical, and environmental challenge (Dodson et al. 2012). In France, this topic has notably given rise to the creation of the Laboratory of Excellence LabEx Ressources 21 ‘Strategic metals in the 21st Century’ at the University of Lorraine. In Australia, it is developed within the Sustainable Minerals Institute at The University of Queensland.

In this context, agromining technology may play an important role since it allows the recovery of metals and other compounds from soils or other secondary resources by taking advantage of the natural ability of hyperaccumulator plants to concentrate and purify elements. Processing the biomass to recover products also settles the issue of the fate of metal-contaminated biomass in phytoremediation projects. Agromining feasibility depends on ‘farming for metals’ (van der Ent et al. 2015), that is to say growing hyperaccumulator plants on a large scale. This involves identifying the most promising hyperaccumulator plants and developing agronomic practices that enable a high yield of biomass and high concentration of the target metal to be obtained. These plants must be grown on suitable soils, e.g. soils naturally containing metal concentrations such as serpentine (ultramafic) soils, mine tailings, and secondary resources. The harvested biomass is then converted into a marketable product. Processes must be simple and robust with the ability to take into account biomass variability, and must have a low environmental impact. A further consideration is that the market for the final product, preferably a value-added product, must be established.

One of the most promising applications of agromining is for nickel. One reason for this is that more than 450 Ni HA plants have been identified and agronomic strategies have been developed, particularly with *A. murale*, to reach yields of >100 kg Ni ha$^{-1}$ in extensive conditions (Bani et al. 2009, 2015) or even up to 200 kg ha$^{-1}$ under intensive agricultural conditions (Nkrumah et al. 2016). Efforts are also ongoing with tropical Ni hyperaccumulators (Vaughan et al. 2016b). Another reason why agromining of Ni is of interest is the relatively high value of Ni products compared to those of Cu, Zn, and Cd, for example. As such, Ni agromining has received significant attention and processes for Ni recovery have been designed (Barbaroux et al. 2009, 2011, 2012; Li et al. 2003; Losfeld et al. 2012a; Tang et al. 2012; Zhang et al. 2016).

Agromining has also been considered recently for gold recovery from tailings generated at artisanal and small scale-mining operations in Indonesia, using tobacco (Anderson et al. 2005; Wilson-Corral et al. 2012; Krisnayanti et al. 2016), based on a previous demonstration in Brazil using corn and mustard plants (Anderson et al. 2005).

This chapter begins with an overview of HA biomass processing. The approaches are illustrated with examples for nickel and gold. Developments concerning other metals are summarized. Economic, social, and environmental considerations are also discussed.

## 2 Overview of Ni Products and Production Processes

### 2.1 Main Production Processes for Nickel

According the U.S. Geological Survey, an estimated 2.53 Mt of nickel was mined in 2015 with the producers, in order, being the Philippines, Canada, Russia, Australia, New Caledonia, Indonesia, Brazil and China (USGS 2016). The current production from sulphide ore exceeds that of laterites; about 60% of global Ni reserves are contained in laterite deposits (Gleeson et al. 2003; Mudd and Jowitt 2014). Sulphide ores (0.7–2.2% Ni; e.g. pentlandite) are typically upgraded by mineral flotation then smelted, which takes advantage of the exothermic reaction to provide heat to the reactor (Fig. 1).

In laterite ores, Ni and Co substitute into the crystal structures of oxy-hydroxide or hydroxyl-silicate host minerals (Hellman 2001). They are characterized by two zones:

- Limonite zone (1–2% Ni) near the surface where Ni is mainly hosted in ferric iron oxides and to a lesser extent in manganese oxides;

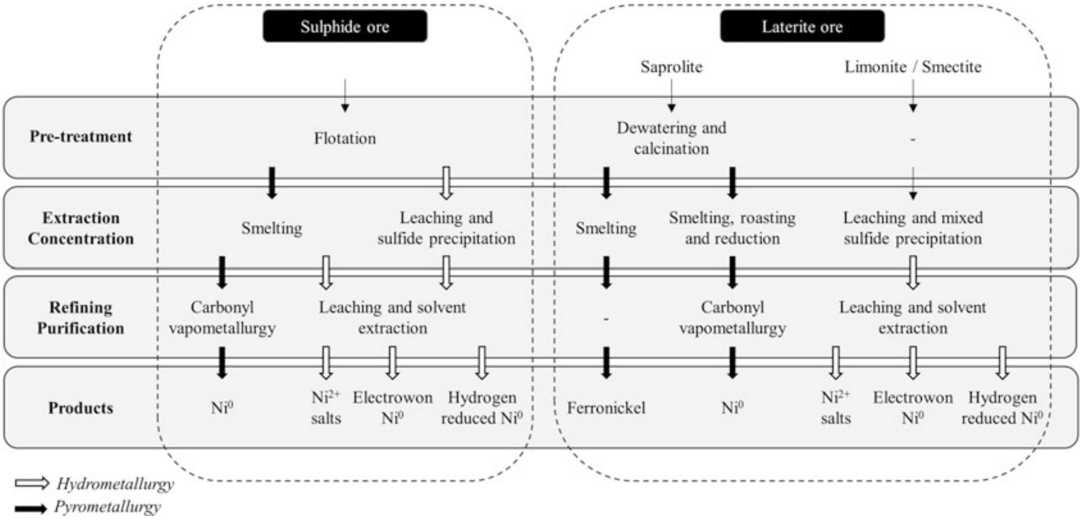

**Fig. 1** Overview of the main processes of nickel ore (adapted from Crundwell et al. 2011)

- Saprolite zone (1.5–2.5% Ni) dominated by hydrous Mg silicates.

Because of the exhaustion of large, high-grade and accessible sulphide ore deposits, laterites are increasingly exploited, despite the relatively costly recovery processes for Ni, in terms of high energy or reagent consumption and high capital equipment costs (Taylor 2013). In addition, such methods often fail to economically recover valuable metals such as Co.

Saprolite ore is usually treated via pyrometallurgical reduction to ferronickel. Limonite is also increasingly being processed to produce low-grade ferronickel (Ni pig iron) by high-temperature reduction, typically in a blast furnace. Limonite is also processed hydrometallurgically via Pressure Acid Leaching (PAL), whereby the host mineral together with nickel and cobalt dissolve at high temperature (~250 °C) in sulphuric acid and the iron largely re-precipitates as hematite or jarosite (Fig. 1). PAL plants have often proven to be a costly option, and the first generation of operations were plagued with technical challenges. Limonite ore is also treated through the Caron process which involves reduction roasting followed by leaching with an ammonia-ammonium carbonate solution (Caron 1924), as implemented at the Votorantim Macedo Refinery in Brazil and the recently closed Queensland Nickel Refinery in Australia (Fittock 1997). The Caron process suffers from high energy costs to dry and reduce the ore, as well as low Ni and Co recoveries (70–85% Ni, 20–50% Co), relative to PAL. The cost and complexity of nickel processing is driven by the need to reject a wide range of impurities including Fe, Al, Si, and Mn, amongst others.

## 2.2 Nickel Products

Ni-containing materials exhibit desirable mechanical and physical properties, corrosion resistance, durability, and ease of use. These materials include stainless steel, a number of alloys, and Ni chemicals for plating and electroforming, for catalysts, for the production of coloured glass, and ceramic glazes (e.g. Ni hydroxide, sulphate, carbonate). Therefore, they are used in many industrial sectors: metallurgy, architecture, building, and construction), automotive and aeronautics, petrochemical and chemical, energy and power, and food and beverage, as well as in electronics and battery production. The main primary Ni products are Ni and ferronickel metal and Ni compounds such as chloride, sulphate, and oxide. Extensive processing is required to recover Ni salts of high purity, because these are generally produced from processes that begin with a feed of high-purity Ni metal.

## 2.3 Principles of Nickel Recovery from HA Biomass

Similar to Ni production from ore, recovery from HA biomass is guided and constrained by the elemental composition of the plants and the distribution of Ni-bearing phases. A general flow sheet of biomass processing is presented in Fig. 2.

The first approach is to extract Ni directly using high-temperature processing such as ferronickel smelting (Li et al. 2003). Biomass can then be integrated into an existing smelter feed. Constraints for this option are the fact that the natural purification that the HA plant afforded are being negated by recombining Ni with impurities in the bio-ore. Other practical aspects are the propensity for the biomass to cause dusting issues, or that the alkaline components of the biomass react with the refractory brick liners at the smelter.

The second approach is to ash the dry biomass, which appears to be quite promising. Once ashed, Ni is extracted and refined using hydrometallurgy by leaching, solution-phase purification, and then recovering of a product by precipitation.

A third approach is to directly leach Ni from the dried biomass. However, the reaction is hindered by the distribution of Ni throughout the organic matter, and questions exist about how to manage dissolved organic compounds.

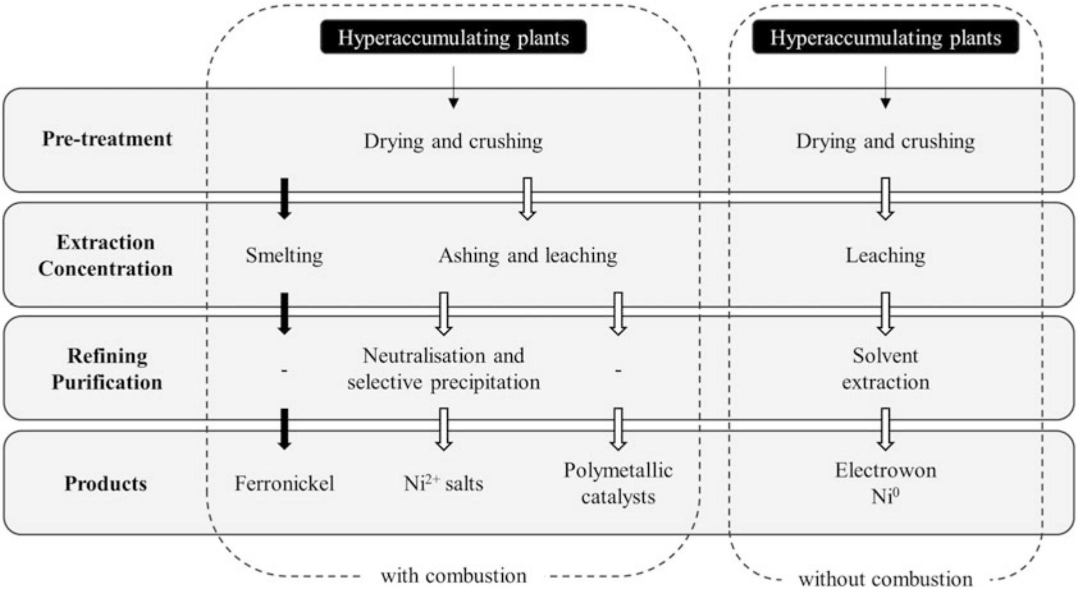

**Fig. 2** General flow sheet of hyperaccumulator treatment to obtain Ni and Ni compounds

Ahead of the chemical processes, the freshly harvested biomass is sun-dried to obtain a dry biomass containing 1–2% Ni together with other major elements such as Ca, Mg and K (Barbaroux et al. 2012; Zhang et al. 2014). Differences between Ni ore and HA biomass are that in HA biomass there is a large amount of organic carbon and impurities such as Cl and P, whereas major impurities in the conventional ore such as Fe, Al, Si and Mn are substantially lower. These significant compositional differences mean that the type of processing tailored for the bio-ore will not necessarily be the optimal route for HA biomass.

## 3 Thermal or Chemical Nickel Extraction from Biomass

### 3.1 Ashing

Biomass is ashed to remove organics, dispose of contaminated biomass, generating heat and energy, and to chemically liberate and concentrate elements (Robinson et al. 1997b; Li et al. 2003; Koppolu and Clements 2003; Boominathan et al. 2004; Koppolu et al. 2004; Sas-Nowosielska et al. 2004; Keller et al. 2005; Barbaroux et al. 2012; Zhang et al. 2014, 2016; Houzelot et al. 2017). Concentrating metal is important to minimize costs of transporting the ashed intermediate product of the bio-ore, and the size of the downstream processing equipment. Examples of major elements in the ore ash are listed in Table 1. It is worth noting that Ni ash concentrations of 5–13% are much higher than the grades of mined Ni ore from laterites. Nickel ore grades of ca. 1% are currently being processed by PAL and for production of nickel pig iron.

Biomass ashing has been extensively investigated at the laboratory scale using small amounts of *A. murale* and other Brassicaceae such as *Leptoplax emarginata* and *Bornmuelllera tymphaea* (Barbaroux et al. 2012; Zhang et al. 2014, 2016; Houzelot et al. 2017). Ashing was conducted in an electrical furnace. The ashing temperature is an important process parameter, because temperatures of >550 °C are required to effectively degrade organic matter but if the temperature is too high, certain metals may be lost to volatilization. Ashing time is also an important variable as a shorter time is desired to maximize furnace utilization, whereas a certain period of

**Table 1** The major element composition of Ni hyperaccumulator ash

| g (kg-ash)$^{-1}$ | Ni | K | Ca | Mg | References |
|---|---|---|---|---|---|
| *A. murale* | 126–168 | 126–128 | 131–172 | 42–71 | Barbaroux et al. (2012), Zhang et al. (2014), Houzelot et al. (2017) |
| *P. securinegioides* | 127 | 72 | 108 | 14 | Vaughan et al. (2016a) |
| *R. bengalensis* | 55 | 85 | 254 | 12 | |

time is required to ensure that the organic residues have been thoroughly combusted. From dry biomass to ash, the solid mass is decreased by a factor of about 15, resulting in a Ni concentration increase from 1–2% to 10–20%. The ash contains a complex mixture of phases including oxides (NiO, MgO) and carbonates ($K_2CO_3$, $CaCO_3$, $K_2Ca(CO_3)_2$).

Ash has also been characterized in terms of particle size distribution. Typically, it is quite fine, less than a few hundred micrometers. This material needs to be sieved in order to remove unburnt carbon particles to improve leachate quality; it cannot be treated by conventional physical sorting methods used with conventional ores.

Ashing dry biomass on a large scale has some practical challenges. The simplest approach is to burn the biomass in an open-air fire. However, this procedure would be difficult to control and there would be no effective method for energy recovery and off-gas treatment. Specialized equipment is available for biomass combustion, such as industrial biomass furnaces. For example, the KWB Multifire MF2 D/ZI has a continuous track, with the combustion being monitored by the oxygen content of the exhaust fumes. This unit can process 7 kg per hour of dry biomass. Temperature monitoring during the ashing process in this furnace indicated that the average temperature is ~900 °C. The properties of the ash from this furnace can differ from that obtained in a small-scale furnace, but still meets process requirements (Houzelot et al. 2017).

In future trials, metal concentrations and particle emissions in the flue gas should be monitored. Government regulations on biomass combustion are often unclear and vary depending on the country. If needed, boilers can be equipped with efficient gas filters (Delplanque et al. 2013).

## 3.2 Ash Leaching

Ash leaching aims at transferring Ni from the solid to the aqueous solution. Acid leaching has been the focus of most reported studies, usually using hydrochloric or sulphuric acid (Barbaroux et al. 2012; Losfeld et al. 2012b). Sulphuric acid has advantages of being less expensive, less volatile, less corrosive to equipment materials of construction, and easily neutralized with limestone yielding gypsum. To minimize the amount of acid used, it is desirable to extract water-soluble salts such as potassium carbonate ahead of the leaching stage using a water wash. Potassium salt solution can be reused as a fertilizer. Water washing of ash has been shown to be very effective for selective solubilizing of K salts (Barbaroux et al. 2012; Vaughan et al. 2016b; Zhang et al. 2016). From an acid leaching study of *A. murale*, the optimal conditions were 10% solids in 2 M $H_2SO_4$, at 95 °C for 2 h, which enabled nearly complete Ni extraction (Zhang et al. 2016). The leachate is then neutralized and partly evaporated to obtain a concentrated solution suitable for further purification and refining to a final product. The unleached components of the ash and precipitated gypsum, free of Ni, can be re-used during metal farming. This operation is easy to scale up, following conventional methods of chemical engineering.

## 3.3 Direct Leaching

Direct leaching of biomass has been conducted with *A. murale*, to avoid the ashing stage. Acid leaching allows the recovery of a Ni-rich solution (Barbaroux et al. 2009, 2011), and Ni can be extracted from the biomass by water at room temperature (Zhang 2014). It has been suggested that water HA-extracted Ni could qualify as an organic Ni fertilizer for some crops such as Pecan (Chaney et al. 2007). The leachates contain the other major elements as well as the organic compounds of the plants, which can complicate product recovery. Recovering both Ni and biofuel was proposed, involving the hydrothermal treatment of HA in water over the temperature range of 200–350 °C based on experimental research using an extract of *Berkheya coddii* (630 mg Ni $kg^{-1}$) (le Clercq et al. 2001).

## 4 Recovery of Nickel Products

### 4.1 Nickel Metal

A method of recovering metallic Ni from HA ash was put forward by Chaney and co-workers (Li et al. 2003; Chaney et al. 2007) feeding the biomass or ash of *A. murale* and *A. corsicum* directly into a smelter. Nickel metal can also be recovered from a leachate solution (Barbaroux et al. 2011). Direct leaching of *A. murale* by a sulphuric acid solution produces a multicomponent leachate containing organic compounds. Selective precipitation or electroplating did not allow Ni recovery. Selective recovery of Ni from *A. murale* leachate was processed by solvent extraction with bis (2,4,4-trimethylpentyl) phosphinic acid (Cyanex 272). Versatic 10 solvent extractant, which was used at the Bulong Nickel Refinery, could be considered for this step (O'Callaghan 2003). Alternatively, ion exchange resin could be used, which might be an easier process to implement for a small-scale operation (Littlejohn and Vaughan 2012). Following solvent extraction or ion exchange upgrading and purification, high-purity nickel can be recovered by electrowinning.

### 4.2 Nickel-Based Catalysts

Nickel-based catalysts have been produced at the laboratory scale from the ash of two HA: *Psychotria douarrei* and *Geissois pruinosa* harvested in New Caledonia (Losfeld et al. 2012a; Grison et al. 2013). HAs were first ashed at 500 °C, then leached with HCl. The leachates were dispersed on montmorillonite K10 to produce Lewis acid catalysts. These catalysts have been tested and compared to commercial $NiCl_2$ for use in the acetylation of anisole (Losfeld et al. 2012a) and green organic synthesis (Grison et al. 2013). Good performance has been attained, especially with *P. douarrei*. The issue of performance versus plant variability should be addressed.

### 4.3 Nickel Compounds

The synthesis of ammonium nickel sulphate hexahydrate (ANSH) salt from the acid leachate of ash of *A. murale* has been demonstrated (Barbaroux et al. 2012; Zhang et al. 2016). This synthesis takes advantage of the very low aqueous solubility of ANSH at 0 °C. The process is presented in Fig. 3. The acid leachate is neutralized by $Ca(OH)_2$, in order to remove iron by precipitation of iron hydroxide. Magnesium is removed from solution by precipitation of $MgF_2$ using NaF. The solution is then evaporated before crystallizing ANSH by addition of ammonium sulphate. Salt purity is increased by a second crystallization step. Salt with a purity of >99.1% was obtained (Zhang et al. 2016). This process was a pilot-scale system to produce a few kg of ANSH per week. The economics of the HA-ANSH process appears to be promising (Barbaroux 2010). This approach can be extended to other Ni salts. A lower cost process yielding a relatively crude, $Ni(OH)_2$ intermediate product has been demonstrated at the laboratory scale using *R. bengalensis* ash (Vaughan et al. 2016b). An elegant aspect of the $Ni(OH)_2$ route is that nickel is precipitated using the ash water-wash solution containing $K_2CO_3$.

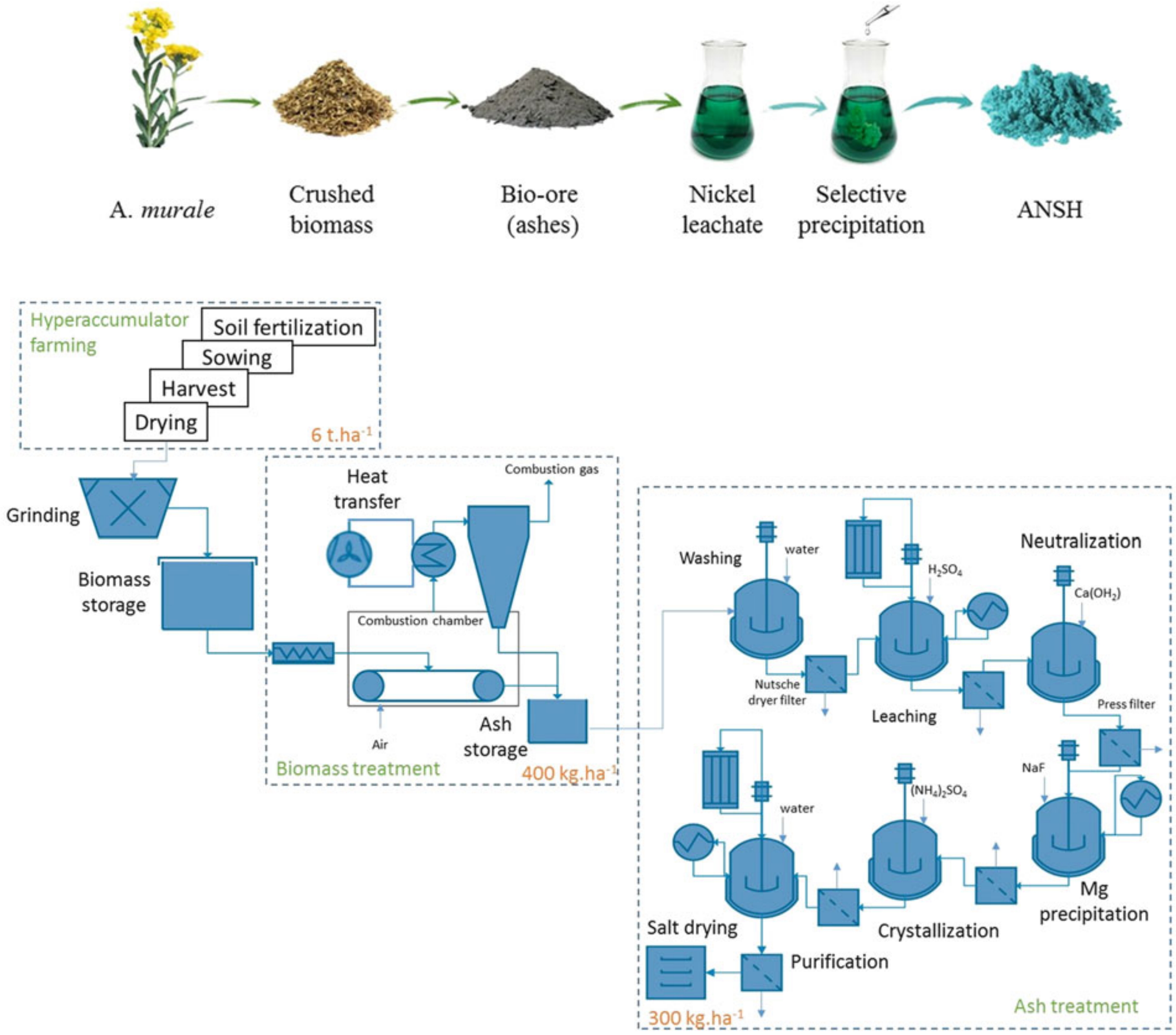

**Fig. 3** Process to produce Ammonium Nickel Sulphate Hexahydrate (ANSH) from the hyperaccumulator plant *Alyssum murale*

## 5 Extraction and Recovery of Other Elements

### 5.1 Gold

Gold can be found at relatively high concentrations in mine tailings and mine wastes, representing extremely large volumes, and can be recovered by phytoextraction as demonstrated by Anderson et al. (1998). To overcome the low solubility of gold in most soils, chemicals are often used to enhance phytoextraction (Wilson-Corral et al. 2012). Phytoextraction is generally performed with plants native to the parent mining sites. Data on plants, mechanisms of gold uptake, and field trials are given in review articles (Sheoran et al. 2009; Wilson-Corral et al. 2012).

Depending on the plants, Au concentration in biomass ranges from ten to a few tens of μg $g^{-1}$. The first route for gold recovery from plant biomass was: ashing, leaching by 2 M HCl, and solvent extraction into methyl isobutyl ketone, addition of a reducing agent (sodium borohydrate) causing the formation of a black precipitate, and heating this precipitate at 800 °C (Lamb et al. 2001). This method has been simplified to save reagents and decrease the costs. Recently, a field trial of the Au phytomining has been conducted in Indonesia to assess the relevance of this technology to artisanal mining. Tobacco has been grown, harvested (100 kg), and ashed. Borax and Ag (used as collector metal) were added to the ash and smelted (Krisnayanti et al. 2016). Despite the low yield in this case, this trial has shown the technical feasibility of the method.

Several economic evaluations have been performed (Anderson et al. 2005; Sheoran et al. 2009; Wilson-Corral et al. 2012), showing that gold phytomining could be economically viable in many cases.

### 5.2 Recovery of Different Metals

Hyperaccumulator plant species that target a range of elements are known to exist, thus in principle agromining could be considered widely. Calculations by Robinson et al. (1997a, b), considering the price of the specific metal, provided an estimate of the biomass metal concentration required to make a return of US $500/ha as a function of biomass production (t $ha^{-1}$). The concentration required increased with decreasing metal value, for which the order at the time was: Au > Pd > Ag > Ag > Co > Tl > Ni > Sn > Cd > Cu > Mn > Zn > Pb. The table was updated in 1999 and the order shifted to: Pt > Au > Pd > Tl > Ag > Co > U > Sn > Ni > Cd > Cu > Mn > Zn > Pb, which reflects changes in metal price (Anderson et al. 1999). Other important considerations are the cost of processing the biomass and the actual value to be obtained from the final product, if not metal. Whereas it would be more profitable to produce a value-added product, the market risk in selling a bulk commodity is lower.

Catalyst synthesis has been explored with other metals using the same approach as for Ni (Losfeld et al. 2012a): ashing and leaching with HCl, followed by deposition on montmorillonite K10 or another purification process. Lewis catalysts for the Diels-Alder reaction have been prepared from the Zn hyperaccumulator plants *Noccaea caerulescens* and *Anthyllis vulneraria* (Escande et al. 2014a). Mn-derived catalysts have been produced from *Grevillea exul* ssp. *rubiginosa* (Escande et al. 2015). Eventually, polymetallic catalysts will be prepared (Escande et al. 2014b), given that hyperaccumulator plants often contain several metals. These catalysts are effective in organic chemistry for different types of reactions, enhancing developments in 'green chemistry'.

Aside from catalysts, Zn recovery has also been investigated. Leaching of the biomass of *Sedum plumbizincicola* (1.2% Zn) (Yang et al. 2009a) and of *S. alfredii* (1.3% of Zn) (Yang et al. 2009b) in ammonia/ammonium chloride was effective. Following a systematic study of the leaching parameters, it was recommended to use a $NH_4Cl$:$NH_3$ molar ratio of 0.6 and temperature of 60 °C. A recent contribution has shown the possibility of recovering Zn and Cd from *N. caerulescens* biomass by cementation

(Hazotte et al. 2017). Another potential opportunity is to recover nanoparticles from plant tissues (e.g. Pt) (Parker et al. 2014) for specialized end uses.

## 6 Environmental and Economic Aspects

### 6.1 Green Products and Green Processes?

It is commonly believed that products made from plants are 'green' or at least 'bio-sourced' and therefore 'gentle.' However, metal products prepared by agromining have the same properties as products prepared by conventional technologies. In the case of Ni, metal and compounds were selected for hazard and risk review by the EU in the 1990s and now are regulated by REACH and CLP, in terms of health and safety, worker and consumer protection, protection of the environment, and/or human health safety through environmental exposure. Risk management option analyses (RMOAs) have been performed. Nickel metal is not classified. Concerning the other compounds, regulation may be reinforced, particularly for worker protection. However, Ni substitution is difficult, and Ni is used to replace other hazardous metals (e.g. Cd, Cr). Therefore, the obligation of authorization would be more feasible than restriction.

Agromining is sometimes confused with 'green chemistry.' Some of the 12 principles of green chemistry are upheld but not all of them. Some products made from plants, namely the catalysts, may open the way to green synthesis in organic chemistry.

In order to evaluate the environmental impacts of the agromining chain, multi-criteria and multi-stage assessment has to be conducted, mainly by Life Cycle Assessment (LCA). Recently, the first Life Cycle Assessment of an agromining chain has been done, and has proved the relevance of agromining for elaboration of the nickel salt ANSH produced from *A. murale* grown in Albania (Rodrigues et al. 2016). This approach is fully described in chapter "Life Cycle Assessment and Ecosystem Services of Agromining". Such evaluations should be conducted for all possible situations.

### 6.2 Agromining as a New Economic Activity

Aside from its scientific interest, agromining has led to new economic activity. Technico-economic evaluations of the process have been made for Ni (Angle et al. 2001; Li et al. 2003; Barbaroux 2010) and Au (Wilson-Corral et al. 2012). For example, from an order of magnitude economic study considering the following scenario: a plant in Canada, 180 days per year, transforming 5 t $h^{-1}$ of *A. murale* biomass to produce ANSH, the annual profit would be 8.5 million Canadian dollars. A dedicated economic study must be performed for each situation. However, these calculations indicate the economic potential of processing HA biomass.

With a series of scientific, technical, economical, and environmental assessments, the whole chain of agromining could be implemented at the commercial scale as there are many applications for the potential products. Initiatives have been taken to transfer this chain to include socio-economic activities. The newly created company, Econick, aims to produce Ni compounds from a large array of hyperaccumulator plants and offer to the market a series of high-value chemicals. This activity is designed for recovering metals from serpentine soils, contaminated soils, and industrial wastes. In addition to the production of metal compounds, energy and by-products are produced.

## 7 Conclusions and Perspectives

The concept of agromining has been proposed to recover metals dispersed in soils or other resources at low concentrations that cannot be efficiently exploited by conventional metallurgical processes. Much of the effort has been focused on nickel agromining, as many Ni HAs are known and vast areas of serpentine soils and mine tailings are available. Furthermore, agronomic practices have been developed to produce high yields of Ni-containing plants, particularly *A. murale*.

For Ni, several processes have been designed and up-scaled to produce metal or a variety of Ni compounds. Life Cycle Assessment has proved that agromining to produce ANSH and recover energy has low environmental impacts (Rodrigues et al. 2016). After an economic study proving the potential benefit of the approach, a start-up company has been launched. Other developments are ongoing for Ni agromining, particularly with a focus on tropical hyperaccumulators.

For Au, agromining feasibility studies have indicated potential to recover Au dispersed in mine tailings at low concentrations. A number of contributions have been reported to recover Zn, Cd, and Mn. Research is also ongoing on the recovery of Co, REEs and Pt.

Farming of hyperaccumulator plants is an alternative to conventional mining of ore. High metal selectivity and concentration factors, together with the opportunity to recover energy and by-products are compelling reasons why agromining should be considered. Whilst agromining will not replace conventional mining practice, there appears to be scope for mainstream implementation of the technology.

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# Life Cycle Assessment and Ecosystem Services of Agromining

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**Abstract**

Starting from the concept of sustainability and the need for metrics to assess it, this chapter presents the basics of the Life Cycle Assessment (LCA) methodology and its different versions (attributional versus consequential, static versus dynamic). Key issues related to the application of LCA to agromining, either on natural soils (such as ultramafic soils) or on anthropogenically polluted areas (such as mine tailings) are highlighted. Land use impacts are described more specifically: these are linked to the ecosystem services rendered by land systems and characterized via indicators that attempt to quantify soil organic carbon content, biodiversity, and land erosion by rainfall and wind. In spite of the actual limitations on quantification of land use impacts, which are not specific to agromining projects, Life Cycle Assessment offers a framework for agrominers to discuss assess their projects in terms of sustainability.

## 1 Introduction

In 1987, the Bruntland Report (*Our Common Future*) marked a turning point in sustainability awareness (World Commission on Environment and Development (WCED) 1987). Different sustainability paradigms have been proposed (Neumayer 1999; Schlör et al. 2015) such as the weak sustainability concept developed by Hartwick (1977) and Solow (1993), and the strong sustainability concept proposed by Holling (1973, 1986). The Holling concept corresponds to a pessimistic vision of sustainability, where the loss or over-exploitation of natural capital, especially abiotic resources (fossil fuels (Shafiee and Topal 2009), minerals (Yellishetty et al. 2011; Boryczko et al. 2014)) is considered irremediable and cannot be compensated by human or human-made capitals. On the contrary, the Solow-Hartwick concept is more optimistic, stating that it is possible to compensate the loss of natural resources by saving them, i.e. improving the efficiency of their usage and increasing their recycling (Habib and

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Wenzel 2014), or by developing new technologies (Moran et al. 2014; Middlemas et al. 2015). In the weak sustainability concept, the consumption of non-renewable resources can be kept on a non-declining path, that is, as long as resource use efficiency and recycling are high. Agromining of natural ultramafic soils or of human-made tailings sites is part of this weak sustainability strategic vision, as it is a new technology designed to efficiently use low-grade and secondary resources.

But whatever the definition of sustainability, metrics are needed to assess the environmental impact caused by any human activity. Among the various environmental assessment methods that have been proposed (Jeswani et al. 2010), such as Life Cycle Assessment (LCA); Environmental Impact Assessment (EIA); (Tukker 2000; Cashmore 2004), Strategic Environment Assessment (SEA) (Finnveden et al. 2003), Material Flow Analysis (MFA) (Brunner and Rechberger 2004), Substance Flow Analysis (SFA) (Yellishetty and Mudd 2014), Water FootPrint (WFP) (Hoekstra 2003), Carbon FootPrint (ISO/TS 14067 2013; Wan et al. 2016), LCA is the most comprehensive (Guinée and Heijungs 1993; Guinée et al. 1993a, b; Rebitzer et al. 2004; Pennington et al. 2004). The 'footprint' approach is attractive for exchanges with stakeholders, but these focus on only one issue (carbon, water, land), when assessing globally the environmental impact of a complex system requires examining several impact categories simultaneously. In order to overcome this difficulty, the development of a framework for sound footprint metrics has been proposed recently (Ridoutt et al. 2016). In the meantime, LCA remains the most encompassing method for environmental impact assessment: without being a course on the details of LCA, the aim of this chapter is to describe the issues in applying LCA to agromining, and to provide theoretical and practical recommendations to assess agromining properly.

## 2 What Is Life Cycle Assessment?

Life Cycle Assessment is a standardized method (ISO 40040, 2006; ISO 40044, 2006) that allows quantification of potential impacts of a product, a process, a service, or an activity on its environment (Heijungs et al. 2010), taking into account the entire life cycle, i.e. from the extraction of raw materials to its end of life (waste treatment and disposal), as well as the necessary commodities (electricity, water, etc.). This cradle-to-grave (Fig. 1) vision allows one to anticipate the possible transfers of impacts among life cycle stages. Energy generation (Sanz Requena et al. 2011; Turconi et al. 2013; Amponsah et al.

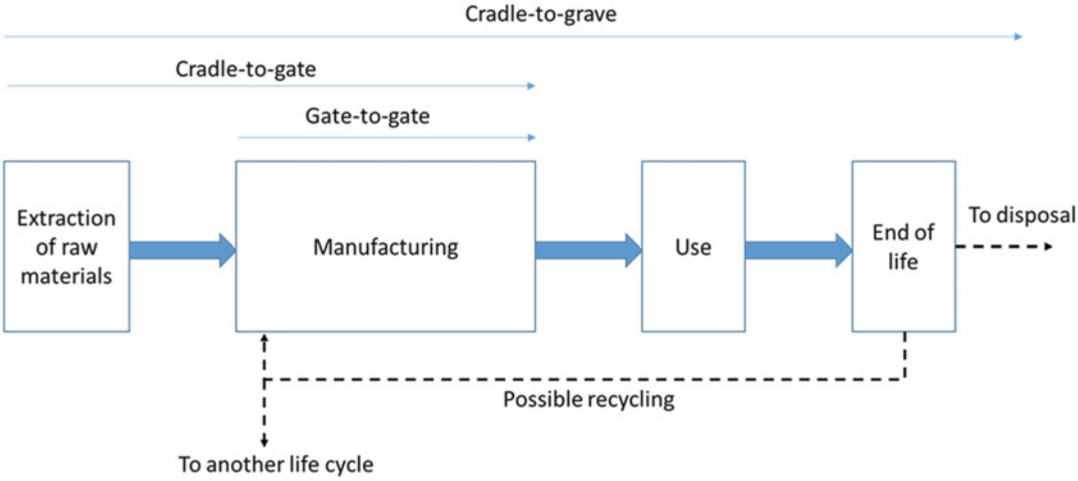

**Fig. 1** Types of LCA according to the selected system boundaries

2014; Asdrubali et al. 2015; Petrillo et al. 2016), exploitation of abiotic resources (Moran et al. 2014), water (Duić et al. 2015), metals (Table 1), and manufacturing of food products (Thomassen et al. 2008; Roy et al. 2009; Cellura et al. 2012; Ahamed et al. 2016) are among the industrial sectors where LCA has been largely developed. Its potential applications to agromining are, for instance: comparison with conventional supply chains, identification of potential hotspots, and assessment of the potential environmental consequences of massive development. Those applications are discussed below.

LCA operates in general by comparison between two or more products, processes, etc., one of which is used as a reference. In the case of Ni agromining, the reference process is based on extractive metallurgy (pyrometallurgy or hydrometallurgy). Through the life cycle analysis of two scenarios for remediation of Pb-polluted soils in Spain, Vigil et al. (2015) concluded that

**Table 1** LCA examples related to metals

| Metal | Reference | Note |
|---|---|---|
| Gold (Au) | Mudd (2007) | Mining |
| | Norgate and Haque (2012) | Cradle-to-gate |
| Nickel (Ni) | Norgate et al. (2007) | Cradle-to-gate |
| | Mudd (2010) | Mining |
| | Norgate and Jahanshahi (2011) | Cradle-to-gate |
| | Schmidt et al. (2015) | Production route for Ni class 1 |
| Copper (Cu) | Norgate et al. (2007) | Cradle-to-gate |
| | Fthenakis et al. (2009) | Cradle-to-gate, for photovoltaics |
| Lead (Pb) | Norgate et al. (2007) | Cradle-to-gate |
| Zinc (Zn) | Norgate et al. (2007) | Cradle-to-gate |
| | Fthenakis et al. (2009) | Cradle-to-gate, for photovoltaics |
| Aluminium (Al) | Norgate et al. (2007) | Cradle-to-gate |
| | Fthenakis et al. (2009) | Cradle-to-gate, for photovoltaics |
| | Liu and Müller (2012) | Cradle-to-grave |
| | Ciacci et al. (2014) | Cradle-to-gate |
| | Ding et al. (2012) | Cradle-to-gate, primary and recycled aluminium |
| | Paraskevas et al. (2015) | Cradle-to-gate, aluminium recycling |
| | Paraskevas et al. (2016) | Cradle-to-gate, primary aluminium |
| Titanium (Ti) | Norgate et al. (2007) | Cradle-to-gate |
| | Middlemas et al. (2015) | Cradle-to-gate |
| Steel | Norgate et al. (2007) | Cradle-to-gate |
| | Burchart-Korol (2013) | Gate-to-gate |
| Stainless steel | Norgate et al. (2007) | Cradle-to-gate |
| | Olmez et al. (2016) | Cradle-to-gate |
| Iron (Fe) | Yilmaz et al. (2015) | Gate-to-gate |
| | Olmez et al. (2016) | Cradle-to-gate |
| Cobalt (Co) | Norgate and Jahanshahi (2011) | Cradle-to-gate |
| | Schmidt et al. (2015) | Production route for Co class 1 |
| Cadmium (Cd) | Fthenakis (2004) | Cradle-to-gate, for photovoltaics |
| | Fthenakis et al. (2009) | Cradle-to-gate, for photovoltaics |
| Indium (In) | Fthenakis et al. (2009) | Cradle-to-gate, for photovoltaics |
| Germanium (Ge) | Fthenakis et al. (2009) | Cradle-to-gate, for photovoltaics |
| Gallium (Ga) | Fthenakis et al. (2009) | Cradle-to-gate, for photovoltaics |
| Selenium (Se) | Fthenakis et al. (2009) | Cradle-to-gate, for photovoltaics |
| Tellurium (Te) | Fthenakis (2004) | Cradle-to-gate, for photovoltaics |
| | Fthenakis et al. (2009) | Cradle-to-gate, for photovoltaics |
| Molybdenum (Mo) | Fthenakis et al. (2009) | Cradle-to-gate, for photovoltaics |

for the phytoremediation scenario, *Morus alba* biomass valorization (in the present case by anaerobic digestion) was necessary to obtain a sustainable benefit. Suer and Andersson-Sköld (2011) reached a similar conclusion in their comparison of phytoremediation by *Salix viminalis* of a former oil depot used for biofuel production with a dig-and-dump scenario. Witters et al. (2012) compared the cultivation of three energy crops (*Salix* spp., *Zea mays* L., *Brassica napus* L.) on soils hosting diffuse pollution by Cd, Pb, and Zn; digestion of silage maize combined with combustion of the resulting contaminated digestate was the best scenario in terms of energy production and $CO_2$ abatement perspectives. The two other considered scenarios were willow and combustion with electricity, and heat production and rapeseed with biodiesel or oil production.

LCA can also be used on a single item (product, process, activity, etc.): the goal is then to compare the stages of its life cycle (manufacturing, operation/use, end-of-life disposal, decommissioning, etc.), and to identify potential issues that may require improvements to minimize environmental impacts (Nielsen and Wenzel 2002; Benini et al. 2015). This corresponds to the definition of classical or 'attributional LCA' (aLCA), and can be used as a management decision tool by companies willing to meet market expectations concerning the sustainability of their product (Buxel et al. 2015).

But what will happen if there is a high demand for agromined nickel products (Buxel et al. 2015)? If, in a given region, all of the ultramafic soils actually used for the production of food crops are devoted to Ni production, where would the food for local inhabitants and their cattle come from? This type of question is tackled in 'consequential life cycle assessment' (cLCA), which aims to help policy makers deal with large-scale and complex problems (Finnveden et al. 2009; Earles and Halog 2011; McManus and Taylor 2015). In aLCA, the models describing the processes are essentially static and do not take into account any variations in the market during the operation time (during several decades sometimes for an industrial plant), either for commodities (change of the energy production model, etc.), or for the products themselves (desire for the public to use more biosourced products (Meyerding 2016), etc.). Rebound effects, where an increase in process efficiency does not result in a decrease in energy or resource consumption because of an increase in product availability, of changes in user behaviour and/or income, etc., are other aspects that can be tackled by cLCA (Benedetto et al. 2014; Vivanco and van der Voet 2014).

In cLCA, the system boundaries, i.e. limits between the technical system under consideration and its environment, are extended with respect to those in aLCA. The assessment is supposed to be more prospective than in aLCA. This is not always true in practice, because many aLCA studies are not restricted to a posteriori problems, i.e. those dealing with fully established industrial systems. aLCA has been applied to new technologies only available at the lab or at pilot scale, and that are compared to established industrial approaches. For example, Vocciante et al. (2016) discuss the benefits of an electrokinetic remediation process to remove metals from a polluted site. This is a rather new technology with few demonstration studies available, and the authors compare it to landfilling.

In cLCA, technology changes and maturation over forthcoming years with yield changes, market response times, etc., should be predicted. Alvarez-Gaitan et al. (2014) have examined the effect of increases of potable water demand, due to the increase of population and availability of a better infrastructure, for chlor-alkali chemicals used in drinking water production: taken into account, up to 2030, were possible changes in the production mode of chlorine gas and of the electricity mix (taking into account energy prices), the effect of climate change on hydroelectricity production and the social impacts (no nuclear power plants, no municipal solid waste incinerators). cLCA also includes some economic information not needed in aLCA. However, the rules on how to apply cLCA are not yet fully defined. Marvuglia et al. (2013) proposed a framework to help practitioners to implement cLCA, and described its application in the case of biogas production from crops: the indirect changes in land use are issues frequently addressed in cLCA involving agricultural resources (Kløverpris et al. 2008a, b; Alvarenga et al. 2013; Vázquez-Rowe et al. 2014).

## 3 From Scope Definition to the Life Cycle Inventory

According to the ISO norm 14044, LCA is based on four steps (Fig. 2):

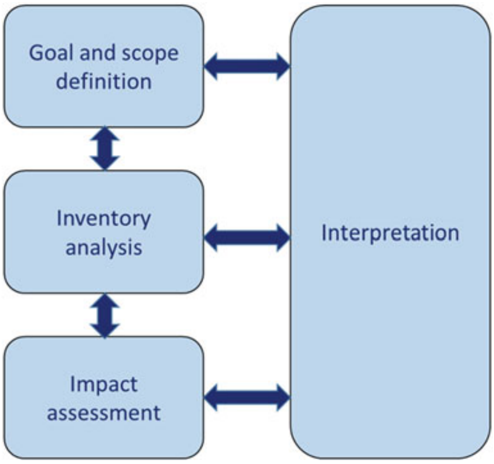

**Fig. 2** The Life Cycle Assessment framework

- *Goal and scope definition*: the goal and scope of the study are explicitly stated. The function (s) fulfilled by the system under study are identified and the functional unit defined: it is on the basis of the functional unit (surface of land to remediate, mass of metal to produce, etc.) that quantification of the impacts will be based. The system boundaries are set and the allocation method needed when several co-products share the same process is selected.
- *Inventory analysis*: processes and activities required to produce the studied good/service are described as thoroughly as possible. Input flows may originate from other processes/activities, which should be described as well, or directly from the environment. Output flows are the studied good, emissions of substances to the environment, and possibly co-products, by-products, or waste to be disposed. Data regarding these exchanged flows may be collected either during the study (and constitute the system foreground), or already be available within Life Cycle Inventory (LCI) databases such as EcoInvent (which would constitute the system background). Eventually, LCI sums up all of the flows exchanged with the environment, and is the basis for which potential impacts are calculated in the next step.
- *Impact assessment*: the potential impacts of the flows inventoried in the previous step are estimated. Quantities of these flows are multiplied by characterization factors, which express the potential impact of an elementary quantity of flow. These characterization factors are calculated with environmental models.
- *Interpretation*: this step allows comparisons among different systems, and answering the questions formulated in the goal and scope definition. The processes and life cycle steps that contribute the most to a given impact are identified. Main hypotheses and control parameters of the studied system may be modified during a sensitivity analysis in order to test their influence on the results. The reliability of the results regarding data uncertainties may be tested through an uncertainty analysis, and their effect on the results documented.

The following sections discuss some methodological issues to bear in mind when doing an LCA.

### 3.1 Goal and Scope Definition

As an illustration, Fig. 3 summarizes the steps necessary to produce ANSH (ammonium nickel sulphate hexahydrate) by agromining on ultramafic soils in the Balkans (Barbaroux et al. 2012; Bani et al. 2015; Zhang et al. 2016; Rodrigues et al. 2016). Figure 4 presents a

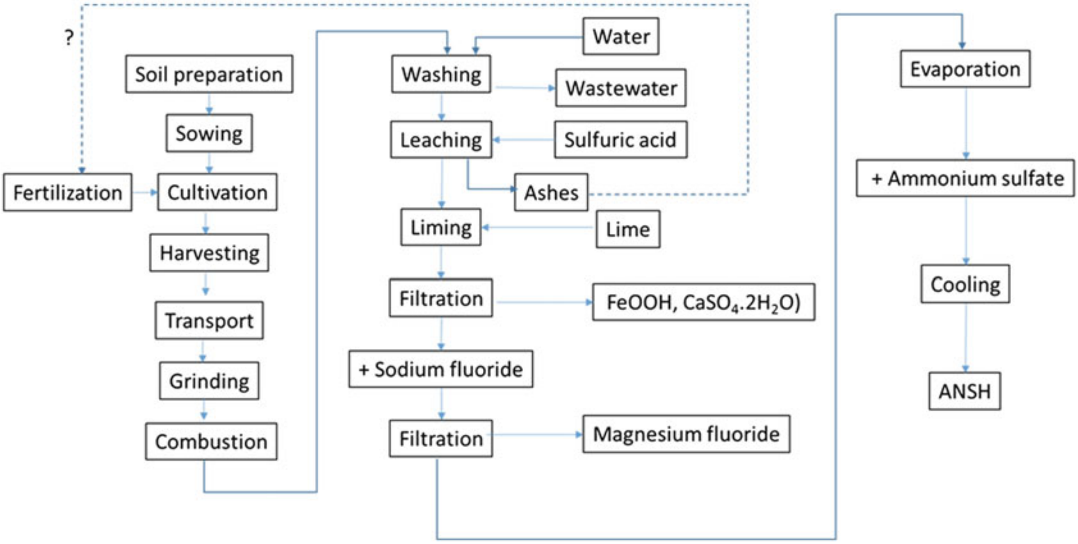

**Fig. 3** Process tree for the production of ANSH based on agromining

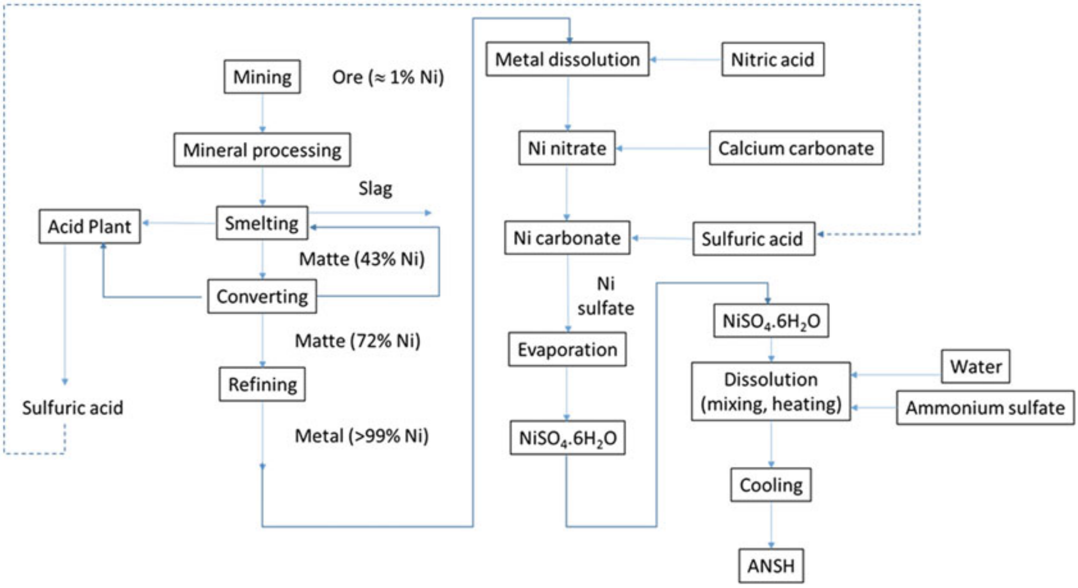

**Fig. 4** Process tree for the production of ANSH from sulphidic nickel ore (adapted from Norgate et al. (2007) for the metal production)

simplified process tree for the conventional production of the same product, ANSH being obtained by mixing nickel sulphate hexahydrate with ammonium sulphate. Several steps are required to obtain nickel sulphate hexahydrate from Ni metal. The Class 1 Ni metal (>99% Ni) that is used in this process is produced mostly by pyrometallurgy from sulphidic ores (Norgate et al. 2007). For sake of simplicity, not all of the inputs or all of the emissions have been indicated. But the full process tree should contain all of the emission routes (to soil, atmosphere, and aquatic environment) and all of the inputs (water, fertilizers and pesticides for the agricultural process, reagents for the chemical processes, energy).

In particular, waste treatment should be described in detail. Most mines around the world store their slurries (mixture of process water and tailings) in dams, with risks of dyke failure or mine drainage seepage. The effect of acid mine drainage (AMD) dispersion (e.g. Aznalcóllar, Spain, dam failure) can be severe to adjacent environments, because of the extremely low pH and high concentrations of metals that result from the high environmental availability of released metals. Reid et al. (2009) tested six scenarios for an underground hard rock Cu-Zn mine, based either on sending the tailings to a disposal area where they were submerged or using them to partially backfill the mine galleries. According to these authors, there is no consensus on methods to describe the land use and the long-term impacts. As the rate of mining increases (due to economic development and decrease in ore grade; Mudd 2007, 2010), new technologies to reduce environmental impact of tailings are developed (Edraki et al. 2014; Asif and Chen 2016). Tailings phytoremediation (or phytostabilization) has been proposed—mostly at the laboratory scale (Han et al. 2016; Yan 2016; Burges et al. 2016; Vargas et al. 2016), with some field-scale testing (Gil-Loaiza et al. 2016; Perkins et al. 2016; Davies et al. 2016). However, phytomining (i.e. recovery of metals from plants) is not an issue and no LCA seems to have been performed on phytoremediation technologies (Morais and Delerue-Matos 2010). In the Ecoinvent database, which provides process data for thousands of products to help the data inventory phase in LCA, models for the treatment of sulphidic ores tailings (e.g. Ni, Cu, Pb, Zn, etc.) have been considered too uncertain, although the effects of AMD on the environment (water and soil acidification, trace metal transfers in adjacent ecosystems and to humans) are recognized (Gutierrez et al. 2016; Romeiro-Freire et al. 2016; Taludkar et al. 2016). Only impacts related to land use are taken into account and not those related to the effects on ecosystems (Althaus and Classen 2005).

Possible recycling should be considered. In the conventional process for production of ANSH, sulphuric acid is produced during the smelting/converting step and can be used later, at least partly, to transform Ni carbonate into Ni sulphate. In agromining, recycling of minerals (Ca, K, Mg and P) could be proposed for fertilization, but the concentrations of metals other than Ni should be taken into account and may prevent such a reuse. The production of sulphuric acid concomitantly to Class I nickel in the conventional process with sulphidic ore helps to illustrate a key issue in LCA: the allocation problem, which should be solved any time an activity generates several co-products. There are basically three ways to deal with this problem:

- *System subdivision*: identify which subsystems generate the co-products and record only the flows related to the product under study.
- *System expansion*: perform the full inventory on the system with all of the co-products and do the same inventory for the other ways that produce the co-products. In our specific example, the conventional manufacturing process for sulphuric acid is the contact process that combines sulphur, oxygen and water in the presence of a catalyst (vanadium oxide). In the agromining process, some minerals can be recovered as fertilizers: conventional processes for the concerned fertilizers should be inventoried. Then theses flows will be subtracted from those of the process under study, as they will be avoided.
- *Allocation*: perform the full inventory on the system with all of the co-products and use allocation factors to weigh the importance of the flows with respect to each co-product. The key issue is then the calculation of the allocation factors: these are usually based on mass, economical value, or energy content, but there is no general consensus on the best allocation procedure (Reap et al. 2008; Schrijvers et al. 2016).

The localization of the process (and of the different stages of the process) should be specified in order to take the transport modes and distances properly into account, as well as the local energy mix. The local energy mix is the recipe used locally to produce energy from different sources such as coal, gas, etc. For the conventional route, ANSH is not necessarily produced at the mining site. In the agromining scenario, one option could be the production of the plants and ashes in one country and the metal salts in another country. In both cases, the mode of transport (ground, air, water) and the distances, should be specified.

## 3.2 Life Cycle Inventory

Mass and energy balances are the core of LCI and data collection is a key issue in LCA, on which the accuracy of the results depends. It is in general a combination of generic information available in databases, especially for commodities (upstream of the process under study), waste treatment (downstream of the process under study), and transport, and information collected on specific sites (laboratory, pilot or field/full scale).

Laboratory and pilot-scale processes are not optimized and different technological choices may be made for the final industrial process, with different yields and recycling possibilities. These will influence the quality of the LCA results and should be considered during the sensitivity analysis.

LCA practitioners apply three main types of methods for LCI (Islam et al. 2016): process-based modelling, basic balances based on inputs and outputs to the system (IO LCI), and hybrid methods that combine at various degrees both previous approaches. LCI based on a full process-based model is very complex and time-consuming, requiring mathematical expertise: in agromining such an approach will require a deep knowledge on all agricultural and chemical steps, as well as of ancillary processes (energy and reagents production, waste treatment). IO LCI is suitable for a fast assessment but its level of detail is low. The best solution is an integrated hybrid approach, especially in the case of a new product or new technology development (Islam et al. 2016) such as agromining.

In LCI, not all of the flows are inventoried or characterized. Some flows are considered as negligible, because they are consumed or produced in small volumes with respect to the main flows. However, it is uncertain whether the fluxes below the cut-off value, i.e. the limit value under which the flows are not taken in account, will not have finally a large impact. In agromining, the mass flow of dust particles from biomass could be considered as negligible and taken out of the inventory. Dust, however, is a diffuse component for human toxicity (usually considered because of its concentration in heavy metals), and it would be better to evaluate its contribution to the final impacts before being discarded (Lundie et al. 2007; Rodrigues et al. 2016).

It is important to define the localization of every process step as some impacts may depend upon it. Electrical energy is a good example of the importance of the regionalization. First, the greenhouse gas emissions related to electricity generation depend upon the mode of production. Table 2 summarizes emission factors for various sources of energy. Obviously, the emission factors for renewable energy (sun, wind, biomass, etc.) are much lower than those for

**Table 2** $CO_2$ emission factors per mode of electricity production

| Nature | Emission factor (kg$CO_2$ eq kW $h^{-1}$) |
|---|---|
| Hard coal | 0.29 (France) |
| Brown coal | 1.22 (Germany) |
| Crude oil | 0.955 (France) |
| Natural gas (combined cycle) | 0.57 (France) |
| Diesel (cogeneration) | 0.69 (Switzerland) |
| Biogas (cogeneration) | 0.03 (France) |
| Wood (cogeneration) | 0.056 (France) |
| Hydroelectricity | 0.0067 (French Alps) |
| Solar (ground solar panels, low voltage) | 0.077 (France) |
| Wind (<1 MW, onshore) | 0.014 (France) |
| Nuclear (pressurized water reactor) | 0.013 (France) |
| Geothermal | 0.075 (Germany) |

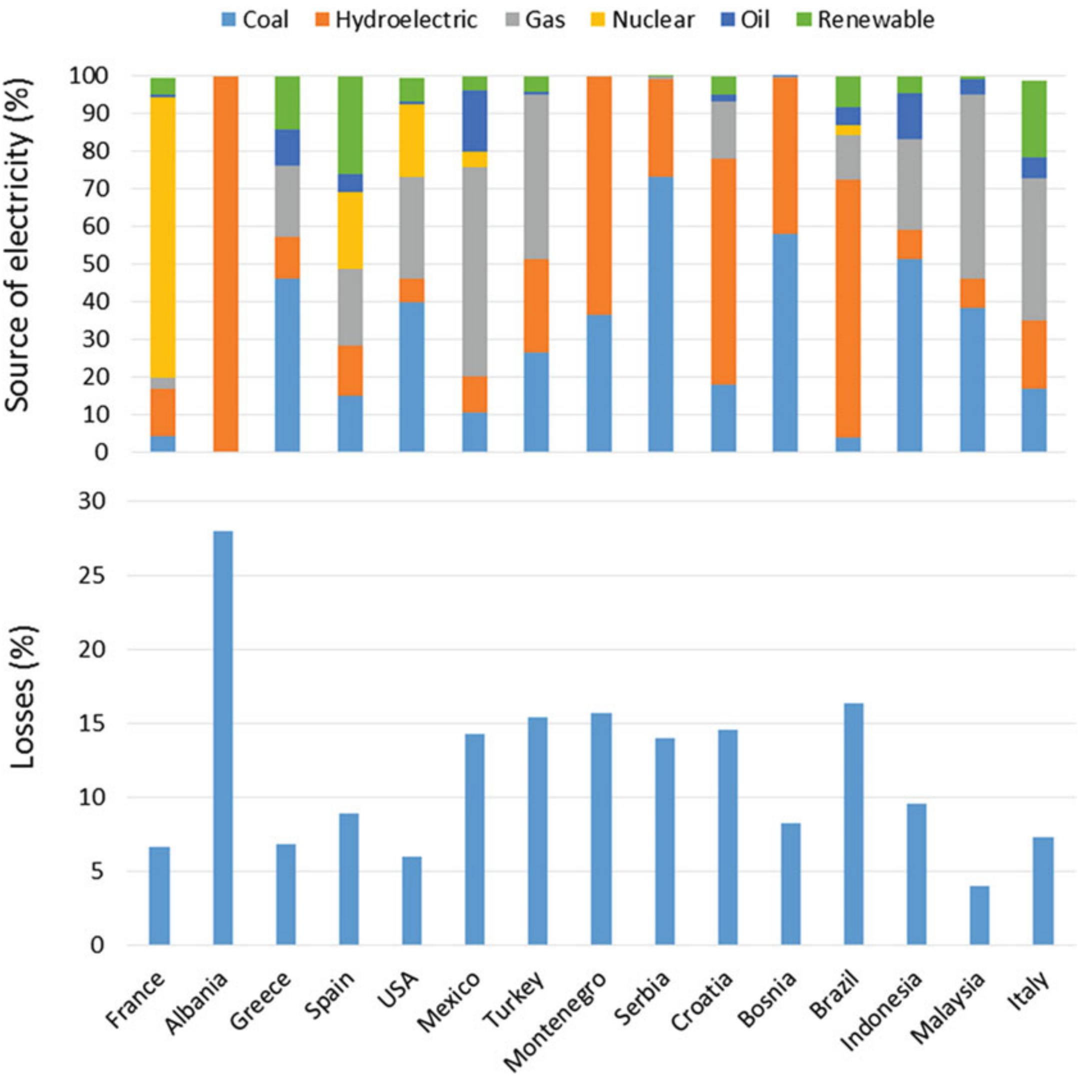

**Fig. 5** Energy mix and electricity loss in the network for some countries susceptible to develop agromining on ultramafic soils (Source of data: World Bank 2016)

fossil combustibles (coal, oil, natural gas). Therefore, depending on how the electricity is produced at the process location, the greenhouse gas emissions can vary. In general, the electricity mix is country dependent, but in some cases a company specific mix can be preferred, when it is largely different from the country mix (Gediga et al. 2015), or when the local distribution network is unreliable.

In practice, Fig. 5 summarizes the electricity mix for different countries that are amenable to develop agromining on ultramafic soils (World Bank 2016). However, these data do not into take into account the possible failure of the electricity grid. The loss index (World Bank 2016) is an indicator of the reliability of the electricity network. It can be seen that if electricity in Albania is theoretically of hydroelectric origin (hence with very limited $CO_2$ emissions), the loss index is very high and the reliability of the network could be questionable. In such a case, another source of electricity, such as a portable generator running on diesel fuel, could be used. This plan would increase locally the $CO_2$ emissions for electricity generation (Rodrigues et al. 2016).

## 4 Impact Assessment

In theory, the number of impacts that may be considered in LCA is not limited. The damages can be classified into areas of protection (depletion of natural resources, human health, and impact on ecosystems) and sub-categories, with for each specifying the relevant scale, an indicator, and a model for its characterization (Fig. 6). Three criteria are applied to select the most appropriate impacts for a given problem:

- *Completeness*: all of the environmental problems that can be linked to the goal to be achieved should be taken into account.
- *Non-redundancy*: the categories should not be redundant and the indicators should be independent one from another, to avoid double-counting.
- *Validity*: the environmental mechanism leading to each indicator should be clearly identified in its category. The characterization models should be accepted and validated by the scientific community.

All of the process flows are transformed into elementary flows (what is taken from/emitted to the environment), such as $CO_2$ for greenhouse gases or $SO_2$ for acidifying substances, as a result of characterization factors (Table 3).

Recommendations were issued in the European context in 2011 to help identify the best practice for the selection of the most relevant impacts and the most mature methods to evaluate them (EC-JRC 2011). Besides problem-oriented methods aimed at midpoint impacts, i.e., CML2002 (Guinée et al. 2002), EDIP (Hauschild and Potting 2005), TRACI (Bare 2002), or LUCAS (Toffoletto et al. 2007), and damage-oriented methods developed for the end-point areas of protection, i.e. Eco-Indicator 99 (Goedkoop and Spriensma 2000), EPS2000 (Steen 1999a, b), or LIME (Itsubo et al. 2004), methods such as Impact 2002+ (Jolliet et al.

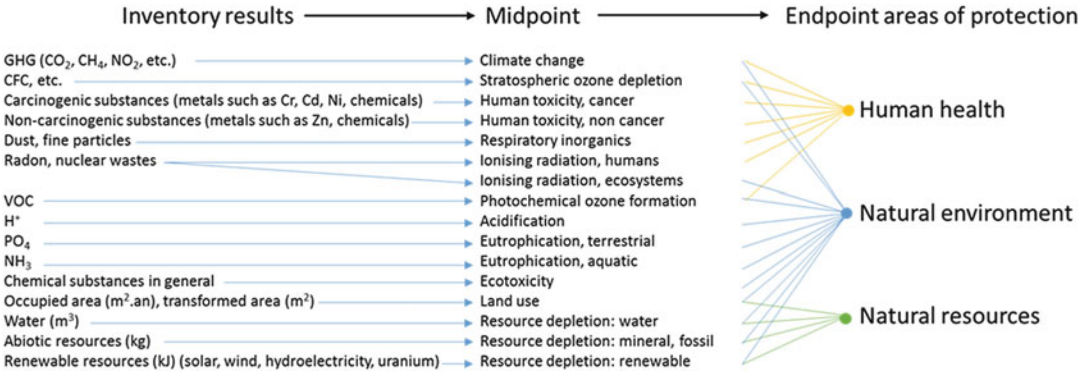

**Fig. 6** Characterization framework linking the elementary flows from the LCI to indicator results at midpoint and end point. Adapted from Hauschild et al. (2013)

**Table 3** Some characterization factors for three categories of impacts (Source: EC-JRC 2011)

| Warming potential (kg $CO_2$ eq 100 years) | | Acidification (mol $H^+$ eq/kg) | | Marine eutrophication (kg N eq/kg) | |
|---|---|---|---|---|---|
| $CH_4$ | 25 | $NH_3$ | 3.02 | $NH_3$ | 0.092 (air) |
| $N_2O$ | 298 | $NO_2$ | 0.74 | $NH_3$ | 0.824 (water) |

2003), ReCiPe (Goedkoop et al. 2009), or Swiss Ecoscarcity 2013 (Frischknecht and Büsser Knöpfel 2013) cover the full spectrum of impacts. USETox deals exclusively with ecotoxicity and human toxicity (Westh et al. 2015).

The proper selection of impact assessment methods remains a key issue in LCA because the results will depend upon assumptions and models embedded in the methods. If the situation is relatively clear for greenhouse gases emissions as the various methods are based on the IPCC recommendations, impacts related to abiotic resource depletion (ADP), human toxicity, ecotoxicity, and land use are still subject to discussion (Renou et al. 2008; Owsianak et al. 2014). These impacts are important in the case of agromining, in its comparison with traditional mining of ores. Frischknecht et al. (2016) recently provided updated guidance for four impacts: global warming, fine particulate matter emissions, water use, and land use.

## 4.1 Abiotic Resource Depletion

The disputes related to ADP come primarily from the definition of this impact. Some workers argue that abiotic resource depletion is more an economic problem than an environmental problem. However, the depletion becomes an environmental problem when the energy demand required to extract abiotic resources from lower-grade ores or to recover them from recycled materials is increasing. Currently, the ADP of a given substance (e.g. Ni) is obtained by comparison with the depletion of a reference substance (Sb) and takes into account the element reserve, according to the following equation:

$$\mathrm{ADP_{Ni}} = \frac{\mathrm{DR_{Ni}}/(R_{\mathrm{Ni}})^2}{\mathrm{DR_{ref}}/(R_{\mathrm{ref}})^2}$$

Where $\mathrm{DR_{ref}}$ is the extraction rate of the reference resource (i.e. Sb), $R_{\mathrm{ref}}$ is the reserve of the reference resource, $\mathrm{DR_{Ni}}$ is the extraction rate of Ni, and $R_{\mathrm{Ni}}$ the reserve of Ni. But which type of reserve, ultimate or economic, should be considered? The ultimate reserve is estimated from the average element concentration in Earth's crust and the economic reserve is the part of the natural reserve base that can be economically extracted at the time of determination. The natural reserve base encompasses reserves that can effectively be extracted economically, but also that some extraction is only marginally economic or even sub-economic (Van Oers and Guinée 2016). However, other ways to assess abiotic resources have been proposed, such as Cumulative Exergy Extraction from the Natural Environment, or the ratio of industrial to ecological Cumulative Exergy Extraction (Alvarenga et al. 2016). Developments are on-going to clarify the different frameworks and to end with a shared vision of ADP (Drielsma et al. 2016).

## 4.2 Land Use (LU)

The impacts related to land use (LUI) consider land use changes (transformation of one land use to another due to human activity) and land occupation (use of a given land for some time under a given activity). The land use changes (LUC) are further divided into either direct (dLUC) or indirect (iLUC). Direct land use change refers, for example, to the creation of mine tailing deposits on natural land or to agromining on natural land. Indirect land use changes are induced when agromining is developed on a cropland or on forestland: new surfaces will be needed or yields would be improved (intensification by an increase of fertilization or irrigation), in order to compensate for the loss of actual production (food, wood). Land use changes can affect several other impact categories such as GHG emissions and ecosystem health (Garraín et al. 2016).

LUIs are not only defined in terms of surface used for a given activity but in terms of functions of the land, i.e. the ecosystem services rendered by the land. A set of nine land use functions

(LUF), divided into three categories, has been proposed by Pérez-Soba et al. (2008). These cover societal (provision of work, human health, recreations, and culture), economic (residential and land-independent production, land-based production, and transport), and environmental (provision of abiotic resources, support and provision of biotic resources, and maintenance of ecosystem processes) issues. Anthropogenic land use affects supporting processes (soil formation, primary production, photosynthesis, nutrient and water cycling) and regulating processes (maintenance of biodiversity, pollination, water purification, waste treatment, and regulation of climate, erosion, air quality, water, diseases and natural hazards) (Taelman et al. 2016). The type of land cover is one parameter to consider, as well as its geographical position.

LUI is generally viewed as negative. To evaluate the impacts related to land use change for natural land and compare different situations, Koellner et al. (2013) proposed an indicator related to duration of the damage, intensity of soil degradation, and extent of the damage (i.e. surface). As depicted in Fig. 7, in their modal, the land use change supposedly induces an immediate loss of quality, related to the land functions. During the new land use, the quality of the ecosystem would remain constant, but at a level lower than that of the reference state. After the end of the land use, the ecosystem would return naturally and at a constant rate to its initial (i.e. reference) state. The reference state is defined as the natural land cover existing under the prevailing soil and climate conditions. The characterization factors are equivalent to the areas depicted in Fig. 6: the impact of sol transformation is proportional to the loss of quality with respect to a reference state and to the duration of natural regeneration of the ecosystem.

The duration of natural regeneration of the ecosystem is the time needed for the ecosystem to recover properties analogous to the reference state. It can be very short (a few years) for the natural reforestation of old agricultural parcels, to centuries (for grasslands, swamps, etc.), or kiloyears (for peatlands, primary forest, tropical forest) in the case of severely disturbed sites (Koellner and Scholz 2007; Brandão and Milà I Canals 2013; Saad et al. 2013). Altitude and latitude are among the factors that influence the natural regeneration (Lindeijer et al. 1998; Saad et al. 2013).

Several indicators (emitted GHG, soil organic carbon content, biodiversity, etc.) have been proposed to quantify LUI but there is no consensus yet on the most adequate metric (Perminova et al. 2016; Taelman et al. 2016). Soil organic carbon (SOC) promotes plant productivity and increases water retention. Depending upon the land management, it can increase or decrease during land use. It is also influenced by the type of soil as well as climatic conditions. If LCA impacts are often calculated at the country level, this level of detail is not well adapted to LUI as limits on ecosystems (characterized by soil type, climate

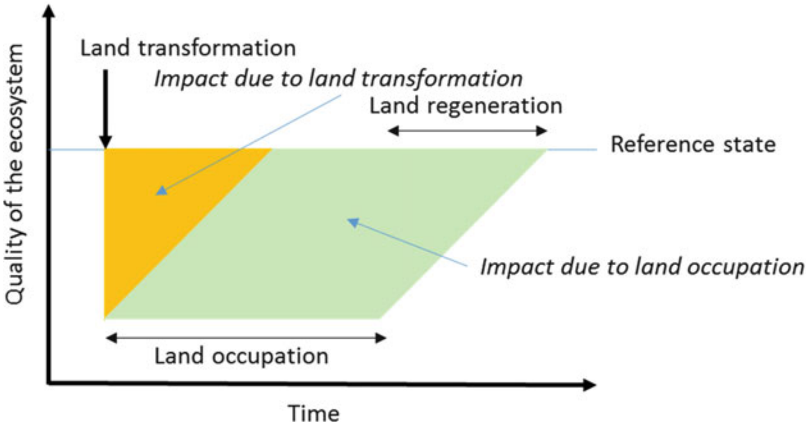

**Fig. 7** Schematic representation of the evaluation of LUC according to Koellner et al. (2013)

conditions, etc.) do not always correspond to political boundaries (Saad et al. 2013). Morais et al. (2016) have calculated characterization factors for SOC depletion in Europe at three spatial scales: climate region, ecoregion (i.e. main terrestrial natural regions as defined by Olson et al. 2001) and NUTS II regions, but a higher degree of scrutiny might be needed for local projects. This is even more critical for large countries such as Brazil (Pavan and Ometto 2016). In Europe, ultramafic soils can be found in several biomes, each biome comprising a number of ecoregions: deserts and xeric shrublands and Mediterranean forests, woodlands and scrub for the Balkans (Greece, Albania, etc.) and Italy, temperate broadleaf and mixed forests for Galicia (Spain), Austria, or Poland. The sequestration of carbon in soil also affects the impacts from climate change due to delayed $CO_2$ emissions in the atmosphere: the time span depends upon the climate, from 20 years in a tropical climate up to 100 years in cold climates (Goglio et al. 2015).

Biodiversity is not easily measured by a single indicator. de Baan et al. (2013) tested the relative change of species richness (SR) (i.e. the ratio between the specific species richness in a natural reference and in the used land) in order to assess LUI, but recognized that this is a rough approximation, first because it records only the number of species and not the number of individuals per species. Furthermore, some species are more tolerant to pollution than others. Lindqvist et al. (2016) have applied the method proposed by de Baan et al. (2013) to spruce monoculture in Sweden, and insist on the constraint of data availability and on the critical choice of the reference. Reflecting on the inability of SR to reflect all of the complexity of biodiversity, de Souza et al. (2013) added functional diversity of three taxonomic groups (birds, mammals, and plants) based on functional traits such as leaf area, height, fruit type and length, foliage, growth form, etc., for plants. In their LUI assessment of the production of kiwifruit in New Zealand, Coelho and Michelsen (2014) preferred to combine naturalness and ecosystem scarcity and vulnerability, but these indices are also difficult to obtain, especially at a regional scale. Large data gaps exist to evaluate the effect of land use activities, and more information on the regeneration time of ecosystems are especially needed. In the future, land surface phenological traits obtained by the analysis of remotely sensed data provided by satellite platforms may help to monitor spatial and time variations of vegetation biodiversity (Viña et al. 2016).

Given many outstanding discussions related to LUI, the situation is not better for assessing LUC: De Rosa et al. (2016) reviewed the different models, and classified these as follows:

- *Economic Equilibrium Models (EEMs)*: based on the fluctuations between supply and demand until an equilibrium point is reached (Starr Ross 1997): modification of any activity (increase or decrease) that require land use will induce LUC.
- *Causal-Descriptive Models (CDMs)*: these forecast patterns of future supply and demand based on predicted trajectories for goods and agriculture supply–demand.
- *Normative models*: which are simpler than EEMs and CDMs, and are based on statistics such as how much GHG are emitted and how much land is necessary, on average, to produce a given food product. A good example is given by Audsley et al. (2009) in the investigation of the reduction of GHG emissions for the UK food system.
- *Conceptual model*: Schmidt et al. (2015) have proposed a conceptual model to relate the iLUC impact to an increase of GHG emissions, based on land markets: this model includes effects related to deforestation and cultivation intensification. One of the soil properties that can be modified with a new land use is organic carbon content (Njakou Djomo et al. 2015; Bosco et al. 2016).

Erosion of land, by rain or wind, is one of the ecosystemic regulating processes affected by land use. There are still large gaps in the modelling of soil erosion by wind: it depends upon many factors such as climatic erosivity (resulting from the stress due to wind and weather conditions), soil erodibility (i.e. the ability of a soil to be eroded), vegetation cover and surface roughness (Borrelli et al. 2016), but recent research brings promises: this LUI could be incorporated in the near future for European studies (Borrelli et al. 2014). Losses of elements (heavy metals and phosphate) through erosion related to runoff on soils can be calculated using the Revised Universal Soil Loss Equation (Wischmeier and Smith 1978; Renard et al. 1997), as follows:

$$A_{\text{soil loss}} = R \cdot k \cdot LS \cdot C \cdot P$$

This equation calculates $A_{\text{soil loss}}$, the potential amount of soil lost through erosion, as the product of different factors describing the erosive force of rainfall (erosivity $R$), the type of soil (erodibility $k$), and field slope and length ($LS$), implemented cropping and anti-erosion practices ($C$ and $P$, respectively). The latter are further described by Panagos et al. (2015a, b) as the product of different parameters, characterizing for example land cover and tillage practices. As emphasized by Rodrigues et al. (2016), it is important to be able to decrease the land erosion in metal agromining in order to limit the dissemination of heavy metal to the aquatic environment. According to Nemecek et al. (2014), heavy metal emissions to groundwater due to infiltration of rainwater through the soil are assumed to be constant: the contribution is calculated with an allocation factor accounting for both direct anthropogenic inputs and atmospheric deposition.

In the case of agromining, the framework depicted by Koellner et al. (2013) is more complex. Metals that are naturally present in soils and can constitute a health risk, either by transfer to edible crops or to groundwater (Tang et al. 2012; Rivera et al. 2016), will be removed slowly (Harris et al. 2009). Therefore, the quality of the soil will be theoretically better after agromining and will never go back to its initial (i.e. reference) state (Fig. 8). When some decrease of soil quality can be foreseen during the initial crop installation, a global improvement will result from the decreased metal concentrations. However, the question remains open in terms of biodiversity: it will be probably modified, but it is difficult to say whether it will be improved or not.

In the case of phytomining of mine tailings, the reference state should be selected carefully, depending upon the system frontiers:

- Either it is limited to the treatment of tailings, without considering how the tailings were produced, and in such case the framework will be similar to the case of agromining on ultramafic soils (Fig. 8);

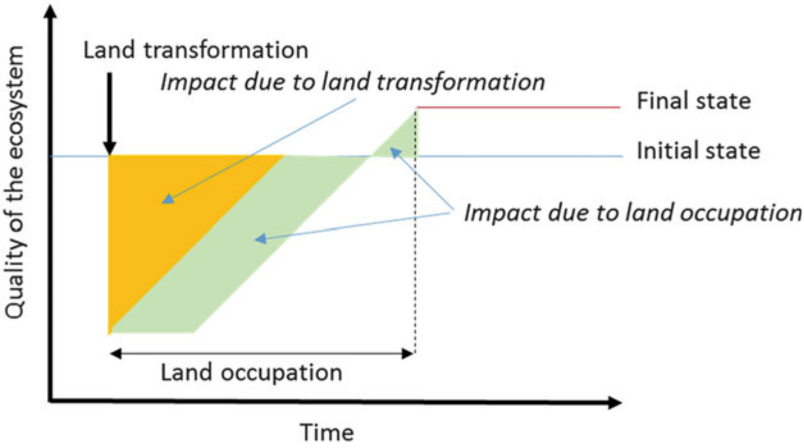

**Fig. 8** Schematic representation of a possible scheme for LUC evaluation in case of agromining on ultramafic soils

- Or they encompass the entire mining implementation, and in such case the reference state would be the pristine natural ecosystem (Fig. 7). It can be foreseen in such a case that regeneration time will be very long.

## 5 Space and Time Dependence

The regionalization of impacts or of their source, as for the electricity mix, is now a well-accepted idea in LCA, although it takes time to build the relevant databases, especially for toxicity related emissions (Huijbregts et al. 2003; Sleeswijk et al. 2008 for Europe; Lautier et al. 2010 for Canada and USA; Lundie et al. 2007 for Australia), and for developing countries.

Another need for improvement in impact evaluation is the time dependence, which is not yet taken into account. In the case of greenhouse gas emissions, the classical approach is to model the effect of GHG emissions occurring along the time horizon considered in a project, as if they were taking place at the beginning of the time horizon. Such a time-distributed emission of $CO_2$ occurs in systems involving agriculture (including agromining) and forestry. Time-adjusted warming potentials have been proposed to solve this problem. By taking into account the year of emission, Kendall (2012) demonstrated that $CO_2$ emissions of a commercial building were significantly reduced (by 30%) for a 100-year span, but that the decrease became less significant when the time span was increased. A similar dynamic LCA has been developed by Levasseur et al. (2010) in their comparison of GHG emissions (including those related to LUC), between fossil fuels and biofuels: under some conditions, corn ethanol was less favourable, compared to gasoline, when the dynamics of the emissions were taken into account, than in classical (static) LCA.

Factors that influence plant growth such as temperature, water availability, or $CO_2$ concentration in the atmosphere are expected to vary in the future owing to climate change, inducing possible decreases in yields (Ingvordsen et al. 2015; Niero et al. 2015). It is not straightforward to model the effect of climate change on any plant, but time-adjusted LU characterization factors could be developed in the future to better evaluate the impact on land functions.

## 6 Conclusions

The concept of Life Cycle Assessment has been introduced as a tool for environmental assessment of agromining, either on natural ultramafic lands or mine tailings. LCA is a powerful tool that has been used successfully to study various supply chains. In particular, in the case of site phytoremediation, it highlighted that valorization of the produced biomass is crucial in reducing environmental impacts. Metrics related to land use and land use change need refinement, in terms of selection of the most appropriate indicators of ecosystem services, of development of the models to evaluate these indicators, of access to regionalized, and to time-dependent databases to feed these model. More recently, and more specifically to agromining, LCA demonstrates that ensuring soil erosion control could be a key advantage of agromining—plant growth ensures soil cover and function—as opposed to leaving soils bare. Considering the potential toxicity of metal-rich soil particles, this characteristic is of crucial importance. However, the still-pioneering state of agromining has many uncertainties. Co-product valorization, cropping practices, biodiversity integration, and local social and economic consequences of land use for agromining, remain unknowns. This chapter gives an overview of how these features can be assessed in LCA, as well as some current methodological issues and considerations that one should take into account while assessing agromining supply chains with this methodology. Nevertheless, even with known limitations, the LCA methodology gives to developers of agromining a solutions framework to assess the sustainability of their projects.

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# Global Distribution and Ecology of Hyperaccumulator Plants

Roger D. Reeves, Antony van der Ent, and Alan J.M. Baker

**Abstract**

A large body of analytical data is available on the inorganic composition of many thousands of plant species, for which typical concentration ranges have been tabulated for major, minor, and trace elements. These elements include those that have been shown essential for plant growth, as well as others that lack this status, at least universally. Metalliferous soils, having abnormally high concentrations of some of the elements that are generally present only at minor (e.g. 200–2000 μg $g^{-1}$) or trace (e.g. 0.1–200 μg $g^{-1}$) levels, vary widely in their effects on plants and have attracted increasing attention during the last 50 years. The effects depend on the species, the relevant elements, and soil characteristics that influence the availability of metals to plants. Some of these soils are toxic to all or most higher plants. Others have hosted the development of specialized plant communities consisting of a restricted and locally characteristic range of metal-tolerant species. These plants often show a slightly elevated concentration of the elements with which the soil is enriched, but in places a species exhibits extreme accumulation of one or more of these elements, to a concentration level that may be hundreds or even thousands of times greater than that usually found in plants on the most common soils. These plants, now widely referred to as hyperaccumulators, are a remarkable resource for many types of fundamental scientific investigation (plant systematics, ecophysiology, biochemistry, genetics, and molecular biology) and for applications such as phytoremediation and agromining, and are discussed in detail below.

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## 1 Introduction

It has been known since the 1850s and 1860s that certain plant species then found on the zinc (Zn)-rich ‘calamine’ soils near Aachen in Germany accumulated Zn to very high concentrations. Although the first record referred to *Viola calaminaria* (Violaceae), a later report presented data showing that *Thlaspi alpestre* var. *calaminare,* now classified as *Noccaea caerulescens* (Brassicaceae), contained at least 1% Zn in the dry leaf tissue, or 10% in the inorganic ash (Sachs 1865). Plants of the genus *Noccaea* (earlier included in *Thlaspi sensu lato*) are discussed in more detail below. During the last 100 years, unusual accumulation of other metals and metalloids has been found: selenium (Se) in the 1930s; nickel (Ni) in the 1940s; cobalt (Co), copper (Cu), and arsenic (As) in the 1960s; and cadmium (Cd) and manganese (Mn) in the 1970s.

## 2 Hyperaccumulation

Normal concentration ranges in plants have been tabulated for major, minor, and trace elements in many reviews (e.g. Reeves and Baker 2000). The term ‘hyperaccumulation,’ describing a highly abnormal level of metal accumulation, was first applied by Jaffré et al. (1976) in the title of their paper on Ni concentrations in the New Caledonian tree *Sebertia acuminata* (Sapotaceae), now classified as *Pycnandra acuminata*. In discussing Ni concentrations in species of *Homalium* (Salicaceae) and *Hybanthus* (Violaceae) from various parts of the world, Brooks et al. (1977a) used the term to indicate a defined concentration threshold ($>1000\ \mu g\ g^{-1}$) for Ni. A similar concept was used earlier by Jaffré and Schmid (1974), who referred to certain Ni-rich plants from the ultramafic soils of New Caledonia as ‘hypernickelophores,’ i.e. ‘extreme nickel-bearers.’

The choice of the 1000 $\mu g\ g^{-1}$ criterion was not entirely arbitrary. In many reports on Ni-rich soils, plant Ni concentrations are generally 5–100 $\mu g\ g^{-1}$; levels of 100–1000 $\mu g\ g^{-1}$ are quite rare; the local cases of accumulation to $>1000\ \mu g\ g^{-1}$ seem to represent a distinct form of plant response, implying some characteristic and unusual physiological behaviour. Greater precision in the definition of hyperaccumulation was provided by Reeves (1992) for Ni: *“a hyperaccumulator of Ni is a plant in which a Ni concentration of at least 1000 mg/kg has been recorded in the dry matter of any above-ground tissue in at least one specimen growing in its natural habitat.*” The criteria defining hyperaccumulation should therefore not be based on analyses of whole plants or subterranean plant parts, largely because of the difficulty of ensuring that the samples are free of soil contamination, but also because plants that immobilize metals in the root system, and fail to translocate them further (Baker 1981), are of less interest for many purposes than those that actively accumulate metals into all tissues.

Definitions of hyperaccumulation have been extended to elements other than Ni. Malaisse et al. (1978) used the 1000 $\mu g\ g^{-1}$ criterion for Cu accumulation, and Brooks et al. (1980) applied this to Co. Reeves and Brooks (1983b) used the same criterion in discussing lead, but for Mn and Zn, which are normally present at higher and more widely varying concentrations (~20–400 $\mu g\ g^{-1}$), a 10,000 $\mu g\ g^{-1}$ threshold was suggested by Baker and Brooks (1989), following use of the term ‘hypermanganèsophore’ for plants having this level of Mn accumulation (Jaffré 1980).

Extensive recent discussions of appropriate criteria for defining hyperaccumulation of many elements are those of Baker et al. (2000), Baker and Whiting (2002) and van der Ent et al. (2013) who summarized the history of the development of this topic. These papers also pay attention to the limitations of hydroponic experiments in relation to hyperaccumulation, because these experiments have often involved the use of unrealistic concentrations of free metal ions that are not relevant to the continuing life cycle of naturally occurring metallophyte populations living on metalliferous soils.

## 3 Ecology and Conservational Status of Hyperaccumulator Plants

The soils produced from the weathering of surficial ore deposits or naturally enriched metalliferous country rocks (e.g. ultramafics, Cu-Co mineralization, calamine deposits) can be regarded as primary habitats for most hyperaccumulator plants. In certain cases, as in some of the ultramafic terraines of Cuba and New Caledonia, such soils are believed to have been continuously available for plant life and evolution for millions of years (Reeves et al. 1996, 1999). Other naturally occurring metalliferous soils are much younger, having been subjected to more recent geological processes such as erosion and re-deposition, hydrothermal alteration, or glaciation. Secondary habitats (on the scale of decades to a few thousand years) have resulted from the exploitation of mineral deposits via metalliferous mining and ore processing activities. A tertiary category of distribution results from the superficial deposition of dusts and particles derived from smelting operations and the beneficiation of processed ores where effluents are discharged into river systems leading to metal enrichment of alluvial floodplains (Baker et al. 2010).

Present-day plant species that show metal tolerance through occurrence on metalliferous soils may therefore have experienced any of this wide variety of soil histories. In relation to species that appear to be endemic to metalliferous soils, there has been extensive discussion of the concepts of palaeo-endemism and neo-endemism (Stebbins 1942; Kruckeberg 1954; Antonovics et al. 1971; Brooks 1987, pp. 70–71). Palaeoendemics are supposed to be relics of formerly widespread species that have survived in the metalliferous environment, restricted by competitive pressures and often having no or few closely related surviving species. Neo-endemic metallophytes are species that have evolved from a parent in the metalliferous environment, leading to morphological characteristics now recognized as distinctive. The concept as applied generally to metallophytes can also be used in discussion of the particular case of hyperaccumulator species and their putative origins. Some Ni hyperaccumulators, for example, in genera consisting of only one or two species on ancient soils and without close relatives, may be palaeo-endemics. Examples include *Shafera platyphylla* (Asteraceae) and *Phyllomelia coronata* (Rubiaceae) from Cuba. These phylogenetically isolated hyperaccumulators contrast with the situation in some genera where actively evolving speciation appears to be continuing, as shown by the large numbers of Ni hyperaccumulating endemics present in genera such as *Alyssum* (Brassicaceae) in Mediterranean Europe, Turkey, and nearby parts of Asia—Fig. 1; *Buxus* (Buxaceae) and *Leucocroton* (Euphorbiaceae) in Cuba; and *Phyllanthus* (Phyllanthaceae) in several tropical parts of the world.

Mineral wastes have enabled locally endemic species that are both hypertolerant and hyperaccumulating to extend their distributions regionally, such that the current distributions of some hyperaccumulator plants have extended well beyond primary habitats. Additionally, some species are known from both non-metalliferous and metalliferous locations, exhibiting hyperaccumulation solely from the latter. This situation, described as 'facultative hyperaccumulation' has been discussed in detail by Pollard et al. (2014). Further, some species have been reported to show extreme accumulation of some elements (e.g. Zn, Mn, Se) from normal soils or those with only modest elevations of the element concerned. Examples include: *Noccaea caerulescens* (Brassicaceae) that hyperaccumulates Zn from both metalliferous and non-metalliferous soils in France and elsewhere in Europe (Reeves et al. 2001); *Gossia* (formerly *Austromyrtus*) *bidwillii* (Myrtaceae)

**Fig. 1** The field spot test based on dimethylglyoxime applied to the Ni hyperaccumulator *Alyssum murale* (Brassicaceae) from Albania

from eastern Australia (Bidwell et al. 2002; Fernando et al. 2009), and *Alyxia rubricaulis* (Apocynaceae) from New Caledonia (Jaffré 1980) that hyperaccumulates Mn from soils having only a slightly elevated Mn content; and species of *Astragalus* (Fabaceae) in the USA (Rosenfeld and Beath 1964) that hyperaccumulate selenium from soils in which the elevated Se content is commonly $<50\ \mu g\ g^{-1}$.

In temperate regions, the plant assemblages on metalliferous soils generally consist of a limited range of absolute and pseudo-metallophytes (Baker 1987) that may or may not include hyperaccumulators. On ultramafic soils, in particular in Mediterranean Europe, there can frequently exist a nearly monospecific community of a Ni hyperaccumulator, e.g. *Alyssum* spp. in Greece, Turkey, and Albania. By contrast, in the tropics ultramafic soils regularly show a high density of woody species where hyperaccumulators and non-hyperaccumulators may grow side by side. Often the most ancient and undisturbed metalliferous environments support the richest assemblages of hyperaccumulator plants (e.g. Reeves et al. 1996, 1999).

In spite of the rapidly increasing number of hyperaccumulator plants being discovered (especially for Ni), the overall rarity of this resource must be stressed. Furthermore, anthropogenic and environmental factors threaten the habitats of many hyperaccumulator plants. These include: ongoing mineral exploration and mining activities, reworking of ancient mine spoils, land reclamation and improvement for agricultural production, urbanization and development of brownfield sites, natural fire events, and probably climate change (Whiting et al. 2004; Baker et al. 2010; Wulff et al. 2013). Urgent conservation and management steps are clearly needed in areas under threat, in order to ensure the persistence of the valuable phytotechnological resource. Appropriate options are the maintenance of living materials in botanical gardens and seed in germplasm banks, and regeneration in situ using ‘seed orchards’ on mining lands. Exploitation of the hyperaccumulator resource base for agromining must be considered with due caution and with appropriate management practices in place. An unfortunate incident has been reported in southwestern Oregon, where poor crop management led to the extension of the distribution of *A. murale* well outside of the operational area, to the extent that it is now regarded as a noxious weed in Oregon and future use has been banned (Strawn 2013). Invasions such as this may also affect the status of other local endemics native to the area that have been selected for agromining.

## 4 Instances of Hyperaccumulation

The following discussion outlines instances of hyperaccumulation of selected trace elements (Ni, Zn, Cd, Pb, Co, Cr, Cu, Mn, Se, As and Tl) for which a substantial body of reliable plant analysis data exists. Further exploration of various types of metalliferous environments, both natural and man-modified, will certainly uncover more examples. The exact enumeration of metal hyperaccumulator species is made difficult by the lack of recent and complete Floras for many tropical regions, in particular. The exact identification of some specimens of interest is still in doubt. In addition, since the first hyperaccumulator species were identified, numerous name changes have occurred, some species have been grouped into synonymy, whereas others have been split into several taxa (species, subspecies and varieties). Some of the earlier information was published in periodicals that are difficult to access, and much useful detail has been omitted because of the space limitations of most journals. All of these difficulties have justified the initiative to create a Global Hyperaccumulator Database (www.hyperaccumulators.org), an ongoing project to encompass as much of the knowledge as possible on identified hyperaccumulator species, including synonymies and other taxonomic changes.

### 4.1 Nickel

Unprecedented Ni concentrations (up to about 10,000 µg $g^{-1}$ or 1%) were discovered in the Italian serpentine plant *Alyssum bertolonii* (Brassicaceae) (Minguzzi and Vergnano 1948). In the 1960s, two additional Ni-accumulating *Alyssum* species—*A. murale* from Armenia (Doksopulo 1961) and *A. serpyllifolium* ssp. *lusitanicum* from Portugal (Menezes de Sequeira 1969)—were reported to behave similarly. These observations were followed by studies in Zimbabwe (Wild 1970) and two independent discoveries of high Ni concentrations (3000–9800 µg $g^{-1}$) in *Hybanthus floribundus* from Western Australia (Severne and Brooks 1972; Cole 1973).

Beginning in 1974, concerted attempts were made to discover the extent of Ni hyperaccumulation, both geographically and in terms of distribution in the plant kingdom. Detailed studies of the flora of ultramafic soils were carried out in New Caledonia (Jaffré and Schmid 1974; Jaffré et al. 1976, 1979a, b; Jaffré 1980). Particularly notable was the discovery that the latex of the New Caledonian tree *Pycnandra* (formerly *Sebertia*) *acuminata* contained about 10% Ni, yielding a dried solid with 20–25% Ni (Jaffré et al. 1976), in which citrate was a major organic constituent (Lee et al. 1977)—Fig. 2.

During the next 25 years, R.R. Brooks, R.D. Reeves, A.J.M. Baker, and co-workers in many other parts of the world collected and analyzed plant material from ultramafic areas in the search for further examples of Ni hyperaccumulation. Extensive use was made initially of leaf fragments from herbarium collections, but later this gave way to field studies. Brooks et al. (1977a) identified several species of *Homalium* and *Hybanthus* in New Caledonia as hyperaccumulators. A comprehensive survey of nearly all of the 170 known species of *Alyssum* (Brooks and Radford 1978; Brooks et al. 1979) established the existence of 48 Ni hyperaccumulators, all in one section (Odontarrhena) of the genus, distributed from Portugal across Mediterranean Europe to Turkey, Armenia, Iraq, Iran, and Russia. Most are ultramafic-endemic species, and many have a very restricted distribution. Several additions to the list of *Alyssum* Ni hyperaccumulators have been made subsequently.

Further work by various groups has focused on other genera of the Mediterranean region, on species of ultramafic outcrops in the European Alps, southern Africa, Newfoundland, and the Pacific Northwest of the United States, and on plants of tropical ultramafic soils of Brazil, Cuba, and other Caribbean islands, Queensland, Costa Rica, Sri Lanka, and Southeast Asia (especially certain islands of Indonesia and the Philippines). Hyperaccumulators discovered from temperate-

**Fig. 2** The Ni hyperaccumulator *Pycnandra acuminata* (Sapotaceae) from New Caledonia has a peculiar blue-green latex with up to 25% Ni

**Fig. 3** The Ni hyperaccumulator *Bornmuellera tymphaea* (Brassicaceae) from Greece can accumulate up to 3% foliar Ni

zone areas include *Leptoplax* (formerly *Peltaria*) *emarginata* from Greece (Reeves et al. 1980), species of *Bornmuellera* —Fig. 3 and *Cochlearia* (*Pseudosempervivum*) from Turkey and the Balkans (Reeves et al. 1983a; Reeves and Adıgüzel 2008), *Streptanthus polygaloides* from California (Reeves et al. 1981), and species of *Thlaspi* (*Noccaea*) from Europe (Reeves and Brooks 1983a), Turkey, and Japan (Reeves 1988; Reeves and Adıgüzel 2008), and California (Reeves et al. 1983b). Discoveries from tropical areas include several species from Palawan (Baker

**Fig. 4** The Ni hyperaccumulator *Psychotria gabriellae* (Rubiaceae) from New Caledonia can accumulate in excess of 4% Ni

et al. 1992) and other parts of Southeast Asia (Wither and Brooks 1977), *Stackhousia tryonii* from Queensland (Batianoff et al. 1990), and numerous species from Brazil (Reeves et al. 2007).

The ultramafic soils of Cuba host the largest number of Ni hyperaccumulators reported from any one country. Following initial observations by Berazaín (1981), a survey of much of the Caribbean ultramafic flora revealed 128 such species in Cuba, as well as *Phyllanthus nummularioides* from the Dominican Republic (Reeves et al. 1996, 1999). *Psychotria grandis* is a Ni hyperaccumulator where it occurs on ultramafic soils in Puerto Rico (Reeves 2003; Campbell et al. 2013; McAlister et al. 2015). Other major sources of Ni hyperaccumulator plants, with more than 50 species each, are New Caledonia (see Fig. 4 for an example) and Turkey. Substantial additions to the list are being made from ongoing work in New Caledonia (Jaffré et al. 2013), Brazil, Indonesia (Halmahera and some of the smaller islands), Sabah (Malaysia) (van der Ent et al. 2015), and the Philippines (Fernando et al. 2013).

The most recent information brings the worldwide total of known Ni hyperaccumulator plant species to more than 450. Developments can be followed through earlier summaries, some of which deal with hyperaccumulators of other elements (Brooks 1987, 1998; Baker and Brooks 1989; Reeves et al. 1996, 1999; Reeves and Baker 2000; Reeves 2003, 2005); more recent results can be found in reports on work in Brazil by Reeves et al. (2007), and in Turkey by Reeves and Adıgüzel (2004, 2008). Ongoing investigations in Sabah (Malaysia) and New Caledonia continue to reveal numerous hyperaccumulator plants new to science (van der Ent et al. unpublished data).

Most Ni hyperaccumulators belong to two groups, geographically: (1) the Mediterranean region, extending from Portugal through Italy and the Balkans to Turkey and adjacent countries; and (2) tropical and subtropical areas worldwide, particularly Cuba, New Caledonia, and various islands of Indonesia and the Philippines. The plant family most strongly represented in the first group is Brassicaceae, whereas in tropical areas there is strong representation from Euphorbiaceae, Phyllanthaceae, Salicaceae, Buxaceae, and Rubiaceae. Within Violaceae, species of *Hybanthus* (Severne and Brooks 1972; Brooks et al. 1974; Jaffré 1980) and *Rinorea* (Brooks and Wither 1977; Brooks et al. 1977b; Proctor et al. 1994) are notable as having potentially suitable biomass for agromining purposes. Hyperaccumulators in the Asteraceae occur in South Africa (*Berkheya* and *Senecio*; Morrey et al. 1989, 1992), in the

Mediterranean-Turkey region (*Centaurea*; Reeves and Adıgüzel 2004), and in the neotropics (e.g. *Pentacalia* and *Senecio* in Cuba, Reeves et al. 1999; and in species in several genera from Brazil, Reeves et al. 2007). The Ni hyperaccumulator plants reported to date belong to about 40 different families, distributed widely throughout the plant kingdom; this property is therefore presumed to have evolved independently many times.

The relatively large number of Ni hyperaccumulators discovered (compared with those of other elements) may be partly the result of the concerted attention to analytical work on ultramafic floras and partly to the ability to detect high Ni concentrations ($>1000$ μg $g^{-1}$) in leaf tissue by a simple test with dimethylglyoxime. However, among various types of metalliferous soils, the Ni-enriched ultramafics are the most widespread on a global scale, and in places continuous ultramafic areas of tens or even hundreds of $km^2$ can be found (e.g. New Caledonia, Cuba and Turkey). Where such areas have been continuously available for plant colonization for millions of years, as appears to be the case in New Caledonia and eastern Cuba, a long-term opportunity has existed for the evolution of a characteristic flora with numerous endemic species, including some that have developed Ni accumulation as a particular response to growth on high-Ni soils.

Most of the known Ni hyperaccumulator species are endemic to ultramafic rocks, but some occur on a wider variety of soils and exhibit facultative hyperaccumulation, i.e. high Ni concentrations are found only in those specimens from Ni-rich soils. A tabulation of facultative hyperaccumulators, covering Ni and other elements, has been given by Pollard et al. (2014). In a few cases, ultramafic-endemic species may show a wide variation in Ni uptake, apparently being sensitive to parameters other than total soil Ni concentration, such as soil pH; this 'erratic' Ni hyperaccumulation occurs, for example, in the Queensland ultramafic endemic *Pimelea leptospermoides* (Reeves et al. 2015). It is certain that many further examples of Ni hyperaccumulation remain to be discovered. These will include species not yet discovered or described, and known species that have never been analyzed. Further studies of plants growing on ultramafic areas in several islands of the Philippines and Indonesia, in Central America, mainland Asia, and possibly West Africa, are particularly likely to be fruitful.

When the focus is specifically on agromining potential, the interest logically moves towards those species that contain consistently $>1\%$ Ni in their leaves (and ideally $>1\%$ in the total harvestable biomass). This property needs to be considered in conjunction with the rate of biomass production, and a number of other agronomic features as reviewed by Nkrumah et al. (2016). The observation that the Californian *Streptanthus polygaloides* (Brassicaceae) could accumulate Ni to 1.5% of the dry plant matter (Reeves et al. 1981) stimulated a study by Nicks and Chambers (1995, 1998) of the use of this plant for phytomining. This included investigations of various fertilization regimes and the optimization of harvest time. They estimated that a crop of nearly 5 t $ha^{-1}$ could be obtained with unfertilized plants in a small-scale trial in the native environment, and predicted that fertilization could double that yield. Work elsewhere has been carried out with species capable of producing a larger biomass. The discovery of Ni hyperaccumulation by the South African *Berkheya coddii* (Morrey et al. 1989, 1992; Howes 1991) has been followed by extensive work on its cultivation and extraction of the accumulated Ni (Brooks and Robinson 1998; Robinson et al. 1997a). Yields in excess of 20 t $ha^{-1}$ were calculated, again by extrapolation from studies involving small plots.

Several of the *Alyssum* hyperaccumulators have attracted attention for their phytoextraction potential. Although some work has been done on *A. bertolonii* (Robinson et al. 1997b), more investigations have centred on species that have higher biomass such as *A. corsicum* and *A. murale*, in particular (Li et al. 2003; Bani et al. 2015a, b) Other species of the Brassicaceae in the Mediterranean region, such as *Leptoplax* *emarginata* and *Bornmuellera tymphaea* (both Brassicaceae), have also been studied (Chardot et al. 2005). These authors concluded that *L. emarginata* compared favourably with *A. murale* and *Noccaea caerulescens* in its phytoextraction performance.

About 70 tropical hyperaccumulator taxa with $>1\%$ Ni have been listed by Reeves (2003). These include the facultative hyperaccumulator *Rinorea bengalensis* (Violaceae) of Southeast Asia, a large number of Cuban species in the Buxaceae, Phyllanthaceae and Rubiaceae, and several New Caledonian species. Many of these are shrubs or small trees, probably with good rates of biomass production, but in many cases no further information is available about cultivation requirements and reproduction. Some of these species are rare, and in most cases agronomic studies are lacking or are in early stages.

## 4.2 Zinc, Lead, and Cadmium

Since the early discovery of Zn accumulation by certain *Thlaspi* species (noted above), further work, particularly on *Thlaspi* from German and Belgian calamine soils and from British mine wastes, has been reported frequently, as discussed with detailed references by Baker et al. (1994), Reeves and Baker (2000), and Reeves et al. (2001). This species, often referred to as *Thlaspi calaminare* or *T. alpestre* in earlier work, and later as *T. caerulescens*, is now classified as *Noccaea caerulescens* after a taxonomic revision by Meyer (1973) and DNA analysis (Koch and Mummenhoff 2001; Al-Shehbaz 2014).

Following the observation of Rascio (1977) that *T. rotundifolium* ssp. *cepaeifolium* from Zn-polluted soils near the border of Italy and Austria was also a hyperaccumulator of Zn, surveys of the genus *Thlaspi sensu lato* (Reeves and Brooks 1983a, b; Reeves 1988) revealed that many species of this genus are hyperaccumulators of Ni from ultramafic soils and often have Zn levels above 1000 $\mu g\ g^{-1}$, even from soils of background Zn content. Reeves and Baker (1984) showed that the ability of the Austrian species *T. goesingense* to accumulate Ni and Zn was an innate or 'constitutional' property, not dependent on the geochemistry of the area from which the seed originated. Baker et al. (1994) showed that *T. caerulescens* grown in amended nutrient solutions had the ability to accumulate to high concentrations a wide variety of elements (Zn, Cd, Co, Mn and Ni throughout the plant; aluminium, chromium, Cu, iron, and lead largely into the root system).

There are several other examples of accumulation of Zn to the level of 10,000 $\mu g\ g^{-1}$ set as the criterion for Zn hyperaccumulation by Baker and Brooks (1989), but this was lowered to 3,000 $\mu g\ g^{-1}$ by Broadley et al. (2007), Krämer (2010) and van der Ent et al. (2013). The most notable example is probably *Arabidopsis* (formerly *Cardaminopsis*) *halleri* (Brassicaceae) (Ernst 1968). Other examples, mainly from the Zn-rich soils around mine sites or from the vicinity of smelters, are listed elsewhere (e.g. Reeves and Baker 2000).

Lead is usually present in foliar concentrations below $<10\ \mu g\ g^{-1}$. Even where concentrations of 1–10 $\mu g\ g^{-1}$ are measured in aboveground plant parts, it is likely that much of this comes from various forms of environmental contamination. Plant root systems restrict severely the uptake of this element and significant translocation to the upper parts is uncommon in plants in natural environments. There have been several reports of very high lead concentrations in plants from areas of Zn-Pb mineralization, or from mine or smelter wastes; notably, these have not generally been subjected to rigorous scrutiny in relation to washing procedures and contamination possibilities. Increased lead uptake can be achieved in hydroponic experiments or by various treatments of soil with complexing agents (Raskin and Ensley 2000). However, such soil treatments designed to mobilize relatively insoluble elements such as lead into harvestable plants, as promoted by several groups, are now regarded as being both economically and environmentally unfavourable.

Elevated levels of Cd (10–200 $\mu g\ g^{-1}$, locally higher) can be found in soils containing waste materials from the mining of Zn ores, but may also occur in soils treated with industrial wastes or Cd-rich phosphate fertilizers. Plant Cd is generally $<3$ μg g$^{-1}$, but may reach 20 μg g$^{-1}$ or more in the flora of Cd-rich soils. A plant concentration of $>100$ μg g$^{-1}$ has been proposed as the threshold for hyperaccumulation of this element (van der Ent et al. 2013); such a level is exceptional, even on a Cd-contaminated site. However, on some Zn-Pb mine waste sites in the south of France and in Slovenia, *Noccaea* species such as *N. caerulescens* and *N. praecox* have been found to typically contain $>100$ μg g$^{-1}$ Cd, and $>1000$ μg g$^{-1}$ locally, with very large variations existing among sites and populations, and considerable intra-site variability (Robinson et al. 1998; Escarré et al. 2000; Lombi et al. 2000; Reeves et al. 2001; Schwartz et al. 2006). Similar observations have been made for *Arabidopsis halleri* in Europe (Bert et al. 2002) and for *Sedum alfredii* (Crassulaceae) and *Viola baoshanensis* (Violaceae) in PR China (Liu et al. 2004; Deng et al. 2008). As stressed by van der Ent et al. (2013), further claims of hyperaccumulation of Cd (and other elements) should be restricted to the behaviour of self-sustaining natural populations. Extensive investigations of the behaviour of selected *N. caerulescens* populations have generally been carried out with a focus on phytoremediation rather than agromining (e.g. Chaney et al. 2005).

## 4.3 Cobalt and Copper

An earlier threshold of 1000 μg g$^{-1}$ for plants to be considered as hyperaccumulators of Cu and Co (Baker and Brooks 1989) has been modified to 300 μg g$^{-1}$ (Krämer 2010; van der Ent et al. 2013) in the light of the apparent rarity of genuine accumulations of these elements in plants. Most reports of Co and Cu exceeding 1000 μg g$^{-1}$ are derived from studies of the metalliferous soils of the Democratic Republic of the Congo, where the two metals occur together at elevated levels in the soils, although in widely varying proportions. Elsewhere, there are local early records of plants having $>1000$ μg g$^{-1}$ Cu from Cu-mineralized areas (Blissett 1966; Dykeman and De Sousa 1966; Ernst 1966). These reports, and the plant species involved, need more detailed investigation, particularly in view of the potential for soil and dust contamination and the difficulty of its removal from many plant surfaces. The problem is exacerbated in the case of Cu mineral exposures by the common occurrence of more or less pure Cu compounds as secondary mineralization products: a very small amount of such contamination remaining on the plant material can elevate the analytical result considerably. A similar problem arises in the case of plants sampled from the vicinity of smelters.

Normal concentrations of Co and Cu in plants are in the ranges of 0.03–2 μg g$^{-1}$and 5–25 μg g$^{-1}$, respectively. Plant Cu concentrations are controlled within a remarkably narrow range, even in the presence of high soil Cu; plant Cu concentrations above 100 μg g$^{-1}$ are rare. Even on Co-rich soils, such as those derived from ultramafic rocks, Co in plants rarely exceeds 20 μg g$^{-1}$. However, the black gum of the southeastern United States (*Nyssa sylvatica* var. *biflora* and var. *sylvatica*) (Nyssaceae) shows exceptional Co accumulation (as much as 845 μg g$^{-1}$) from normal soils (Beeson et al. 1955; Kubota et al. 1960; Brooks et al. 1977c). Brooks (1977) reported as much as 10,220 μg g$^{-1}$ foliar Co in *Haumaniastrum robertii* (Lamiaceae) from The Democratic Republic of the Congo.

Extensive studies of the vegetation of many sites of mining and smelting activity throughout the Democratic Republic of Congo by F. Malaisse, R. R. Brooks, A.J.M. Baker, and co-workers identified 30 hyperaccumulator plants of Co and 32 of Cu, with 12 species being common to the two lists. The species involved have been summarized and updated in several papers and chapters (Brooks 1977; Malaisse et al. 1979; Brooks et al. 1978, 1980, 1987, 1995; Brooks and Malaisse 1985; Reeves and Baker 2000; Lange et al. 2017). Assessment of these data is difficult for several reasons: (1) numerous changes have been made to the classification and nomenclature of the species involved; (2) uncertainties exist surrounding the pre-treatment of the samples prior to analysis, and in particular the efficacy of the washing regimes; (3) few of the Co- and Cu-accumulating species appear to be absolutely restricted to metalliferous soils, although some have had local or regional uses as indicator plants; (4) there are wide variations in the apparent metal concentrations occurring within many species, even from the same area; (5) there has been a lack of reproducibility in cases where the plants from a given location have been re-examined later; and (6) difficulties have been reported in attempting to reproduce the metal accumulating behaviour in plants in cultivation. A detailed re-assessment of several putative hyperaccumulators was presented by Faucon et al. (2007), who concluded that at least part of the previously reported elevated metal levels could be ascribed to inefficient washing of sample materials prior to analysis. However, in spite of the suspicion that the last of these possibilities is sometimes relevant, many records of Cu and Co hyperaccumulation represent some degree of abnormal uptake by the plant from the soil: Malaisse et al. (1994), for example, have presented iron data that indicate little likelihood of soil contamination (e.g. *Anisopappus davyi* (Asteraceae) with 3504 μg $g^{-1}$ Cu, 3 μg $g^{-1}$ Co, and 67 μg $g^{-1}$ iron). A re-examination of putative Cu hyperaccumulation by *Millotia myosotidifolia* (Asteraceae) from a Cu mine site in South Australia (R.D. Reeves, unpublished data), has not supported the earlier finding of 4% Cu in the plant ash or 2400 μg $g^{-1}$ in the leaves (Blissett 1966), but instead showed Cu levels averaging 516 μg $g^{-1}$. This concentration is still abnormally high, and much higher than found in other species from the same site, apart from *Arctotheca calendula* (Asteraceae) that averaged 779 μg $g^{-1}$ Cu. Extensive analyses of plants from some unusually Cu-rich ultramafic soils in Malaysia and Brazil have not shown any instance of Cu concentrations reaching 300 μg $g^{-1}$ (van der Ent and Reeves 2015).

Even with the adoption of a 300 μg $g^{-1}$ threshold in defining hyperaccumulation of Cu and Co, and with the addition of reports of Cu accumulation from Sri Lanka, China and Indonesia, we conclude that Cu and Co hyperaccumulation is very rare. From the point of view of agromining applications, it is scarcely relevant whether the threshold is set at 300 or 1000 μg $g^{-1}$, because the levels of 5000–10,000 μg $g^{-1}$ of interest for agromining of these elements have rarely, if ever, been observed. The high specificity of Ni hyperaccumulation, relative to uptake of Co by Ni accumulator plants on ultramafic soils, also implies that extracting Co as a by-product of Ni agromining will rarely be economically feasible.

## 4.4 Manganese

Jaffré (1977, 1979, 1980) found that 98 out of 445 species (22%) growing on ultramafic soils of New Caledonia had mean Mn concentrations above 1000 μg $g^{-1}$; six species had means exceeding 10,000 μg $g^{-1}$, and nine had at least one specimen above this level. The Mn concentrations within these soils ranged from about 1000–5000 μg $g^{-1}$, only a little above the range determined for many types of soils worldwide. Recognizing that normal levels of Mn in plant dry matter fall within the rather wide range of 20–500 μg $g^{-1}$, Baker and Brooks (1989) chose a level of 10,000 μg $g^{-1}$ to define Mn hyperaccumulation. This criterion has been maintained in the review by van der Ent et al. (2013). After accounting for synonymies and changes of nomenclature for several species, data are now available for 24 species that have been found to meet this threshold in at least one specimen. These include a single species of *Alyxia* (Apocynaceae), *Beaupreopsis*, and *Grevillea* (Proteaceae) (Jaffré 1977, 1979; Losfeld et al. 2015), all from New Caledonia; *Chengiopanax* and *Tieghemopanax* (Araliaceae) from Japan and New Caledonia, respectively (Mizuno et al. 2008; Losfeld et al. 2015); *Garcinia* (Clusiaceae) from New Caledonia (Jaffré 1980); two species each of *Phytolacca* (Phytolaccaceae) (Xue et al. 2004); and *Polygonum* (Polygonaceae) from China (Deng et al. 2010; Liu et al. 2016), three species of *Denhamia* (formerly in *Maytenus*—Celastraceae), from New Caledonia and Australia, (Jaffré 1977;

Fernando 2008), two of *Virotia* (formerly in *Macadamia*—Proteaceae) from New Caledonia (Jaffré 1979), and nine of *Gossia* (formerly in *Austromyrtus* and *Eugenia*—Myrtaceae) from eastern Australia (Bidwell et al. 2002; Fernando et al. 2008, 2009; Jaffré 1980; Losfeld et al. 2015). Because of the extreme levels of Mn, locally reaching 2–5% in dry matter in some of these species, the plant ash may contain 10–25% Mn, which should make agromining for Mn worthy of further study and field trials.

## 4.5 Chromium

Even on ultramafic soils having high chromium (Cr) concentrations (500–5000 μg $g^{-1}$) it is normal to find Cr concentrations in plant material in the range of 1–30 μg $g^{-1}$. Occasional reports of much higher concentrations are believed to reflect contamination by wind-blown dusts or smelter fallout, or analytical problems. In fact, high Cr concentrations in plants from ultramafic soils have been used as an indicator of soil contamination (see, e.g. Jaffré et al. 1979b; Brooks and Yang 1984). In the absence of evidence for consistently high Cr concentrations existing in any species, there seems to be little hope of finding a future for agromining of this element.

## 4.6 Selenium

Selenium is essential for animal and human health, and exhibits a narrow range between the levels required to prevent deficiency diseases and those that produce symptoms of toxicity. The Se content of soils is typically below 2 μg $g^{-1}$, but can reach several hundred μg $g^{-1}$ in soils derived from certain Cretaceous shales. In plant dry matter, Se concentrations are generally below 1 μg $g^{-1}$, and may even be <0.01 μg $g^{-1}$ in areas of Se-poor soils. However, the accumulation of Se to high levels (locally >1000 μg $g^{-1}$) by legumes in the genus *Astragalus* (Fabaceae) from seleniferous soils in the western United States was found to be responsible for poisoning of livestock (Byers et al. 1938).

A detailed account of the discovery of Se-accumulating plants in the western United States can be found elsewhere (Rosenfeld and Beath 1964). Reeves and Baker (2000) tabulated values and references for 20 species that have shown maximum Se concentrations above 1000 μg $g^{-1}$. Because of the very low levels of Se that normally occur in plants, a case can be made (Reeves 2005; van der Ent et al. 2013) for taking 100 μg $g^{-1}$ as the threshold for Se hyperaccumulators. The use of plants showing some degree of Se accumulation for economic extraction of elemental Se has not yet been proposed. However, there are potential applications in: (1) phytoremediation of soils that have become Se-contaminated through extensive use of Se-rich irrigation waters (Parker et al. 2003), (2) harvesting crop plants suitable for stock feed from high-Se areas and transport of this material to areas of Se deficiency (Bañuelos and Mayland 2000), and (3) Se biofortification for improving human health (Bañuelos et al. 2014).

## 4.7 Arsenic

Normal As concentrations in igneous rocks and soils are in the range of 1–10 μg $g^{-1}$. Higher soil As concentrations can be found in areas of polymetallic sulphide mineralization and of some pyritic black shales, in places contaminated through the smelting of chalcophile element ores, in areas of geothermal activity, and where As compounds have been used as horticultural sprays or timber preservation agents. Plant As concentrations are normally on the order of 1 μg $g^{-1}$, but higher values can be found in contaminated areas. As hyperaccumulation (based on a 1000 μg $g^{-1}$ dry matter criterion) has been known for more than 50 years. Warren et al. (1964) found As in the ash of growing tips of Douglas fir (*Pseudotsuga menziesii*) to be 2500–10,000 μg $g^{-1}$ over soils containing 1000–5000 μg $g^{-1}$ As. The highest of these values almost certainly corresponds to >1000 μg $g^{-1}$ on a dry weight basis.

Studies by several groups on the behaviour of aquatic plants in the Waikato River in the North Island of New Zealand showed that three aquatic plants act as As hyperaccumulators. Natural geothermal activity, together with borefield drainage and waste water from the Wairakei geothermal power plant that opened in 1953, combined to raise the As concentration in the river from ca. 0.01 mg $L^{-1}$ to as much as 0.08–0.09 mg $L^{-1}$ before dilution and sedimentation processes lower the concentrations downstream. The adventive aquatic weeds *Ceratophyllum demersum* (Ceratophyllaceae), *Egeria densa,* and *Lagarosiphon major* (Hydrocharitaceae) act as As hyperaccumulators (Lancaster et al. 1971; Aggett and Aspell 1980; Liddle 1982; Reeves and Liddle 1986), yielding As concentrations in the plant dry matter from ca. 100 μg $g^{-1}$ to 1000–1500 μg $g^{-1}$. The bioaccumulation factor, taken as the plant/substrate concentration quotient, can be as high as 30,000, e.g. where the plants contain 1500 μg $g^{-1}$ in water with 0.05 mg $L^{-1}$ As.

More recent attention has been paid to As accumulation by fern species, particularly those growing in areas of As contamination from waste disposal related to timber preservation processes or mining. Ma et al. (2001) reported As at 3280–4980 μg $g^{-1}$ in *Pteris vittata* (Pteridaceae) plants from soils containing 19–1603 μg $g^{-1}$ As. As hyperaccumulation was also found by Vittoottiviseth et al. (2002) in the fern *Pityrogramma calomelanos* (Pteridaceae). A number of fern species may possess this capability of As accumulation as a constitutive property (Meharg 2002). However, applications of As hyperaccumulators seem likely to lie more in the area of remediation of As-contaminated waters and land, rather than in economic extraction of the As itself.

### 4.8 Thallium

Currently only a small number of thallium (Tl) hyperaccumulator plants have been reported, mainly from France: *Biscutella laevigata* (Brassicaceae) with up to 15,200 μg $g^{-1}$ Tl (Anderson et al. 1999), and *Iberis intermedia* (Brassicaceae) (now regarded as a synonym of *I. linifolia*) with up to 2810 μg $g^{-1}$ Tl (LaCoste et al. 1999; Leblanc et al. 1999). Van der Ent et al. (2013) proposed a threshold value of 100 μg $g^{-1}$ to define Tl hyperaccumulation. The substantial value of Tl metal might justify Tl agromining, but the locations at which this could take place appear to be rather limited.

## 5 Hyperaccumulators, Phytoremediation, and Agromining

Major potential uses of hyperaccumulator plants focus on the possibility of removal of large amounts of a particular element from the soil without significant chemical intervention, other than the application of conventional fertilizers. Such uses include phytoremediation (removal of an undesirable metal to restore contaminated soil for any of a variety of uses), and agromining (plant extraction of elements to provide a feedstock from which a valuable metal, or one of its compounds, can be extracted economically). For these purposes, important factors are the rate of biomass production and the concentration of the desired element that can be achieved in harvestable plant matter.

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# Physiology and Molecular Biology of Trace Element Hyperaccumulation

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## Abstract

Some trace elements are essential for plants but become toxic at high concentration. Remarkably, about 500 plant species worldwide are able to accumulate tremendous amounts of metals in their leaves and are therefore called metal hyperaccumulators. In the context of sustainable development, there is a regain of interest for metal hyperaccumulation mechanisms that may become instrumental for improving metal phytoextraction from contaminated soils to produce metals with a lower net impact on the environment. In addition, studying the molecular mechanisms of hyperaccumulation in diverse plant species is necessary in order to understand the evolution of this extreme and complex adaptation trait. Our current knowledge of metal hyperaccumulation is mostly based on the analysis of a few species from the Brassicaceae family, and suggests that the underlying mechanisms result from an exaggeration of basic mechanisms involved in metal homeostasis. However, the development of Next Generation Sequencing technologies opens today the possibility for studying new hyperaccumulator species that therefore may reveal more diversity in these mechanisms. The goal of this chapter is to provide background information on metal hyperaccumulation and give a clear picture of what we know currently about the molecular mechanisms involved in this trait. We also attempt to outline for the reader the future scientific challenges that this field of research is facing.

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# 1 Introduction

Investigating the mechanisms involved in metal hyperaccumulation allows us to observe extreme adaptation of metal homeostasis networks in plants and identify key players in metal distribution and tolerance in plant tissues. The study of metal hyperaccumulator species also permits examination of mechanisms underlying the evolution of this extreme adaptive trait (Shahzad et al. 2010; Hanikenne and Nouet 2011; Hanikenne et al. 2013). Several comprehensive reviews have been published recently on this topic (Verbruggen et al. 2009b; Krämer 2010; Hanikenne and Nouet 2011) and we refer readers to those reviews. Whereas hyperaccumulator species typically accumulate specifically one metal when growing in their natural environment, some species have the ability to tolerate and accumulate several metals when grown *ex situ*. This is well documented for the hyperaccumulator species of the Brassicaceae family *Noccaea caerulescens*, in which ultramafic-adapted accessions such as Puy de Wolf (France), Monte Prinzera (Italy), or Puente Basadre (Spain) are able to accumulate Ni but also Zn and Cd (Assunção et al. 2003; Peer et al. 2003; Escarré et al. 2013; Gonneau et al. 2014; Callahan et al. 2016). This ability to tolerate and accumulate several metals likely reflects the relatively low specificity of some mechanisms involved in metal transport and chelation.

Several metals that are accumulated in hyperaccumulator species (e.g. Zn, Ni, Mn) are essential nutrients but become toxic at high concentrations for most plants (i.e. non-accumulating species). Therefore, all plant species have developed mechanisms to regulate essential metal homeostasis according to their needs and metal availability in soils (Burkhead et al. 2009; Palmer and Guerinot 2009; Thomine and Vert 2013). Our current knowledge suggests that the molecular mechanisms involved in metal hyperaccumulation are derived essentially from the mechanisms involved in metal homeostasis. In several examples, genes involved in metal homeostasis are differentially expressed in hyperaccumulators compared to related non-accumulator species, as a result of gene duplication and/or changes in promoter activity (Talke et al. 2006; van de Mortel et al. 2006; Krämer et al. 2007; Hanikenne et al. 2008; Shahzad et al. 2010). However, specific genes linked to hyperaccumulation may be discovered as molecular analysis of hyperaccumulation will extend in the future to additional non-model species from various plant families owing to the development of high-throughput sequencing technologies (Verbruggen et al. 2013; Halimaa et al. 2014b; Merlot et al. 2014). For most metals, the hyperaccumulation trait appeared independently in distant plant families. Therefore, some of the mechanisms involved in metal hyperaccumulation may be specific to a plant family or a species, whereas others may be convergent among distant hyperaccumulators. For instance, several examples of convergent evolution have been identified between the Brassicaceae *Arabidopsis halleri* and *N. caerulescens* (see below and Krämer et al. 2007; Hanikenne et al. 2008; O'Lochlainn et al. 2011; Craciun et al. 2012), suggesting important functional constraints in the metal homeostasis network.

Because of its singularity, metal hyperaccumulation may appear as an exception having minimal relevance. However, from a scientific point of view, metal hyperaccumulation in plants is fascinating, and understanding the mechanisms involved in this trait may provide tools for producing metals with lower impact on the environment in the near future. The goal in this chapter is to outline our current knowledge about the molecular mechanisms of metal hyperaccumulation in plants and to highlight possible future developments in this important field of research.

# 2 Molecular Physiology of Metal Hyperaccumulation

## 2.1 Main Steps of Metal Hyperaccumulation

When exposed to excess metals, most plant species adopt a so-called excluder strategy to prevent metal accumulation in photosynthetically active shoot tissues (Krämer 2010). This goal can be achieved by limiting metal absorption by roots, increasing metal efflux from root tissues, and/or increasing metal storage in root cell walls and vacuoles. In contrast, achieving metal hyperaccumulation and hypertolerance requires modifications at specific nodes of the metal homeostasis network to ensure that the metal flux in the plant is directed towards shoot tissues (Fig. 1; Clemens et al. 2002). At the physiological level, these alterations include some or all of the following steps:

1. An enhanced metal mobilization and uptake in roots;
2. An efficient radial metal transport towards the root vascular tissues; this includes a reduction of metal storage in root vacuoles;
3. An increased transport of metal from the root to the shoot, with efficient xylem loading; this

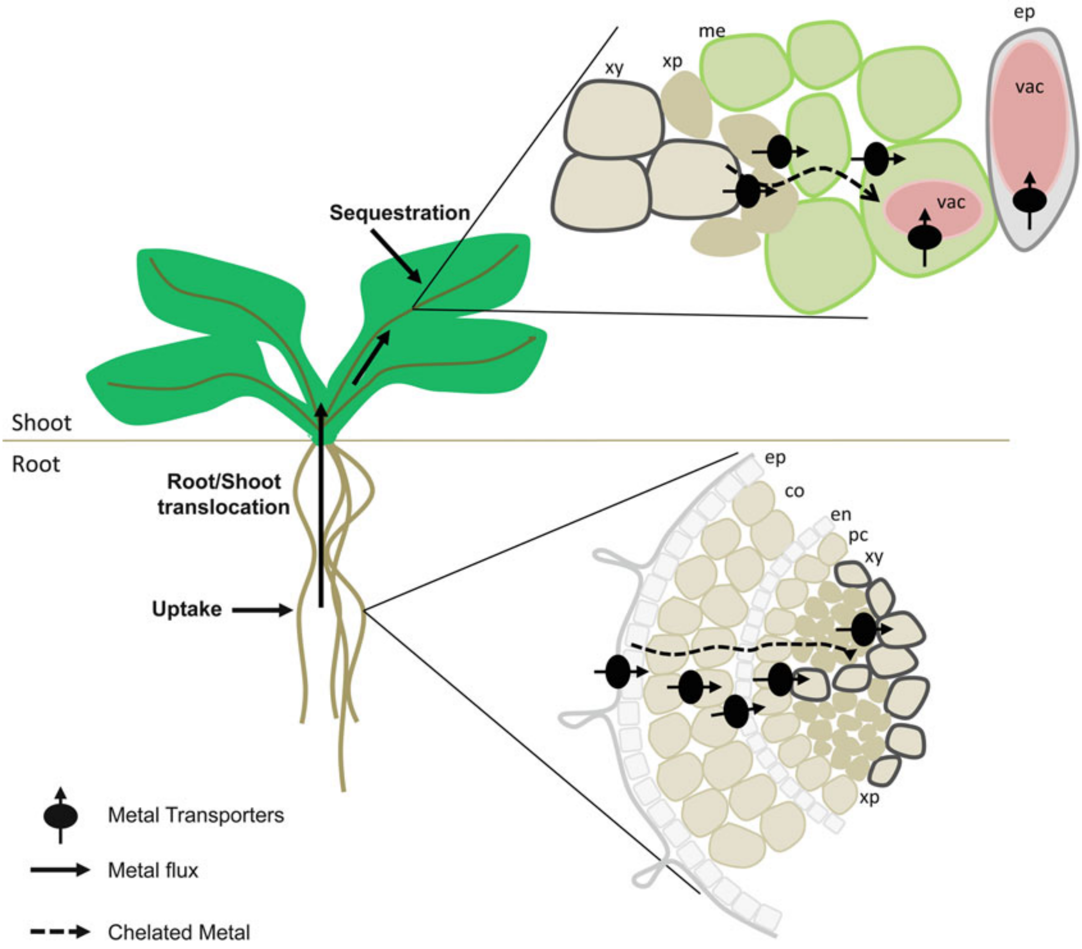

**Fig. 1** Model of the physiology of metal hyperaccumulation and hypertolerance. Enhanced metal uptake and radial transport in roots, xylem loading/unloading and vacuolar storage in shoots all make major contributions to the traits. In roots, it is possible that the metal can travel in the apoplasm up to the endodermis cell layer before cellular uptake (not represented). Note that the tissue (epidermis or mesophyll) involved in metal storage varies depending on the species and the metal (see text). *co* cortex, *en* endodermis, *ep* epidermis, *me* mesophyll, *Zinc-NA* Zinc-Nicotianamine chelates, *pc* pericycle, *vac* vacuole, *xp* xylem parenchyma, *xy* xylem. Figure modified from Hanikenne and Nouet (2011)

step contributes to metal tolerance by enabling metal storage (and thus detoxification) in shoot tissues;

4. An efficient mechanism for xylem unloading and metal distribution in shoots together with a high vacuolar storage capacity.

Processes occurring in both roots and shoots thus contribute to metal tolerance and hyperaccumulation. Root-based processes play an essential role in hyperaccumulation whereas shoot-based processes are required for hypertolerance, as suggested from grafting experiments between *N. caerulescens* and the non-Zn-accumulating *Microthlaspi perfoliatum* (Guimarães et al. 2009).

### 2.2 Metal Distribution in Shoots

Metal distribution in shoot tissues is specific to both the species and the metal considered. This topic has been extensively reviewed recently (Fernando et al. 2013; Leitenmaier and Küpper 2013). Briefly, in most cases, metals (Zn, Cd, Ni or Se) accumulate at the base of the trichomes and in the vacuoles of epidermal cells. In contrast, mesophyll cells that are the main site of photosynthesis accumulate lower amounts of metals (Küpper et al. 1999, 2001; Lombi et al. 2002; Cosio et al. 2005). In the vacuoles of epidermal cells, metals can reach very high concentrations (e.g. several hundred mM; Küpper et al. 1999; Fernando et al. 2006b). There are, however, exceptions. For instance, Zn and Cd are stored in the vacuoles of mesophyll cells in both Zn- and Cd-hyperaccumulators *A. halleri* and *Sedum alfredii*, in which Zn is mostly bound to malate (Küpper et al. 2000; Sarret et al. 2002, 2009; Tian et al. 2011; Lu et al. 2014; Isaure et al. 2015). In the hyperaccumulator *Sedum plumbizincola,* Zn accumulates mostly in leaf epidermal cells, but also in large amounts in mesophyll cells of young leaves (Cao et al. 2014). In this species, Cd is mostly bound to cell walls in leaves (Peng et al. 2017). Accumulation of Mn in mesophyll cells is also observed in several Mn-hyperaccumulators (Fernando et al. 2006a, b, 2013).

## 3 Identification of Molecular Processes Involved in Metal Hyperaccumulation

In the last 15 years, a number of complementary approaches have been used to identify the molecular actors underlying hyperaccumulation and hypertolerance. These approaches included:

I. Screens of cDNA libraries in yeast in order to isolate genes contributing to metal transport and tolerance (e.g. Lasat et al. 2000; Pence et al. 2000; Bernard et al. 2004; Papoyan and Kochian 2004);
II. Quantitative genetics analyses aiming to identify Quantitative Traits Loci (QTLs) co-segregating with the traits in progenies of crosses between an hyperaccumulator and a related non-accumulator species (e.g. Dräger et al. 2004; Deniau et al. 2006; Filatov et al. 2007; Courbot et al. 2007; Willems et al. 2007, 2010; Frérot et al. 2010; Baliardini et al. 2015).
III. Transcriptomic studies comparing gene expression levels in hyperaccumulator and related non-accumulator species (e.g. Becher et al. 2004; Weber et al. 2004, 2006; Chiang et al. 2006; Craciun et al. 2006; Filatov et al. 2006; Hammond et al. 2006; Talke et al. 2006; van de Mortel et al. 2006; van de Mortel et al. 2008; Gao et al. 2013; Han et al. 2015).

These candidate genes are mostly involved in metal transport, metal chelator synthesis, or metal-induced oxidative stress response. Note that the last is not discussed in this chapter. Several candidate genes were further characterized functionally (e.g. Pence et al. 2000; Persans et al. 2001; Dräger et al. 2004; Kim et al. 2004; Hanikenne et al. 2008; Gustin et al. 2009; Lin et al. 2009; Shahzad et al. 2010; Ueno et al. 2011; Milner et al. 2012; Deinlein et al. 2012; Merlot et al. 2014; Baliardini et al. 2015; Nouet et al. 2015; Charlier et al. 2015). However, only a few candidates (e.g. *HMA4*, see below) were confirmed by reverse genetics in hyperaccumulator species as major players in metal hyperaccumulation and tolerance; their functions are described in detail in the following sections.

A large part of our knowledge on metal hyperaccumulation comes from the study of two model Zn and Cd hyperaccumulating species of the Brassicaceae family, *A. halleri* and *N. caerulescens*, which are related to the sensitive and non-accumulating species *A. thaliana* (Yogeeswaran et al. 2005; Clauss and Koch 2006). These two species have been instrumental in successfully improving our understanding of the physiological, molecular, and genetic bases of metal hyperaccumulation and associated hypertolerance (see Krämer et al. 2007; Milner and Kochian 2008; Pauwels et al. 2008; Roosens et al. 2008; Verbruggen et al. 2009b; Krämer 2010; Hanikenne and Nouet 2011). Those successes relied on availability of the *A. thaliana* genome sequence (The Arabidopsis Genome Initiative 2000), and on dedicated tools and resources combined with relatively high gene sequence conservation among Brassicaceae species (94% and 88% identity with *A. thaliana* for *A. halleri* and *N. caerulescens*, respectively) (Talke et al. 2006; van de Mortel et al. 2006). It is expected that our knowledge will rapidly broaden with new species becoming accessible to molecular and genomic analyses (Gao et al. 2013; Verbruggen et al. 2013; Merlot et al. 2014).

# 4 Mechanisms of Zn and Cd Hyperaccumulation

As mentioned above, most of our knowledge about Zn and Cd hyperaccumulation was acquired using *A. halleri* and *N. caerulescens* (Fig. 2). More recent models include (i) the Crassulaceae *S. alfredii* and *S. plumbizincola* from Asia, which are the only species reported to hyperaccumulate Cd other than the Brassicaceae (Yang et al. 2004, 2006; Deng et al. 2007; Krämer 2010; Wu et al. 2013; Cao et al. 2014; Ma et al. 2015; Peng et al. 2017); and (ii) the Amaranthaceae *Gomphrena claussenii* from South America, which is highly tolerant to Zn and Cd and presents indicator levels of Zn and Cd accumulations (Villafort Carvalho et al. 2013, 2015).

*Arabidopsis halleri* and *N. caerulescens* display constitutive Zn hyperaccumulation and hypertolerance, although intraspecific variation for those traits has been reported (Bert et al. 2000, 2002; Reeves et al. 2001; Assunção et al. 2003; Molitor et al. 2005; Besnard et al. 2009). Similarly, hyperaccumulation of Cd shows substantial intraspecific variation (Escarré et al. 2000; Bert et al. 2002; Roosens et al. 2003; Verbruggen et al. 2013; Meyer et al. 2015). Metal hyperaccumulation evolved independently in the two species (Krämer 2010). However, both share a set of alterations of their metal homeostasis networks in comparison to the non-accumulator *A. thaliana*, which is described in detail below.

## 4.1 Uptake of Zn and Cd

Prior to uptake, it is suggested that metals are actively mobilized from the soil, by acidification and/or chelate secretion (Clemens et al. 2002). A recent report, however, suggested that the roots of *A. halleri* secrete elevated levels of nicotianamine (NA), a metal chelator able to form NA-Zn complexes (Curie et al. 2009; Clemens et al. 2013), which may reduce root Zn uptake and increase tolerance (Tsednee et al. 2014). Another report indicated higher organic acid levels and Zn mobilization in the dissolved organic matter within the rhizosphere of hyperaccumulator compared to non-accumulator accessions of *S. alfredii* (Li et al. 2012).

Several divalent metal transporters of the ZIP (Zrt-Irt-like Protein) family are highly expressed in roots and/or shoots of both *A. halleri* and *N. caerulescens* (Talke et al. 2006; Krämer et al. 2007; Lin et al. 2009, 2016). It presumably results in enhanced rates of root metal uptake or mobilization from root storage sites. By contributing to Zn radial transport towards the xylem in roots, it also may contribute to metal partitioning between root and shoot tissues. Several

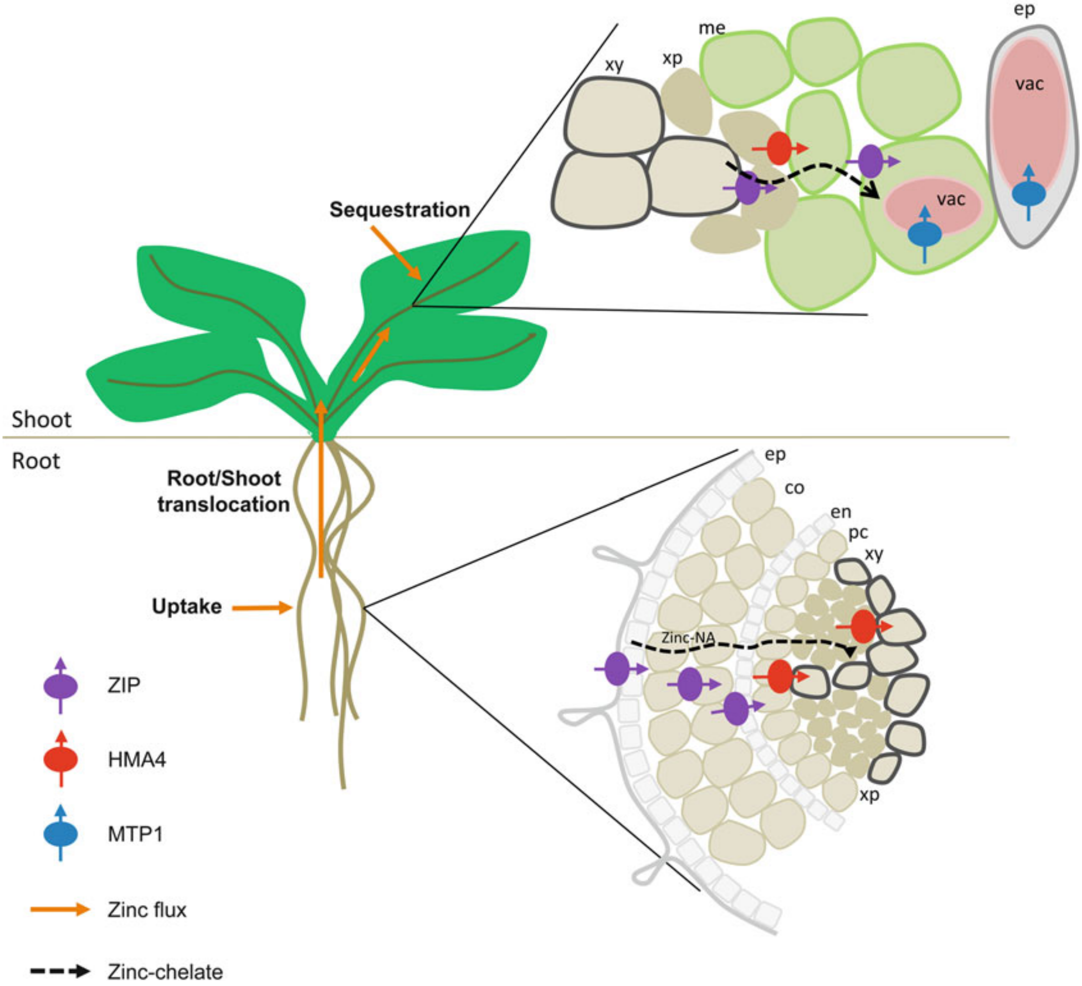

**Fig. 2** Model for Zn hyperaccumulation and hypertolerance in the Brassicaceae *A. halleri* and *N. caerulescens*. Enhanced functions of ZIP transporters in cellular uptake, of the P-type ATPase HMA4 in xylem loading/unloading and of MTP1 in vacuolar storage all make major contributions to the traits. The exact functions and localizations of individual ZIPs are unknown. In roots, the metal chelator nicotianamine (NA) possibly favours Zn radial transport towards the xylem by symplastic inter-cellular mobility of Zn by either allowing Zn movement through plasmodesmata and/or preventing vacuolar storage. Vacuolar storage in shoots occurs in the epidermis in *N. caerulescens* and in the mesophyll in *A. halleri*. Additional metal homeostasis genes that are highly expressed in both hyperaccumulators are discussed in the text. Note that similar mechanisms have been identified as more active in a metal hyperaccumulating population of the Crassulaceae *S. alfredii* compared to a non-accumulating population. *co* cortex, *en* endodermis, *ep* epidermis, *me* mesophyll, *Zinc-NA* Zinc-Nicotianamine chelates, *pc* pericycle, *vac* vacuole, *xp* xylem parenchyma, *xy* xylem. Figure modified from Hanikenne and Nouet (2011)

ZIP genes are induced by Zn deficiency under the control of the bZIP19 and bZIP23 transcription factors in *A. thaliana* (Assunção et al. 2010). Their high expression in *A. halleri* and *N. caerulescens* roots could be the direct consequence of the high activity of HMA4 (Heavy Metal ATPase 4; see below), which depletes Zn in roots (Talke et al. 2006; Hanikenne et al. 2008; Gustin et al. 2009). Note that several ZIP genes are also highly expressed in *S. plumbizincola* (Peng et al. 2017). Further work will be required to determine their individual function in Zn hyperaccumulation and to assess whether Cd uptake is determined by specific mechanisms or via Zn- and/or Fe-transport mechanisms (Meyer and Verbruggen 2012).

## 4.2 Root-to-Shoot Transfer of Zn and Cd

Increased rate of root-to-shoot metal transfer is key to achieving metal hyperaccumulation in shoots. It requires enhanced radial transport to xylem, decreased vacuolar storage in root cells, and efficient xylem loading. Several *NAS* (nicotianamine synthase) genes are highly expressed in *A. halleri* and *N. caerulescens* (Weber et al. 2004; van de Mortel et al. 2006; Deinlein et al. 2012). *NAS* transcript levels are also higher in roots of a hyperaccumulator accession compared to those of a non-hyperaccumulator accession in *S. alfredii* (Liang et al. 2014). Elevated levels of NA have been measured in roots of *A. halleri* compared to *A. thaliana* (Weber et al. 2004; Deinlein et al. 2012). It was further shown, using *A. halleri* RNAi lines, that high expression of the *NAS2* gene provides increased NA levels for Zn symplastic mobility towards the xylem and for controlling the rate of Zn xylem loading in roots (Deinlein et al. 2012; Cornu et al. 2015). The amino-acid histidine (His), whose concentration weakly correlates with Zn content in *N. caerulescens*, was shown to enhance Zn xylem loading and thus contribute to reduce Zn storage in roots (Callahan et al. 2007; Kozhevnikova et al. 2014).

In *A. halleri*, Zn and Cd loading into the xylem is driven by the HMA4 protein (Talke et al. 2006; Courbot et al. 2007; Hanikenne et al. 2008), which is a plasma membrane P-Type ATPase pump that uses the energy released from the hydrolysis of Adenosine triphosphate (ATP) to transport metal against the electro-chemical gradient (Hussain et al. 2004; Wong and Cobbett 2009; Pedersen et al. 2012; Hanikenne and Baurain 2014). The *HMA4* gene co-segregates with QTLs for Zn and Cd tolerance and accumulation (Courbot et al. 2007; Willems et al. 2007, 2010; Frérot et al. 2010; Meyer et al. 2016). High expression of *HMA4* is required for both hyperaccumulation and hypertolerance in *A. halleri* (Talke et al. 2006; Hanikenne et al. 2008). Increased gene dosage of *HMA4* was selected during the evolutionary history of *A. halleri* and evolved through tandem triplication and activation in *cis* of the promoters of all three copies (Hanikenne et al. 2008, 2013). The *A. halleri HMA4* locus was shaped by positive selection, resulting in a selective sweep and in ectopic gene conversion (Hanikenne et al. 2013). The three *HMA4* copies are active mainly in vascular tissues of *A. halleri*, which allows acting in xylem metal loading in roots and possibly in metal distribution in leaves. It also may ensure metal exclusion from metal-sensitive tissues (e.g. root tip, cambium). By controlling highly active Zn xylem loading, HMA4 also acts as a physiological regulator: it depletes the root Zn pool, which triggers a Zn-deficiency response resulting in high expression of several *ZIP* genes (Hanikenne et al. 2008). In agreement, modeling of the Zn supply-dependent spatio-temporal evolution of Zn concentration in root symplast and apoplast of *A. thaliana* predicted that slight changes in *HMA4* transcript levels have a major impact on the radial distribution of Zn in roots and the root-to-shoot Zn gradient (Claus et al. 2013). It was further shown that a certain extent of functional differentiation exists among the three *AhHMA4* copies when expressed in *A. thaliana*, stemming from differences in expression levels rather than in expression profile. Interestingly, *AhHMA4* copy 3 was subjected to the strongest, possibly most recent, positive selection during the evolutionary history of *A. halleri* (Hanikenne et al. 2013), thus linking sequence diversity patterns and function *in vivo* (Hanikenne et al. 2013; Nouet et al. 2015).

*HMA4* is also highly expressed in *N. caerulescens* as well as in Zn- and Cd-hyperaccumulator accessions of *S. alfredii* and *S. plumbizincola*, where it very likely plays similar roles to the *A. halleri HMA4* (Bernard et al. 2004; Papoyan and Kochian 2004; van de Mortel et al. 2006; O'Lochlainn et al. 2011; Craciun et al. 2012; Zhang et al. 2016; Peng et al. 2017). Moreover, the gene coding for the ZIP transporter *ZNT1* of *N. caerulescens* is highly expressed in cortex, endodermis, and pericycle root cells. When expressed in *A. thaliana*, it contributes to Zn and Cd tolerance and accumulation. The *NcZNT1* gene may therefore be involved in Zn and Cd influx into cells responsible for xylem loading, providing metals for transport by HMA4 (Milner et al. 2012; Lin et al. 2016). The ortholog of *ZNT1* in *A. halleri*, *ZIP4*, is also highly expressed and may contribute to a similar function (Talke et al. 2006). Note that NRAMP1 (Natural Resistance-Associated Macrophage Protein 1) may also play a similar role for Cd in *N. caerulescens* (Milner et al. 2014). Moreover, the vacuolar metal efflux transporters NRAMP3 and NRAMP4 are highly expressed in *N. caerulescens* and *A. halleri* roots and were proposed to limit vacuolar storage and increase metal mobility (Weber et al. 2004; Oomen et al. 2009). NRAMP3 is also highly expressed in *S. plumbizincola* (Peng et al. 2017).

Once in the xylem sap, metals are transported to the shoot owing to the evapo-transpiration stream. In this compartment, Zn is mainly bound to organic acids such as malate and citrate (Monsant et al. 2011; Lu et al. 2013; Cornu et al. 2015).

## 4.3 Storage of Zn and Cd in Leaves

It is suggested that HMA4 and ZIP transporters play an important role in Zn unloading and distribution in shoot tissues (Krämer et al. 2007; Hanikenne and Nouet 2011). However, their exact contribution, as well as that of metal ligands or other transporters, to these processes remains to be detailed. Zn storage in vacuoles is most likely ensured by the MTP1 (Metal Tolerance Protein 1) protein in *A. halleri* (Dräger et al. 2004; Talke et al. 2006; Shahzad et al. 2010), although its role remains to be formally established through the analysis of knock-down plants. MTP1 is a vacuolar transporter implicated in Zn tolerance (Krämer 2005). The MTP1 gene is constitutively highly expressed in both root and shoot of *A. halleri*, and is present in four to five copies that are located on three distinct linkage groups in the genome (Dräger et al. 2004; Talke et al. 2006; Willems et al. 2007; Shahzad et al. 2010). The two most highly expressed copies each co-segregate with QTLs for Zn tolerance (Dräger et al. 2004; Talke et al. 2006; Willems et al. 2007; Shahzad et al. 2010).

*MTP1* is also highly expressed in *Noccaea* (formerly *Thlaspi*) *goesingense*, another Zn and Ni hyperaccumulator, in *N. caerulescens*, and in Zn-hyperaccumulating populations of *S. alfredii* (Milner and Kochian 2008; Gustin et al. 2009; Zhang et al. 2011). It likely plays in these species a similar role as in *A. halleri*.

Finally, no detailed information is currently available on the molecular mechanisms of Cd storage in *A. halleri* shoot vacuoles (Meyer and Verbruggen 2012). Indeed, MTP1 is not associated with high Cd tolerance or accumulation in *A. halleri* (Courbot et al. 2007; Willems et al. 2010), although MTP1-related proteins were shown to transport Cd in several species, including the Ni hyperaccumulator *N. goesingense* (Persans et al. 2001; Migocka et al. 2015). In *N. caerulescens*, HMA3 (Heavy Metal ATPase 3) may contribute to this process (Ueno et al. 2011). In addition, differential regulation of *MTP1* by Cd in related *Noccaea* species suggests that it may contribute to Cd handling (Martos et al. 2016).

## 4.4 Additional Candidate Genes for Zn and Cd Accumulation and Tolerance

A few additional candidate genes for a role in Zn or Cd tolerance have been functionally characterized. In *A. halleri*, the PDF1.1 (Plant Defensin 1.1) protein was identified through a cDNA screen in yeast as a contributor to Zn tolerance. It also confers Zn tolerance when ectopically overexpressed in *A. thaliana* and is more highly expressed in shoots of *A. halleri* compared to *A. thaliana* (Mirouze et al. 2006). It localizes in intracellular compartments (Oomen et al. 2011). PDFs were initially known as secreted antifungal proteins and are characterized by a cysteine-stabilized, α-helix β-sheet, three-dimensional structure (De Coninck et al. 2013; van der

Weerden and Anderson 2013). Family wide comparison of *A. halleri* and *A. thaliana PDF1* genes revealed that the molecular function of the *A. thaliana* and *A. halleri* proteins in Zn tolerance and antifungal activity is conserved, and that functional differences in the two species may result from differential expression levels and regulation (Shahzad et al. 2013; Nguyen et al. 2014).

The fine mapping of a QTL in *A. halleri* allowed the identification of *CAX1* (cation/hydrogen exchanger 1) as a candidate gene for Cd tolerance (Courbot et al. 2007; Baliardini et al. 2015). CAX1 is localized in the vacuolar membrane and plays a key role in Ca homeostasis (Conn et al. 2011). *CAX1* is more expressed in the roots of *A. halleri* compared to those of *A. thaliana*, and high expression of *CAX1* co-segregated with Cd tolerance in a back-cross 1 population of an *A. halleri*/*A. lyrata* cross. The CAX1 QTL is conditional on Ca supply in the medium and is detected at low Ca supply only. The *A. thaliana cax1* mutant analysis suggests that, at low Ca supply, CAX1 may be required to tolerate Cd-induced oxidative stress (Baliardini et al. 2015, 2016).

## 5 Mechanisms of Ni Hyperaccumulation

Today, more than 400 nickel (Ni) hyperaccumulator species have been identified worldwide. These species are scattered in more than 40 plant families, mostly dicotyledons (Krämer 2010; van der Ent et al. 2013; Cappa and Pilon-Smits 2014). Despite this large diversity and the interest in understanding the underlying mechanisms, as they may be instrumental to improve Ni phytoextraction, only a limited number of studies have focused on the molecular mechanisms of Ni hyperaccumulation. Ni is an essential microelement for plants because it is required for urease activity (Polacco et al. 2013). Plants have, therefore, evolved mechanisms for the regulation of Ni homeostasis and Ni hyperaccumulation that likely derive from these mechanisms (Fig. 3). In *A. thaliana*, the mechanisms involved in Ni homeostasis are strongly linked to Fe homeostasis (Schaaf et al. 2006; Morrissey et al. 2009; Nishida et al. 2011). Interestingly, in Ni hyperaccumulators of the *Alyssum* genus, it was shown that Mn treatment reduces Ni accumulation, thus suggesting that in some species Ni hyperaccumulation may also use mechanisms primary involved in Mn homeostasis (Leigh Broadhurst et al. 2009; Ghaderian et al. 2015).

### 5.1 Uptake of Ni

Efficient uptake of Ni by the roots of hyperaccumulators requires divalent metal importers (e.g. ZIP, NRAMP) or transporters able to carry conjugated forms of Ni (e.g. Yellow Stripe-Like (YSL) family). However, the identity of the transporters involved in Ni uptake in hyperaccumulators is still not clearly established.

In *A. thaliana*, the metal transporter IRT1 that is required for the uptake of Fe from soil was shown to also be involved in Ni uptake (Vert et al. 2002; Nishida et al. 2011, 2012). Interestingly, the high expression of the *IRT1* ortholog in the roots of *N. caerulescens* (*NcIRT1*) is correlated with Ni hyperaccumulation in the Monte Prinzera accession (Halimaa et al. 2014b). In addition, *de novo* sequencing of *NcIRT1* in Monte Prinzera revealed sequence polymorphism in the large cytoplasmic loop of IRT1 that may have a role in transport specificity and/or regulation (Halimaa et al. 2014a). However, in other Ni hyperaccumulator accessions of *N. caerulescens* (i.e. Puy de Wolf and Bergenbach), we were unable to detect correlation between *NcIRT1* expression and Ni hyperaccumulation (V. S. Garcia de la Torre, S. Merlot, unpublished data). These data suggest that diverse transporters may be involved in the uptake of Ni in hyperaccumulators. Indeed, several members of the ZIP and NRAMP families from *Noccaea* species have been linked to Ni transport or accumulation, but further studies will be required to support their implication in an efficient uptake of Ni (Mizuno et al. 2005, 2007; Wei et al. 2009; Halimaa et al. 2014b).

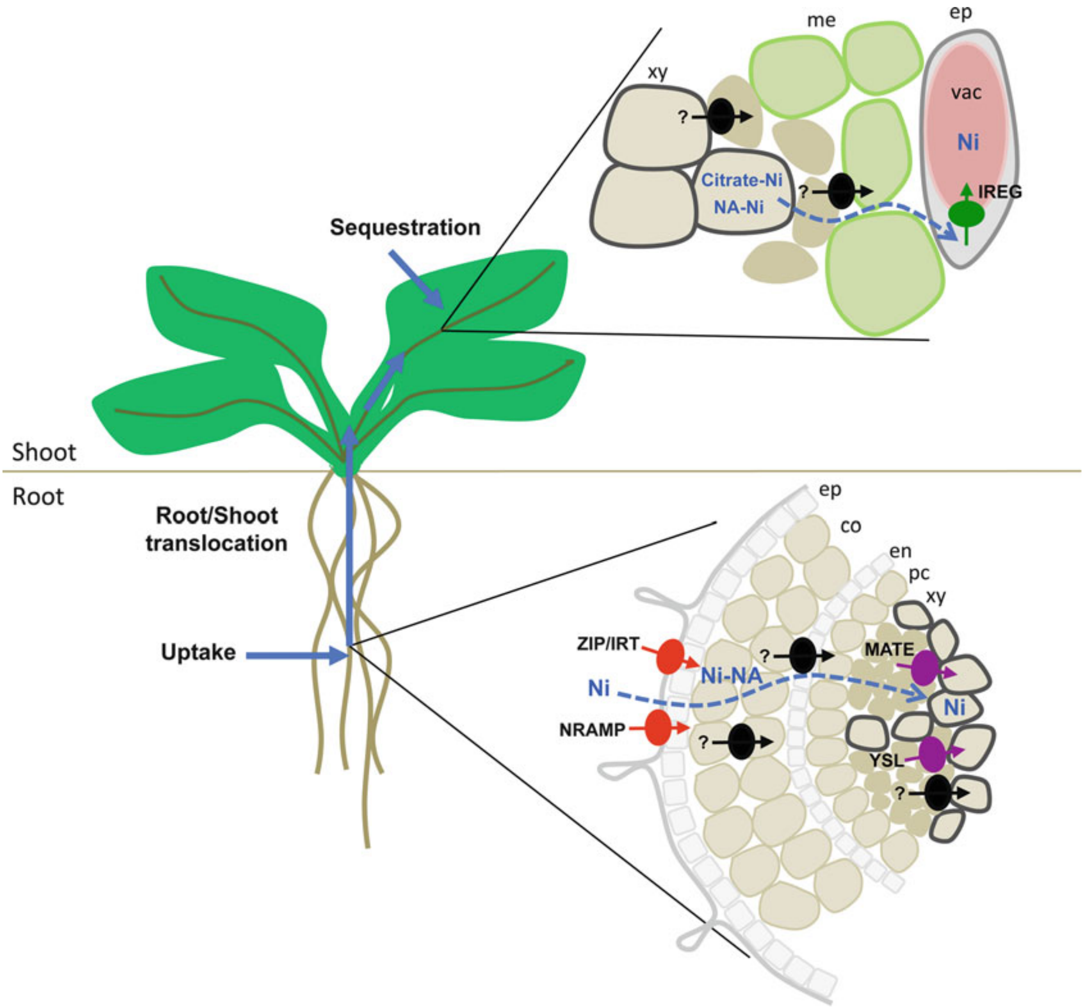

**Fig. 3** Proposed mechanisms of Ni transport in Hyperaccumulators. Efficient Ni uptake is mediated by metal transporters (ZIP/IRT, NRAMP…) located at the plasma membrane of root epidermal cells (ep). Ni is then transported through the cortex (co) and the endodermis (en) by a combination of Ni export and import transporter activities. During this step, Ni is chelated [e.g. by nicotianamine (Ni-NA)] to reduce its reactivity in the cytoplasm. Binding to His might prevent vacuolar sequestration to favor radial transport. In the pericycle (pc), Ni is loaded in the xylem (xy) together with chelator molecules (e.g. NA, Citrate…) by YSL and MATE transporters and transported to the shoot. The mechanisms involved in xylem unloading and transport to the epidermal cell are not well known but may be similar as the one involved in Ni transport in roots. In epidermal cell, Ni is transported and stored in the vacuole (vac) by IREG transporters. Figure modified from Hanikenne and Nouet (2011)

## 5.2 Root-to-Shoot Transfer of Ni

The long-distance transport of Ni from roots to shoots requires several steps that involve metal transporters and chelators that are able to bind Ni in different pH environments. In hyperaccumulators, a large proportion of Ni is found as complexes with carboxylic acids including citrate and malate (for reviews see Callahan et al. 2006; Sarret et al. 2013). These organic acid complexes are stable in acidic compartments such as vacuoles and xylem. In particular, citrate-Ni was identified in the xylem sap of the Ni hyperaccumulator *Alyssum serpyllifolium*

(Alves et al. 2011). Interestingly, an ortholog of the *A. thaliana* citrate transporter FRD3 of the Multidrug And Toxic compound Extrusion family (MATE) is more expressed in the hyperaccumulator *N. caerulescens* than in the related non-accumulator *A. thaliana* (van de Mortel et al. 2006). AtFRD3 and its orthologue in rice, OsFRDL1, are involved in the translocation of Fe from roots to shoots (Rogers and Guerinot 2002; Yokosho et al. 2009). Therefore, high expression of MATE transporters in the root pericycle of hyperaccumulators would increase the loading of xylem with citrate and therefore favor the translocation of Ni-citrate complex from root to shoot. However, to date no direct correlation has been established between MATE transporters and Ni hyperaccumulation. It is interesting to note that *FRD3* is also highly expressed in *A. halleri*, which hyperaccumulates Zn. FRD3 transporters may, therefore, have a general function in metal hyperaccumulation, favouring long-distance transport of metal from root to shoot (Talke et al. 2006; Charlier et al. 2015). NA also has a strong affinity for Ni over a wide pH range and is proposed to bind Ni in more neutral compartments such as cytoplasm or phloem (Callahan et al. 2006; Rellan-Alvarez et al. 2008; Alvarez-Fernandez et al. 2014). Accordingly, over-expression of NA synthase in transgenic *A. thaliana* increases Ni tolerance but is not sufficient to improve Ni accumulation (Pianelli et al. 2005). The NA-Ni complex was identified in the xylem sap of *N. caerulescens* (Mari et al. 2006), in the latex of the Ni hyperaccumulator *Pycnandra acuminata* (Schaumölffel et al. 2003), and in extracts of several hyperaccumulator species (Callahan et al. 2012). Transporters of the YSL family have been shown to transport NA-metal complexes (Curie et al. 2009; Conte and Walker 2012). Several genes coding for YSL transporters are more expressed in the hyperaccumulator *N. caerulescens* than in the related non-accumulator *A. thaliana* (Gendre et al. 2007). Among these transporters, NcYSL3 that is able to transport the NA-Ni complex is expressed in the vasculature of roots and leaves, suggesting a role in long-distance Ni transport.

Finally, the amino acid histidine (His), whose concentration in some hyperaccumulators of the *Alyssum* and *Noccaea* genera correlates with Ni accumulation, is proposed to play a role in the radial transport of Ni (Krämer et al. 1996; Richau et al. 2009). His has a strong affinity for Ni and the Ni-His complex, and has been identified in samples from *Alyssum* and *Noccaea* hyperaccumulators (Krämer et al. 1996; Persans et al. 1999; Callahan et al. 2006; McNear et al. 2010). In the Ni hyperaccumulator *A. lesbiacum*, genes involved in His biosynthesis are constitutively and highly expressed compared to the non-accumulator *A. montanum*. The over-expression of the first enzyme of the His biosynthetic pathway, ATP-phosphoribosyltransferase, in *Arabidopsis thaliana* increases Ni tolerance but not Ni content, thus suggesting that other mechanisms are necessary for accumulation of this metal (Wycisk et al. 2004; Ingle et al. 2005). Treatment of plants with Ni-His increases xylem loading and inhibits Ni uptake from root vacuoles (Richau et al. 2009). It was therefore proposed that the high concentration of His in roots of Ni hyperaccumulators prevents vacuolar storage, favoring radial transport and xylem loading (Kerkeb and Krämer 2003; Richau et al. 2009). However, the mechanisms responsible for the inhibition of Ni vacuolar sequestration by His are still unknown. Moreover, it is currently not known if this strategy is widely conserved in Ni hyperaccumulators and if this is relevant in natural environments (e.g. ultramafic-derived soil), where N is limiting (Alves et al. 2011; Centofanti et al. 2013).

## 5.3 Storage of Ni in Leaves

In most hyperaccumulators that have been studied, Ni is stored in the vacuole of leaf epidermal cells (for review see Sarret et al. 2013). Several lines of evidence indicate that Ferroportin (FPN)/Iron Regulated (IREG) transporters play an essential role in the sequestration of Ni in vacuoles.

In *A. thaliana*, *AtIREG2* is expressed in roots in response to Fe-starvation and the AtIREG2 protein localization on the vacuole. The analysis of the *ireg2* mutant indicated that AtIREG2 is involved in the storage of Ni excess in the vacuole of root cells (Schaaf et al. 2006; Morrissey et al. 2009). On the contrary, a second IREG transporter in *A. thaliana*, named FPN1/IREG1, is localized on the plasma membrane and is proposed to play a role in xylem loading of metals in roots (Morrissey et al. 2009). Interestingly, the ortholog of *AtIREG2* in *A. lyrata* is genetically linked to ultramafic adaptation (Turner et al. 2010). Recent comparative transcriptomic analysis using RNA-Seq technology revealed that a high expression of the ortholog of *AtIREG2* in roots of *N. caerulescens* Monte Prinzera is linked to Ni accumulation (Halimaa et al. 2014b). Independent RNA-Seq analyses also indicate that *NcIREG2* is strongly and constitutively expressed in leaves of the *N. caerulescens* accessions Puy de Wolf and Bergenbach (V. S. Garcia de la Torre, S. Merlot, unpublished results).

The PgIREG1 transporter from the Ni hyperaccumulator *Psychotria gabriellae* (Rubiaceae) localizes in the vacuolar membrane and is able to transport Ni when expressed in yeast. PgIREG1 therefore seems to be the functional homolog of AtIREG2. Interestingly, *PgIREG1* is highly expressed in leaves of *P. gabriellae* in environmental conditions and is more expressed than in the closely related, non-accumulator *P. semperflorens* (Merlot et al. 2014). These data further suggest that the high expression of IREG transporters in leaves is a convergent mechanism for Ni hyperaccumulation. However, whereas over-expression of *AtIREG2* and *PgIREG1* in transgenic *Arabidopsis* plants significantly increases Ni tolerance, it does not increase Ni accumulation, further supporting the hypothesis that other mechanisms are required for hyperaccumulation (Schaaf et al. 2006; Merlot et al. 2014). Other families of divalent metal exporters such as MTP transporters could mediate the transport of Ni in vacuoles, but their role in hyperaccumulation needs to be further supported (Persans et al. 2001).

## 6 Hyperaccumulation of Other Trace Elements

Species that are able to hyperaccumulate Mn have been identified in more than 10 genera mostly in the Myrtaceae (e.g. *Gossia*) and Proteaceae (e.g. *Virotia*) families (Fernando et al. 2013; Losfeld et al. 2015). As for other metals, Mn hyperaccumulation likely evolved from basic mechanisms involved in Mn homeostasis; however, these mechanisms are poorly investigated to date in Mn hyperaccumulators (Pittman 2005; Fernando et al. 2013; Socha and Guerinot 2014). In hyperaccumulators, Mn was found to accumulate in the vacuole of non-photosynthetic epidermal cells, but also more surprisingly in photosynthetic palisade mesophyll cells in the hyperaccumulator *Virotia neurophylla* (Fernando et al. 2012). These differences in Mn localization suggest that some mechanisms involved in Mn accumulation and detoxification might be divergent among hyperaccumulators. The high concentration of Mn found in leaves of several species is linked to their strategy to acquire P. For example, Proteaceae species excrete carboxylates in their rhizosphere that solubilize not only P but also micronutrients including Mn (Lambers et al. 2015). Several families of metal transporters, such as NRAMP, ZIP, YSL, and MTP have been shown to be able to transport Mn in plants, but their role in hyperaccumulation is not clearly established (Fernando et al. 2013; Socha and Guerinot 2014). Most NRAMP transporters are able to transport Mn in the cytoplasm, either from the external medium or the vacuole, and therefore could participate in several steps of Mn hyperaccumulation. In particular, the NRAMP1 transporter from *A. thaliana* was shown to be the main transporter involved in Mn uptake in roots (Cailliatte et al. 2010). The ShMTP8 transporter (previously known as ShMTP1) was isolated from the Mn-tolerant species *Stylosanthes hamata* (Fabaceae) as a transporter that confers Mn resistance when expressed in yeast, and was proposed to mediate accumulation of Mn in the vacuoles of plant cells (Delhaize et al. 2003). Further molecular studies on Mn hyperaccumulators will be required to identify those mechanisms that are key to Mn hyperaccumulation.

In addition to the above-mentioned metals, mechanisms involved in the hyperaccumulation of the metalloid element Se in plants are extensively studied because of their relevance for improving Se phytoremediation and biofortification. We only briefly address Se hyperaccumulation here and refer interested readers to thorough reviews on Se homeostasis and hyperaccumulation (Zhu et al. 2009; Barillas et al. 2011; Schiavon and Pilon-Smits 2016; White 2016). Selenium hyperaccumulation has been described for 45 taxa scattered among six families. More than half of Se hyperaccumulators have been described in the genus *Astragalus* (Fabaceae), but other well-described Se hyperaccumulators have been found in the genera *Stanleya* (Brassicaceae), *Oonopsis*, and *Xylorhiza* (Asteraceae). The distribution of Se hyperaccumulators among plant families suggests that Se hyperaccumulation likely evolved independently at least six times (Cappa and Pilon-Smits 2014). Selenium is available to plants mostly as selenate ($SeO_4^{2-}$), a structural homologue of sulphate, or selenite ($SeO_3^{2-}$), depending on the nature of the soil (Elrashidi et al. 1987). In cultivated soils, selenate uptake by root cells is catalyzed by high-affinity sulphate transporters of the SULTR family (Shibagaki et al. 2002; El Kassis et al. 2007; Barberon et al. 2008). Transporters of this family are highly and constitutively expressed in Se hyperaccumulators of the *Astragalus* and *Stanleya* genera (Freeman et al. 2010; Cabannes et al. 2011; Schiavon et al. 2015). The Se hyperaccumulation trait is also linked to the capacity of Se hyperaccumulators to preferentially take up selenate over sulphate. Sequence analysis of SULTR1 transporters from *Astragalus* identified a Gly to Ala polymorphism linked to the hyperaccumulation trait; however, it is not demonstrated yet if this difference explains the preferential uptake of selenate over sulphate (Cabannes et al. 2011). In rice growing in anaerobic soils (e.g. paddy fields), selenite forms are transported in root cells by the phosphate transporters OsPT2 (Zhang et al. 2014) and by aquaporins (Zhao et al. 2010). Long-distance transport of selenate to the shoot is also proposed to be mediated by SULTR transporters (Takahashi et al. 2000). In shoots, the main fraction of selenate is metabolized into organoselenium compounds (SeCys and SeMet) in chloroplast (Zhu et al. 2009; Barillas et al. 2011; White 2016). In hyperaccumulator species, genes involved in the synthesis of organoselenium compounds and in the methylation of SeCys to produce the non-toxic form MeSeCys were shown to be constitutively more expressed than in non-accumulator species (Pickering et al. 2003; Freeman et al. 2010; Schiavon et al. 2015), indicating that the metabolism of Se is key for its hyperaccumulation.

## 7 Perspectives and Conclusions

### 7.1 Interaction of Hyperaccumulators with Biotic Environment

The so-called ‘elemental defense’ hypothesis proposes that metal hyperaccumulation provides a defense against pathogens and/or herbivores by direct toxicity (Boyd and Martens 1992; Hörger et al. 2013). Indeed, it has been shown that Ni and Zn accumulation can protect different Brassicaceae species from bacterial and fungal infection (Boyd et al. 1994; Ghaderian et al. 2000; Fones et al. 2010). However, it is also well documented that in environmental conditions, specific populations of bacteria are associated with the root system of metal hyperaccumulators (Aboudrar et al. 2012; Cabello-Conejo et al. 2014; Lucisine et al. 2014; Muehe et al. 2015). Endophytic bacteria have also been identified in the shoots and roots of metal hyperaccumulators (Idris et al. 2004; Mengoni et al. 2009a; Nonnoi et al. 2012). However, since the majority of these bacteria are not cultivable, current studies mostly describe endophytic populations using metagenomics approaches (Chen et al. 2014; Luo et al. 2011; Sessitsch et al. 2012; Visioli et al. 2014). Interestingly, it was shown that the inoculation of

*N. caerulescens* and *A. serpyllifolium* with cultivable endophytic bacteria increases Ni translocation to shoots (Ma et al. 2011b; Visioli et al. 2015). Little is known about the interactions between metal hyperaccumulators and associated bacteria. Metal hyperaccumulators represent an extreme niche for metal-tolerant bacteria (Mengoni et al. 2009b). On the other hand, metal-tolerant bacteria can improve plant growth and confer protection against abiotic stress by the production of hormones (auxins, cytokinins, etc.), or protect the host plant against other pathogens by the production of antagonistic substances or by competition for space and nutrients (Ma et al. 2011b; Reinhold-Hurek and Hurek 2011). Metal-tolerant bacteria can also produce organic acids and metal chelators that can favor metal solubility, transport, and tolerance (Idris et al. 2006; Ma et al. 2011a; Visioli et al. 2015). These observations suggest intimate interactions between hyperaccumulators and associated bacteria; however, mechanisms and genes involved in these interactions are mostly unknown. The development of next-generation sequencing technologies and associated meta-transcriptomic (or dual-transcriptomics) analyses will uncover those genes expressed by plants and associated bacteria involved in their interaction (Camilios-Neto et al. 2014; Pankievicz et al. 2016). Understanding these mechanisms will be instrumental to improving metal phytoextraction or to producing secondary metabolites such as metal chelators of bacterial origin that can be used in metal-based therapies (Franz 2013).

## 7.2 Evolution of Hyperaccumulation Mechanisms

After this brief review of our current knowledge of the molecular mechanisms of Zn, Cd, or Ni hyperaccumulation, it is apparent that several candidate genes involved in these processes are involved in the control of metal homeostasis in non-accumulator plants. These genes display an enhanced function in hyperaccumulators, through gene-copy number amplification and/or altered regulation, which profoundly modifies the metal flux in the plants towards shoot accumulation. Many examples have also been presented that highlight the high level of convergent evolution between *A. halleri*, *N. caerulescens*, and *S. alfredii*. This convergent evolution likely reflects—and sheds light on—functional constraints of the metal homeostasis network (Krämer et al. 2007; Krämer 2010; Verbruggen et al. 2009a; Hanikenne and Nouet 2011). The key function of HMA4 in several hyperaccumulator species represents a potent example of this convergent evolution (Hanikenne et al. 2008; O'Lochlainn et al. 2011; Craciun et al. 2012).

Metal hyperaccumulation and associated tolerance in plants are complex traits that have required the fine-tuning of multiple mechanisms during the course of evolution. If several key players have now been identified, how tolerance and hyperaccumulation traits evolved remains an open 'chicken or egg' question. Hence, Bayesian inference suggested that speciation between *A. halleri* and *A. lyrata* closely coincided with *HMA4* duplication (Roux et al. 2011). The complex signature of selection detected at the *HMA4* locus of *A. halleri* further supports the key role of this gene in the evolution of the hyperaccumulation trait (Hanikenne et al. 2013). Recent adaptations to anthropogenic metal-polluted sites possibly occurred independently within distinct phylogeographic units of the *A. halleri* European distribution (Pauwels et al. 2012). Hypertolerance of metallicolous populations thus potentially evolved using a variety of genetic mechanisms (Meyer et al. 2009, 2010; Pauwels et al. 2012). Moreover, the recent study by Meyer et al. (2016) suggests that, if HMA4 contributes to Zn tolerance in both metallicolous and non-metallicolous populations of *A. halleri* (Hanikenne et al. 2013), the function of MTP1 in Zn tolerance may have evolved later in metallicolous populations that colonized recently polluted soils. Indeed, the co-segregation of *MTP1* with Zn tolerance is only observed in a backcross 1 population of a cross between a French metallicolous individual (i.e. living on metal-polluted soil) of *A. halleri* and *A. lyrata,* and is lost when a Slovakian non-metallicolous (i.e. living on non-polluted soil) *A. halleri* individual is used as parent (Meyer et al. 2016). In contrast, the co-segregation of *HMA4* with Zn tolerance is independent of the edaphic origin of the *A. halleri* populations used in the analysis (Willems et al. 2007; Meyer et al. 2016). This interpretation partially contradicts the hypothesis that MTP1 is required for metal detoxification accommodating the high HMA4-dependent metal flux into *A. halleri* shoots, which was proposed based on the observation that expression of *AhHMA4* in non-accumulator plants resulted in increased sensitivity to excess Zn (Hanikenne et al. 2008; Barabasz et al. 2010).

The study of metal hyperaccumulation in distant plant families combined with the comparison of distinct accessions with contrasting accumulation capabilities within a species will likely shed light on the evolution of the hyperaccumulation and hypertolerance traits. Such study may indeed reveal the commonalities and differences in the mechanisms underlying these traits, highlighting evolutionary divergence and convergence. It also may reveal evolutionary 'intermediates' (i.e. genotypes that do not display the full extent of hyperaccumulation or tolerance), which may allow ordering the evolutionary events that took place during the adaptation of the metal homeostasis network.

## 7.3 How Can Phytoextraction Technologies Benefit from Molecular Knowledge?

To date, most of our knowledge on metal hyperaccumulation arises from studies on a few model hyperaccumulator species (i.e. *A. halleri* and *N. caerulescens*) of the Brassicaceae family. In the future, it will be necessary to pursue molecular studies and improve genetic manipulation of these species, in order to identify and demonstrate the role of key mechanisms involved in metal hyperaccumulation. However, these species have a low biomass and a relatively restricted distribution worldwide. Therefore, one of the coming challenges for the development of agromining/phytoextraction will be to transfer knowledge of the mechanisms involved in metal hyperaccumulation to species having a high potential for phytoextraction. As mentioned above, the development of Next Generation Sequencing technologies opens the possibility for study of 'non-model' species at the genomic and transcriptomic levels.

As for other crop plants, this molecular knowledge will be instrumental for the selection of genotypes having the best potential for metal phytoextraction. The level of expression of key genes involved in metal hyperaccumulation can be used as markers to predict metal accumulation capacities. These marker genes can also be used to study the interaction between metal accumulation and agricultural practices (e.g. fertilization), for improving biomass production while maintaining efficient metal accumulation.

Finally, genome-editing technologies such as CRISPR-CAS9 are currently implemented in plants to specifically modify the sequence of target genes (Doudna and Charpentier 2014). This technology offers several advantages compared to traditional transformation technologies used to produce Genetically Modified Organisms (GMO), and therefore could be better accepted by the civil society and political stakeholders to engineer crop plants for metal phytoextraction. Using this recent technology, we can, for example, imagine specifically introducing point mutations in the sequence of genes involved in metal accumulation in order to increase their activity, improve specificity, or modify selectivity towards metals of interest (Rogers et al. 2000; Menguer et al. 2013; Pottier et al. 2015).

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# Tools for the Discovery of Hyperaccumulator Plant Species and Understanding Their Ecophysiology

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## Abstract

Globally the discovery of hyperaccumulator plants has been hindered by systematic screening of plant species, and is highly biased towards Ni hyperaccumulators. This is mainly due to the existence of a reagent paper test that is only specific to nickel (based on dimethylglyoxime) such that more than 400 of the approximately 500 known hyperaccumulators species are for Ni. New technical advances now permit massive screening of herbarium specimens using non-destructive, portable X-Ray Fluorescence Spectroscopy (XRF), an approach that has already led to the discovery of numerous hyperaccumulator species new to science. The elemental distribution in selected hyperaccumulator plant tissues can then be further studied using techniques such as desktop or synchrotron micro-XRF, nuclear microprobe (PIXE), scanning/transmission electron microscopy with energy-dispersive spectroscopy (SEM/TEM-EDS), secondary ion mass spectrometry (SIMS) or laser ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS). The use of histochemical dyes combined

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with light microscopy further aids in the identification of anatomical and structural features of the studied plant tissues.

# 1 Introduction

## 1.1 Hyperaccumulator Plant Discovery

The high proportion of identified Ni hyperaccumulator species in comparison to hyperaccumulators that accumulate other elements is primarily due to the fact that ultramafic soils are by far the most widespread metalliferous anomaly worldwide (Echevarria 2017). Furthermore, the availability of an easily deployed, field-testing method for Ni using dimethylglyoxime (DMG)-treated paper has helped identification of Ni hyperaccumulator plants substantially. After initial field screening using a spot test (e.g. DMG-paper, see Fig. 1 for an example), confirmation of hyperaccumulator status has traditionally been achieved using Atomic Absorption Spectrophotometry (AAS) (e.g. Kelly et al. 1975; Jaffré et al. 1979b; Kersten et al. 1979) and Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) (e.g. Reeves et al. 1996, 2007; Fernando et al. 2009; van der Ent and Reeves 2015) after acid digestion of dried leaf material samples. Although the analysis with AAS or ICP-AES itself takes only minutes, the sample preparation is time and resource consuming (e.g. 100 samples take 2–3 days to digest). Table-top XRF (X-ray fluorescence) was also used for this purpose in the past (e.g. Mesjasz-Przybylowicz et al. 1994). XRF analysis can be done on intact specimens, but for more reliable results homogenisation and preparation of pellets is recommended. Recent technological advances in more sensitive and compact X-ray fluorescence (XRF) instruments enable non-destructive elemental screening of a vast numbers of samples, including herbarium specimens, in a relatively short period of time. This chapter introduces the use of portable XRF as an efficient tool for hyperaccumulator species discovery in herbaria, and shortly summarizes which micro-analytical techniques can be used to gain further insights into the ecophysiology of hyperaccumulator plant species. The test case of New Caledonia is used to illustrate the application of XRF screening for the discovery of hyperaccumulator plants.

# 2 Handheld X-Ray Fluorescence Technology for Herbarium Screening

In order for hyperaccumulator species to be discovered, systematic screening of plant species must be conducted. The use of handheld XRF instruments is a time- and cost-effective method. It has the ability to measure a range of different elements simultaneously within 30–60 s in dry samples. The latest generation of such XRF instruments (equipped with an Ag anode 6–50 kV, 0–200 μA) have detection limits in the range of ~100–300 μg $g^{-1}$ for most transition elements (i.e. Co, Ni and Zn). The XRF instrument works by subjecting the sample to a beam of focused, high-energy X-rays generated from an X-ray tube in the device. The spectrum of excited fluorescent X-rays is then analyzed to determine the presence of different elements and to calculate their relative concentrations in the sample.

Recently, handheld XRF systems have been validated for the measurement of elements in plant samples, although this has involved powdering and pelletisation of sample material before measurement (McLaren et al. 2012; Reidinger et al. 2012; Guerra et al. 2014). However, XRF can be employed for non-destructive analysis of plant material, and with the advent of portable instruments utilizing the latest type of fast (Silicon drift detectors or SDDs) detectors, has made it possible to perform measurements of tens of thousands of samples in a relatively short time span (see Fig. 2 for the setup of XRF scanning of herbarium specimens). As such, elements such as Ni or Zn in herbarium specimens can be

**Fig. 1** Field spot test based on the Ni-specific colorimetric reagent dimethylglyoxime demonstrating the reaction with Ni from the fresh leaf of the Ni hyperaccumulator species *Psychotria gabriellae* (Rubiaceae) in the field in New Caledonia. The test is fast and sensitive and has aided in the identification of numerous Ni hyperaccumulators globally

**Fig. 2** The typical setup during XRF scanning of herbarium specimens, here conducted at the Forest Research Centre herbarium in Sabah, Malaysia. The herbarium specimen sheet is placed on a titanium metal sheet that serves as a X-ray radiation block, with the portable XRF device fitted with a backscatter shield placed. The XRF device is connected to a computer to read out the acquired data

measured at a rate of ~300 specimens per day (translating to ~6000 specimens per month). Hence, this makes it feasible to scan the entire phylogenetic lineages, as well as specimens of the same species from many different collections or localities. Additionally, XRF screening may be combined with the digitisation process of herbarium specimens, an effort already underway in many global herbaria. From such initial mass screening, species may be selected for further investigation to elucidate elemental distribution at the tissue and cellular level, after dedicated field collection of samples.

The results of XRF measurements of herbarium specimens may vary somewhat within a specimen, depending on the exact region of the leaf surface measured (e.g. mid-vein or lamina), and it is advisable to measure multiple spots

**Fig. 3** Examples of herbarium specimens from the IRD herbarium in New Caledonia. The panels illustrate some of the challenges encountered with measuring such specimens, including small leaves, clumped leaves and uneven plant parts

within a specimen (see Fig. 3). Another consideration when undertaking XRF measurements is that some herbarium specimens were treated with $HgCl_2$ for long-term insect protection and therefore readings for Hg can be extremely high for such samples (i.e. $>500$ µg $g^{-1}$). Furthermore, surficial contamination with soil particles must also be considered (easily gauged from Fe and Cr concentrations), as this also raises the apparent concentrations of Ni, Co and Mn. In order to obtain quantitative data, the XRF system should be properly calibrated (Markowicz 2008). In the example provided in the case study below, a set of parallel foliar samples were analyzed by

ICP-AES to obtain element-specific correlation coefficients for calibrating the XRF concentration values obtained by a handheld instrument.

## 2.1 The Case of New Caledonia

New Caledonia is located in the southwest Pacific, approximately 1500 km east of Australia, and consists of one main island (Grande Terre) and a group of smaller islands with a total area of approximately 19,000 km$^2$ (Pillon et al. 2010). It is a biodiversity hotspot (Myers et al. 2000; Mittermeier et al. 2004), harbouring over 3371 vascular plant species of which nearly 75% are endemic (Morat et al. 2012). In addition, there is 16% endemism at the generic level for vascular plant species, and New Caledonia has three endemic families, namely the Amborellaceae, Oncothecaceae, and Phellinaceae (Morat et al. 2001; Heads 2010). The highly distinctive plant diversity of New Caledonia results in part from its geological history (Pelletier 2006), its isolation (Morat 1993; Murienne 2009; Morat et al. 2012), and the climatic stability (Murienne 2009), although the latter is yet to be proven (Pillon 2012). The high species richness and endemicity also raise questions on their origins, with both vicariance (e.g. Ladiges et al. 2003; Barker et al. 2007), and long-distance dispersal purported as possible mechanisms for speciation (Setoguchi et al. 1998; Swenson et al. 2001; Bartish et al. 2005; Pillon et al. 2014). The geological evidence suggests that the species diversity is dominated by neo-endemism and disharmonic colonization, hence not of ancient relictual Gondwanan origins (Murienne et al. 2005; Grandcolas et al. 2008). Pillon et al. (2010) showed that plant families with low levels of endemism but high effective dispersal capacities were under-represented on the island of New Caledonia. Therefore, they concluded that dispersal was not a significant factor in sorting the species in the flora, but instead that ultramafic soils may have been a major force for speciation, as previously emphasized by Jaffré (1993).

More than 70% of the 2492 endemic plant species in New Caledonia grow on ultramafic soils, of which over 1200 species are ultramafic soil obligates, representing ~50% of the endemic flora of the island (Isnard et al. 2016). New Caledonia has extensive outcrops of ultramafic soils (totalling approximately 5600 km$^2$) that are strongly weathered (i.e. laterization) (L’Huillier and Edighoffer 1996). The parent ultramafic rocks contain 0.16–0.4% Ni, but the portion of Ni increases during soil weathering up to complete laterization, mainly in saprolitic horizons (Echevarria 2017). The deficiency of essential plant nutrients (e.g. P, K, Ca) in ultramafic soils and the bioavailability of potentially toxic elements (i.e. Co, Mn, Ni) may cause stresses for plants to survive (Alexander and DuShey 2011). This so-called ‘serpentine syndrome’ in general is more pronounced on soils developed on serpentinite, i.e. Magnesic Cambisols (Isnard et al. 2016; Echevarria 2017).

## 2.2 Herbarium XRF Scanning in New Caledonia

In New Caledonia, currently 65 Ni and 11 Mn hyperaccumulator plant species are known (van der Ent et al. 2015a). Jaffré et al. (1976) discovered that the New Caledonian endemic tree, *Pycnandra acuminata* (formerly *Sebertia*) has a latex with more than 25 wt% dry weight Ni. XRF herbarium screening was undertaken with a portable XRF instrument at the Herbarium of the Institute for Research and Development (IRD) in New Caledonia on over 7500 dried herbarium specimens. The selection of herbarium specimens to scan was based on families that were already known to contain numerous hyperaccumulator species (Jaffré et al. 1976, 1979a, 2013; Swenson and Munzinger 2010) (e.g. Cunoniaceae, Phyllanthaceae, Salicaceae, Sapotaceae and Violaceae), as well as a systematic screening of 1–4 specimens (depending on availability) of species known to occur on ultramafic soils in New Caledonia (3996 specimens covering 1087 species). The measurements led to the recording of numerous new hyperaccumulators: restricting to high range records only (i.e. Ni >5000 μg $g^{-1}$, Mn >20 000 μg $g^{-1}$, Co >1000 μg $g^{-1}$, and Zn 10 000 μg $g^{-1}$), there were 87 taxa for nickel (including 22 new records), 68 taxa for manganese (including 59 new records), 8 taxa for cobalt (none previously recorded), and 4 taxa for zinc (none previously recorded). This demonstrates that XRF screening of herbarium specimens has the potential to discover vast numbers of new hyperaccumulator species, even in well-studied floras such that of New Caledonia.

### 2.3 Knowledge Gaps: Priority Regions for Exploration and Discovery

Currently it is estimated that hyperaccumulation occurs in 0.2% of Angiosperms and 1–2% of the known global ultramafic flora (Baker 1981; Baker and Brooks 1989; van der Ent et al. 2015b). Systematic herbarium specimen XRF scanning, combined with auxiliary collection data, can provide insights into phylogenetic patterns of hyperaccumulation, and has the potential to complement and add insights to biogeographical and phylogenetic studies. This approach also facilitates the selection of plant species targeted for more detailed investigations, using the methods described below.

## 3 Sample Preparation for Micro-Analytical Technique Investigations

The preparation of plant tissue samples for physiological investigations is critical to achieve preservation of the intrinsic elemental distribution. Proper preservation of plant tissues for micro-analytical investigations principally involves using cryo-techniques–various forms of cryo-fixation followed by analysis of frozen hydrated or freeze-dried plant tissue material. There are three main approaches to cryo-fixation:

1. ***Rapid plunge cryo-fixation:*** fast plunging of small samples into a liquid cryogen bath (usually propane, ethane or isopentane) cooled by liquid nitrogen. Liquid nitrogen should not be used as primary cryogen due to the Leidenfrost phenomenon (i.e. the formation of an insulating gas layer around the warm object that results in a slow freezing speed).
2. ***Metal mirror fixation*****:** quickly pressing small excised samples against a solid block of Cu (sometimes covered by Au) cooled by liquid nitrogen. This method is possible in the field.
3. ***High pressure freezing:*** requires specialized equipment, with several restrictions regarding the method of preparation and the specimen size. This method is mainly recommended for further cryo-ultramicrotomy.

The recommended starting point for investigations is collecting samples from plants growing in their natural habitat. Collecting plant tissue samples in the field is, however, challenging, but two strategies have been successfully used: (i) collecting whole plants with soil in pots from the metalliferous outcrops and transport to a suitably equipped laboratory (Koosaletse-Mswela et al. 2015), or (ii) freezing tissue samples in the field using metal-mirror freezing and transport to laboratory in a cryoshipper at liquid nitrogen temperature (Mesjasz-Przybylowicz et al. 2016; van der Ent et al. 2017). It is important that cryo-fixation is done as soon as possible (i.e. within seconds after excision) to minimise artefacts associated with sample deterioration. Air drying, or chemical fixation followed by resin embedding (including freeze-substitution) should be avoided for micro-analytical investigations because of the very substantial risks for elemental re-distribution.

Once cryo-fixed, the samples can be analyzed in either frozen-hydrated state (e.g. Tylko et al. 2007a, b; Wang et al. 2013) or in freeze-dried state, employing a very slow, temperature controlled freeze-drying regime. Samples may also be sectioned for analysis of specific tissues and cells. This approach is advisable because the penetration depth of the X-ray is greater than the thickness of a cell layer, and where there is more than one overlapping layer it becomes impossible to ascertain from which layer the fluorescence signal is originating (Donner et al. 2012). A cryomicrotome can easily produce sections ~30 μm thick which typically corresponds to a single cell layer. When using a cryo-ultramicrotome, semi-thin sections of the order of 1–2.5 μm in thickness and thinner, can be obtained. Cryo-sections need to be kept at cryogenic temperature (−130 °C or colder) until micro-analytical examination, or they can be freeze-dried.

## 4 Elemental Localization Within Plant Tissues

A range of approaches can be utilized for assessing elemental distribution within plant tissues. Below, a number of techniques are discussed, including those that illuminate the sample with photons (desktop micro-XRF, synchrotron XFM), irradiate the sample with electrons (SEM/TEM-EDS) or ions (PIXE and SIMS). The elemental detection is based upon either the emitted fluorescent X-rays (desktop micro-XRF, synchrotron XFM, SEM/TEM-EDS and PIXE) or by mass spectrometry (SIMS and LA-ICP-MS). In addition, we also describe the use of light microscopy and histochemistry, as well as autoradiography. The various techniques differ in their sensitivity (i.e. the limits of detection), lateral resolution, and sample preparation requirements. Although discussed in detail below, desktop micro-XRF, synchrotron XFM, SEM-EDS, PIXE, and LA-ICP-MS potentially allow analysis of (frozen) hydrated samples, thereby reducing the likelihood of experimental artefacts. Synchrotron (XFM) and microprobe (PIXE) facilities are often not easily accessible for many users, which is in contrast to SEM/TEM-EDS which is comparatively readily accessible within most large research institutions.

### 4.1 SEM-EDS

In SEM-EDS, electrons are used to excite core electrons of the elements of interest within the plant tissue sample, with the subsequent measurement of emitted fluorescent X-rays. Of the techniques considered here, SEM-EDS has relatively poor detection limits, in the order of 1000–5000 μg $g^{-1}$ for most transition elements. ‘Traditional’ SEM analysis is conducted in a vacuum, requiring samples that are dehydrated, although samples can be analyzed in frozen state where a cryogenic stage is available. More recently, the development of Environmental SEM has facilitated the analysis of hydrated/fresh samples. Although it is possible to obtain high resolution when imaging with SEM, elemental information is limited to a resolution of ca. 1–5 μm due to the interaction of the electrons with the sample, resulting in a tear drop-shaped penetration of the electrons into the sample. Figure 4 shows SEM-EDS elemental maps of a petiole cross-section.

### 4.2 Desktop Micro-XRF

Desktop micro-XRF instruments uses a focused beam of high-energy X-rays as the excitation source. These types of instruments provide high spatial resolution with spot sizes as small as 25 μm and detection limits in the order of 5–50 μg $g^{-1}$ for most transition elements. It relies on the same principle as a handheld XRF, but has the ability to raster-scan a sample, thereby producing elemental distribution maps. Figure 5 shows a micro-XRF image of a hyperaccumulator plant leaf with the distribution of different elements marked by different colours. The sample preparation for desktop micro-XRF analysis is minimal (samples of living

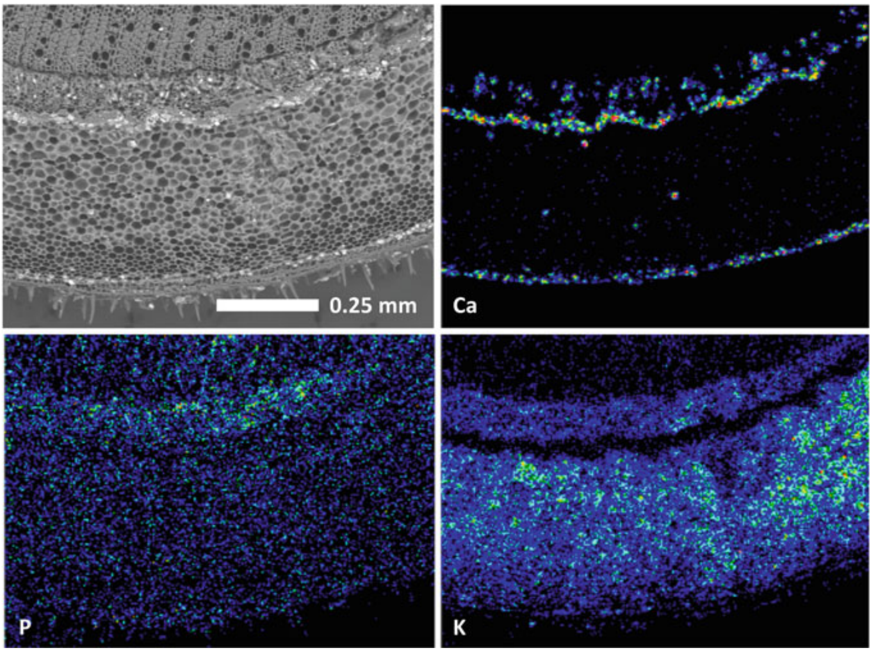

**Fig. 4** Examples of elemental maps obtained with SEM-EDS analysis. The top left panel shows the typical SEM image, with the other panels showing the elemental maps of Ca, P and K respectively. The sample is a freeze-dried petiole cross-section of the Ni hyperaccumulator *Rinorea bengalensis* (Violaceae)

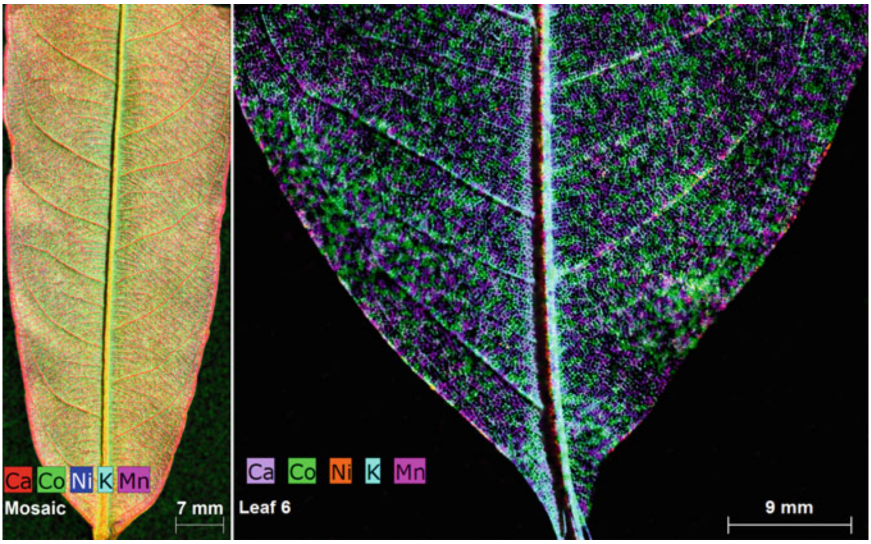

**Fig. 5** Colour-composite elemental maps of a hyperaccumulator (*Rinorea javanica*, Violaceae) leaf. The data was obtained with a Bruker microXRF instrument that was fitted with a 30 Watt X-ray source coupled to a polycapillary X-ray optics permitting down to 25 μm spot size

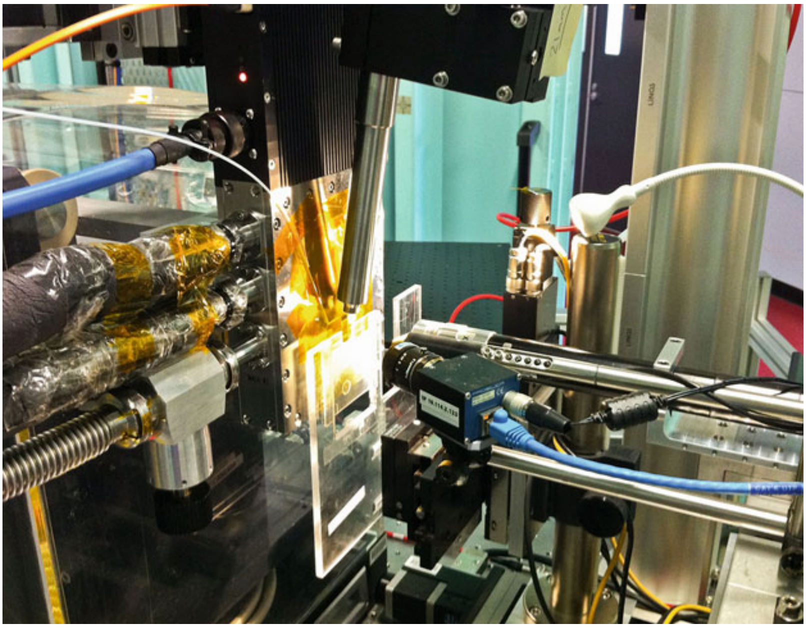

**Fig. 6** The analytical measurement conditions of the XFM beamline at the Australian Synchrotron. The system consists of the Maia detector and motion stages with the samples mounted on Perspex frame (between sheets of Kapton thin film). In this experiment, a nitrogen 'cryo-stream' is used to keep the hydrated sample frozen

leaves or other plant parts can be analyzed directly), however, it is preferable that the leaf samples are dehydrated to increase relative elemental concentration during analysis, and to reduce scattering due to water content.

## 4.3 Synchrotron Micro-XRF Microscopy

Synchrotron light is emitted by electrons when affected by a magnetic field resulting in photons that range from infrared and ultraviolet to X-ray wavelengths (Watson and Perlman 1978). The electromagnetic radiation is delivered to 'end stations' in individual beamlines. At 'end stations' the X-rays illuminate samples in a spectrometer, and fluorescence spectra may be recorded by different types of detectors (Miller and Dumas 2006; Sarret et al. 2013; Vijayan et al. 2015). Techniques that are possible using synchrotron XRF microscopy (XFM) beamlines include X-ray fluorescence microscopy (XFM), X-ray fluorescence micro-computed tomography (XRF-µCT); X-ray absorption Spectroscopy (XAS), and differential phase contrast imaging of low-*Z* ultrastructure (DPC), amongst others. The XFM beamline at the Australian Synchrotron achieves a spatial resolution of ~1 µm, with an energy range of 4–25 keV operating on an in-vacuum undulator source (Paterson et al. 2011). XFM beamlines have the ability to generate fine-scale elemental maps with spatial resolutions and quantification within plant tissues, cells, and organelles (McRae et al. 2009). Fluorescence emitted from the irradiated spots is detected as a function of the location on the specimen, when the specimen is moved relative to the stationary X-ray beam. Synchrotron XRF is a highly sensitive technique and hydrated samples can be analyzed in vivo, and it has the capacity to perform simultaneous, multi-elemental analysis (Sarret et al. 2013). Figure 6 shows the analytical measurement conditions at the XFM beamline of the Australian Synchrotron with the Maia detector system, with Fig. 7 showing the elemental maps of a mid-vein portion of a Ni hyperaccumulator leaf.

New-generation fast detector systems such as the Maia detector system, which comprises of an array of 384 detectors can process photon rates exceeding $10^7$ $s^{-1}$, with pixel transit times as small as 50 µs, thereby enabling the production of megapixel elemental maps (Ryan et al. 2010, 2014; Kirkham et al. 2010). The use of these new-generation detector systems permits new

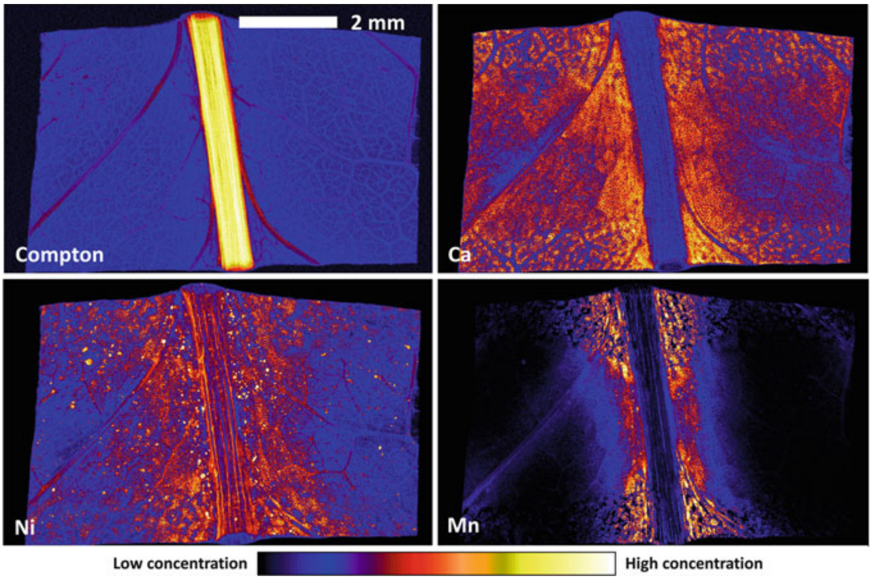

**Fig. 7** Examples of elemental maps obtained with XFM analysis. The sample is a freeze-dried portion of a Ni hyperaccumulator leaf (*Actephila alanbakeri*, Phyllanthaceae). The top left panel shows the Compton map (which represents scattered X-rays and provides information on the sample structure and density), and the other panels show the elemental maps of Ca, Ni and Mn respectively

insights in both hyperaccumulators and other plant species. It is now possible to image living plants repeatedly following exposure to elevated levels of trace metals (such as Ni or Mn) to examine progressive changes in metal distribution within plant tissues. For example, using the non-accumulating plant species, cowpea (*Vigna unguiculata*), leaves were scanned repeatedly for up to 48 h after exposure to 30 µM Mn. In this study, images were obtained six times over 48 h, with the images having 4 µm × 4 µm pixels collected over an area of 25 mm × 5 mm (see Fig. 8). An essential component of such studies, however, is to determine that such repeated scanning does not cause damage to the living leaf tissues and hence experimental artefacts.

## 4.4 PIXE

In micro-PIXE, characteristic X-rays of elements present in analysed samples are excited with focused ion beams, typically protons with energies of 2–3 MeV (Johansson et al. 1995; Mandò and Przybyłowicz 2009). This technique allows quantitative studies of concentration and distribution of elements from Na to U with detection sensitivity down to 1–10 µg g$^{-1}$ for transition elements, and lateral resolution of the order of 1 µm. Such a broad elemental range enables quantification of a wide range of elements such as S, P, K, Ca, Mn, Fe, Cu and Zn (Mesjasz-Przybyłowicz and Przybyłowicz 2002, 2011). Simultaneous use of at least one additional analytical technique is a common practice and the facility is referred to as a nuclear microprobe or a proton microprobe, because a proton beam is most often used. All research related to hyperaccumulator plants using PIXE has been carried out using protons and beam spot sizes of the order of a few µm. Typically, areas up to 2.5 mm × 2.5 mm can be analysed in a single scan, but mapping of larger structures requires specimen movement and mosaicking of maps. Specimens of any thickness can be analysed. Fundamentally PIXE is a standardless method that uses a fundamental parameter model approach. Once the experimental system has been calibrated,

**Fig. 8** Live plants (cowpea, *Vigna unguiculata*) to be analyzed with the Maia detector system at the XFM beamline of the Australian Synchrotron. The leaves were scanned (4 μm pixels, area of 25 mm × 5 mm) repeatedly for up to 48 h after exposure to 30 μM Mn

no additional standards matching the composition of biological samples are necessary. Quantification of analyses is obtained by direct measurements of accumulated charge deposited by protons or a value proportional to it. Simultaneously used proton backscattering spectrometry (BS) allows for the determination of specimen thickness (areal density) and the concentrations of main components of a biological material–C, O, N and indirectly H. For samples fully penetrated by the proton beam, the thickness can be also determined using the scanning transmission ion microscopy (STIM) method (Pallon et al. 2009). Numerous studies of hyperaccumulators have been conducted mainly at the three laboratories–iThemba LABS in South Africa, MIC in Slovenia and ANSTO in Australia. The first two laboratories offer facilities suitable for analysis of frozen-hydrated samples and have reported results of elemental mapping of hyperaccumulator plants (Tylko et al. 2007a, b; Wang et al. 2013; Vavpetič et al. 2013). However, the majority of studies were conducted on freeze-dried samples. The first PIXE elemental mapping of hyperaccumulator plants was undertaken in 1993 (Mesjasz-Przybylowicz et al. 1993, 1994). Overviews of numerous micro-PIXE studies related to hyperaccumulation of Ni, Co, As, Mn, Zn and Cd can be found e.g. in Przybylowicz et al. (1997) and Mesjasz-Przybyłowicz and Przybyłowicz (2011). Studies in which micro-PIXE was employed extended from elemental mapping of organs, tissues and plant cells, to the role of mycorrhizas in hyperaccumulating plants and the relations between these plants and herbivorous insects feeding on them (Mesjasz-Przybyłowicz and Przybyłowicz 2011).

## 4.5 NanoSIMS

Secondary ion mass spectrometry (SIMS) uses ions as the incident beam that collides with the sample surface and causes atoms and molecules from the upper layers of the sample to be ejected into the vacuum (Dé Rue et al. 2006; Hoppe et al. 2013). Ionized particles, referred to as secondary ions, are extracted to a mass spectrometer for analysis (which also enables isotopic identification). Given that the sputtering depth is 5–20 nm

(Hoppe et al. 2013), NanoSIMS is a surface-sensitive technique, allowing for a spatial resolution as low as <50 nm. The primary beam is typically $O^-$ or $Cs^+$, with a negatively charged primary beam ($O^-$) tending to favour the production of positively charged secondary ions; a positively charged primary beam ($Cs^+$) tends to favour the production of negatively charged secondary ions. Thus, for metals (such as Ni), an $O^-$ beam is generally preferable, whereas for metalloids (such as As) a $Cs^+$ beam is generally preferable. For some elements, such as Zn, Cd, and Mn, a poor secondary ion yield makes analyses more difficult (Zhao et al. 2014). NanoSIMS analyses are conducted within an ultra-high vacuum, and hence plant samples must first be processed and dehydrated prior to examination (Moore et al. 2011). In the hyperaccumulator *Alyssum lesbiacum*, Smart et al. (2007) used NanoSIMS to examine leaf sections, with Ni found to accumulate in the peripheral regions of large unicellular trichomes and in the epidermal cell layer. In a similar manner, Smart et al. (2010) examined the distribution of Ni and other elements in highly vacuolated leaf tissue of *A. lesbiacum*.

## 4.6 LA-ICP-MS

Laser ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS) uses a focused laser beam to ablate the surface, with ablated particles then transported to the mass spectrometer. Elemental and isotopic analyses are both possible, as for NanoSIMS. However, the spatial resolution of LA-ICP-MS is 2–3 orders of magnitude worse than for NanoSIMS, being 10–50 μm. However, in contrast to NanoSIMS, it is possible to analyze hydrated samples using LA-ICP-MS, in which analyses are performed at atmospheric pressure and room temperature. Moradi et al. (2010b) examined Ni in cross sections of fresh (hydrated) roots of the hyperaccumulator *Berkheya coddii*, with Ni concentrations found to be higher in the cortex compared to the stele. Using LA-ICP-MS, Tian et al. (2011) examined Cd distribution in *Sedum alfredii* using fresh (entire) leaves and freeze-dried sections taken from both leaves and stems. These authors reported that there was preferential accumulation of Cd within parenchyma cells, especially in stems. Wu et al. (2009) used LA-ICP-MS to examine elemental distribution in fresh (hydrated) leaves of *Elsholtzia splendens*.

## 4.7 Autoradiography and MRI

The use of radioactive isotopes in autoradiography is a powerful technique for the visualisation of specific radiotracers within intact living plants. It offers high resolution and the ability for time-resolved studies, but its application is limited primarily due to occupational health and safety concerns. The Cd distribution has been elucidated in *N. caerulescens* and *Arabidopsis halleri* using the $^{109}$Cd radiotracer (Cosio et al. 2005; Huguet et al. 2012). An alternative approach that does not involve radioactive isotopes, but has many of the same advantages, is the use of Magnetic Resonance Imaging (MRI), which has been successfully demonstrated for in situ Ni distribution in the rhizosphere of *Berkheya coddii* (Moradi et al. 2010a).

# 5 Histochemical Methods Localisation and Light Microscopy

Knowledge of anatomical features is crucial for accurately interpreting the elemental distribution in the hyperaccumulator plant tissues being studied. For bright-field light microscopy samples are usually fixed in 1–3% glutaraldehyde ($C_5H_8O_2$) buffered in cacodylate or phosphate-buffered saline (PBS). Fixation is often followed by dehydration in a graded ethanol ($C_2H_6O$) series, with or without propylene oxide ($C_3H_6O$), followed by embedding in a low-viscosity resin [such as Spurr's resin (Spurr 1969)]. Sections 0.5–2 μm thick can then be cut, stained with dyes, and imaged. If subcellular information is needed on the cells in which elemental distribution is being investigated, ultrathin sections of gold interference colour (80–90 nm) can be cut from the same resin blocks and mounted onto grids, stained with 2% uranyl acetate ($UO_2(CH_3COO)_2$) and lead citrate ($(C_6H_5O7)_2Pb_3$) (Reynolds 1963), and then examined by TEM. It is better, however, to use tissue samples that are rapidly frozen and then freeze-substituted and treated with a secondary fixative.

## 5.1 Use of Chelating Dyes and Light Microscopy

Chelating dyes have also been used to stain metal (loids) in plant tissues. Dimethylglyoxime ($C_4H_8N_2O_2$) forms a crimson complex with $Ni^{2+}$ and has been used as a histochemical stain for the localization of Ni within tissues of hyperaccumulator plants (Mizuno et al. 2003; Bhatia et al. 2004). However, artefacts are evident, as demonstrated by Bhatia et al. (2004) who found re-distribution of Ni. Similarly, Zincon ($C_{20}H_{15}N_4NaO_6S$), which forms a blue complex with $Zn^{2+}$, has been used for staining Zn in *N. caerulescens* (Macnair and Smirnoff 1999; Kozhevnikova et al. 2016).

Autometallography is another histological technique in which target ions (such as $Cu^{2+}$, $Cd^{2+}$, $Pb^{2+}$, $Zn^{2+}$) are precipitated with sulphides upon which metallic Ag is subsequently deposited by use of a reductant (Danscher 1984). The Ag clusters can then be imaged by light microscopy or SEM/TEM. The main disadvantage is that the sulphide reaction is non-specific and precipitates a range of transition elements, although at neutral pH the selective formation of ZnS nanocrystals is favoured (McRae et al. 2009). Selectivity may also be achieved using immunohistochemical methods via monoclonal anti-bodies to specifically bind chelated target ions, as demonstrated for $Cd^{2+}$ in plant tissue (Gao et al. 2015).

## 5.2 Use of Fluorophores and Confocal Microscopy

Fluorescence microscopy is sometimes employed for further interpretation of plant structure that may be pertinent for interpreting elemental distribution patterns in specific tissues. These include, for example, the identification of apoplastic barriers such as Casparian bands in roots stained with the fluorophore berberine ($C_{20}H_{18}NO_4+$) (Brundrett et al. 1988). Ion-selective fluorophores have been used to image the distribution of Ni and Zn in hyperaccumulator plants. Zinpyr-1 ($C_{46}H_{36}Cl_2N_6O_5$) was used to image $Zn^{2+}$ in *N. caerulescens* (Kozhevnikova et al. 2014, 2016). The high sensitivity of this fluorophore also enabled imaging of $Zn^{2+}$ in non-hyperaccumulator plants, such as in the roots of *Arabidopsis thaliana* (Sinclair et al. 2007). Leadmium Green was used to map $Zn^{2+}$ and $Cd^{2+}$ in the hyperaccumulators *Sedum alfredii* and *Picris divaricata* (Lu et al. 2008; Hu et al. 2012). Newport Green ($C_{43}H_{30}Cl_2N_4O_8$) has been applied for imaging $Ni^{2+}$ in cells of *A. murale* (Agrawal et al. 2013) and *A. lesbiacum* (Ingle et al. 2008).

Although fluorophores have a number of merits, including high selectivity, the ability to be used in live tissues in time-resolved visualization, and an inherently high resolution via visible light microscopy, there are also limitations related to unknown penetration and binding to target metal(loid) ions. Fluorophores will only bind to free ions that are not already strongly ligated; for example, $Al^{3+}$ binds strongly to the cell wall but the fluorophore morin ($C_{15}H_{10}O_7$) does not bind to this wall-bound Al, and this method was thus unable to identify this major compartment of Al (Eticha et al. 2005). Another concern relates to the application method of fluorophores to the sample: loading vibratome-sectioned samples in a solution with the fluorophore will ostensibly result in removal of any soluble metal(loid) ions present. Delivery of cell-permeable fluorophores into a nutrient solution, followed by uptake in the plant, could circumvent this issue, but avoiding re-distribution during sectioning remains difficult. Attempts have also been made at fixing mobile target ions ($Cd^{2+}$ or $Zn^{2+}$) by in situ precipitation as metal sulphides or with strong chelators before microtoming and microscopy (Hu et al. 2009, 2012; Gao et al. 2015). There is a need for independently validating the use of fluorophores for mapping metal(loid) ions in hyperaccumulator plants, for instance by mapping of fluorophore-labelled tissues with other micro-analytical techniques, such as XFM or PIXE.

## 6 Conclusions and Outlook

The use of a portable XRF-instruments enable mass screening of herbarium collections that will likely lead to the discovery of substantial numbers of hyperaccumulator plant species. The data obtained from systematic screening of entire phylogenetic lineages can lead to better insights in the evolution of hyperaccumulator plants.

The major limitation for researchers in using XFM and PIXE in their experiments is the restrictive nature of access to synchrotron and accelerator facilities. There is, therefore, a need to bridge the gap between what is currently possible in the laboratory environment and the capability of large analytical facilities. Technological developments, including very high flux X-ray sources (Hemberg et al. 2003), and even table-top accelerators (Graves et al. 2014), will not replace synchrotron-based XFM, but will permit combining their individual strengths, for example, by whole organism mapping at the local laboratory followed by investigation of target cells at a major synchrotron or accelerator facility.

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# Genesis and Behaviour of Ultramafic Soils and Consequences for Nickel Biogeochemistry

Guillaume Echevarria

**Abstract**

Ultramafic outcrops represent less than 1% of the terrestrial surface but their unusual geochemistry makes them a global hotspot for biodiversity. Ultramafic soils are a peculiarity for soil scientists in all climatic zones of the world. These soils lack essential pedogenetic elements: Al, Ca, K, and P. Whereas serpentinites will most likely give birth to Eutric Cambisols with little influence by climate, peridotites will induce an acceleration of weathering processes; this over-expressed weathering is due to their deficiency in Si and Al and lack of secondary clay formation. Soils evolve towards Ferralsols in tropical conditions. Results from isotopic dilution techniques show that Ni borne by primary minerals is unavailable. Secondary 2:1 clay minerals (e.g. Fe-rich smectite) and amorphous Fe oxyhydroxides are the most important phases that bear available Ni. Therefore, smectite-rich soils developed on serpentinite and poorly weathered Cambisols on peridotite (only in temperate conditions) are the soils with highest availability of Ni. Although soil pH conditions are a major factor in controlling available Ni, the chemical bounds of Ni to containing phases are even more important to consider. Plants take up significant amounts of Ni, and its biogeochemical recycling seems an essential factor that explains Ni availability in the surface horizons of ultramafic soils.

## 1 Introduction

Globally ultramafic (or ultrabasic) outcrops represent less than 1% of the terrestrial surface. They have focused attention for decades because of their unusual geochemical composition at the surface of continents. This peculiar geochemistry (lack of most essential elements for plant nutrition: K, Ca and P) makes them a global hotspot for biodiversity. To soil scientists (Alexander 2009), ultramafic soils have also been of a particular interest because the pedogenetic processes involved in their formation are usually

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different from those taking place in soils on all other type of bedrock in the same area. It is quite common that ultramafic soils in warm regions display soil types that are common in colder regions, or the opposite. Ultramafic soils are also the most important source of nickel (Ni) and cobalt (Co) to terrestrial ecosystems (Estrade et al. 2015). Nickel and Co hyperaccumulation by plants have evolved a great deal from ultramafic soils, mostly in relation to metal bioavailability within these soils (van der Ent et al. 2016a). This chapter discusses the most important trends of soil genesis and evolution on ultramafic bedrock worldwide. It then focuses on the main Ni-bearing phases resulting from soil genesis patterns in the most important cases, providing a foundation for better understanding the factors controlling Ni availability in ultramafic soils.

## 2 Properties of Ultramafic Bedrock

### 2.1 What Are Ophiolites and Peridotites?

Ophiolites, which were long seen as continental analogs of oceanic crust, are now viewed as out-of-place fragments of oceanic lithosphere (Dilek and Furnes 2009). They generally consist of some or all of the following components: lherzolite or harzburgite commonly altered to serpentinite, gabbro, 'sheeted' basic dykes, pillow-bearing basic volcanic rocks, chert, and sedimentary rocks (Vaughan and Scarrow 2003). Ultramafic outcrops occupy <1% of the Earth's land surface (Fig. 1), but are locally abundant in ophiolite belts along tectonic plate margins (Coleman and Jove 1992; Vaughan and Scarrow 2003; Dilek and Furnes 2009). One of the most studied ophiolite complex is that of the ancient Tethyan Ocean, which spread from the Alps to the Himalayas, and was subducted onto the margin of the continental Eurasian plate from Jurassic to Cretaceaous time (Dilek and Furnes 2009). Of course, ophiolites worldwide, including upper mantle peridotites, vary because of differences in compositions of magmas over time and according to regional conditions (Dilek and Furnes 2009).

### 2.2 Variety of Ultramafic Rocks and Their Composition

Peridotite (or ultramafic) rocks are defined as igneous rocks that contain more than 90% mafic minerals (Le Bas and Streckeisen 1991), of which are olivines (nesosilicates), and pyroxenes (inosilicates). There are several types of peridotites based on vertical position in the original ophiolite, being distinguished according to the ratio of olivine to clino- and ortho-pyroxene (Fig. 2) as officially defined by the International Union of Geological Sciences, i.e. IUGS (Le Bas and Streckeisen 1991). The most common ultramafic rocks found in outcrops include dunite, harzburgite, lherzolite, and pyroxenite.

All of these rocks can undergo metamorphism in varying conditions according to the history of metamorphism of mid-ocean ridges and subduction zones (O'Hanley 1996; McCollom et al. 2016). This process includes serpentinization, which essentially consists of a hydration of olivine and pyroxene that results in the formation of serpentine clay minerals (Coleman and Jove 1992; McCollom et al. 2016); however, other minerals are also formed during the serpentinization process, e.g. magnetite, brucite, talc, chlorite, etc. (Coleman and Jove 1992; McCollom et al. 2016). As an example, the formation of serpentine through the weathering of olivine is described in Eq. (1) (McCollom et al. 2016):

$$\underset{\text{Olivine (Forsterite)}}{\mathrm{Mg_{1.82}Fe_{0.18}SiO_4}} + \mathrm{wH_2O} \rightarrow \underset{\text{Serpentine}}{0.5\left(\mathrm{Mg,Fe^{II},Fe^{III}}\right)_3\left(\mathrm{Si,Fe^{III}}\right)_2\mathrm{O_5(OH)_4}} + \mathrm{x}\underset{\text{Brucite}}{\mathrm{(Mg,Fe)(OH)_2}} + \mathrm{y}\underset{\text{Magnetite}}{\mathrm{Fe_3O_4}} + \mathrm{zH_2} \quad (1)$$

*A.P.M. Vaughan, J.H. Scarrow / Earth and Planetary Science Letters 213 (2003) 407–416*

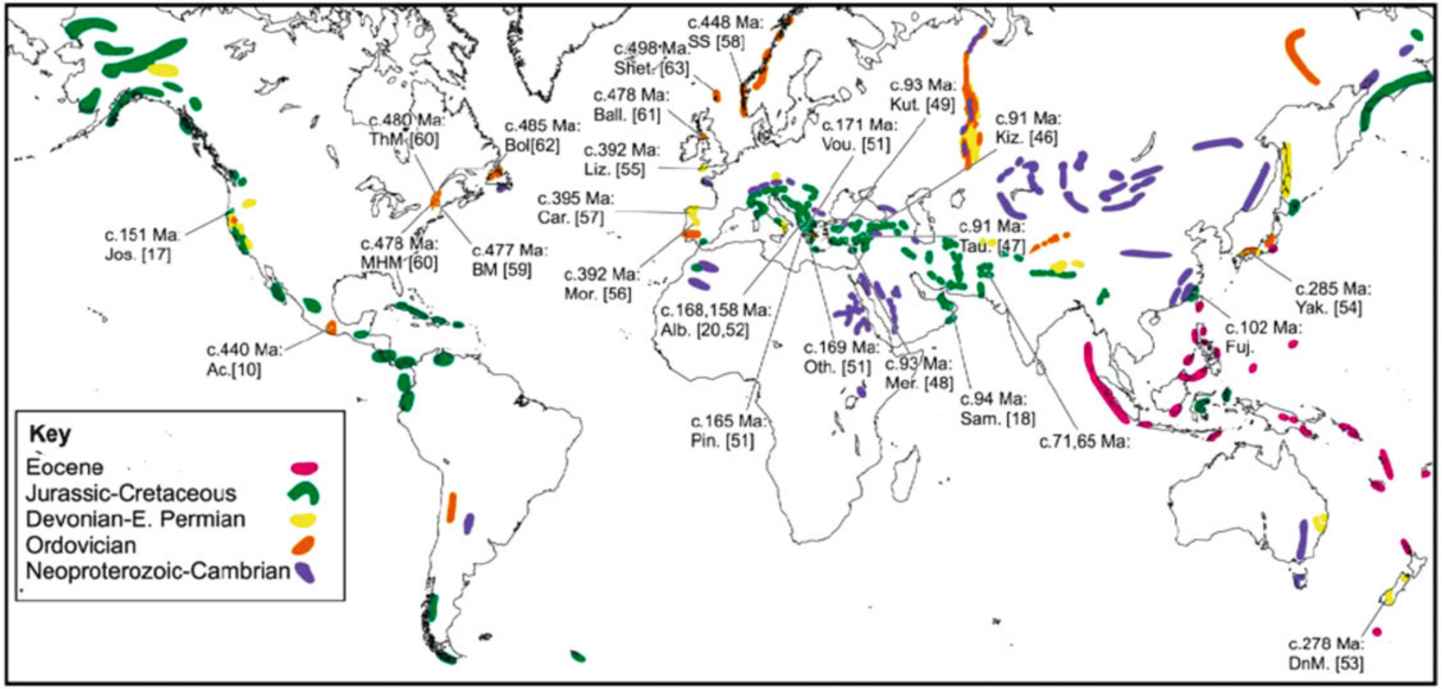

Fig. 1. Distribution of belts of Phanerozoic ophiolites cited in the text, including major Neoproterozoic and Phanerozoic occurrences. Note that ophiolites of several age pulses are represented in most belts. Scandinavian ophiolites [2], Mediterranean and western Asia ophiolite distribution [3], Central Asian ophiolites [4] apart from [5], western Pacific ophiolites [6], Uralian ophiolites [7], Alaskan ophiolites [8], western North American, Central American and Caribbean ophiolites [9] apart from [10,11], southern South American ophiolites [12,13]. Shet.: Shetland; Ball.: Ballantrae; ThM: Thetford Mines; MHM: Mont Ham Massif; BoI: Bay of Islands; BM: Boil Mountain; SS: Solund-Stavfjord; Car.: Careon; Ac.: Acatlán; Mor.: Morais; Liz.: Lizard; Yak.: Yakuno; DnM: Dun Mountain; Vou.: Vourinos; Oth.: Othris; Alb.: Albanian; Pin.: Pindos; Jos.: Josephine; Fuj.: Fujian; Sam.: Samail; Kut.: Kutahya; Mer.: Mersin; Tau.: Tauride; MB: Muslim-Bagh.

**Fig. 1** Distribution of ophiolite belts worldwide (from Vaughan and Scarrow 2003)

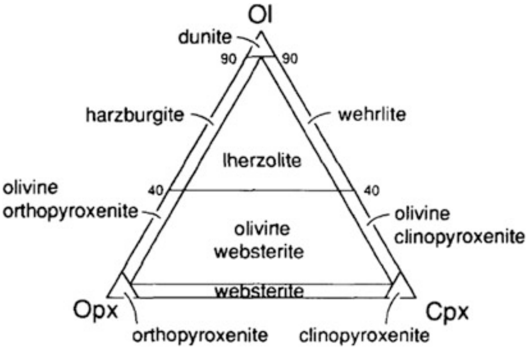

**Fig. 2** Classification and nomenclature of the ultramafic rocks (mafic minerals >90%) based on the modal proportions of olivine (Ol), orthopyroxene (Opx) and clinopyroxene (Cpx) (from Le Bas and Streckeisen 1991)

Serpentines are 1:1 clay minerals and comprise three different types: chrysotile (asbestos form—Fig. 3c), lizardite, and antigorite (platy form). Chrysotile is one of the most common serpentine-group minerals and typically displays long fibres due to self-winding of the clay-like sheets (Fig. 3). When serpentinization results in the transformation of the majority of olivine and pyroxene contained in the peridotite, then the rock is classified as serpentinite (O'Hanley 1996). The difference between peridotite and serpentinite is clear. The former is dark black with an oxidised weathering sheath (Fig. 3a), mainly due to Fe-oxide precipitation, whereas the serpentinite has a greener and uneven aspect (Fig. 3b) owing to particles of serpentine, talc, and other clay minerals, which resembles snake skin and gives the rock its name 'serpentine'.

Secondary geological materials originally derived from ultramafic rocks such as mountain rock slides (Gasser et al. 1995; d'Amico et al. 2008), colluvium (Lee et al. 2004), sedimentary rocks (e.g. conglomerate), river alluvia (Estrade et al. 2015; Rinklebe et al. 2016), might also represent in many cases a significant contribution to ultramafic soils and landscapes. Usually, these materials are not identified as ultramafic rocks on geological maps but are mentioned using different qualifiers based on geomorphological origin and period of deposition (e.g. glacial/fluvial deposits). According to the composition of the original minerals forming peridotites, ultramafic rocks have very high Mg (18–24%) and high Fe (6–9%), but very low Ca (1–4%) and Al (1–2%) contents (Alexander 2004). Also, the main weatherable minerals in peridotite and serpentinite (Lee et al. 2004; White and Buss 2014) are olivine, pyroxene, and clay minerals (mainly serpentine but also talc, chlorite, etc.).

## 3 Pedogenesis of Ultramafic Rocks and Soil Characteristics

The genesis, development, and functioning of soils developed on ultramafic materials have received a great deal of attention over the last 30 years. Firstly, ultramafic soils were studied because of their economic importance related to the genesis of nickeliferous laterites (Colin et al. 1990; Gleeson et al. 2003), but also because of their role in trace metal geochemistry (Becquer et al. 2006; Chen et al. 2011; Bani et al. 2014; Kierczkiak et al. 2016). One of the major factors in influencing the genesis and characteristics of soils developed on ultramafics is the nature of the parent ultramafic rock itself (Alexander 2004, 2009; Alexander and DuShey 2011). Other major factors affecting soil genesis are climatic conditions, topographic situation (position in the toposequence), and vegetation cover (Bonifacio et al. 1997; Alexander and DuShey 2011; Chardot et al. 2007; Kierczak et al. 2007; Cheng et al. 2011; Bani et al. 2014). After compiling available descriptions on major soils developed on serpentinite—*sensu stricto*—(Table 1), and on partially serpentinized peridotite (Table 2), it appears that most such soils reach a limited development stage, whatever the climatic conditions, that confers on them typical cambic and magnesic (exchangeable Ca/Mg <1) properties. These cambic properties are mainly derived from the formation of secondary clays of the 2:1 type—Fe-rich smectite and low-charge vermiculite—that form stable complexes in soils, typically with excess Mg ions (Bonifacio et al. 1997; Lee et al. 2004; Bani et al. 2014).

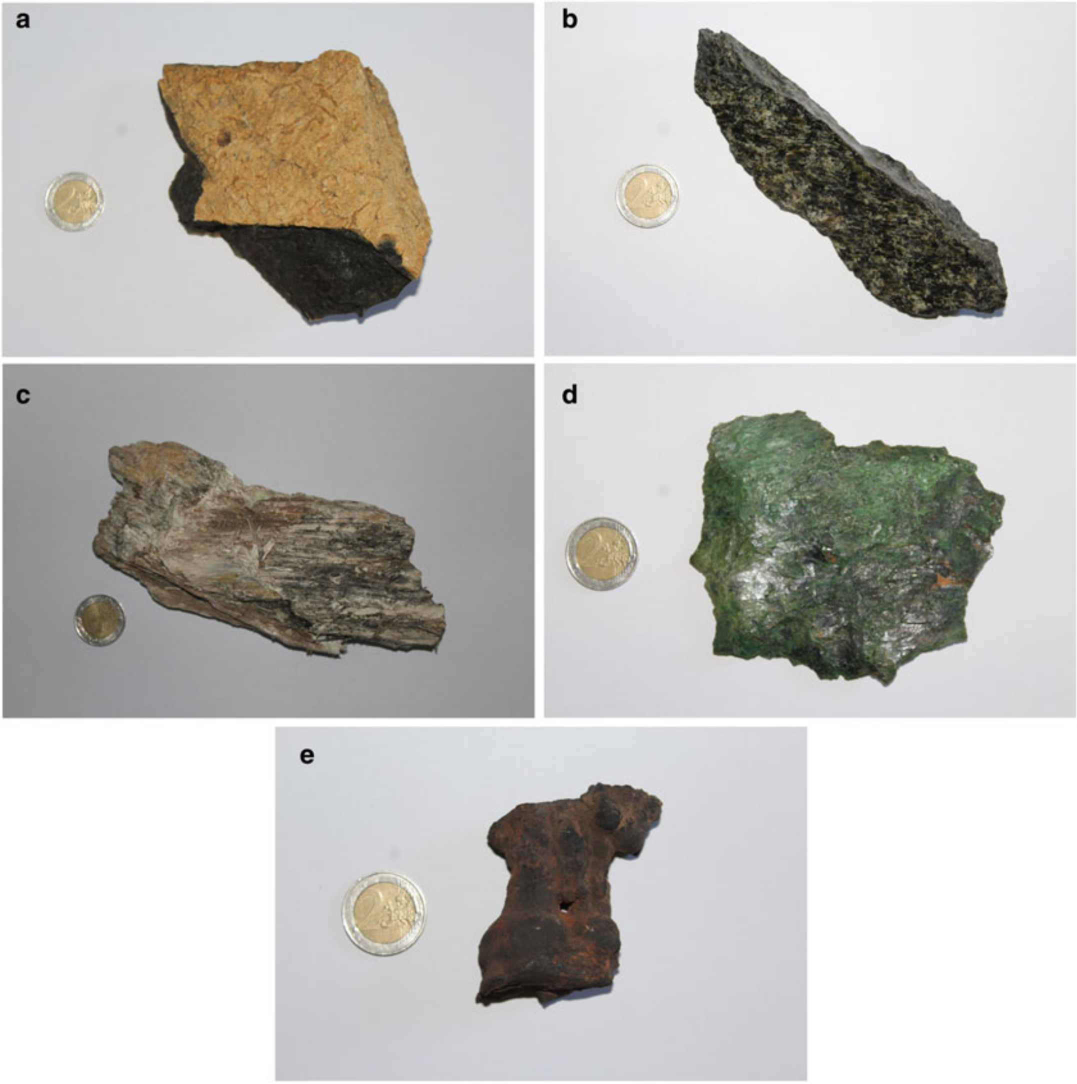

**Fig. 3** (**a**) Olivine-rich peridotite with weathered surface from Shebenik (Albania). (**b**) Highly serpentinized peridotite from Pindus Mountains (Greece). (**c**) Large chrysotile fibers from the Barberton Greenstone belt (South Africa). (**d**) Garnierite: serpentine-like minerals with approx. 20% Ni (*green* colour) from the saprolite horizon of Geric Ferralsols in Vulcain Mine, La Tontouta (New Caledonia). (**e**) Fragment taken from the Petroplinthic horizon (ferricrete or iron pan) of a Geric Ferralsol in Yaté (New Caledonia) where two parallel earthworm galleries have been fossilised through Fe-oxide crystallisation

## 3.1 Common Steps of Pedogenesis on Ultramafic Bedrock

Olivines in the bedrock are not only weathered into clay minerals but a significant fraction transforms into Fe-oxyhydroxides with loss of Si and Mg. Formation of secondary 2:1 clay is the main difference between serpentinized and non-serpentinized ultramafic bedrock (Alexander 2009). The influence of draining conditions seems to strongly influence the outcome of pedogenesis: formation and accumulation of Fe- or Mg-rich smectite in poorly drained conditions (Lee et al. 2004; Bani et al. 2014) or low-charge vermiculite in well-drained soils (Bonifacio et al. 1997). Serpentine-group

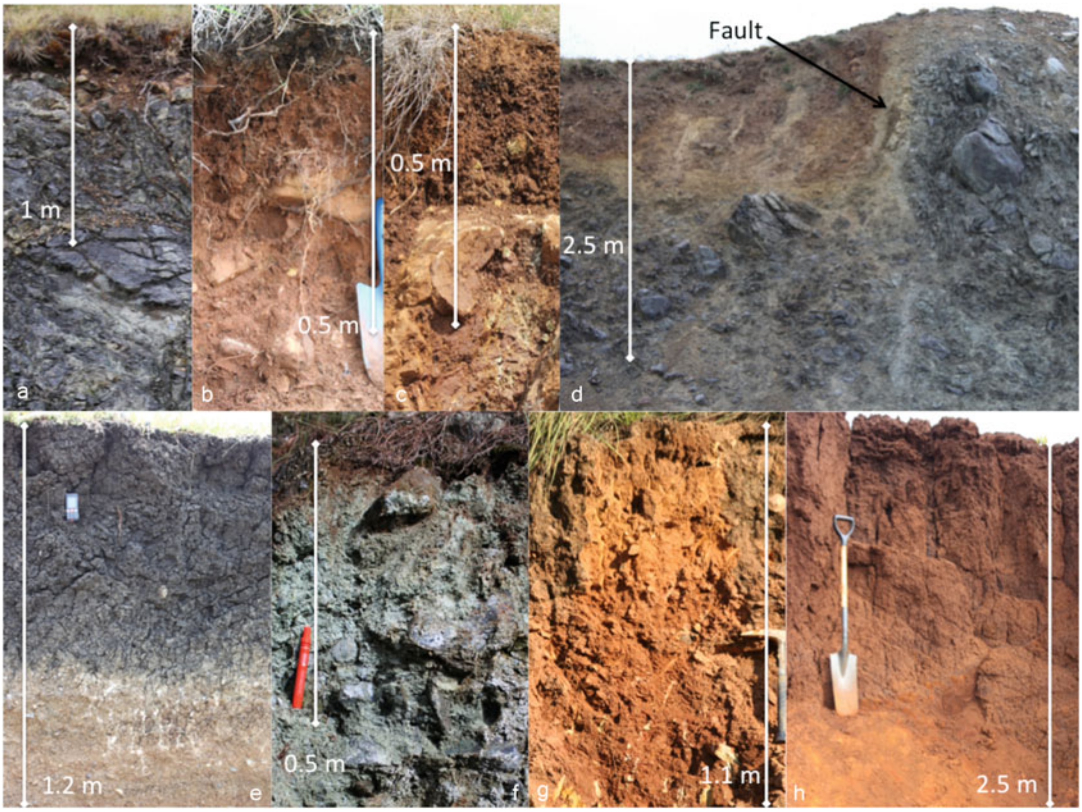

**Fig. 4** Caracteristic pedons on ultramafic rocks globally: (**a**) Cambic Leptosol (Serpentinite 1700 m, Katara Pass, Greece). (**b**) Eutric Cambisol (Dunite, 1200 m, Qaftë Shtamë, Albania). (**c**) Chromic Cambisol (Dunite, 890 m, Trygona, Greece). (**d**) A fault between partially-serpentinized (*left*) and fully-serpentinized peridotite (*right*) (1700 m, Katara Pass, Greece). (**e**) Hypermagnesic Vertisol (Serpentinite, 650 m, Prrenjas, Albania). (**f**) Folic Leptosol (Serpentinite, 800 m, Kinabalu Park, Sabah, Malaysia). (**g**) Chromic Luvisol (Peridotite, 1100 m, Barberton, South Africa). (**h**) Geric Ferralsol (peridotite, 100 m, Goro, New Caledonia). Photographs: G. Echevarria

minerals are commonly unstable in soil conditions and form secondary 2:1 phyllosilicates as well (Bonifacio et al. 1997; Lee et al. 2003; Cheng et al. 2011). Iron released from olivine and serpentine during weathering can be bound in a great range of secondary minerals according to temperature and drainage conditions. Under a temperate climate and even in the case of moderately weathered tropical soils, Mg plays a leading role among exchangeable cations although Ca binds preferentially relative to Mg to soil Cation Exchange Capacity (CEC; Lee et al. 2001; Chardot et al. 2007; Kierczak et al. 2007; Cheng et al. 2011). Magnesium is partially leached during soil formation in temperate climates (incomplete hydrolysis), and a variety of secondary clay phases such as vermiculite and smectite, is produced; in contrast in tropical lateritic soils Mg is almost totally leached (complete hydrolysis) as is Si, leading finally to the accumulation of free Fe oxides (Becquer et al. 2001).

Ultramafic soils share a number of chemical particularities including a low Ca/Mg quotient with Ca existing at significantly lower concentrations than in other soils of surrounding areas. The ultramafic soils also typically contain elevated levels of metals including Fe, Mn, Ni, Cr and Co, which could induce toxicity for most of the plants. Serpentine soils are commonly deficient in essential plant nutrients such as N, K and P (Brooks 1987; Proctor and Woodell

**Table 1** Soil types derived from serpentinite according to the location, climatic conditions, mineralogy of the bedrock and position in landscape. Soil types are given according to the latest version of soil taxonomy (IUSS Working Group WRB 2014)

| Bedrock | Climate | Location | Mineralogy (order of predominance) | Soil type (WRB Soil Resources) (as described or adapted from descriptions) | Reference |
|---|---|---|---|---|---|
| Serpentinite | Semi-continental | Slovakia | Serpentine, magnetite | Technosol (Hypermagnesic) | Massoura et al. (2006) |
| Augite-serpentinite | Sub-alpine | Swiss Alps | Serpentine, augite, spinels | Dystric Cambisol— rock slides | Gasser et al. (1995) |
| Serpentinite | Sub-alpine | Italian Alps | Serpentine, magnetite | Haplic Regosol (Dystric)—mid-slope | D'Amico et al. (2008) |
| « | « | « | « | Epileptic Cambisol (Protospodic)—high-slope | « |
| Serpentinite | Temperate montane | Poland | Serpentine, spinels | Eutric Cambic Leptosol (Magnesic) | Kierczak et al. (2016) |
| Serpentinite | Semi-continental | Poland | Serpentine, spinels | Eutric Skeletic Leptic Cambisol (Magnesic) | Kierczak et al. (2016) |
| Serpentinite | Temperate oceanic | France, Centre | Serpentine, chlorite, magnetite | Hypereutric Cambisol (Hypermagnesic) | Caillaud et al. (2004) |
| Serpentinite | Mediterranean montane | Albania | Serpentine | Chromic Hypereutric Cambisol (Magnesic)—slope | Bani et al. (2014) |
| « | « | « | Serpentine (alluvium) | Eutric Cambic Vertisol (Hypermagnesic)—downslope or alluvial terrace | Bani et al. (2014), Estrade et al. (2015) |
| Serpentinite | Mediterranean montane | California | Serpentine (no pyroxene) | Lithic Mollic Leptosols (Magnesic)—summit | Alexander and Dushey (2011) |
| « | « | « | « | Epileptic Luvic Phaeozem (Magnesic)—slope | |
| « | « | « | « | Endoleptic Luvisol (Magnesic)—downslope | « |
| Serpentinite<br>« | Mediterranean<br>« | Italy<br>« | Serpentine, magnetite<br>« | Leptosol (Hypermagnesic)—upslope<br>Leptic Cambisol (Hypermagnesic)—backslope | Bonifacio et al. (1997)<br>« |
| «<br>« | «<br>« | «<br>« | «<br>« | Chromic Luvisol (Magnesic)—downslope<br>Dystric Cambisol (Magnesic)—bottom | «<br>« |
| Serpentinite | Humid subtropical | Taiwan | Serpentine, chlorite, enstatite | Cambic Leptosol (Magnesic)—upslope | Cheng et al. (2011) |
| « | « | « | « | Eutric Cambisol (Magnesic)—slope | « |
| « | « | « | « | Haplic Luvisol—downslope | « |
| « | « | « | Serpentine, amphibole, talc, chlorite | Haplic Vertisol—shoulder | Hseu et al. (2007) |
| Serpentinite | Tropical short dry season | Brasil, Minas | Serpentine, tremolite, chlorite | Eutric Regosol (Magnesic) | Vidal-Torrado et al. (2006) |
| Serpentinite | Humid tropical | Sabah | Serpentine, magnetite | Eutric Mollic Leptosol (hypermagnesic)—slope | van der Ent et al. (2016b) |

(continued)

**Table 1** (continued)

| Bedrock | Climate | Location | Mineralogy (order of predominance) | Soil type (WRB Soil Resources) (as described or adapted from descriptions) | Reference |
|---|---|---|---|---|---|
| Serpentinite | Humid tropical | Sulawesi | Serpentine, olivine, augite | Posic Ferralsol (Eutric, Rhodic) | Anda (2012) |
| Serpentinite | Humid tropical | Cameroon | Antigorite, magnetite, (olivine) | Geric Ferralsols | Yongoué-Fouateu et al. (2006) |
| Serpentinite | Humid tropical | New Caledonia | not described | Eutric Cambisols (hypermagnesic) > Vertisols | Proctor (2003) |

**Table 2** Soil types derived from non- or slightly-serpentinized peridotite according to the location, climatic conditions, mineralogy of the bedrock and position in landscape. Soil types are given according to the latest version of soil taxonomy (IUSS Working Group WRB 2014)

| Bedrock | Climate | Location | Mineralogy (order of predominance) | Soil type (WRB Soil Resources) (as described or adapted from descriptions) | Reference |
|---|---|---|---|---|---|
| Hornblende-Peridotite | Semi-continental | Poland | Serpentine (32%), enstatite, forsterite, amphibole | Eutric Skeletic Leptic Cambisol (Magnesic, Siltic) | Kierczak et al. (2016) |
| Partially serp. harzburgite | Temperate montane | France, Vosges | Serpentine, olivine, pyroxene, chlorite | Hypereutric Cambisol (Magnesic)—slope | Chardot et al. (2007) |
| Peridotite | Mediterranean montane | California | Olivines, Pyroxenes | Abruptic Luvisol—summit | Alexander and Dushey (2011) |
| « | « | « | « | Endoleptic Chromic Luvisol—downslope | |
| Peridotite | Humid Mediterranean montane | « | « | Ferritic Luvisol (Rhodic) | Alexander (2014) |
| Dunite | Tropical montane | Sabah | Olivines | Dystric Folic Cambisol (Magnesic) | van der Ent et al. (2016b) |
| Peridotite | Humid tropical | New Caledonia | not described | Geric Ferralsol (Pisolithic Rhodic)—slope and downslope | Becquer et al. (2001) |
| Partially serp. peridotite | Humid tropical | New Caledonia | Olivines, serpentines, enstatite | Geric Ferralsol (Pisolithic) | Dublet et al. (2014) |
| Peridotite | Humid tropical | Philippines | not described | Geric Ferralsol | Fan and Gerson (2011) |
| Peridotite | Humid tropical | « | not described | Geric Ferralsol (Plinthic Rhodic) | « |
| Pyroxenite | Tropical with dry season | Brazil, Cerrado | Enstatite, serpentine, spinel | Vertic Eutric Cambisol (Magnesic Rhodic)—backslope | Garnier et al. (2009b) |
| « | « | « | « | Geric Ferralsol (Pisolithic Rhodic)—downslope | « |
| Pyroxenite | Tropical with dry season | Brazil, Cerrado | Enstatite, diopside | Geric Ferralsol with smectitic saprolite | Colin et al. (1990) |
| Dunite | « | « | Olivines | Geric Ferralsol (Petroplinthic Rhodic) | « |

**Fig. 5** (**a**) Formation of hematite on the upper part of the lateritic profile in the background hill due to surface dehydration of Fe oxyhydroxides (Niquelândia, Brazil). (**b**) A fault between pyroxenite (*left*) and dunite (*right*) allows seeing the difference of composition and mineralogy: Ni-rich smectites (*left*) and goethite (*right*) (Niquelândia, Brazil). Photographs: G. Echevarria

1975). In ultramafic soils, Cr and Ni distribution and mineral speciation strongly depend on the mineralogy of the bedrock (Garnier et al. 2006, 2009b; Raous et al. 2013), as well as on climatic conditions (Massoura et al. 2006), on the position in the toposequence (Cheng et al. 2011), and more precisely on thermodynamic conditions of the soils (Antić-Mladenović et al. 2011). However, the degree of serpentinization is known to be a major discriminant factor in ultramafic pedogenesis (Alexander 2004, 2009). Photographs of the most common pedons mentioned in the following paragraphs are shown in Fig. 4.

## 3.2 Soils on Serpentinite Bedrock

Serpentinite is one of the most amazing bedrocks for soil development worldwide because soils developed on this substrate tend to be similar in pedogenesis and functioning regardless of latitude or elevation (Table 1): They are mostly classified as Cambisols. If we take a close look at the genesis and characteristics of these soils in both cold and temperate climates, they commonly range from high pH Regosols/Leptosols with cambic properties with CEC dominated by Mg over Ca, to Cambisols with neutral to slightly acidic pH (Caillaud et al. 2004; Chardot et al. 2007; Kierczak et al. 2007, 2016). Temperate ultramafic Cambisols typically contain serpentine as the predominant mineral phase, although 2:1 clays either derive from the bedrock (e.g. chlorite) or form from serpentine weathering and loss of Mg (Caillaud et al. 2009; Chardot et al. 2007). The formation of Fe-rich smectite from serpentine is evidenced in temperate serpentinite soils (Caillaud et al. 2004, 2009). The Mg-chlorite is weathered to trioctahedral vermiculite (Caillaud et al. 2009). During weathering, free Fe is released and oxyhydroxides are partly crystallized (Chardot et al. 2007). The chroma from serpentinite soil in temperate conditions is generally redder than that in surrounding soils on non-ultramafic substrates, owing to the high content of free Fe (Chardot et al. 2007) although not as high as for soils developed on non-serpentinized peridotite (Kierczak et al. 2016).

In Mediterranean conditions, weathering intensity is higher and magnesic Cambisols have been shown to form after the loss of 85–90% of the initial bedrock material (Estrade et al. 2015). Also, further pedological evolution may occur if topographic conditions are suitable, including the formation of Luvisols where the pedons get thicker (Bonifacio et al. 1997). However, the soils remain dominated by magnesic Cambisols where landscapes are dominated by slopes. As in temperate environments (Caillaud et al. 2004, 2009), the primary serpentine is easily weathered into smectite (Bonifacio et al.

1997) that typically is Fe-rich (Ece et al. 1999; Caillaud et al. 2004). Further evolution of the soil transforms smectite into low-charge vermiculite if drainage conditions of the soil are favourable (Istok and Harward 1982; Bonifacio et al. 1997). In the absence of sufficient drainage (downslope or in alluvial zones), Vertisols form due to a significant accumulation of neoformed smectite (Lee et al. 2003; Bani et al. 2014), some of which are Mg-rich smectite that is not found in Cambisols (Bani et al. 2014). Upslope, the soil cover is still very similar as in colder regions (Bonifacio et al. 1997; Alexander and DuShey 2011), and Leptosols are still found on shallower portions of the toposequences.

In humid climates of subtropical to tropical areas, serpentinite soils are still dominated by 2:1 clays (e.g. smectite) and mostly dominated by Cambic Leptosols/Cambisols having relatively high pH and magnesic/hypermagnesic properties (Cheng et al. 2011; Isnard et al. 2016; van der Ent et al. 2016b). These soils are typically developed on upslope and midslope landscape positions (Hseu et al. 2007; Cheng et al. 2011). Within these soils, the main pedogenic process is weathering of primary clay minerals into smectite after a significant loss of Mg (Hseu et al. 2007) and formation of a stable organo-mineral complex. Vertic properties have been mentioned in several Cambisols in such regions, because of the high proportion of 2:1 clays (e.g. smectite), including even the true formation of Vertisols (Proctor 2003). Soils tend to be rejuvenated because of the Vertic properties and erosion where a slope is present that could explain the predominance of Cambisol-related soils even in humid-tropical conditions. Under conditions that are favourable for leaching, the loss of Si from smectite/vermiculite can lead to accumulation of kaolinite and Fe-oxides (Hseu et al. 2007). Luvisols are present in such areas (i.e. downslope) where weathering products of the toposequence accumulate (Hseu et al. 2007; Cheng et al. 2011), which again shows a great similarity with those of colder regions (Bonifacio et al. 1997). Only one record of a Ferralsol has been reported to develop on pure serpentinite, in Sulawesi (Anda 2012), but the author did not mention if lateritic material had accumulated from the surrounding soil cover on peridotite, or if that particular soil was several millions of years old. According to Gleeson et al. (2003), laterite formation on wholly serpentinized peridotite is common and leads to smectite-rich saprolite owing to poor drainage of the serpentinite. The presence of deep Geric Ferralsols with possible ferricrete is reported on serpentinite that contains only traces of olivine (Yongue-Fouateu et al. 2006). The deep laterite profiles developed on this 240 km$^2$ outcrop in the Equatorial Forest have probably developed under stable climatic conditions for tens of millions of years; lateritization is still active. If the presence of serpentinite reduces the speed of lateritization, the endpoint of pedogenesis on ultramafic material (serpentinized or not) seems to be Petroplinthic Geric Ferralsols. However, this type of soil is seldom found in tropical regions because many local factors apparently block pedogenesis at an early stage (e.g. steep slopes, Vertic properties).

## 3.3 Soils on Non-serpentinized Peridotite

It is difficult to differentiate ultramafic soils developed on serpentinite versus peridotite in cold environments (Kierczak et al. 2007, 2016) because their geochemistry is generally similar and the end products of weathering (i.e. soils) do not differ much in chroma, soil depth, and weathering intensity. In warmer climates, the differences become more obvious (Alexander and DuShey 2011) and in tropical environments, we face two different ecosystems (Proctor 2003). Cuba is a good example, where Cambisols occur on serpentinite that are covered with maquis-like vegetation (high occurrence of Ni-hyperaccumulators); Ferralsols occur on peridotite, which host a rainforest. The latter contain very few hyperaccumulator species.

In temperate environments, the predominant soil type on non- or slightly serpentinized peridotite is again Cambisol (Table 2) that displays slight differences with Cambisols developed on serpentinite, such as the qualifier 'chromic' that can be applied as a consequence of iron segregation (easily weatherable olivine can produce significant amounts of Fe-oxyhydroxides). Soil chroma of such soils can be typical of those found on Mediterranean soils with values situated in the YR range (whereas surrounding soils are in the Y range), locally reaching YR 7.5 or redder (Chardot et al. 2007; Kierczak et al. 2016). In soils developed on serpentinite bedrock, the amount of 'free' Fe oxides (amorphous and crystallized) measured by dithionite-bicarbonate-citrate extraction (McKeague and Day 1966) is significantly different from that of surrounding soils formed on other types of bedrock and can exceed 5% (Chardot et al. 2007). Also, the amount of smectite in $B_W$ horizons of peridotite-derived Cambisols is less significant than in soils formed on serpentinite (Caillaud et al. 2004; Chardot et al. 2007).

In Mediterranean environments, Fe segregation becomes a significant pedogenic process on peridotite (Alexander and DuShey 2011). The amounts of free Fe in the well-drained soils of the Klamath Mountains transect (i.e. a typical ultramafic region of northern California) are related to the degree of serpentinization of the parent peridotite and its weathering (Alexander 2014). Goethite in the peridotitic soil is predominant in the wetter soil having higher free Fe contents (Alexander 2014). In the peridotitic soil, the accumulation of Fe oxyhydroxides (up to 25%; Massoura et al. 2006; Alexander 2014) is associated with chroma values redder than YR 5; the soils can be defined as Chromic. Such soils are mostly Luvisols with properties that typically include Magnesic and Chromic/Rhodic. The high amount of free Fe, and the reduced concentrations of 2:1 clay minerals in the B horizons of these soils, induce the lack of CEC with net positive charges such as in Ferralic horizons that are typical of tropical lateritic soils. This feature found in Mediterranean climatic conditions on peridotite is unique for non-tropical soils (Alexander 2014), and means that pedogenesis on peridotite under Mediterranean conditions involves the first steps of lateritization.

Soil genesis occurring on peridotite in tropical environments is probably the most peculiar and intense type of weathering of rock occurring on the surface of Earth. It has been extensively documented over the last 40 years because of the economic value of nickel laterites (Trescases 1975; Nahon et al. 1982; Colin et al. 1990; Gleeson et al. 2003). Ultramafic laterites are divided into three main categories that mostly differentiate in the saprolitic horizon, although the type of Ni laterite deposit is only partially controlled by lithology (Gleeson et al. 2003). Each of the three laterite classes may be developed on peridotite, but on dunite protoliths oxide deposits predominate. However, in terms of soil properties, these three types present only slight differences as all of them are classified as Geric Ferralsols. Only a few papers have focused on biological and active soil functioning at the toposequence level and hence provide elements of soil taxonomy (Becquer et al. 2001; Garnier et al. 2009b). Most ultramafic laterites studied worldwide are paleo-laterites that are not actively forming, such as Cuban or Dominican ultramafic laterites that formed in the Tertiary (Aiglsperger et al. 2016). Below we therefore emphasize on soil behaviour rather than try to describe the complete development of laterites. Some of the most developed ultramafic laterite profiles result in 60–100 m (Colin et al. 1990) of weathered material with the formation of a full lateritic horizon, i.e. goethite-dominant limonite that ranges in thickness from 4 m in Niquelândia on pyroxenite (Colin et al. 1990) to 30 m in Goro (New Caledonia) on peridotite (Dublet et al. 2014) or in Moa Bay (Cuba) on non-weathered harzburgite (Aiglsperger et al. 2016). On pyroxenite, the high silica content (twice as high as in other peridotite) allows the formation of smectite at a relatively low depth (Fig. 5b) and therefore limits the development of laterite. Such soils are relatively similar to those found on serpentinite where smectite minerals predominate (Gleeson et al. 2003; Garnier et al. 2009b), also showing a high CEC (high proportion of 2:1 clays) – that is incompatible with Ferralic properties – and Vertic properties due to swelling-shrinking properties of smectite.

Finally, saprolitic horizons (fine and coarse saprolite) are the main resources for Ni mining (Colin et al. 1990). They usually have Ni concentrations higher than 2% (Dublet et al. 2014) but they can reach more than 10% in the case of pyroxenites (Colin et al. 1990). In pedological terms, saprolite corresponds to B/C and C horizons. When the landscape is eroded, what was originally saprolite may reach the surface and the soil may have similar properties as Cambisols developed on serpentinite (Garnier et al. 2009b; van der Ent et al. 2016b). Also, in foothills, colluvial material may accumulate and thus artificially develop the Ferralic horizons as in the case of the Ouénarou toposequence in New Caledonia (Becquer et al. 2001). In the lateritic horizon usually described by miners as 'limonite' or 'laterite,' the mineralogy is dominated by goethite. Hematite is formed at the surface ('red limonite or laterite') as a consequence of extremely hot temperatures reached at the soil surface where affected by sunlight (Fig. 5a). Mineralogical and geochemical changes of iron oxides occur through the distance gradient from the bedrock (Dublet et al. 2014). The formation of ferricrete (iron pan) after crystallization (Fig. 3e) and dehydration of Fe oxides (typically as a result of successive dissolution-crystallization cycles during lateritization) in a significant loss of Ni (Dublet et al. 2015) and a relative gain of trivalent elements (e.g. Al, Cr, Sc), especially in hematite particles (Aiglsperger et al. 2016). Where developed on dunite, the ferricrete can be continuous with hematite being the predominant mineral, whereas goethite is a secondary phase (Colin et al. 1990). On olivine-poor ultramafic bedrock, the formation of ferricrete does not seem to be significant (Colin et al. 1990). Ferricrete dominates in the southeastern part of the main island of New Caledonia where it is continuous over many square kilometres (Fig. 6). The formation of ferricrete is the final evolution of Petroplinthic Rhodic Geric Ferralsols and, after the sealing of soil surfaces, a landscape of Hyperskeletic Leptosols, a few decimetres deep (Fig. 6), develops on a large scale. A rather low vegetation (stunted secondary forest dominated by *Nothofagus* spp., *Arillastrum gummiferum*, and *Gymnostoma* spp.) develops on these soils (Isnard et al. 2016) where most of the nutrients are brought to plant roots by the cycling of organic matter decay.

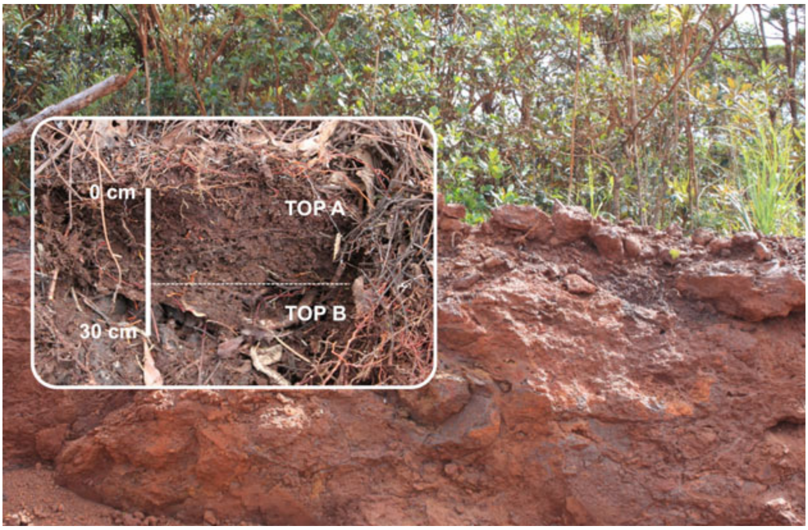

**Fig. 6** Hyperskeletic Mollic Sideralic Leptosol on ancient laterites with a stunted forest dominated by *Gymnostoma* species (Goro, New Caledonia). Photographs: G. Echevarria

## 4 Mining Ultramafic Technosols

After removing the lateritic cover, mining operations are required to rehabilitate the stripped areas by preferentially using the spoil materials (Echevarria and Morel 2015). The construction of mining Technosols should lead the future soil cover in order to fulfil essential ecological and environmental functions (Echevarria and Morel 2015). Ultramafic raw materials (spoils, stripped soils, tailings) are the main geochemical driving force of the new soil cover. Limonitic spoils composed mainly of goethite and hematite, saprolitic spoil composed of a high fraction of clay minerals and chalcedony, are the main materials available for soil construction (Raous et al. 2013). Topsoil is also frequently used to cover the spoil materials after storage, for periods that vary from a few days to several years (Echevarria and Morel 2015). The evolution of topsoil biogeochemistry and biology during storage has been thoroughly studied in a recent project called BIOTOP, which was funded by the New Caledonian Research Center on Nickel (CNRT). The evolution of topsoil geochemistry after stripping and storing (0–-24 months) showed several trends. The storage of topsoil developed on ferricrete does not affect strongly its chemical and biochemical fertility (Echevarria and Morel 2015). Typically, organic amendments or even mineral fertilizers (hydroseeding) are also added for the reconstruction of soil cover on stripped land.

## 5 A Global Model of Ultramafic Soil Evolution

Summarizing the pedogenetical features of all soils developed on ultramafic bedrock worldwide cannot be done on a single figure. However, in most sites from cold to tropical climates, it is possible to highlight the most significant pedogenic processes and the predominant mineral phases that characterise the ultramafic soils known on both non-serpentinized peridotite and serpentinite (Fig. 7). A comparison of the different evolutionary patterns developed in cold,

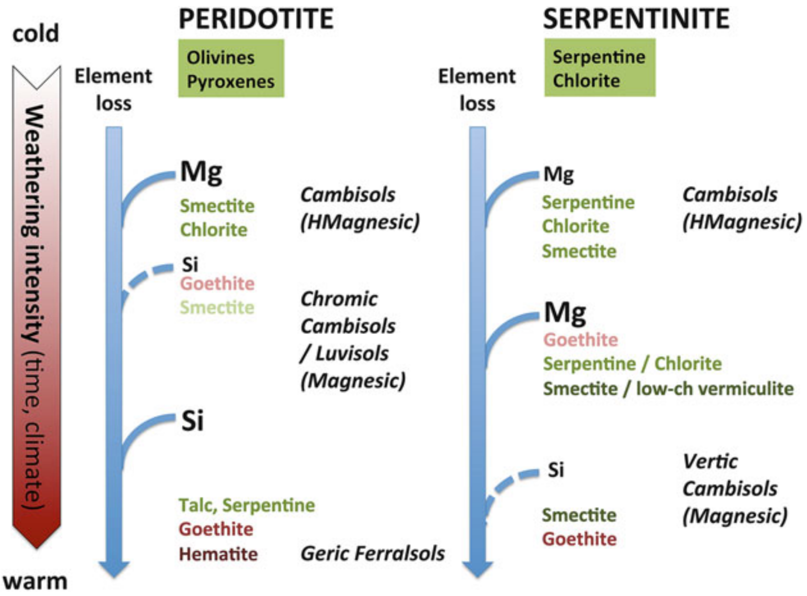

**Fig. 7** A simplified description of soil genesis and evolution on ultramafic bedrock: non-serpentinized peridotite and serpentinite. In warmer climates, the difference between the two types of ultramafic bedrocks becomes more pronounced. The high abundance of serpentine minerals impedes the formation of a lateritic (Ferralic) horizon and gives to the soil Cambic and Vertic properties, mainly inherited from the high proportion of smectites. Pyroxenite will produce a similar type of pedogenesis than serpentinite

temperate, and Mediterranean regions, based on results from the literature, showed that Mg is extremely depleted and reduces from nearly 20% of the mass of the bedrock to less than a few percent (Kierczak et al. 2007). Silicon is also depleted, especially in Mediterranean conditions. As a result, the relative concentrations of Fe and Cr increase in the soil (with a strong impact on soil redness), as well as those of other stable metals (i.e. Ti and Nb), whereas Ni is slightly depleted relatively in temperate soils and is stable in Mediterranean soils. As a matter of fact, 85–90% of the Ni is lost during pedogenesis (Estrade et al. 2015). The presence of serpentine will considerably reduce the loss of Si, and most mineral phases will be primary and secondary clay minerals.

## 6 The Fate of Nickel During Ultramafic Soil Evolution

Nickel in the ultramafic bedrock is borne by serpentine, olivine, and pyroxene grains with an average concentration of 1200–3800 μg $g^{-1}$ for serpentine grains. In Magnesic Cambisols, both poorly crystallized Fe-oxides (Chardot et al. 2007) and possibly Mn-oxides (Alves et al. 2011), and secondary 2:1 clays such as Fe-rich smectite (Bani et al. 2014) will hold most Ni in the soil. However, weathered serpentines will contain higher concentrations of Ni than those present in the bedrock, i.e. 6000 μg $g^{-1}$ (Bani et al. 2014). Progressively, the formation of smectite will produce Ni-rich particles that may in places reach several percent, including under non-tropical conditions (Bani et al. 2014). When lateritization occurs, Ni from the first few meters is leached down the profile when Fe-oxides crystallize and is transferred to clay minerals in deep saprolite (Colin et al. 1990; Dublet et al. 2014). These saprolite clays that trap Ni can be smectite (Colin et al. 1990; Raous et al. 2013), talc (Becquer et al. 2006), or serpentine (Dublet et al. 2012). On top of the saprolite layer, Mn-oxides (e.g. asbolane, lithiophorite) also trap Ni and Co (Llorca and Monchoux 1991; Dublet et al. 2014). However, Ni is also concentrated in goethite from the yellow limonite horizon that has not undergone such pervasive dissolution-recrystallization processes (Dublet et al. 2015).

## 7 Nickel Availability in Ultramafic-Bearing Minerals and Soils

### 7.1 Methods of Evaluating Nickel Availability

Current methods to assess and characterize Ni chemical availability in ultramafic soils are described by numerous workers (Echevarria et al. 1998, 2006; Chaney et al. 2005; Garnier et al. 2006; 2009a; Raous et al. 2013; Zelano et al. 2016a, b), and include:

- Single chemical extractions using salts—$CaCl_2$ and $Sr(NO_3)_2$;
- Single chemical extractions using chelating agents—DTPA and EDTA;
- Sequential chemical extraction procedures; and
- Isotopic exchange and dilution techniques (IEK).

The last are useful and complete tools for measuring the true phytoavailability of trace elements in soils (Echevarria et al. 1998; Massoura et al. 2004). Measured by IEK, Isotopically-exchangeable Ni during time t ($E_t$) refers mostly to the element retained through sorption and surface-complexation onto soil particles. Soil labile Ni—$E_t$—is highly dependent on the chemical state of the element, i.e. inclusion in crystal lattices (Massoura et al. 2006; Zelano et al. 2016a, b). The exchangeable metal for a very short period of time ($E_{0\text{–}1\ \text{min}}$) corresponds to the fraction that is weakly bound to the soil solid phase (e.g. CEC). Isotopically exchangeable pools having longer periods of exchange (i.e. 1 min to 3 months) are typically linked to diffusion-limited sorption sites (Massoura et al. 2006; Zelano et al. 2016b).

The DTPA extraction of Ni in soils is a test that provides a good field tool for assessing ecotoxicological risk (L'Huillier and Edighoffer 1996; Echevarria et al. 1998, 2006). The Ni pool extracted by DTPA was significantly correlated with medium-term, isotopically exchangeable, i.e. $E_{0\text{–}3\ \text{months}}$, which is the same source of Ni uptake for plants with contrasted demand for Ni (Echevarria et al. 2006). Recently, it was shown that DTPA-extracted Ni has a similar stable isotope composition as soluble Ni, whereas soil Ni has a much lighter isotope composition (Estrade et al. 2015). The DTPA extraction thus accesses only exchangeable and surface-complexed Ni that is eventually available and accessible to plants (Echevarria et al. 1998).

In 100 very contrasted soil samples that represent the range of origin and total content of Ni in soils worldwide, including ultramafic soils, IEK methods and DTPA extractions have shown that the availability of Ni is mainly controlled by soil pH (Echevarria et al. 2006). As expected, soil pH controls the intensity (i.e. Ni concentration in solution). In 16 naturally and anthropogenically Ni-rich soils selected from various weathering conditions, Ni was found to be present in either primary phyllosilicates (i.e. serpentine, chlorite, talc), secondary clay minerals or Fe-Mn oxyhydroxides (Massoura et al. 2006; Echevarria et al. 2006). The availability of Ni from primary clay minerals was low and attributed to the presence of Ni in the crystal lattice (Zelano et al. 2016b). Nickel in secondary clay minerals (e.g. smectite) was probably sorbed onto the mineral surfaces and its availability was very high (Massoura et al. 2006; Raous et al. 2010, 2013). Bioavailability of Ni in goethite-dominant soils was extremely poor, because these minerals act as a stable sink for the metal in both natural and contaminated Ni-rich soils (Massoura et al. 2006; Raous et al. 2010, 2013; Zelano et al. 2016a, b). The response of excluder, accumulator and hyperaccumulator plants to Ni availability assessed by IEK methods showed that all plants took up Ni from the same labile pools of Ni in soils, regardless of mechanisms of root uptake, translocation, and storage (Massoura et al. 2004; Echevarria et al. 2006). This finding validates IEK as a universal method for assessing Ni availability in soils and other environmental solid matrices (Zelano et al. 2013, 2016a, b).

## 7.2 Main Ni-Bearing Phases in Ultramafic Soils and Ni Availability Properties

The use of isotope exchange kinetics to describe Ni availability properties of pure Ni-bearing minerals were introduced over decade ago (Echevarria et al. 2006; Massoura et al. 2006), as Ni-bearing phases in ultramafic soils were shown to almost be the sole control on Ni availability, with only a limited effect by pH. The Ni availability properties of single pure minerals were described for chrysotile, which was purified from an asbestos mine spoil (Chardot-Jacques et al. 2013), and for Ni-rich synthetic goethite (Massoura 2003), and fully explained the resulting Ni transfer to plants in both cases. Recently, Zelano et al. (2016a) detailed isotopically exchangeable properties of single pure mineral phases that are commonly found in ultramafic soils, with $E_t$ values being a first-order kinetic of time, and specific for each Ni-bearing phase. Furthermore, these workers modelled the bulk Ni availability of soils by computing the linear combination of properties of single minerals according to their relative proportion in the studied soils (Zelano et al. 2016a). It was verified, in most cases, that the computed values were accurate and that Ni-bearing phases contributed to soil Ni availability in this manner. This approach was successfully applied to natural ultramafic soils and mining of Technosols (Zelano et al. 2016b), thus assigning to each phase its contribution in Ni availability in the bulk soil.

No study has been performed on primary olivine and pyroxene assuming that the only release of Ni from these silicates comes from their dissolution (Chardot-Jacques et al. 2013). Serpentine minerals in unweathered bedrock have been reported to contain from 0.12 to 0.38 wt% Ni (Caillaud et al. 2009; Yongue-Fouateu et al. 2006; Quantin et al. 2008; Bani et al. 2014;

Kierczak et al. 2016), with a surprising value of 1.99 wt% reported in specific conditions (Quantin et al. 2008). In cold temperate conditions, the serpentine Ni content in the BW horizon remains similar to that in the bedrock: 0.18 wt% (Chardot et al. 2007). However, in the B horizons of Cambisols and Vertisols developed on serpentinite under Mediterranean conditions, it seems that the Ni content in serpentine minerals is slightly higher (e.g. 0.60–0.85 wt%) than in the original bedrock (0.30 wt%), probably because these serpentines underwent the first steps of weathering (Bani et al. 2014). In the saprolite layers of Geric Ferralsols, serpentine minerals such as nepouite and garnierite can contain 20 wt% Ni as a result of neoformation (Fig. 3d). The availability of Ni in primary phyllosilicates such as chrysotile is very limited (Chardot-Jacques et al. 2013), with Ni concentrations in solution that are very low at ca. a few μg $L^{-1}$, exchangeable sites that are very limited (a few % of total Ni), and a strong diffusion-limited component that slowly releases soluble and exchangeable Ni over time (Zelano et al. 2016a). Where plants are grown on this sole substrate, Ni absorption by roots is mainly fed by chrysotile dissolution instead of by labile pools (Chardot-Jacques et al. 2013).

Chlorite and talc in ultramafic soils can be both primary (Lee et al. 2003) or secondary (Becquer et al. 2006) minerals. Talc is a 1:1 clay mineral that is extremely resistant to pedological weathering and in places is the only clay mineral present in Ferralic horizons of ultramafic laterites (Becquer et al. 2006). The CEC of such talcs is almost null, which explains that the availability of Ni in talc (e.g. deweylite) is so scarce that it is lower than that of chrysotile (Zelano et al. 2016a). The availability of Ni in chlorite minerals is more than twice that of chrysotile (Zelano et al. 2016a), as it is a 2:1 clay mineral having much higher CEC. Although chlorite is more common at the surface than in the $B_W$ and C horizons of Hypereutric Cambisols in the Vosges Mountains (Massoura et al. 2006; Chardot et al. 2007), Ni availability is much higher in the lower horizons and negatively correlates with chlorite content. Chlorite is, therefore, not a significant contributor to Ni availability in ultramafic soils (Massoura et al. 2006).

Smectite is a high-exchange, high-specific, surface secondary clay. The Ni content of smectite in ultramafic soils is also highly variable and no specific trend in terms of geochemical composition (Mg-rich *vs*. Fe-rich), weathering intensity, or climatic conditions seems to influence the Ni content. Nickel concentrations in smectite range from 0.49 to 3.12 wt% in Temperate B horizons (Caillaud et al. 2009; Bani et al. 2014), whereas they range from 0.40 to 14.9 wt % in a tropical $B_W$ horizon of a Cambisol developed on pyroxenite (Garnier et al. 2009b). In the saprolite horizons of Geric Ferralsols, Ni typically ranges from 1.06 to 1.33 wt% (Yongue-Fouateu et al. 2006; Raous et al. 2013), as in temperate soils. But concentrations can exceptionally reach 13.3–25.9 wt% Ni in Fe-rich smectite of Geric Ferralsols developed on pyroxenite in Niquelândia (Decarreau et al. 1987). Ni-bearing smectite is a major source of available Ni in ultramafic soils (Bani et al. 2007; Raous et al. 2013; Zelano et al. 2016a), and its availability is similar to that of Mg released under similar conditions (Raous et al. 2010). The concentration of Ni measured in solution during IEK of smectite is one of the highest of all Ni-bearing minerals, with 0.25–0.5 μg $L^{-1}$ (Massoura 2003; Bani et al. 2007). Instantaneously exchangeable pools (i.e. $E_{0\text{–}1\ \text{min}}$) are also the highest, reaching more than 100 μg $g^{-1}$ after one minute (Massoura 2003; Bani et al. 2007; Zelano et al. 2016a) as a result of the high content of exchangeable Ni present in the CEC (Raous et al. 2013).

Fe- and Mn-oxyhydroxides are important scavengers of Ni in ultramafic soils. When free Fe is released during the first stages of weathering, Ni is rapidly sorbed onto, and incorporated in, amorphous Fe-hydroxides (Chardot et al. 2007). When goethite (a significant mineral in ultramafic Cambisols) is formed, the affinity to Ni seems to decrease (Massoura et al. 2006; Chardot et al. 2007; Bani et al. 2009) and the ratio of Nio/Nid is usually the double as Feo/Fed (Chardot et al. 2007);

however, Ni contents in goethite can range from 0.8 to 4.3 wt% in these soils. Goethite in the lateritic horizons of Geric Ferralsols ranges from 1.7 wt% Ni% in the upper layers to 3.8 wt % in the deeper parts of the Ferralic horizon (Becquer et al. 2006). The same concentrations have been reported in other Geric Ferralsols (Yongue-Fouateu et al. 2006). Nickel concentration in Fe-oxides decreases from the bottom of the Ferralic horizons up to the surface, by about a factor 5–10 as a result of goethite aging (Dublet et al. 2015). This process involves dissolution and recrystallization. Hematite particles are even poorer in Ni than the surface goethite (Raous et al. 2013). The isotopically exchangeable pools of Ni in ultramafic soils were shown to correlate with concentrations of amorphous Fe-oxyhydroxide or with the ratio of amorphous to well-crystallized Fe-oxides (McKeague and Day 1966; Massoura et al. 2006). Manganese oxides, although a minor phase quantitatively in temperate soils, also have significant concentrations of Ni that are suspected to be highly available (Alves et al. 2011). In tropical laterites, Mn-oxides play a significant role in that Ni-bearing minerals and heterogenite can hold up to 20 wt% Ni, followed by lower Ni contents in lithiophorite and asbolane (Llorca and Monchoux 1991; Decrée et al. 2015). These minerals are the most important scavengers for cobalt; little is known about Ni availability in such well-characterised Mn-oxides.

Amorphous Fe-oxides are not easily isolated in pure form from bulk soils, so it is impossible to assess the specific availability of Ni in these phases. The available properties of Ni borne by goethite particles have been studied by several workers (Massoura 2003; Raous et al. 2013; Zelano et al. 2016a, b). Nickel availability associated with synthetic goethite can be nearly null (Massoura 2003), with no proof of isotopic exchange between Ni included in the crystal lattice and free Ni present in solution. This is confirmed by the absence of Ni in the CEC of limonite where Ni is contained mostly in goethite particles (Raous et al. 2013). Goethite has some potential to bear isotopically exchangeable Ni as surface-complexed Ni (Raous et al. 2013; Zelano et al. 2016a). However, Ni availability associated with synthetic goethite is extremely low relative to other ultramafic Ni-bearing phyllosilicates (Zelano et al. 2016a). Unsurprisingly, the availability of Ni from hematite is null (Zelano et al. 2016a).

Available Ni (DTPA-extractable) is strongly positively correlated to organic C in an ultramafic toposequence in Albania (Bani et al. 2014). The labile pool associated with organic matter in a Brazilian ultramafic soil also accounts for most isotopically exchangeable Ni of the whole soil, 3 wt% of the soil mass provide 60 wt% of labile Ni (Zelano et al. 2016b). Little attention has been devoted to the role of organic-borne Ni in ultramafic soils, but recently it was shown that plants and litter are a major contributor to total Ni in surface soils (Estrade et al. 2015), which is in turn highly available. It is, therefore, crucial to describe better the pools of available Ni associated with organic matter and detail the speciation of Ni and the chemical bounds that exist to organic matter.

## 8 The Biogeochemical Cycling of Nickel in Ultramafic Soils

The use of nickel stable isotopes has recently been introduced in studies of ultramafic environments to elucidate the pathways and mechanisms of nickel biogeochemistry (Estrade et al. 2015; Ratié et al. 2015): mineral weathering, nickel fluxes, root uptake, and plant translocation. Typically, the weathering of primary minerals during pedogenesis induces a loss of heavy isotopes by leaching processes, so the resulting soil has isotopically lighter Ni than the parent bedrock. The soluble and available (i.e. DTPA-extractable) fraction of Ni in soils is therefore much heavier, isotopically, than Ni in the soil solid phase (Estrade et al. 2015; Ratié et al. 2015). In a Chromic Cambisol, the upper $A_1$ (0–3 cm) horizon presents a significantly lower degree of fractionation from bedrock than the deeper horizons (i.e. A/Bw and Bw). The isotopic compositions of Ni in litter are all heavier than in corresponding rhizosphere soils, suggesting a significant contribution from decaying plant material that contains a much greater proportion of isotopically heavier Ni than the bulk soil (Estrade et al. 2015). The reason for this is that plants take up soluble Ni from the soil, which is the heaviest isotopic pool of Ni present in the soil. In a ploughed ultramafic Vertisol, this relationship could not be observed because the organic matter residues in this soil are diluted in the Ap horizon (0–20 cm).

In May 2013, five plants of the Ni-hyperaccumulator species *Alyssum murale* were sampled at flowering stage on a Eutric Vertisol (Hypermagnesic) in Prrenjas (Albania) where no other vegetation had been growing for at least 12 months. Plants were distant from each other with at least a 1 m distance (details in Bani et al. 2015). In this study, a careful sampling of plant leaves, deposited as litter beneath each plant, and surface soil samples clear of any litter particles (from a 0–3 cm depth) were taken (five replicates). In parallel, soil samples (from a 0–3 cm depth) were taken randomly in bare soils at a minimal distance (2 m) from each plant to ensure that there would be no influence of *A. murale* on the geochemistry of the soil sample. All plant and litter samples were analyzed for total element concentrations after complete digestion with concentrated $HNO_3$. Soil samples were digested with concentrated HF for total dissolution of minerals. Digestion solutions were analyzed using ICP-MS after appropriate dilutions. The results (Table 3) clearly showed that total Ca, K, and Ni were significantly enriched in the soil underneath *A. murale* after only one growth cycle of the plant (9–12 months). Moreover, the Ni contained in plant leaves after decaying was leached away after only a few months (by almost 50%), which was the time equivalent to the age of the deposited litter. Potassium was even more affected by leaching (>66% of initial K). This experiment brings additional understanding to what had been previously shown with stable isotope studies on Ni in topsoil and litter (Estrade et al. 2015): the contribution of plants to the building up of Ni concentrations in topsoil. Furthermore, the majority of Ni present in the primary minerals is leached away during pedogenesis (Kierczak et al. 2007; Estrade et al. 2015), thus resulting in a Ni pool that is enriched in lighter isotopes. So, the existing Ni contents in topsoils, which have a Ni isotopic composition strongly influenced by plant recycling (Estrade et al. 2015), probably result from significant biogeochemical recycling. Nickel hyperaccumulator plants, which can be quite common in these environments, likely contribute in a major way to this phenomenon. More detailed studies should be carried out by tracing Ni fluxes using stable isotopes (Deng et al. 2016) in soil-plant systems that are representative of various edaphic situations worldwide, in order to shed more light on the importance of Ni biogeochemical cycling in ultramafic soils and related ecosystems.

## 9 Conclusions: What Are Trends of Nickel Availability in Ultramafic Soils Globally?

At first glance, it seems that soils developed on serpentinite are much richer in available Ni than those developed on non-serpentinized peridotite (van der Ent et al. 2016a). However, it is not that simple, because extremely well-developed Geric Ferralsols may occur on serpentinite (Yongue-Fouateu et al. 2006), at the surface of which is a

**Table 3** Element concentrations in leaves, litter and soils under the influence of the Ni-hyperaccumulator *Alyssum murale* grown on a Eutric Vertisol (Hypermagnesic) in Albania. Mean values of five replicates that are followed by different letters indicate a significant difference at the $p < 0.05$ level (ANOVA)

| Compartment | Nickel (g $kg^{-1}$) | Calcium (%) | Potassium (%) |
|---|---|---|---|
| Leaves | 19.0 | 3.57 | 1.50 |
| Litter (current year) | 9.22 | 2.06 | 0.53 |
| Soil under litter at 0–3 cm | 3.03 a | 0.72 a | 0.34 a |
| Bare soil (no litter) at 0–3 cm | 2.84 b | 0.69 a | 0.32 a |

very low availability of Ni. At the same time, Cambisols with extremely high Ni availability may exist on non-serpentinized pyroxenite (i.e. peridotite) (Garnier et al. 2009b). Nickel is a relatively mobile metal in ultramafic environments and its resulting total concentration in most temperate and Mediterranean soils, although of the same order of magnitude as the bedrock concentration, results from the loss of more than 85% of its initial mass in the bedrock (Kierczak et al. 2007; Estrade et al. 2015). It is frequently seen that Ni is as easily released and mobilized as magnesium during the steps of soil weathering and pedogenesis (Raous et al. 2010; Chardot-Jacques et al. 2013). The difference between these elements is the fact that Ni forms stable complexes with organic matter and Fe- and Mn-oxyhydroxides (especially amorphous Fe-oxyhydroxides). Therefore, Ni is retained in a certain number of pedological situations in which Mg is not, thus explaining the differences in losses between the two elements during pedogenesis (Kierczak et al. 2007). The availability of Ni in soils is therefore favoured by the following mechanisms that may occur in various edaphic conditions:

- Dissolution/weathering of primary minerals holding Ni (Chardot-Jacques et al. 2013).
- Building up a CEC from the neoformation/transformation of primary silicates into 2:1 clays and from the incorporation of organic matter in the substrate (Lee et al. 2003; Bani et al. 2014), and the subsequent uptake of Ni released by mineral dissolution.
- The formation of amorphous Fe-oxyhydroxides that have a CEC at high pH values (typically above 7–8, which is the usual pH of slightly weathered ultramafic soils), and to which Ni is easily and reversibly sorbed (Chardot et al. 2007) after being released during weathering.

Under tropical conditions, the intense loss of Si during lateritisation favours the formation of Fe-oxides (e.g. goethite) that incorporate high concentrations of Ni during the phases of dehydration of Fe-hydroxides, despite a lower affinity of Ni to crystallized Fe-oxyhydroxides (Massoura et al. 2006). Further evolution of goethite can lead to an important loss of Ni (Dublet et al. 2015) in which Ni contents in hematite may reach those of the bedrock. Nickel is known to be unavailable from well-crystallized Fe-oxides (e.g. hematite) that occur in ferricrete (Zelano et al. 2016a). In the saprolite layers of Geric Ferralsols, Ni that is progressively leached by lateritization and the maturation of Ni-bearing Fe-oxides is accumulated to high concentrations in phyllosilicates (serpentine, talc, smectite), which commonly represent the best Ni lateritic ore. The prime example is saprolitic smectite (Decarreau et al. 1987) found on the pyroxenite of Niquelândia (Brazil), where Ni concentration reaches >20 wt%. The Ni borne by saprolitic smectite is one of the most available sources of Ni in soils globally (Raous et al. 2013). Vertic Cambisols Hypermagnesic described on many serpentinite outcrops in the tropics, such as in Cuba, Borneo, and New Caledonia (Proctor 2003), also display very high Ni availability, for the same geochemical and mineralogical reasons. Where natural erosion or mining has removed the lateritic horizons of ultramafic Ferralsols, the resulting soils have similar geochemical properties as the natural Vertic Cambisols Hypermagnesic found on serpentinite (Garnier et al. 2009b). Tropical Ni hyperaccumulators are generally native to these two types of environments (van der Ent et al. 2016a). Geric Ferralsols having well-established ferricrete lack hyperaccumulator species owing to the absence of available Ni (Raous et al. 2010, 2013; Zelano et al. 2016a; van der Ent et al. 2016a).

Finally, vegetation and possibly Ni-hyperaccumulators, are very active at recycling Ni in topsoil (Estrade et al. 2015). As such, hyperaccumulator plants provide organic matter that increases the CEC and releases significant amounts of Ni through litter and the decay of organic matter. The result is a very mobile pool of Ni in the surface of soils (Bani et al. 2014). The role of biogeochemical recycling for maintaining sufficient levels of essential elements in acidic forest top soils have been elucidated recently (Laclau et al. 2010). It is probable that the same processes control Ni geochemistry in ultramafic soils and ecosystems worldwide.

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# The Role of the Rhizosphere and Microbes Associated with Hyperaccumulator Plants in Metal Accumulation

Emile Benizri and Petra S. Kidd

**Abstract**

Phytomining can be limited by low biomass productivity by plants or limited availability of soil metals. Ongoing research attempts to overcome these potential constraints and to make phytomining a successful commercial technique in the recovery of metals from polluted or naturally metal-rich soil by (hyper)accumulating plants. Recently, the benefits of combining phytoremediation with bioremediation, which consists in the use of beneficial microorganisms such as endophytes or rhizosphere bacteria and fungi, for metal removal from soils have been demonstrated. Metal-resistant microorganisms play an important role in enhancing plant survival and growth in these soils by alleviating metal toxicity and supplying nutrients. Furthermore, these beneficial microorganisms are able to enhance the metal bioavailability in the rhizosphere of plants. An increase in plant growth and metal uptake increases the effectiveness of phytoremediation processes coupled with bioremediation. Herein, we highlight the specificity of the rhizosphere and the critical roles in soil nutrient cycling and provision of ecosystem services that can be brought by rhizosphere microorganisms. We discuss how abiotic factors, such as the presence of metals in polluted sites or in naturally rich (ultramafic) soils modulate activities of soil microbial communities. Then we introduce the concept of microbe-assisted phytomining, and underline the role of plant-associated microorganisms in metal bioavailability and uptake by host plants that has attracted a growing interest over the last decade. Finally, we present various techniques, including phenotypic, genotypic, and metagenomic approaches, which allow for characterising soil microbial community structure and diversity in polluted or naturally metal-rich soils.

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# 1 Introduction

## 1.1 Physico- and Bio-chemical Properties

The rhizosphere is defined as the volume of soil adjacent to, and influenced by, living plant roots (Hiltner 1904; Nguyen 2003). Generally, it has a thickness of 1–2 mm, but functionally, the rhizosphere can be defined as the soil portion that is physically and chemically influenced by root growth and activity. Its dimensions can therefore vary depending on the mass and architecture of plant roots. As a result of various biotic and abiotic factors, such as root exudation, competition for Fe and carbon, inorganic nutrients, and soil pH and temperature, rhizosphere microbial communities are distinct from those inhabiting non-rhizosphere or bulk soil (Baudoin et al. 2003).

The rhizosphere is a region of intense microbial activity, which is mainly driven by root exudation. Between 40% and 90% of the carbon transferred to the root is lost through rhizodeposition in the soil and represents an important flux of carbon in the rhizosphere (Nguyen 2003). Five kinds of rhizodeposits are recognized: diffusates, secretions, lysates, gases, and mucilage (Nguyen 2003). Diffusates are water-soluble compounds of low molecular weight such as sugars, organic acids or amino acids that diffuse passively through the cell envelope or among epidermal cells, due to concentration gradients between the exterior and interior of the root. Secretions are made up of high molecular weight compounds that are actively secreted by the roots. Lysates are the organic material released into the soil by dead cells after autolysis. Gases are mainly ethylene, carbon dioxide, and hydrogen cyanide. Mucilage, used for improving root penetration into soil, is composed of polysaccharides.

The role of root rhizodeposits as a nutrient source for microorganisms has been demonstrated many times as well as their implication as key determinants of rhizosphere microbial community structure (Baudoin et al. 2003). The type and composition of root secretions can alter soil microbial dynamics and diversity, favouring the growth of microorganisms that can benefit plant health and crop productivity, or in other cases act against harmful microbes (Chaparro et al. 2012; De-la-Peña et al. 2014). Indeed, the plant provides microbial substrates, together with hormones and vitamins, as well as toxins and other antagonistic compounds that may exert a selective effect on microorganisms (Warembourg 1997). Phytochemicals collected from the root exudates of *Arabidopsis thaliana* added to the soil have shown how important these compounds are to the modulation of microbe composition (Badri et al. 2013). Moreover, rhizodeposition is known to display quantitative and qualitative variations bewteen different plant species, and it has long been hypothesised that the diversity of microorganisms present in different plant rhizospheres, and especially their functional abilities, may be linked to these variations in rhizodeposits (Grayston et al. 1998; Baudoin et al. 2002).

## 1.2 Microbial Activity and Communities

Soil microbes including bacteria, archaea, and fungi, play diverse and often critical roles in soil nutrient cycling and the provision of ecosystem services (Table 1). Compared to bulk soil, the rhizosphere soil is characterized by higher concentrations of nutrients and labile organic C (Duineveld et al. 2001). Consequently, it represents a unique 'hot spot' in terms of microbial ecology, because soil microorganisms are stimulated by root activity (Hinsinger et al. 2006). Soil microorganisms drive, via their enzyme activities, the cycling of all major elements (e.g. C, N, P and S). This cycling affects the structure and functions of soil ecosystems as well as the ability of soils to provide ecosystem services.

Microbial enzyme activities in the rhizosphere can be of intracellular origin, released

**Table 1** Roles of soil microbes in provisioning and regulating ecosystem services

| Role of soil microbes | Soil services |
|---|---|
| Microbial products are critical to soil aggregation, improved soil structure making soil more habitable for plants | Physical support |
| Soil microbes mobilize nutrients from insoluble minerals to support plant growth | Growth medium for plants |
| Soil microbial activity drives nutrient cycling and organic matter production, thus contributing to soil formation | Nutrient cycling |
| Beneficial species include bacteria that support plant growth through increasing nutrient availability and by outcompeting invading pathogens | Biological control of pests, weeds and pathogens |
| By mineralizing soil carbon and nutrients, microbes are major determinants of soil carbon storage capacity | Carbon storage |

*Adapted from* Dominati et al. (2010), Aislabie and Deslippe (2013)

**Table 2** Role of soil enzymes

| | Organic matter Substances | | | |
|---|---|---|---|---|
| Enzyme | Acted on | End product | Significance | Predictor of soil function |
| Beta glucosidase | Carbon compounds | Glucose (sugar) | Energy for microorganisms | Organic matter decomposition |
| FDA hydrolysis | Organic matter | Carbon and | Energy and nutrients for microorganisms, measure | Organic matter decomposition |
| | | Various nutrients | Microbial biomass | Nutrient cycling |
| Amidase | Carbon and nitrogen compounds | Ammonium ($NH_4$) | Plant available $NH_4$ | Nutrient cycling |
| Urease | Nitrogen (urea) | Ammonia ($NH_3$) and | Plant available $NH_4$ | Nutrient cycling |
| | | Carbon dioxide ($CO_2$) | | |
| Phosphatase | Phosphorus | Phosphate ($PO_4$) | Plant available P | Nutrient cycling |
| Sulphatase | Sulphur | Sulphate ($SO_4$) | Plant available S | Nutrient cycling |

*Modified from* Tabatabai (1994), Dick (1997), Bandick and Dick (1999)

after microbial cell disruption, and may be associated with soil colloids and cell debris, or enzymes may be actively secreted by root-associated microorganisms. Soil enzyme activities have been reported to be used as a unique integrative biochemical assessment of soil function and condition (Naseby and Lynch 2002), and to be useful indicators of soil functional diversity (Bending et al. 2002; Sowerby et al. 2005; Epelde et al. 2008). The polymeric C and N components of plants (and microbes and animals) are both structurally complex and highly diverse, and their breakdown requires the combined activities of many different microorganisms. Some enzymes only facilitate the breakdown of organic matter (e.g. hydrolase, glucosidase), whereas others are involved in nutrient mineralization (e.g. amidase, urease, phosphatase, sulphatase) (Table 2). The presence of rhizosphere microorganisms increases the ability of plants to acquire nutrients, by either increasing the extent of the root system (through fungal hyphae), or by solubilizing macronutrients such as P or S (Smith and Read 1996). In natural systems, the action of soil microorganisms is a major determinant of efficient nutrient cycling. However, soil microorganisms also influence plant-pathogen interactions and/or plant growth by producing antibiotics or hormone compounds (discussed in the following sections). The activities of soil microorganisms are influenced by various environmental parameters, e.g. soil type, nutrient status, pH, texture, organic matter content, moisture, and their interactions, as well as plant factors such as species, age and root zone (Baudoin et al. 2002).

Among abiotic factors, metals present in contaminated sites or in naturally rich (such as ultramafic) soils also modulate activities of soil microorganisms and interactions among them (Krumins et al. 2015). The interaction of metals with native soil communities and their activities is an important area of research as scientists strive to understand the effects of metal pollution or enrichment on soil properties (Hagmann et al. 2015). Effects of trace elements on enzyme activity in the rhizosphere are complex and contrasting effects can be observed (Egamberdieva et al. 2011). Kandeler et al. (2000) found that C-acquiring enzymes (cellulase, xylanase, b-glucosidase) were the least affected by soil pollution, phosphatase and sulphatase being the most affected; finally, N-acquiring enzymes (urease) had an intermediate response. In a study of metal-polluted grassland soil (Kuperman and Carreiro, 1997), b-glucosidase was the most depressed, whereas phosphatase and endocellulase activities were the least. Renella et al. (2011) reported that soil arylesterase activity was negatively correlated with exchangeable and soluble Cd fractions, although soil pH values and organic matter were important covariates. Nonetheless, in general, arylsulphatase appears to be most sensitive to metal pollution, whereas acid phosphatase and urease are less affected (Dick 1997).

An excess of metals can negatively affect the metabolic function of soil microorganisms. For instance, both the decomposition of organic matter and microbial activity were inhibited in soils polluted with trace metals (Fritze et al. 1997). The addition of trace elementss such as Cd, Cr, Cu, Ni, Pb, and Zn to soil significantly inhibited N mineralization and nitrification (Liang and Tabatabai 1978). Influences of trace elements on soil microbial activities vary with the kind of trace metal and soil type and properties. Doelman (1985), who reviewed the literature on the effects of Cd, Cr, Cu, Hg, Ni, Pb and Zn on soil microbial activities, showed that the effect of Hg was the most significant and that of Pb was the least significant. These variations may be ascribed to differences in toxicity of the metals to soil microorganisms and the form of the metals in the soil. High Cd concentrations (40 μg $g^{-1}$ soil) inhibited soil acid phosphatase, alkaline phosphatase, and urease activities (Renella et al. 2006). In another study, the presence of Cd (12 μg $g^{-1}$ soil) and Zn (300 μg $g^{-1}$ soil) negatively affected ammonifying microorganisms in loamy sand soil (Wyszkowska et al. 2009). A higher concentration of Cu (150–450 μg $g^{-1}$ soil) significantly inhibited the activities of soil dehydrogenase, urease, and catalase (Wyszkowska et al. 2008).

However, such toxic effects may not always be observable owing to limited soil metal bioavailabilty or the development of metal resistance by the bacterial community (Nannipieri et al. 2012). Niklinska et al. (2006) found no differences between Zn- and Cu-polluted and non-polluted sites regarding the activity of microbial communities. Similarly, Pessoa-Filho et al. (2015) concluded that activities of enzymes related to C, P and S cycles were unaffected by high Ni concentrations; changes in soil microbiological functioning observed in their study were more related to organic matter content than to Ni availability. Epelde et al. (2008) confirmed that metal pollution did not cause a clear inhibition of soil enzyme activities. Metals are toxic to living organisms, primarily due to their protein-binding capacity and hence ability to inhibit enzymes (Dick 1997). However, the nature and degree of this inhibition is strongly related to soil type, pH, organic matter content, and interactions with other soil minerals and organic matter (Tate 2002). Moreover, Hattori (1989) reported that the influence of Cd on the mineralization of carbon and nitrogen varied with the content of water-soluble Cd in soil.

## 2 The Concept of Microbe-Assisted Phytomining

Experimental evidence clearly shows that root proliferation and effective root uptake mechanisms are among the key processes distinguishing metal hyperaccumulator plants from ‘normal’ plants. On the other hand, the role of plant-associated microorganisms in trace element bioavailability and their uptake by host plants is less well known and has attracted a growing interest over the last decade. Toxic concentrations of trace element have often been suggested to reduce microbial densities and diversity in metal-enriched soils (Pal et al. 2005). In fact, microorganisms are ubiquitous in soils to which metal-hyperaccumulating plants are native, despite the presence of high concentrations of metals (Abou-Shanab et al. 2003a). During the late 1990s and early 2000s, several studies suggested that the accumulation of metals by (hyper)accumulating plants was influenced by their rhizospheric microflora (Mengoni et al. 2001; Lodewyckx et al. 2002; Abou-Shanab et al. 2003a). Comparisons between sterile and non-sterile systems showed that metal accumulators in plants only reached their full accumulation capacity in the presence of indigenous rhizosphere microflora. Since then, a large number of studies have indicated that microbiota are essential players during metal phytoextraction or phytomining (Lebeau et al. 2008; Kidd et al. 2009; Glick 2010; Becerra-Castro et al. 2013; Sessitsch et al. 2013). Moreover, it appears that the plant host can select metal-tolerant microbes out of the enormous pool and diversity of bacteria present in the bulk soil (Mengoni et al. 2001; Becerra-Castro et al. 2009; Alvarez-Lopez et al. 2016a). Plants can also promote the abundance of beneficial microorganisms (such as plant growth-promoting bacteria, PGPB) in their surroundings (Vessey 2003; Thijs et al. 2016). Plants employ various strategies to increase metal bioavailability, such as the secretion of phytosiderophores, carboxylates, and acidification of the rhizosphere (Kinnersley 1993), but soil microorganisms are also known to influence biogeochemical cycling of soil metals. The potential benefits of microbes associated with metal-accumulating plants led to the concept of microbial-assisted phytoextraction or phytomining (MAP) (Thijs et al. 2016). MAP aims to enhance metal recovery rates by inoculating metal (hyper)accumulating plants with plant-associated microbes, which are able to increase the metal accumulation capacity of the phytoextracting plants, by: (i) improving plant biomass and thus metal yield, and/or (ii) increasing the bioavailability of metals and thus plant uptake and bioaccumulation.

Characterizing the structure and genetic diversity of microbial communities associated with hyperaccumulators, and unravelling the complex plant-microbial-soil interactions occurring in the rhizosphere, will contribute towards our understanding of the metal hyperaccumulation process and may help enhance phytomining technology. In this chapter, we focus mainly on the bacterial dimension of the plant-associated microbiome.

## 3 The (Hyper)Accumulator Plant-Associated Microbial Community

Despite the long history of interest in serpentine flora and metal hyperaccumulating plants, the attention of microbiologists towards bacteria from serpentine and metal-enriched soils is more recent, with the relevant exception of Lipman (1926). Soil bacterial community structure and diversity can be categorized on a broad scale using various techniques, including phenotypic, genotypic, and metagenomic approaches.

### 3.1 Cultivation-Dependent Approaches

Several studies have shown that metals influence microorganisms by adversely affecting their growth, morphology, and biochemical activity, resulting in decreased biomass. In their study of microbial flora of serpentine soils in Andaman Islands (India), Pal et al. (2005) used -culture-dependent methods to show a lower microbial density (6.2–11.3 × $10^6$ colony-forming units (cfu) $g^{-1}$ soil) in serpentine soil than serpentine-free outcrops. The serpentine microbial population was dominated by bacteria that represented 5.12 to 9.5 × $10^6$ cfu $g^{-1}$ of soil, whereas the fungal population ranged from 0.17 to 3.21 × $10^6$ cfu $g^{-1}$ of soil (Pal et al. 2005). These results were confirmed by the study of Abou-Shanab et al. (2003a) in which total culturable microbial populations in the rhizosphere of *Alyssum murale* and bulk soil were compared: they found $1.4 \times 10^4$ fungi and $5.5 \times 10^9$ bacteria $g^{-1}$ of rhizosphere soil (and $1.5 \times 10^3$ and $1.8 \times 10^5$, respectively, in the bulk soil). Similarly, Aboudrar et al. (2007) reported that the serpentine population of *Noccaea caerulescens* harboured various types of microorganisms with a predominance of bacteria. Lucisine et al. (2014) showed that the size of the culturable bacterial community appeared to be stable regardless of the rhizosphere soil type, when they compared different covers of hyperaccumulator plants (*Leptoplax emarginata*/*N. tymphaea*/*A. murale*). These authors found no significant difference between the different species (mean values of $1.5 \times 10^7$ cfu $g^{-1}$ dry soil). Moreover, Rue et al. (2015) determined that the ratio between the number of microorganisms in rhizosphere soils (R) and the corresponding number of microorganisms in the unplanted soils (S), the R/S quotient (Benizri et al. 2007), was highest for a mixed multi-species cover of four hyperaccumulator plants, relative to the monospecies covers and the bulk soil (Rue et al. 2015).

On the other hand, there are several reports showing the presence of highly metal-resistant bacteria from serpentine soils, which suggest a potential adaptation of the indigenous bacterial populations (Abou-Shanab et al. 2007). Different nickel salts added to culture media were used to isolate Ni-resistant bacteria, including $NiCl_2$ (Abou-Shanab et al. 2003b, 2007; Turgay et al. 2012) and $NiSO_4$ (Aboudrar et al. 2007; Álvarez-López et al. 2016a; Durand et al. 2016). The concentrations used generally ranged from 0 to 40 mM. Such Ni concentrations might seem elevated (400–1000 times more) when compared with natural Ni concentrations in the pore waters of ultramafic soils (Bani et al. 2015); however, it is known that a significant amount of added Ni is adsorbed onto agar and complexed by other culture media components in Petri dishes. Pal et al. (2005) showed that bacterial isolates from core serpentine areas were more resistant to Ni than those collected from peripheral areas. Analysis of the relative resistance in bacteria revealed that 62.3% of serpentine isolates were able to grow at 4 mM Ni, whereas only 24.8% grew for non-serpentine isolates. At the highest concentration of Ni (8 mM) only 19 bacterial strains (11.7%) from core serpentine outcrops showed visible growth. In their study, Durand et al. (2016) showed that the ratios of Ni-resistant bacteria to total bacteria steadily decreased from 81% at 0.5 mM Ni to 0.76% at 10 mM. Similarly, Aboudrar et al. (2007) found that the ratio of the number of Ni-resistant bacteria (cfu on TSA plate with 1, 3 or 10 mM Ni) to the total number of bacteria (cfu on TSA plate) varied between 4 and 100%, and was about two to three times higher in the rhizosphere than in the bulk soil. Because metal availability and toxicity in soil are related to soil pH, soil acidification in the rhizosphere of *A. murale* and also *N. caerulescens* has been hypothesized to explain the higher proportion of Ni-resistant bacteria in the rhizosphere compared to bulk soil (Abou-Shanab et al. 2003a). However, it should be noted that many studies have shown an increase in soil pH in the rhizosphere of hyperaccumulators growing in ultramafic soils alongside metal-resistant bacteria (e.g. Álvarez-López et al. 2016a).

Changes in microbial communities can be studied using sole-carbon-source tests. The method first described by Garland and Mills (1991) involves a commercially available microtiter plate (Biolog), which can be used to simultaneously test the utilization of 95 substrates as sole carbon sources. Carbon source utilization is indicated by colour development of a redox indicator dye, and changes in the overall patterns of carbon source utilization rates can be assessed by multivariate statistics. The technique has been used to detect differences between microbial communities in soil and the rhizosphere (Baudoin et al. 2001), but only in a few cases have the effects of metal pollution been studied (Cd, Cu or Zn according to Knight et al. (1997); Cu according to Fritze et al. (1997)). The Biolog EcoPlate system was used more recently to characterize the metabolic profile of microbial communities. It contains 31 of the most useful carbon sources for soil community analysis, allowing for community-level physiological profiling (CLPP) of heterotrophic bacterial assemblages. This technique has been widely used to assess the toxicological impacts of different pollutants, including different heavy metals. By studying rhizosphere microbial communities under mono- or multi-species hyperaccumulator plant cover in a serpentine soil, Rue et al. (2015) showed that the phenotypic structure of the bacterial communities appeared to be specific to the type of cover. Rhizosphere bacteria associated with *A. murale*, *N. tymphaea*, or a mixed cover of four hyperaccumulator plants (*L. emarginata*/*N. tymphaea*/*A. murale*/*Bornmuellera tymphaea*) catabolized carbohydrate substrates more intensely than did bacteria from other soils. Similarly, bacteria from the rhizosphere of *L. emarginata* seemed to prefer both carboxylic acids and polymers. Aboudrar et al. (2007) also showed that rhizosphere microorganisms isolated from *N. caerulescens* tended to show a greater utilization of some carboxylic acids than did the bulk soil microorganisms. Interestingly, Epelde et al. (2008) observed that ketobutyric acid and D-malic acid were utilized to a significantly greater extent by the culturable portion of the soil microbial community in metal-polluted and planted soil, than in all other treatments (unplanted soil with or without metals and planted soil without metals). In hyperaccumulators it has also been shown that *N. caerulescens* has constitutively high concentrations of malic acid/malate in their tissues (Boominathan and Doran 2003). Based on Biolog EcoPlate values, some authors estimated the average well colour development (AWCD), determined by calculating the mean of every well absorbance value at each reading time, and also estimated different diversity indexes such as S (richness), H′ (Shannon's diversity), and J′ (Shannon's evenness). Based on the AWCD technique, contradictory results were found. Indeed, Epelde et al. (2009) reported lower AWCD values in metal-polluted than control unpolluted soils, but the same authors highlighted that diversity indices were higher in polluted soils with or without plants (Epelde et al. 2008).

Microbial community characterization by biomolecules other than nucleic acids such as lipids has been used without relying on culturing (Banowetz et al. 2006). Fatty acids are present in a relatively constant proportion of the cell biomass, and signature fatty acids exist in microbial cells that can differentiate major taxonomic groups within a community. The emerging pattern is then compared to a reference FAME database, in order to identify fatty acids and their corresponding microbial signatures by multivariate statistical analyses. For example, Kozdrój and van Elsas (2001a, b) studied the structural diversity of microbial communities in arable soils of a heavily industrialized area.

Generally, bacteria found to be associated with hyperaccumulators using cultivation-dependent methods mainly comprise the Gram-positive genera *Arthrobacter*, *Microbacterium*, *Bacillus*, and *Curtobacterium*, and the Gram-negative genera *Pseudomonas*, *Sphingomonas*, and *Variovorax* (Mengoni et al. 2001; Oline 2006; Pal et al. 2007; Turgay et al. 2012). These genera have been reported in both the rhizosphere and endosphere of hyperaccumulators, regardless of the specific metal composition of the soil (Visioli et al. 2015). Studies focusing on the culturable bacterial community can be useful for obtaining potentially beneficial isolates, which can be used to improve the Ni phytoextraction capacity of Ni-(hyper)accumulating plant species (discussed below). Álvarez-López et al. (2016a) found the culturable rhizobacterial community of the Ni-hyperaccumulators *A. pintodasilvae* and *A. malacitanum* to be dominated by the phyla *Proteobacteria* and, in particular, *Actinobacteria*. Isolates were affiliated with members of genera, such as *Arthrobacter*, *Streptomyces*, *Rhodococcus* or *Microbacterium*, which have been frequently described amongst soil bacteria. Significantly, however, differences were observed in the bacterial communities associated with different populations of the same Ni-hyperaccumulating species.

## 3.2 Cultivation-Independent Approaches

Because only a minor percentage of naturally occurring microorganisms can be cultured,

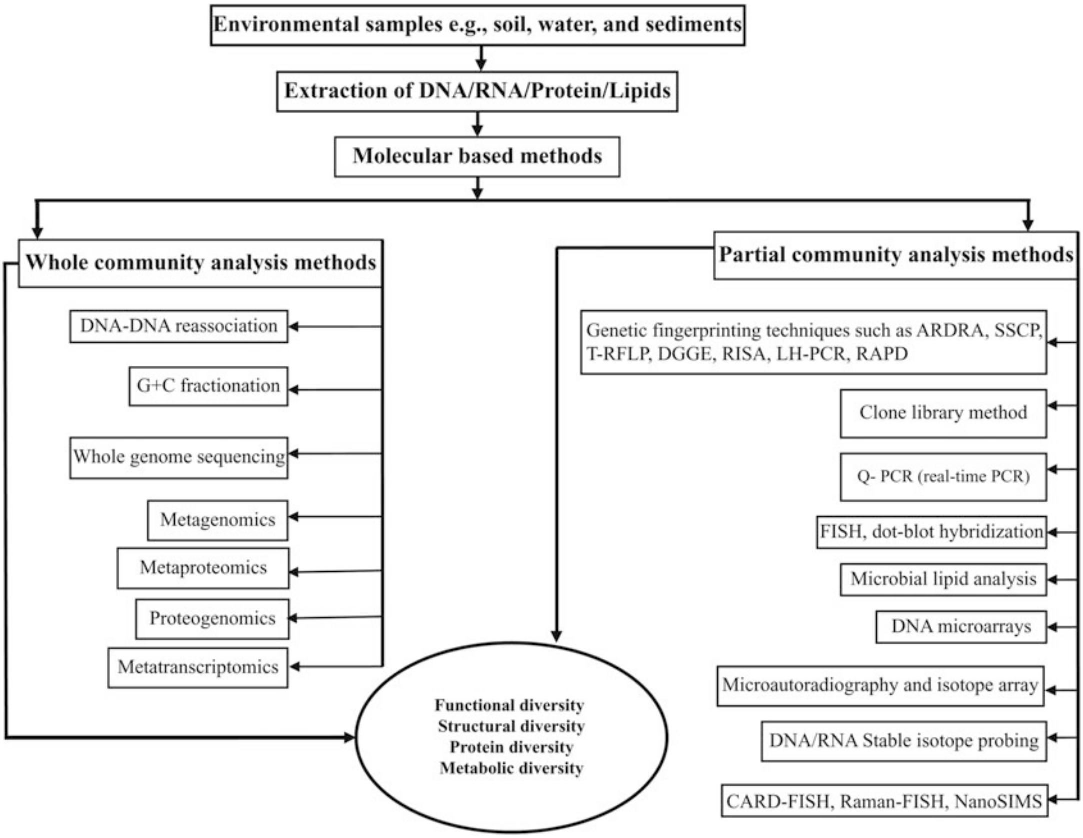

**Fig. 1** Culture independent molecular toolbox to characterize the structural and functional diversity of microorganisms in the environment (Rastogi and Sani 2011)

cultivation-independent approaches are now often used to analyze plant-associated microflora. The molecular techniques generally involve extraction of nucleic acid, directly or indirectly, from soil. These approaches are independent of culture, and according to sensitivity can detect species, genera, families, or even higher taxonomic groups (Rastogi and Sani 2011). Such techniques have been classified into two major categories, depending on their capability of revealing the microbial diversity structure and function: (i) partial-community analysis approaches, and (ii) whole-community analysis approaches (Fig. 1). Despite the recent interest paid to the study of microbial communities in naturally metal-rich soils, few studies have focused on the characterization of genetic diversity of rhizosphere bacterial communities of metal-hyperaccumulator plants (Kumar et al. 2009).

## 3.3 Partial-Community DNA Analysis

Partial genetic approaches have been developed as a result of the polymerase chain reaction (PCR) of specific marker genes. Indeed, these techniques offer direct access to the genomes of different microbial communities inhabiting a given environment. The gene encoding the small subunit ribosomal genes is the most used markers to reveal the structure of microbial communities that inhabit a given environment. The 16S gene (18S respectively), described above, codes for the small ribosomal subunit in prokaryotes (eukaryotes respectively), whereas the 23S gene (28S respectively) codes for the large subunit. The intergenic spacer (IGS or intergenic Spacer) between the two ribosomal genes can also be used to characterize the microbial community structure within one or more samples. Indeed, the length and sequence of the ribosomal IGS is variable depending on the species (between 60 and 1500 base pairs) and the alleles of different species (Gürtler and Stanisich 1996). Therefore, by using specific primers to the conserved sequences of ribosomal genes 16S and 23S (18S and 28S respectively), the IGS can be amplified by PCR to analyze the microbial community structure present in a soil samples (Table 3).

### 3.4 Clone Library

Bacterial populations can also be characterized by cloning and sequencing of 16S rRNA genes. The most widely used method to analyze PCR products amplified from an environmental sample is cloning and then sequencing the individual gene fragments (DeSantis et al. 2007). The obtained sequences are compared to known sequences in a database such as GenBank, Ribosomal Database Project (RDP), and Greengenes. Typically, cloned sequences are assigned to phylum, class, order, family, subfamily, or species at similar sequence cut-off values of 80, 85, 90, 92, 94 or 97%, respectively (DeSantis et al. 2007).

### 3.5 Genetic Fingerprinting

Genetic fingerprinting generates a profile of microbial communities based on direct analysis of PCR products amplified from environmental DNA (Muyzer 1999). These techniques include, for example, DGGE/TTGE, SSCP, RAPD, T-RFLP, RISA, and ARISA, and produce a community fingerprint based on either sequence polymorphism or length polymorphism. In denaturing-gradient gel electrophoresis (DGGE), the PCR products are obtained from environmental DNA using primers for a specific molecular marker (e.g. 16S rRNA gene) and electrophorised on a polyacrylamide gel containing a linear gradient of DNA denaturant such as a mixture of urea and formamide (Muyzer et al. 1999). Temperature-gradient gel electrophoresis (TTGE) is based on the same principle of DGGE, except that a temperature gradient rather than a chemical denaturant is applied. In single-strand conformation polymorphism (SSCP), the environmental PCR products are denatured followed by electrophoretic separation of single-stranded DNA fragments on a non-denaturing polyacrylamide gel. Separation is based on subtle differences among sequences, which results in a different folded secondary structure leading to a measurable difference in mobility in the gel. The RAPD technique uses short random primers (about 10 bp) that anneal at different places on the genomic DNA, generating PCR products of various lengths further resolved on agarose or acrylamide gel. This technique was demonstrated to be rapid and sensitive for revealing differences among prokaryotic genomes of similar complexity (Ranjard et al. 2000). The terminal restriction fragment length polymorphism (T-RFLP) technique use of one 5′ fluorescently labelled primer during the PCR reaction. PCR products are digested with restriction enzyme(s), and terminal restriction fragments (T-RFs) are separated on an automated DNA sequencer. Only the terminally fluorescent-labelled restriction fragments are detected. The ribosomal intergenic spacer analysis (RISA) technique allows the analysis of a ribosomal intergenic region after PCR amplification and migration on acrylamide gel. The automated version (ARISA) consists, for its part, in the use of a fluorescence-labelled forward primer, and ISR fragments are detected automatically by a laser detector. These two methods of analysis of ribosomal IGS are considered highly reproducible and provide a community specific profile, with each band corresponding to at least one organism in the original community. However, these methods require a large amount of microbial DNA and are slow to develop (Kirk et al. 2004).

### 3.6 Whole-Genomic Community DNA Analysis Approaches

The latest advances in molecular biology have allowed, since the early 2000s, the provision of next-generation sequencers (NGS). Unlike Sanger sequencing (Sanger et al. 1977), the next-generation sequencers enable access to the

**Table 3** Advantages and inconveniences of the main analytical techniques used in microbial ecology studies dedicated to ultramafic or metal-polluted soils

| Techniques | Advantages | Inconveniences | Focus of the study | Main results | References |
|---|---|---|---|---|---|
| Partial community DNA analysis | | | | | |
| T-RFLP | – Highly reproducible<br>– Allows studying simultaneously a large number of samples | – Difficult to access to the microbial diversity<br>– Cannot detect the dominant microbial populations<br>– The sequences of the marker gene from two different organisms can share the same restriction site | *Thlaspi goesingense* rhizosphere | In rhizosphere T-RFLP profiles, a total of 112 fragments with fluorescence intensities higher than 50 were detected | Idris et al. (2004) |
| Establishment of 16S rRNA gene clone libraries and clones sequencing | – High phylogenetic resolution | – Time consuming<br>– A very small number of samples can be processed simultaneously<br>– Use several microbiological techniques | *Thlaspi goesingense* rhizosphere | *Holophaga*/*Acidobacterium* (27%), α-*Proteobacteria* (22%), high-G + C gram-positives (16%), *Cytophaga*/*Flexibacter*/*Bacteroides* (10%), γ- and ß-*Proteobacteria* (8 and 4%) | Idris et al. (2004) |
| | | | *Alyssum bertolonii* rhizosphere | Dominance of *Proteobacteria* | Mengoni et al. (2004) |
| | | | Serpentine soils from Northern California and Southern Oregon | *Actinobacteria* (17.3%), *Acidobacteria* (16.7%), *Alphaproteobacteria* (11.3%), *Verrucomicrobia* (8.7%), Green-nonsulfur-bacterium related (8.7%), *Gemmatimonadetes* (8.0%), *Planctomycetes* (7.3%), *Bacteriodetes* (6.6%), *ß-proteobacteria* (5.3%), *δ-proteobacteria* (4.6%) | Oline (2006) |
| | | | Environments located at the Ni-mining sites in New Caledonia | Predominance of *Acidobacteria* in mining sites (without any vegetation), but not in sites revegetated with native plants, where *Proteobacteria* was the most abundant phylum | Herrera et al. (2007) |
| | | | Ultramafic soils from a tropical Savanna (Brazil) | Nine phyla, two of which—*Acidobacteria* and *Actinobacteria*—were the most abundant followed by *Proteobacteria* | Pessoa-Filho et al. (2015) |
| | | | Soils exposed to different metal pollutants | An increase in *Acidobacterium and* decrease in terrestrial non-thermophilic *Crenarchaeota* | Kozdrój and van Elsas, (2001a, b) |

| | | | | | |
|---|---|---|---|---|---|
| DGGE/TGGE | – Microbial diversity can easily be assessed by excision and sequencing of bands<br>– Theoretical discrimination possible between two sequences having a difference of only one nucleotide | – Comparison between two gels difficult<br>– Technique poorly reproducible<br>– Multiple bands for a species can be maintained due to a micro-heterogeneity within the genome,<br>– Possible bias related to PCR amplification | Polluted soils from an explosives factory | *Bacteroidetes* (0–25.9%), *Actinobacteria* (3.4–15.7%), *Firmicutes* (44.5–69.4%), *β-proteobacteria* (0–6.5%), *γ-proteobacteria* (5.6–50.4%) | Ellis et al. (2003) |
| | | | Cu-, Zn-, and Cd-contaminated paddy soils | A decrease in microbial community diversity | Li et al. (2006) |
| | | | Soil collected from a lead and zinc mine | A decrease in microbial community diversity due to the presence of heavy metals such as Cd and Pb | Hu et al. (2007) |
| | | | Soil sample was added with Cd and Pb | The structure of the bacterial community changed in heavy metal-amended soil samples | Khan et al. 2010 |
| | | | Serpentine soils (Ultrabasic Massif of Lanzo and the Piedmont Zone of Calcschist) | *Ascomycota, Verrucariales, Hypocreales, Pleosporales*, *Chaetothyriales* and *Capnodiales* were the more represented orders | Daghino et al. (2012) |
| | | | Polluted Rhizosphere soil with chromium of the metallophyte *Silene vulgaris* | Under Cr pollution, a shift in the relative abundance of specific taxa with dominant phylotypes such as *Variovorax*, *Chitinophaga niastensis*, *Pontibacter* sp., *Ramlibacter* sp. | García-Gonzalo et al. (2016) |
| SSCP | | | *Alyssum murale, Leptoplax emarginata, Bornmuellera tymphaea, Noccaea tymphaea* | The bacterial genetic structure depended on the plant cover composition (mono- or multi-species covers) | Lucisine et al. (2014), Rue et al. (2015) |
| RISA/ARISA | – Highly reproducible | – More of one sequence can be generated for the same organism<br>– A similar size IGS between different organisms may lead to an underestimation of the microbial richness<br>– Possible bias related to PCR amplification | Ultramafic neocaledonian soils | Emergence of a bacterial group closely related to the *Ralstonia/Oxalobacter/ Burkholderia* group in the ß-Proteobacteria and also related to *Actinomycete* group | Héry et al. (2006) |
| | | | Polluted soils with copper | ARISA fingerprinting showed slight but significant modifications of bacterial and fungal communities suggesting a short-term effect of Cu stress | Ranjard et al. (2006) |

(continued)

**Table 3** (continued)

| Techniques | Advantages | Inconveniences | Focus of the study | Main results | References |
|---|---|---|---|---|---|
| Whole genomic community DNA analysis approaches | | | | | |
| Metagenomic | High resolution, very fast | – Requires knowledge of bioinformatics and powerful computers<br>– Expensive equipment and consumables | Cu-polluted field site in Hygum, Denmark | *Proteobacteria*, *Bacteroidetes*, *Verrumicrobia*, *Chloroflexi*, *WS3*, and *Planctomycetes*, decreased with increasing bioavailable Cu, while members of the dominant phylum, the *Actinobacteria*, showed no response and members of the *Acidobacteria* showed a marked increase in abundance. | Berg et al. (2012) |
| | | | Soil pollution with trace metals (Pb, Zn, Cd, or Cu, and Cr) in the vicinity of mines, smelters, and other industrial facilities (Southern Poland) | *Proteobacteria* were the most abundant phylum (27.5–49.5%), followed by *Acidobacteria* and *Actinobacteria*. Other major phyla were *Bacteroidetes, Chloroflexi*, and *Gemmatimonadetes*. The most abundant classes were *α-proteobacteria, Actinobacteria, Acidobacteria, γ-proteobacteria, Sphingobacteria*, and *ß-proteobacteria* | Gołębiewski et al. (2014) |
| | | | Ultramafic soils (New Caledonia) | The most abundant bacterial groups were Proteobacteria (41.38–1.26%) and Acidobacteria (18.49–1.01%), followed by Actinobacteria (10.29–0.77%), Planctomycetes (10.06–0.86%), Verrucomicrobia (6.24–1.38%) and Chloroflexi (<6%) | Bordez et al. (2016) |

structure and diversity of microbial communities from diverse environments, by high-throughput sequencing and simultaneous, multi-million sequences obtained from many samples (Rastogi and Sani 2011). These high-throughput sequencing technologies were developed by different companies (Roche Diagnostics Corp., Illumina, Life Technologies Corp., mainly), and thus allow rapid access, by the analysis of a specific marker gene, to the structure and genetic diversity of microbial communities in any environment (Visioli et al. 2015). NGS has been used to analyze the bacterial community in soils polluted with heavy metals and to determine the impact of heavy metal pollution on the composition of the community (Table 3).

Transcriptomics and proteomics can be useful in revealing the ability of microbes to respond to particular stimuli and the functions of the majority of microbial species. These tools focus on the messenger RNAs produced by the microbial community from a given environment. By analysing these messenger RNAs (called meta-transcriptomic), the information relating to the structure and functional diversity of different populations present in a sample are obtained. Finally, meta-proteomics focuses on the study of all proteins produced by the microbial community and also provides insights to functional diversity.

## 4 Isolation of Plant-Associated Microorgansims and Application in Phytomining

### 4.1 Improving Metal Removal Through Plant Growth Promotion

Microorganisms can enhance plant growth and resistance to biotic and abiotic stresses by various mechanisms (Lebeau et al. 2008; Gadd 2010). Well-known examples include the nitrogen-fixing rhizobia and mycorrhizal fungi that facilitate phosphorus uptake (Richardson and Simpson 2011). The importance of symbionts such as mycorrhizal fungi in supplying nutrients and minerals to the plant, in soil physical structure and aggregate stability, and in the suppression of soil-borne plant pathogens is well recognized and documented (Kapulnik and Douds 2000; van der Heijden and Sanders 2002).

Documented mechanisms through which plant growth-promoting (PGP) bacteria can benefit plant growth and physiology include the provision of plant nutrients, the release of plant hormones or growth regulators, or the suppression of plant stress ethylene levels. PGP bacteria directly influence plant growth by increasing the availability of essential nutrients such as N, P or Fe, thus improving plant nutrition in nutrient-deficient soils. For example, diazotrophs use the enzyme nitrogenase to fix atmospheric nitrogen into ammonia which can then be absorbed by plant roots. Diazotrophic bacteria occur in the form of nodules (symbionts) or free-living cells (Beattie 2007). Another mechanism involves the release of organic acids and/or phosphatases which solubilize inorganic phosphates into plant-available forms (Richardson and Simpson 2011). Inoculating plants with P-solubilizing microorganisms has been shown to improve plant P accumulation and growth (Richardson and Simpson 2011). Microorganisms are also known to exude high-affinity Fe(III)-specific chelating agents (generally $<1000$ Da) or siderophores, in response to limited iron supply, which can mobilize iron associated with soil solid phase minerals or complexed by organic matter (Crowley and Kraemer 2007). Bacterial indoleacetic acid (IAA) stimulates root hair formation while increasing the number and length of lateral and primary roots when in an ideal concentration range (Duca et al. 2014). Some PGP bacteria are also known to synthesize cytokinins which have been suggested to enhance plant growth under stress conditions (Glick 2010). An important mechanism of plant stress alleviation is through the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase. ACC deaminase-producing bacteria can reduce the production of stress ethylene in plants (Dell'Amico et al. 2008). Improvements observed in the growth of plants under various stresses, including flooding, drought, high salinity, phytopathogens or the presence of organic pollutants and metals, have been associated with the activity of ACC deaminase-producing PGP bacteria (Glick 2010). Bacterial production of volatile compounds can also significantly promote plant growth (Ryu et al. 2003). Several studies have focused on screening bacterial-liberated volatile compounds for their effects on the growth of *Arabidopsis thaliana* (Ryu et al. 2003; Blom et al. 2011). A diverse array of bacterial volatile compounds were detected (over 100), and amongst these, compounds such as such as 2,3-butanediol, acetoin, indole, 1-hexanol, and pentadecane were found to have a strong beneficial effect on the growth of this plant species.

The activity of some bacteria can also indirectly inhibit or suppress plant diseases. Biocontrol traits include competion with pathogenic bacteria for nutrients and space (niche exclusion), production of antimicrobial compounds (such as hydrogen cyanide (HCN)), biosurfactants with antimicrobial activity or chitinolytic enzymes (such as quitanases), or the induction of plant defence mechanisms (Compant et al. 2005; Lemanceau et al. 2007). Compant et al. (2005) showed that PGP bacteria could deprive pathogens of Fe through the production of iron-chelating siderophores. In a study by Kurek and Jaroszuk-Scisel (2003), siderophore-producing strains of *P. fluorescens* and *P. putida* strain B10 successfully suppressed the activity of the fungus *Fusarium* sp. Many bacterial genera produce antimicrobial compounds and most of these have been shown to have a broad-spectrum activity. Antibiotics produced by antagonistic bacteria include compounds such as 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide (HCN), kanosamine, phenazines, oomycin A, pyrrolnitrin, viscosinamide, pyoluteorin, butyrolactones, pantocin A and B, xanthobaccins, and zwittermycin A (Raaijmakers et al. 2002). The antimicrobial compounds released by these bacterial biocontrol agents have been shown to act on four main targets: cell wall synthesis, protein synthesis, nucleic acid replication, and cellular membranes (Raaijmakers et al. 2002). Many bacterial biocontrol agents show hyperparasitic acitivty and can attack pathogens through the production of cell wall hydrolases, such as by quitinases or glucanases (Compant et al. 2005). Other PGP bacteria are able to detoxify the virulence factors produced by pathogens (Thangavelu et al. 2001). *Pseudomonas fluorescens* strain Pf10, isolated from the rhizosphere of banana, detoxifies fusaric acid produced by the wilt pathogen, *Fusarium oxysporum* f. sp. *cubense* (Thangavelu et al. 2001). Finally, some bacterial strains can induce plant systemic resistance (ISR) without causing visible symptoms of stress on the host plant, and often simultaneously enhancing plant growth (van Loon et al. 1998). Examples of rhizobacteria-mediated ISR were given by van Loon et al. (1998). Various bacterial determinants of ISR have been described, including lipopolysaccharides, siderophores, salicylic acid, and other macromolecules (van Loon et al. 1998).

The use of microorganisms that influence the availability of plant nutrients (such as N, Fe or P) in agriculture as ‘biofertilizers’ to enhance plant nutrient uptake and alleviate nutrient deficiencies is well established (Vessey 2003). However, the idea of using plant-associated microbes to enhance plant-metal uptake as part of phytoremediation or phytomining strategies is more recent. In fact, over the last decade a growing number of studies have been dedicated to the search for bioinoculants with potential application in phytoremediation techniques (Table 4). The discovery of new microbes can contribute greatly towards optimising the efficiency of these technologies (Haslmayr et al. 2014).

Trace metal-polluted soils or natural metal-enriched soils can be a potential source of metal-tolerant bacteria, including strains that promote plant growth. Numerous metal-tolerant and plant growth-promoting microbial strains have been isolated from trace elements-polluted mine tailings and their use as inoculants in phytoextraction is documented (Becerra-Castro et al. 2012; Pereira et al. 2015). For Ni phytomining, serpentine soils represent a source of potentially beneficial plant-growth promoting and Ni-tolerant microorganisms (Cabello-Conejo et al. 2014;

**Table 4** Effects on plant metal accumulation of plant-associated bacteria isolated for phytoextraction or phytomining purposes

| Plant species | Bacterial strain | Characteristics | Metal | Effect | Reference |
|---|---|---|---|---|---|
| *Alyssum murale* | *Microbacterium oxydans AY509223* | Acid; Siderophore | Ni | ↑ leaf [Ni] | Abou-Shanab et al. (2006) |
| *A. murale* | *Sphingomonas macrogoltabidus*, *Microbacterium liquefaciens*, *Microbacterium arabinogalactanolyticum* | Acid (*M. l*, *M. a*); $PO_4$ (*M. l*, *M. a*); Siderophore (*M. l*) | Ni | ↑ soil $[Ni]_{Sr(NO3)2}$ (*M. a*); ↑ shoot [Ni] | Abou-Shanab et al. (2003a) |
| *Alyssum pintodasilvae* | *Microbacterium* sp., *Arthrobacter* sp., *Streptomyces* sp. | Acid; IAA; Siderophore; $PO_4$ | Ni | ↑ shoot [Ni]; ↑ plant biomass; ↑ Ni yield | Cabello-Conejo et al. (2014) |
| Intercropping of *Bornmuellera tymphaea*–*Noccaea tymphaea* and *B. tymphaea*–*A. murale* | *Variovorax paradoxus* | IAA, ACCD, Siderophore | Ni | ↑ plant biomass; ↑ plant Ni uptake | Durand et al. (2016) |
| *Brassica juncea* | *Enterobacter aerogenes* NBRI K24, *Rahnella aquatilis* NBRI K3 | IAA; Siderophore; ACCD; $PO_4$ | Cr, Ni | ↑ plant growth/ biomass; ↑ [Cr, Ni] uptake | Kumar et al. (2009) |
| *B. juncea* | *Achromobacter xylosoxidans* Ax10 | IAA; ACCD; $PO_4$ | Cu | ↑ plant growth/ biomass; ↑ [Cu]root/ shoot | Ma et al. (2009c) |
| *B. juncea* | *Bacillus biosubtyl*, *Bacillus licheniformis*, *Bacillus thuringiensis* | nd | Cd, Cr, Se | ↑ Cd accumulation (*B.l*) | Hussein (2008) |
| *B. juncea* | *Enterobacter* sp. NBRI K28 | IAA; Siderophore; ACCD; $PO_4$ | Cr, Ni | ↑ plant biomass; ↑ [Cr, Ni, Zn] uptake | Kumar et al. (2008) |
| *B. juncea* | *Pseudomonas* sp. Ps29C, *Bacillus megaterium* Bm4C | IAA; Siderophore; ACCD; $PO_4$ | Ni | ↑ plant biomass plant protection from Ni toxicity | Rajkumar and Freitas (2008a) |
| *B. juncea* | *Pseudomonas* sp. PsA4, *Bacillus* sp. Ba32 | IAA; ACCD; $PO_4$ | Cr | ↑ plant growth protection of Cr toxicity | Rajkumar et al. (2006) |
| *B. juncea* | *Azotobacter chroococcum* HKN-5, *Bacillus megaterium* HKP-1, *Bacillus mucilaginosus* HKK-1 | N-fixing (*A. c*); $PO_4$ (*B. me*); K solubilization (*B. mu*) | Cu, Pb, Zn | ↑ removal of Cu, Pb and Zn | Wu et al. (2006) |
| *B. juncea* | *Bacillus subtilis* strain SJ-101 | IAA; $PO_4$ | Ni | ↑ plant growth/ biomass; ↑ [Ni] shoot | Zaidi et al. (2006) |
| *B. juncea* | 11 bacteria strains, included:<br>*Variovorax paradoxus*,<br>*Rhodococcus* sp.,<br>*Flavobacterium* sp. | Cd-tolerance; IAA; ACCD; Siderophore | Cd | ↑ Co, Cu, Ni, Zn tolerance<br>↑ root elongation | Belimov et al. (2005) |

(continued)

**Table 4** (continued)

| Plant species | Bacterial strain | Characteristics | Metal | Effect | Reference |
|---|---|---|---|---|---|
| *B. juncea* | Se-tolerant rhizobacteria (strains BJ1, BJ2, BJ2, BJ4) | Produce heat-labile bioactive compound | Se | ↑ [Se] shoot | De Souza et al. (1999) |
| *B. juncea*,<br>*Brassica oxyrrhina* | *Bacillus* sp. (strains SN3,SN9,SRS5, SRS15, SRI4, SRI11, SRI14)<br>*Pseudomonas* sp. SRI2,<br>*Psychrobacter* sp. SRS8 | Ni-solubilization; IAA; Siderophore (except SRS5); ACCD (SN9, SRI2, SRI4, SRI11, SRI14); $PO_4$ | Ni | ↑ plant biomass (mainly SRI2, SRS8, SN9)<br>↑ [Ni] shoot and root (SN9) | Ma et al. (2009a) |
| *B. juncea*,<br>*B. oxyrrhina* | *Psychrobacter* sp. (strains SRA1, SRA2),<br>*Bacillus cereus* sp. (strains SRA10, SRP4),<br>*Bacillus weihenstephanensis* SRP12 | IAA; Siderophore (SRA1, SRA10, SRP4, SRP12); ACCD; $PO_4$ | Ni | ↑ plant biomass (SRA2)<br>↑ Ni solubilization (SRA1, SRA10)<br>↑ [Ni] shoot and root (SRA1, SRA10) | Ma et al. (2009b) |
| *B. juncea*,<br>*Lycopersicon esculentum*,<br>*Zea mays L. var. Denhai-11* | *Burkholderia* sp. J62 | Metal-resistance;<br>Antibiotic resistance;<br>IAA; Siderophore;<br>ACCD; $PO_4$ | Cd, Pb | ↑ plant biomass (*L. esculentum*, *Z. mays*);<br>↑ Cd, Pb uptake (*L. esculentum*, *Z. mays*) | Jiang et al. (2008) |
| *B. juncea*,<br>*Brassica napus*,<br>*L. esculentum* | *Kluyvera ascorbata* SUD165,<br>*Kluyvera ascorbata* SUD165/26 | Siderophore (*K. a. SUD165/26)* | Cu, Ni, Zn | ↓ growth inhibition caused by metals | Burd et al. (2000) |
| *Brassica napus* | *Arthrobacter* sp. MT16,<br>*Microbacterium* sp. JYC17,<br>*Pseudomonas chlororaphis* SZY6,<br>*Azotobacter vinelandii* GZC24,<br>*Microbacterium lactium* YJ7 | IAA; Siderophore; ACCD; $PO_4$ | Cu | ↑ root length | He et al. (2010a) |
| *B. napus* | *Firmicutes* sp.,<br>*Actinobacteria* sp.,<br>*Proteobacteria* sp. | IAA; Siderophore; ACCD; arginine decarboxylase production | Cu | ↑ plant biomass<br>↑ [Cu] shoot | Sun et al. (2010) |
| *B. napus* | *Pseudomonas tolaasii* ACC23,<br>*Pseudomonas fluorescens* ACC9,<br>*Alcaligenes* sp. ZN4,<br>*Mycobacterium* sp. ACC14 | Cd-resistance; IAA; ACCD; Siderophore | Cd | ↑ root elongation<br>↑ shoot and root growth | Dell'Amico et al. (2008) |

| | | | | | |
|---|---|---|---|---|---|
| *B. napus* | *Pseudomonas fluorescens* G10,<br>*Microbacterium* sp. G16 | IAA; Siderophore; ACCD; $PO_4$ | Pb | ↑ plant biomass (root elongation)<br>↑ Pb uptake (shoot) | Sheng et al. (2008a) |
| *B. napus* | *Pseudomonas putida* UW4 | ACCD | Ni | ↑shoot biomass<br>↑ Ni tolerance | Farwell et al. 2007 |
| *B. napus* | *Pseudomonas putida* UW4,<br>*Pseudomonas putida* HS2 | ACCD<br>High Ni-tolerance (*P. p* HS2) | Ni | ↑ plant growth<br>↑ Ni yield | Farwell et al. (2006) |
| *B. napus* | Not determined | Cd-resistant | Cd | ↑ [Cd] shoot | Sheng and Xia (2006) |
| *B. napus*,<br>*L. esculentum*,<br>*Z. mays*,<br>*Sorghum sudanense* | *Bacillus* sp. J119 | Metal-resistance;<br>Antibiotic resistance;<br>Biosurfactant; IAA;<br>Siderophore | Cd | ↑ shoot/root biomass of *L. esculentum*; ↑ [Cd] shoot (*B. napus*, *L. esculentum*); ↑ [Cd] root (*B. napus*, *L. esculentum*, *Z.mays*) | Sheng et al. (2008b) |
| *Heliathnus annuus* | *Bacillus weihenstephanensis* SM3 | IAA; $PO_4$; Cu, Ni, Zn mobilization | Cu, Ni, Zn | ↑ plant biomass<br>↑ Cu, Zn uptake | Rajkumar et al. (2008) |
| *H. annuus* | *Pseudomonas fluorescens* | IAA, Siderophore | As | ↑ shoot biomass; ↑ [As] shoot;<br>↑ phloem fluxes | Shilev et al. (2006) |
| *Lycopersicon esculentum* | *Pseudomonas* sp. RJ10,<br>*Bacillus* sp. RJ16 | IAA; Siderophore; ACCD; Cd, Pb mobilization | Cd, Pb | ↑ plant biomass and root length<br>↑ Cd, Pb uptake | He et al. (2009) |
| *Medicago sativa* | *Pseudomonas fluorescens* (strains Avm, U),<br>*Rhizobium leguminosarum* bv *phaseoli* (strains CPMex44, CPMex46),<br>*Azospirillum lipopherum* (strains UAP40, UAP154) | Siderophore<br>Growth promotion | Cu | ↑ Cu, Fe root-shoot translocation (*CPMex46*, *Avm*) | Carrillo-Castañeda et al. (2002) |
| *Nicotiana tabacum* | *Sanguibacter* sp., *Enterobacter* sp.,<br>*Pseudomonas* sp. | nd | Cd | ↑ plant biomass (*Sanguibacter* sp. S-d2 and consortia)<br>↑ Cd translocation<br>↑ Cd, Fe uptake | Mastretta et al. (2009) |

(continued)

**Table 4** (continued)

| Plant species | Bacterial strain | Characteristics | Metal | Effect | Reference |
|---|---|---|---|---|---|
| *Orychophragmus violaceus* | *Bacillus subtilis*,<br>*B. cereus*,<br>*Flavobacterium* sp.,<br>*Pseudomonas aeruginosa* | Zn-tolerance | Zn | ↑ plant biomass and root length<br>↑ Zn solubilization and uptake | He et al. (2010b) |
| *Pteris vittata* | *Rhodococcus* sp. TS1,<br>*Delftia* sp. TS33,<br>*Comamonas* sp. TS37,<br>*Delftia* sp. TS41<br>*Streptomyces lividans* sp. PSQ22 | As-reducing | As | ↑ plant biomass<br>↑ As uptake<br>↑ As solubilization<br>↓ As leaching | Yang et al. (2012) |
| *Populus deltoides* | *Agrobacterium radiobacter* | As-tolerance<br>IAA; Siderophore | As | ↑ plant biomass<br>↑ chlorophyll, enzymatic activity<br>↑ [As] root, stem, leaf<br>↑ As uptake and translocation | Wang et al. (2011) |
| *Ricinus communis* | *Pseudomonas* sp. PsM6,<br>*P. jessenii* PjM15 | IAA; Siderophore; ACCD; Cu, Ni, Zn mobilization | Cu, Ni, Zn | ↑ plant biomass<br>↑ Zn translocation and uptake | Rajkumar and Freitas (2008a) |
| *Salix caprea* | *Agromyces* sp. AR33,<br>*Streptomyces* sp. AR17 | – | Cd, Zn | ↑ soil extractable-Cd/Zn; ↑ plant growth<br>↑ Cd/Zn uptake | Kuffner et al. (2008) |
| *Salix viminalis* and *S. alba x alba* | *Rahnella* sp., *Sphingobacterium* sp., *Caulobacter* sp., *Curtobacterium* sp., *Pseudomonas* sp. | Acid: IAA, ACCD | Cd, Zn | ↑ Twig biomass, ↑ Cd/Zn extraction potential (with *Rahnella* sp.) | Janssen et al. (2015) |
| *Sedum alfredii* | *Burkholderia* sp. D54 | IAA; Siderophore; ACCD; $PO_4$ | Cd, Pb, Zn | ↑ plant biomass; ↑ [Cd] shoot and root; ↑Cd, Pb, Zn uptake | Guo et al. (2011) |
| *S. alfredii* | 5 bacterial strains (unidentified) | nd | Cd, Cu, Pb, Zn | ↑ plant biomass<br>↑chlorophyll and nutrient content<br>↓ Cd, Cu, Pb, Zn toxicity<br>↑ Cd, Cu, Pb, Zn uptake from contaminated water | Xiong et al. (2008) |

| | | | | | |
|---|---|---|---|---|---|
| *S. alfredii* | *Burkholderia cepacia* | – | Cd, Zn | ↑ plant biomass; ↑ [Cd/Zn] shoot; ↑ metal tolerance; ↑ $[Cd/Zn]_{leaf}$:$[Cd/Zn]_{root}$ ratio | Li et al. (2007) |
| *Sedum plumbizincicola* | *Phyllobacterium myrsinacearum* RC6b | Metal-resistance; Metal mobilization; IAA; Siderophore; ACCD; $PO_4$ | Cd, Pb, Zn | ↑ plant biomass<br>↑ [Cd, Zn] root and shoot | Ma et al. (2013) |
| *Sorghum bicolor*,<br>*Z. mays* | *Bacillus subtilis*,<br>*B. pumilus*,<br>*Pseudomonas pseudoalcaligenes*,<br>*Brevibacterium halotolerans* | – | Cu, Cr, Pb, Zn | ↑ plant biomass (with inoculum mixture); ↑ shoot biomass (*Br. h*, *P. p*); ↑ solubility Cr, Cu (*B. s*, *B. p*); ↑ [Cu, Cr, Pb, Zn] shoot on Cu-rich soil; ↑ [Cr] shoot on Cr-rich soil | Abou-Shanab et al. (2007) |
| *S. bicolor* | *Pseudomonas monteillii* | nd | Cd | ↑ plant biomass<br>↑ Cd uptake | Duponnois et al. (2006) |
| *Thlaspi caerulescens* | Mixed inoculum (*Microbacterium saperdae*, *Pseudomonas monteilii*, *Enterobacter cancerogenes*) | – | Zn | ↑ $[Zn]_{water\text{-}soluble}$; ↑ [Zn] shoot | Whiting et al. (2001) |
| *Thlaspi caerulescens* | Mixed inoculum: *Bacillus pumilus* and *Micrococcus* sp. | Ni-resistant | Ni | ↓$[Ni]_{water\text{-}soluble}$; ↑plant biomass; ↑Ni yield | Aboudrar et al. (2013) |
| *Trifolium repens Linn.* | Bacterial strain mix *(Bacillus cereus)* | Metal-tolerance; IAA | Cd | ↑ plant biomass and phytoextraction | Azcón et al. (2009) |
| *Trifolium hybridum*,<br>*Alopecurus pratensis*,<br>*Poa pratensis*,<br>*Hordeum violaceum*,<br>*Ranunculus kotschyi*,<br>*Cerastium* sp. | *Bacillus megaterium* var. *phosphaticum* | Nutrient-solubilization; Pathogens control | B, Ni, Mn, Pb, Zn | ↑ Ni, Pb, Fe, Zn, Na, B desorption from the soil<br>↑ Pb, Ni, B, Mn, Zn uptake | Gullap et al. (2014) |
| *Zea mays* | *Pseudomonas aeruginosa*,<br>*Pseudomonas fluorescens*,<br>*Ralstonia metalidurans* | Siderophore | Cr, Pb | ↑ Cr, Pb exchangeable fraction in the soil (*P. aeruginosa*)<br>↑ [Cr, Pb] shoot | Braud et al. (2009) |

Durand et al. 2016; Álvarez-López et al. 2016a). Schlegel et al. (1991) found that bacterial strains isolated from serpentine soils tolerated up to 10–20 mM Ni (in the culture medium), whereas strains from other soil types tolerated only 1 mM Ni. Turgay et al. (2012) reported that bacterial strains, isolated from Turkish serpentine soils, could tolerate up to 34 mM Ni in the growth medium. Furthermore, as described above, the rhizosphere bacterial communities associated with Ni-hyperaccumulating plants have been shown to differ from those of non-accumulating plants growing at the same site or of non-vegetated soil, but are also known to host a higher number of Ni-tolerant bacteria (Schlegel et al. 1991; Mengoni et al. 2001; Abou-Shanab et al. 2003b; Becerra-Castro et al. 2009; Álvarez-López et al. 2016a). Schlegel et al. (1991) reported a higher occurrence of Ni-resistant bacteria in soil samples collected with increasing proximity to the Ni-hyperaccumulating tree *Sebertia* (= *Pycnandra*) *acuminata*. Mengoni et al. (2001) also found a higher proportion of Ni-resistant cfu in proximity to the Ni-hyperaccumulator *A. bertolonii* than in non-vegetated soil. These authors observed simultaneous resistance to a set of metals and highest resistance from isolates of the rhizosphere. Becerra-Castro et al. (2009) found higher proportions of Ni-tolerant bacteria in the rhizosphere of *A. serpyllifolium* ssp. *lusitanicum*. This selective enrichment of Ni-tolerant bacteria in the rhizosphere was correlated with an increase in soil Ni availability (Becerra-Castro et al. 2009). Álvarez-López et al. (2016a) confirmed higher densities of Ni-tolerant bacteria associated with the Ni-hyperaccumulators *A. serpyllifolium* ssp. *lusitanicum* and *A. serpyllifolium* ssp. *malacitanum*, but observed significant differences in this selective enrichment amongst different plant populations across the Iberian Peninsula.

The screening of bacterial isolates associated with metal (hyper)accumulating plants has led to the identification of candidate inoculants for application in phytomining. Bacterial strains are commonly characterized for the presence of PGP traits such as the capacity to produce phytohormones (IAA), to solubilize inorganic P or K, to fix atmospheric $N_2$, to release siderophores, or for their ACC activity. Most bioaugmentation studies have evaluated the effects of re-inoculating host plants with their associated isolates (Abou-Shanab et al. 2003a; Abou-Shanab et al. 2006; Li et al. 2007; Cabello-Conejo et al. 2014). However, the specificity of these plant-bacterial combinations is not always clear, and some inoculants have been shown to have beneficial effects on a wide range of plant hosts (Grandlic et al. 2008; Ma et al. 2011; Becerra-Castro et al. 2012). The main objective of these studies has been to improve metal yields during phytoextraction. In this technique, a simple improvement in biomass can result in an increase in overall trace element removal (Sessitsch et al. 2013). In a meta-analysis carried out by Sessitsch et al. (2013), including results from more than 70 publications and 738 individual cases, the authors found that bacterial inoculation was generally more successful in promoting plant growth and biomass production (60% of total cases analyzed) than in influencing shoot trace elements accumulation (only 30% of cases showed a significant increase in shoot trace elements concentration, and 16% a decrease in trace elements concentration).

Ma et al. (2009a, b) found that inoculation with PGP rhizobacteria (*Psychrobacter* sp. SRA1, *Bacillus cereus* SRA10 and *Achromobacter xylosoxidans* strain Ax10) improved the growth of *Brassica juncea* and *B. oxyrrhina*, enhanced soil Ni availability, and increased Ni accumulation in both species. Similarly, Rajkumar and Freitas (2008b) observed that inoculation with the PGP rhizobacterial strains *Pseudomonas* sp. PsM6 and *P. jessenii* PjM15 isolated from a serpentine soil in *R. communis* caused an increase in above-ground biomass, mainly due to IAA production and phosphate solubilization, and consequently enhanced phytoextraction efficiency. Zaidi et al. (2006) demonstrated that inoculation with *Bacillus subtilis* strain SJ-101 not only stimulated the growth of, and Ni accumulation in, *Brassica juncea*, but also protected the plant from Ni toxicity. Various authors also reported increases in

Ni uptake by *B. juncea* and other non-hyperaccumulating plant species (*B. napus*, *Ricinus communis*, *Poa pratensis*, etc.) after bacterial inoculation (Rajkumar and Freitas 2008a, b; Kumar et al. 2009; Gullap et al. 2014). With regard to Ni-hyperaccumulating species, Abou-Shanab et al. (2003b) showed that the strain *Microbacterium arabinogalactanolyticum* isolated from the rhizosphere of *A. murale* significantly increased availability of Ni in the soil and enhanced Ni accumulation by *A. murale*. In agreement with these results, a posterior study with *A. murale* grown in artificially Ni-polluted soils demonstrated that inoculation with selected rhizobacteria strains increased Ni extraction from the soil and Ni uptake by *A. murale* (Abou-Shanab et al. 2006). These authors considered the presence of such rhizobacteria to be an important factor that influenced metal hyperaccumulation. Becerra-Castro et al. (2013) used two strains of *Arthrobacter* (SBA82 and LA44) harbouring several plant growth-promoting characteristics are able to mobilize Ni from serpentine rock, as an inoculum for *A. serpyllifolium* ssp. *malacitanum* grown in ultramafic soil, and observed an increase in plant biomass and shoot Ni concentrations. Durand et al. (2016) isolated plant growth-promoting rhizobacteria from the rhizosphere soil of two hyperaccumulator plant associations: *B. tymphaea*–*N. tymphaea* (NB) and *B. tymphaea*–*A. murale* (AB), both being characteristic of a serpentine outcrop in Greece. The screening of the isolates revealed two PGPR strains (AB30 and NB24) that were affiliated to *Variovorax paradoxus* and that were used to inoculate the same plant associations growing in mesocosms. Biomass (root and shoot), shoot Ni uptake, and Ni removal by the *B. tymphaea*–*N. tymphaea* plant association inoculated with strain NB24 was significantly higher than that of the respective non-inoculated association.

Benefits to plants of mycorrhizal inoculation in highly stressed and nutrient-poor environments are well documented. Inoculation with arbuscular mycorrhizae in trace elements-polluted mine tailings has proven particularly effective in enhancing plant survival and performance during restoration (Orłowska et al. 2011; Kohler et al. 2015; Maltz and Treseder 2015). However, fewer studies have found inoculating with mycorrhizal fungi species or strains to enhance metal uptake and accumulation. In fact, until the early 2000s, hyperaccumulating plants were generally considered non-mycorrhizal (Gonçalves et al. 1997; Leyval et al. 1997; Pawlowska et al. 2000). Since then, several studies have found that hyperaccumulators can form symbioses with arbuscular mycorrhizal fungi (AMF). *Pteris vitatta*, an As-hyperaccumulating fern, tended to show higher mycorrhizal colonization on As-polluted soil. Leung et al. (2007) suggested that enhanced P uptake could be linked to increased As absorption and transport. Mycorrhizal colonisation has been observed in *Thlaspi praecox*, a Cd- and Zn-hyperaccumulator of the Brassicaceae family (generally considered to be -non-mycorrhizal) growing on metal-polluted soil in Slovenia (Vogel-Mikus et al. 2005). *Berkheya coddii* was the first Ni-hyperaccumulating plant in which the presence of arbuscular mycorrhizal (AM) symbiosis was reported (Turnau and Mesjasz-Przybylowicz 2003). Arbuscular mycorrhizal fungi (AMF) isolated from serpentine soil significantly enhanced growth and survival of the Ni-hyperaccumulator *B. coddii* when growing in the same soil (Orłowska et al. 2011). AMF inoculation reduced shoot and root Ni concentrations, however, and owing to the higher biomass the total Ni yield was up to 20 times greater in mycorrhizal plants compared to the non-mycorrhizal ones. The authors proposed that AMF enhancement of Ni uptake may help to improve Ni phytomining techniques.

## 5 Improving Soil Metal Removal Using Metal-Mobilizing Microbial Strains

Metal-hyperaccumulating plant species have an extraordinary capacity for trace metal accumulation. Many authors have considered the possibility that such species actively mobilize trace elements from non-labile soil metal pools, as a means of increasing the labile concentrations and consequently plant uptake and accumulation. In accordance, several studies have shown a higher labile fraction of hyperaccumulated metals, such as Ni, at the rhizosphere of these plants compared to those of non-accumulating plant species or the surrounding non-vegetated soil (Puschenreiter et al. 2005). However, most authors have concluded that this effect is not a result of specific metal-mobilizing mechanisms employed by hyperaccumulators. It seems more likely, therefore, that the pronounced depletion of trace elements in the rhizosphere, caused by the high rate of uptake of the element from the soil solution, induces their replenishment from the less-mobile fraction to the mobile pools, and maintains a sustained high metal concentration in the soil solution. Root activity of *N. goesingensis* has been proposed to participate in an enhanced dissolution of Ni-bearing minerals, contributing to higher Ni uptake (Puschenreiter et al. 2005). Similarly, mineralogical studies have shown the presence of smectite in the rhizosphere of *A. serpyllifolium* ssp. *lusitanicum*, which was related to a more intense weathering of Ni-rich ferromagnesium minerals (chlorite, serpentine) and an increase in labile Ni (Kidd et al. 2009). Chardot-Jacques et al. (2013) found that growth of the Ni-hyperaccumulator *L. emarginata* increased the dissolution of chrysotile (a silicate mineral of the serpentine group with low Ni solubility). These authors suggested that the high Ni uptake by the plant causes a decrease in water-soluble Ni, which in turn induces chrysotile dissolution. However, whether this phenomenon is plant- or microbially-induced, or the result of complex plant-microbial interactions, is unknown. It is well established that microbial transformation of soil minerals leads to the solubilization of metals together with essential nutrients, and to the modification of their form and distribution in the solid phase (Quantin et al. 2002). Soil metal availability greatly influences the success of phytomining and its long-term sustainability, and bioaugmentation with metal-mobilizing, plant-associated bacteria could enhance the viability of this technique (Kidd et al. 2009).

Microbes are intimately associated with the biogeochemical cycling of metals. Microbial activity can result in metal mobilization or immobilization depending on the mechanism involved and the microenvironment where the organism(s) are located (Violante et al. 2008; Ehrlich and Newman 2009; Gadd 2010) (Fig. 2). Some metal-tolerant bacterial strains associated with (hyper)accumulating plants have been shown to mobilize metals in soils, and consequently increase the phytoavailable metal fraction in the soil, as well as plant uptake and accumulation. Muehe et al. (2015) found *Arabidopsis halleri* accumulated 100% more Cd and 15% more Zn when grown on natural Cd-polluted soil than on the same soil that had undergone gamma-irradiation. Gamma irradiation affected neither plant growth nor soil metal availability, but strongly altered the composition and density of the soil microbial community. Bacteria can modify trace elements mobility and bioavailability through several mechanisms: the release of chelating agents (such as organic acids, phenolic compounds, and siderophores), and acidification or redox changes in the rhizosphere (Lloyd 2003; Glick 2010). Sessitsch et al. (2013) reviewed potential mechanisms for microbial effects on bioavailability in the rhizosphere environment. Sorbed, precipitated, and occluded trace elements can be solubilized by acidification, chelation, and ligand-induced dissolution. To date, two groups of bacterially produced natural chelators are known: organic acids and siderophores. Low-molecular mass carboxylic acids can play an important role in chemical attack of minerals, providing protons as well as a metal-chelating anion (Jacobs et al. 2002; Huang et al. 2004; Lian et al. 2008).

Oxalic acid can leach metals such as Al and Fe that form soluble oxalate complexes (Strasser et al. 1994). Bacteria that produce trace element-chelating organic acids, such as citric, oxalic, or acetic acid, have been shown to mobilize various trace elements in soil (Becerra-Castro et al. 2013). As mentioned above, siderophores form

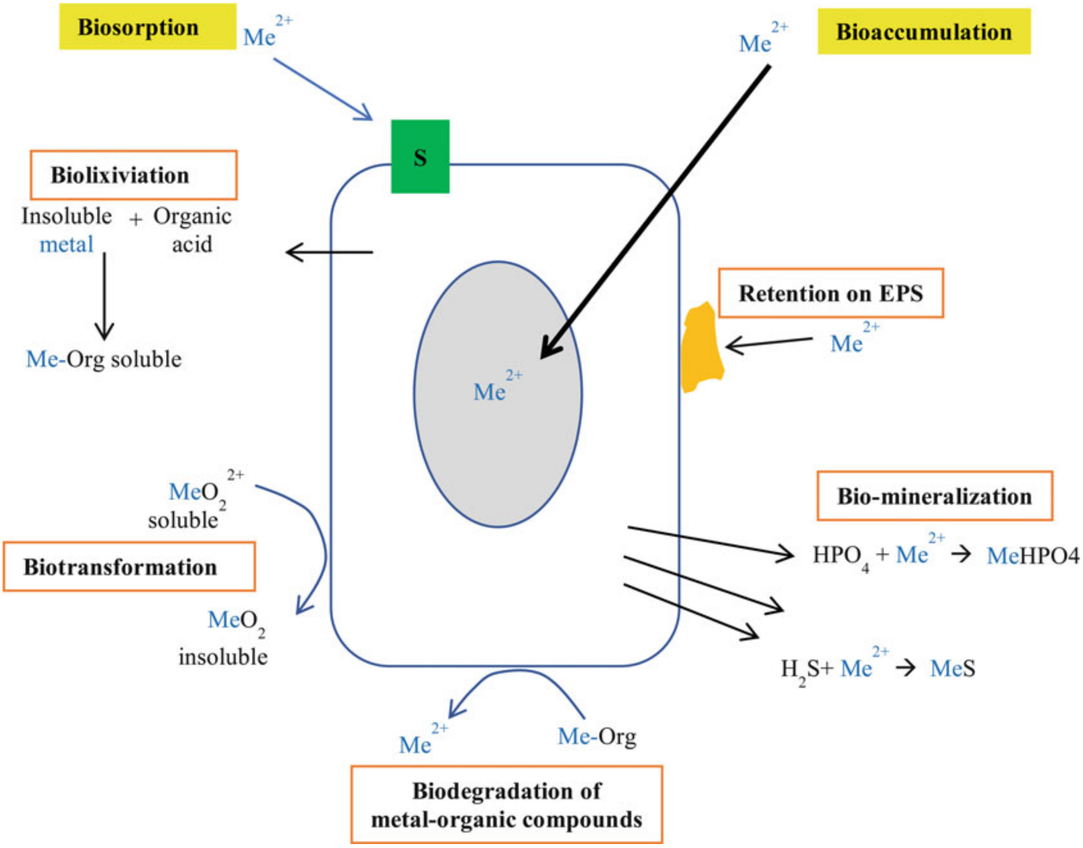

**Fig. 2** Interactions between metals and bacteria (adapted from Ledin 2000). S corresponds to the reactive groups present on the bacterial cell wall. $Me^{2+}$ represents a cation metallic. Org corresponds to an organic compound

high-affinity complexes with Fe(III), but can also form complexes of lower stability with other trace elements (Al, Cd, Cu, Ga, In, Ni, Pb and Zn), thus affecting their bioavailability (Schalk et al. 2011; Rajkumar et al. 2012; Sessitsch et al. 2013). Plants can then take up metals from siderophores via various mechanisms, such as chelate degradation, the direct uptake of siderophore–metal complexes, or by a ligand exchange reaction (Schmidt 1999). Braud et al. (2009) reported that inoculating soils with siderophore-producing *P. aeruginosa* significantly increased the concentrations of bioavailable Cr and Pb compared with non-inoculated controls. Enhanced heavy metal uptake was correlated with the increased production of siderophores. Siderophore-producing bacteria that are present in metal-polluted or metal-enriched soil, and their interaction with hyperaccumulators, could be used in MAP. Several siderophore-producing bacterial strains associated with plants, such as the non-hyperaccumulator *Brassica juncea*, and the hyperaccumulators *T. goesingense, A. bertolonii*, and *A. murale*, have been isolated and characterized from metal-rich soils. They belong to different genera, such as *Staphylococcus* sp., *Microbacterium* sp., *Pseudomonas* sp., *Curtobacterium* sp., *Bacillus* sp., *Arthrobacter* sp., *Paenibacillus* sp., *Leifsonia* sp. *Methylobacterium mesophilicum*, *Methylobacterium extorquens*, *Methylobacterium* sp., *Burkholderia terricola*, *Okibacterium fritillariae*, *Rhodococcus fascians*, *Rhodococcus* sp. (Idris et al. 2004; Barzanti et al. 2007). Becerra-Castro et al. (2013) evaluated the weathering capacities of, and Ni mobilization by, two rhizobacterial strains associated with the

Ni-hyperaccumulator *A. serpyllifolium* ssp. *lusitanicum* and ssp. *malacitanum*. A minimal culture medium containing ground ultramafic rock was inoculated with either of two *Arthrobacter* strains: LA44 (indoleacetic acid [IAA] producer) or SBA82 (siderophore producer, $PO_4$ solubilizer, and IAA producer). Strain LA44 is a more efficient Ni mobilizer, apparently solubilizing Ni associated with Mn oxides, and this appeared to be related to the exudation of oxalate. On the other hand, strain SBA82 also led to the release of Ni and Mn, albeit to a much lower extent. In the latter case, the concurrent mobilization of Fe and Si reflects preferential weathering of Fe-oxides and serpentine minerals, possibly related to siderophore production capacity of the strain. However, some conflicting results showed that the presence of siderophore producers decreased the uptake of metals by plants. Siderophores produced by *Pseudomonas* sp., *Serratia marcescens*, and *Streptomyces* sp. had either no effect or negatively affected Zn and Cd uptake by *Salix caprea* (Kuffner et al. 2010). These contrasting effects suggest that the mechanisms underlying metal uptake are largely plant dependent, but that the efficiency of siderophore producers to either mobilize or immobilize heavy metals from soils is also dependent on several factors such as the binding form of the heavy metals present, the charge of the siderophores, and the pH of the soil and its mineral composition and organic content.

The influence of organic matter content in soil on metal availability and uptake by plants has been extensively studied. It was reported that metal adsorption onto soil constituents declined with decreasing organic matter content in soils (Zeng et al. 2011). Moreover, dissolved organic matter (DOC) in soils may increase the mobility and uptake of heavy metals by plant roots (Du Laing et al. 2008). In fact, organic matter decomposition appears to be one of the most important microbial activities in the rhizosphere, and it is well known that microbes, mainly bacteria and fungi, utilize a wide spectrum of organic compounds such as sugars, organic acids, and amino acids to more complex molecules such as cellulose, pectin, lignin, lignocellulose, chitin, and starch. Consequently, organic matter decomposition by soil microorganisms releases metals such as Fe, Mn, Zn, Cu, Mo, Ni, Co and Se, which are typically found in the tissues of organisms.

## 6 Final Remarks and Future Directions

It seems clear that the rhizosphere microbiome plays an important role in plant trace elements bioaccumulation, given experimental evidence suggesting that by reshaping this microbiome we can further enhance the efficiency of phytoextraction and phytomining. However, the efficacy of plant-associated bioinoculants is dependent on a complex array of interacting factors: plant-microbe specificity, soil type and properties, trace elements concentration and type (mono- or poly-metallic), proliferation and survival of the inoculant, *etc*. Moreover, from the literature it can be seen that inoculation methods differ greatly among studies (inoculation of seed/plant/soil, frequency/timing of inoculation events, bacterial cellular densities, etc.), and these aspects are likely to influence whether or not a beneficial microbially induced effect is observed.

Bioaugmentation can be challenged by the strong competition encountered in the soil when a selected microbe is introduced. Also, the selected host plant species may not necessarily be compatible with the inoculated bacterial or fungal strain, since these are not naturally selected for by the host (Thijs et al. 2016). The importance of rhizosphere competence or effective root colonization in beneficial plant-microbe interactions is underlined by several studies as contributing to the success of bioaugmentation (Lugtenberg and Dekkers 1999). Moreover, inoculation methods should be optimised in orderoptimized to enhance the success of the introduced microorganisms (Álvarez-López et al. 2016b). Pereira et al. (2015) and Álvarez-López et al. (2016b) recently showed that the size of the inoculum applied in the soil rhizosphere, and the mode of application (seed or soil inoculation), are important factors that should be taken into account when planning MAP strategies. Endophytes do not have to compete with the large abundance and diversity of soil microorganisms, which may enhance their chances of establishing a stable and active population. To date, the field of MAP has focused on the use of individual bioinoculants. However, promising results have been found when using mixtures or consortia of different PGP-strains with complementary actions. Visioli et al. (2015) reported that co-inoculating the Ni-hyperaccumulator *Noccaea caerulescens*, when growing in serpentine soil with two root endophytes belonging to the *Arthrobacter* and *Microbacterium* genera, had a more positive effect on plant growth, soil Ni removal, and Ni translocation, than when inoculated individually. Both strains were strong IAA producers and presented ACC deaminase activity. Moreira et al. (2016) showed the benefits of combined inoculation of AMF and PGPR for the growth of maize as an 'energy-crop' in metal-polluted soils and their potential application in phytomanagement strategies.

Ultramafic rocks occupy <1% of Earth's land surface and these outcrops have the potential to provide multiple ecosystem services and contribute to producing renewable raw materials and energy (i.e. agromining). In the same way, polluted soils are an ever-increasing environmental concern due to increased industrialization. Phytoremediation coupled with bioaugmentation could be a solution towards the recovering soil quality, underlining the role of the rhizosphere and microbes associated with hyperaccumulator plants in metal accumulation.

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# Incorporating Hyperaccumulator Plants into Mine Rehabilitation in the Asia-Pacific Region

Peter D. Erskine, Gavin Lee, Bruno Fogliani, Laurent L'Huillier, and Stéphane McCoy

**Abstract**

The identification and use of hyperaccumulator plants in mining projects has been recognised as an important component part of mine planning at several sites around the world. Indeed, mine planning that includes hyperaccumulator plants requires operators to maximize the biological resources of a site by discovering and utilizing these unusual plant species at the early planning stages. These locally adapted hyperaccumulator species can then be used for rehabilitation in and around the mine area, or be utilized for their potential to extract ecocatalysts and metals. Such opportunities should be more widely explored so that these unique plants can become an integrated and valuable part of the mining process. This chapter concentrates on the experiences of integrating hyperaccumulating plants into mine rehabilitation projects in Indonesia and New Caledonia, in order to highlight some of the opportunities and challenges encountered when attempting to incorporate these species into the mining cycle.

## 1 Introduction

The use of metal hyperaccumulating plants has the potential to add significant value to the metal mining cycle and thus create a more sustainable minerals industry. With metal reserves worldwide declining in quality (ICMM 2012), and with cost and technical challenges being faced in the processing of low-grade ores, agromining techniques (i.e. the process of cultivating, harvesting, and processing metal hyperaccumulating plants to produce metals and ecocatalysts), in concert with conventional mineral ore extraction and processing, could improve

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the efficiency of recovery of metal from existing ore reserves. The use of the metal-enriched biomass from agromining to derive Ni, Mn and Zn salts using a 'green technology' appears to be an important opportunity for mining companies to utilise sustainable approaches to mineral extraction (Losfeld et al. 2012). The unique opportunity provided by hyperaccumulator plants to naturally biochemically concentrate and purify metal from ultramafic soils (Kelly et al. 1975; Jaffré et al. 1976, 1979; Jaffré 1979; L'Huillier and Edighoffer 1996; Chaney et al. 2007) and increase recovery of metal from reserves, can also facilitate multiple additional benefits, including:

- Increased metal productivity per unit of biodiversity loss;
- Improved soil fertility in mined-out soils, hence becoming available for a wider range of metal-sensitive plants;
- Remediation of contaminated waste materials;
- Reduced erosion on mined-out soils and wastes; and
- A more productive and viable post-mining use of lands.

These benefits are important and meaningful contributions to mitigating environmental and social impacts from modern mining operations. The use of metal hyperaccumulator plants to increase mineral recovery prior to conventional strip mining and after mine extraction, can also provide a range of low-cost opportunities that facilitate the generation of a sustainable vegetation cover having biodiversity values.

This chapter assesses some of the processes required for the seamless integration of hyperaccumulator plants into mine-site rehabilitation and agromining. It covers the multiple stages of the mining cycle and addresses important issues that need to be considered when approaching a new ore body, particularly in highly biodiverse tropical regions. We then use examples from the Asia-Pacific region to illustrate the process of how hyperaccumulator discoveries and the mining cycle can be combined.

## 2 The Mining Cycle and Agromining Development

The conventional mining cycle can generally be described by a number of specific but overlapping processes. These processes include (i) discovery of the minerals and assessment of their quantity and quality (Exploration and Development); (i) means for extraction of the minerals identified, costed, and a mechanism for funding the development established (Feasibility and Financing); (ii) preparation of infrastructure for extraction and processing of the minerals (Construction); (iv) extraction, processing, and sale of the mineral product to the market (Operations); (v) rehabilitation of disturbed land; and (vi) cessation of extraction and processing (Closure).

If benefits of hyperaccumulator plants are to be attained, then processes need to be developed that identify the potential of these species, demonstrate their effectiveness, ensure their accessibility/viability, and realise their potential to contribute to the mining cycle. There are three steps involved in this agromining process. *Screening and Prioritization* is used to identify local variants of hyperaccumulator species, and which species have the potential for use in agromining. The *Conservation and Propagation* stage ensures the long-term availability and viability of the selected hyperaccumulator species as assets. Finally, the *Recovery and Restoration* stage enables use of the asset and hence the creation of value for the project.

Figure 1 presents the *Mining Cycle* and integration of agromining development within the cycle. The stages of agromining development within the *Mining Cycle* remain fluid and can vary significantly from conceptualization as shown in Fig. 1. However, the commencement of the *Screening and Prioritization* stage to identify potential hyperaccumulating species, as early as possible within the *Mining Cycle*, ensures that subsequent stages can be appropriately planned later. This plan increases the possibility of the strategic objectives of agromining (i.e. maximizing recovery of metals from

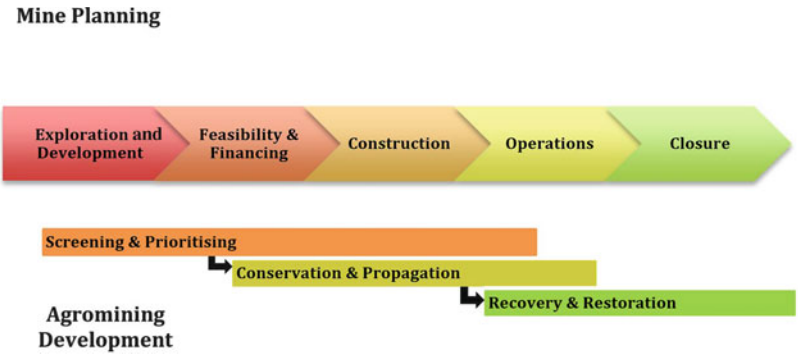

**Fig. 1** The relationship between the mine planning process and the steps required for agromining

existing ore) being met, and the associated benefits being realised.

Agromining extends the recovery of target metals otherwise inaccessible by conventional mining (e.g. ultramafic soils below cut-off grades) that commonly make up a large proportion of mining leases. It provides an opportunity to involve local communities in a ‘metal farming’ cooperative, supported by industry for the required research and development and off-take of the harvested bio-ore. The development of hyperaccumulating vegetation that reduces the metal content of the soil through agromining harvests over time will improve fertility, by allowing more sensitive species to establish biomass owing to the decrease in metal availability. Such a role can be regarded as a significant agronomic or ecosystem service if this is the aim of the rehabilitation. On the contrary, reducing concentrations of metals in environments where the flora is highly adapted to these local conditions may also provide habitat for invasive species (B. Fogliani, pers. observations).

## 3 Screening and Prioritizing

The screening and prioritizing phase should start as early as possible in the *Mining Cycle*. Where the spatial extent of nickel ore bodies is significant, and multiple pit development occurs over a temporal scale, it would be expected that screening could continue until well into the Operation Stage. Where a mining project has made commitments towards assessing biodiversity losses, it remains likely that flora surveys will be required to be ongoing, and as such provide an opportunity for continuation of the screening and prioritizing phase.

### 3.1 Environmental Impact Assessments and Biodiversity Baselines

Environmental Impact Assessments (EIAs) are now a standard regulatory requirement for the mining industry in most countries. A relatively recent trend has been the emergence of EIAs for financing, where the regulatory framework is considered insufficiently rigorous to adequately cover environmental and social risk for investors. Institutional finance and investment agencies, such as International Finance Corporation and Asia Development Bank, encourage their clients to make bold commitments towards management of environmental and social management. Such commitments then place a premium on the development of detailed EIAs. As an example, recognition of the value of local Ni hyperaccumulator species in the EIA process through production and growth trials can provide further proof that the Project Owner is serious in achieving their stated commitments.

EIAs are generally conducted as part of the *Finance and Feasibility* stage, although planning for an EIA may take place in the *Exploration and Development* stage. A key component to the planning of an EIA is development of the biodiversity baseline. One of the initial steps in the generation of such a baseline is the desktop review. Where the flora of a particular location is already well understood and the data are accessible—either through previous EIA studies or in regulatory or other open-source databases—the species list can be cross-checked against known lists of hyperaccumulator species. Given that this is a relatively new field of research and the range of families known to contain hyperaccumulating species is already exceptionally diverse, any species list generated on the basis of known hyperaccumulator species should be taken as a preliminary baseline for further fieldwork, in order to ultimately determine abundance and habitat preference. Floristic surveys conducted as part of the biodiversity baseline provide the opportune time to commence the screening process for hyperaccumulator plants. Benefits of integrating the screening in the flora surveys are as follows:

- Coverage: generally, baseline biodiversity surveys endeavour to cover all habitats in the mining concession, including those areas that may contain deposits of sub-economic ore. These areas should also be recognized as a potentially important source of hyperaccumulator species.
- Expertise: well-planned flora surveys usually include a botanist with local knowledge in order assist with field identifications. Field identification of *in situ*, hyperaccumulator species improves reliability and accuracy of the surveys.
- In cases where EIAs and biodiversity baselines have already been conducted, screening for hyperaccumulator species can be done through targeted survey work. As with the flora surveys of the biodiversity baseline, it is recommended that the survey team include at least one botanist with local knowledge. Plant collections made during the flora survey (whether for the EIA or a targeted hyperaccumulator survey) should ensure that standard field records are maintained, including the location of collections, geospatial coordinates, plant description, habitat description, notes on abundance, ecological role of target species, etc.

## 3.2 Screening

Field screening for candidate plant species to determine their metal content and hyperaccumulating potential is a relatively straightforward process. Concentrations of metals and other elements can be determined in the field or with collected leaves using a handheld X-Ray Fluorescence (XRF) instrument (see chapter "Tools for the Discovery of Hyperaccumulator Plant Species and Understanding Their Ecophysiology"; Gei et al. this book).

In the case of nickel, moistened samples of the identified plant, typically the leaf, but including roots or phloem, are placed on indicator paper containing dimethylglyoxime (Reichen 1951). A positive reaction (i.e. the appearance of a magenta colouration on the indicator paper) indicates Ni hyperaccumulation.

## 3.3 Prioritizing

Prioritising hyperaccumulator species should be based on whether they occur locally, on their metal hyperaccumulating properties, their habitat, and their physiological properties. During the initial EIA, a screening process should identify local native hyperaccumulator species and their habitats. Selection of native species found in open exposed environments are preferred, as they are more likely to match local exposed bioclimatic, geochemical, and physiological conditions of post-mining surfaces. Species vary in metal hyperaccumulating properties, and priority should be given to species that have at least 1% foliar weight of either Ni or Mn (Losfeld et al. 2014a). Other physiological properties of priority are to have high biomass yield and to be relatively straightforward to propagate. The selected species should have ecological characteristics suitable for agromining, such as tolerance to regular harvesting, so that foliage can be removed and reduce metal concentrations for rehabilitation. At the stage of prioritizing, it is likely that only one or two of these criteria will be sufficiently understood for decision making on preferential candidate species. It is recommended that the list of species for the next stage, *Propagation and Conservation*, remain broad in cases where uncertainty exists.

## 4 Conservation

Development of a list of priority hyperaccumulator species is the first step by the project owner in recognising the value of these species. The next phase in the integration of agromining in the *Mining Cycle* is to understand and assess the value of these species grown under exposed harvest conditions.

### 4.1 Conserving Priority Species

Looking broadly at hyperaccumulator species as components of the local biodiversity and assigning a conservation value for development planning is based on how the species is valued by people (local or elsewhere), and on its particular functional importance. This can include rare or threatened species, species of cultural or social importance, species associated with important ecological processes, or species of economic value (Gardner et al. 2013). In this context, hyperaccumulator species have the potential to be recognized as having biodiversity value in at least one of the above-mentioned criteria.

Nickel and Mn hyperaccumulator plant species are known to have a high degree of island endemism. In New Caledonia, studies have documented 65 Ni hyperaccumulator species belonging to 21 genera and 12 families (Jaffré et al. 2013), and 22 Mn hyperaccumulator species belonging to 11 genera and 8 families (Losfeld et al. 2014a). It appears likely that a similar order of magnitude of endemic species will be found through more consolidated survey work in the ultramafic terranes of Southeast Asia. Given economic potential for the exploitation of nickel ore from lateritic soils in this region, and possible high levels of endemism from ‘strict’ hyperaccumulator species for providing information on obligate vs. facultative hyperaccumulators, it is necessary that vulnerability and irreplaceability of the local hyperaccumulator species be assessed, in order to determine their distribution and pinpoint the micro-endemics found in future mine leaseholds.

For project owners with biodiversity objectives such as *No Net Loss*, it will be important to develop specific actions for the conservation of hyperaccumulator species based on their assigned conservation value. Best practices in biodiversity management recommend the application of the mitigation hierarchy (i.e. Avoidance, Minimization, Restoration, Offsetting) as a basis for the development of these plans. Even where project owners have made no specific biodiversity objectives but recognize the value in hyperaccumulator plant species, application of the mitigation hierarchy provides a sound framework for decision making in agromining development. Avoidance, minimization, and offsetting for hyperaccumulator species all provide the benefit of preserving genetic diversity. By maintaining viable populations of hyperaccumulator species *in situ*, the project owner guarantees long-term stock (i.e. seeds, seedlings, cuttings) having greater resilience to pest and disease, and the capability to select specific individuals for propagation/restoration based on several traits deemed suitable for agromining, including:

- Naturally high-metal concentrations (>1%),
- Ability to achieve rapid biomass growth (without a significant loss of target mineral content),
- Ability to grow in open conditions,
- Easily propagated,
- Can tolerate annual harvests, and
- Continues to produce the same amount of aerial biomass after agromining harvests.

Conservation actions related to avoidance, minimization, and offsetting are briefly described below, whilst *Recovery and Restoration* is covered after the section on *Propagation*.

## 4.2 Conservation Actions: Avoidance

It is unreasonable to assume that project owners will avoid clearing in the vicinity of economic ore reserves. However, site selection of supporting infrastructure, such as mine roads, laydown pads, maintenance yards, and fuel storage, can, with careful planning, avoid known stands of hyperaccumulator species in areas where other conservation needs are apparent (e.g. rainforests or rivers). The key to careful planning is that information on hyperaccumulator plants is available at the time of decision making. This fact reinforces the need to start the screening and prioritizing stage as early as possible.

Another form of avoidance is where a particular ore body may be only marginally feasible, either technically or economically, and the project owner decides to set aside this ore body, in order to assist in meeting biodiversity commitments. Setting aside such conservation areas provides a greater potential than other forms of avoidance, by realizing the benefits of hyperaccumulator plant diversity that commonly occur in areas containing high levels of the target minerals.

## 4.3 Conservation Actions: Minimisation

Minimisation of the clearing of hyperaccumulator species typically occurs through implementation of operational activities, as opposed to avoidance during planning. Where avoidance is not feasible, or not 100% certain, and where there will be a loss of hyperaccumulator species, *ex situ* conservation through collection of seeds and cuttings prior to clearing and supervision during land clearing activities is necessary to ensure losses are not greater than anticipated. It is recommended that land clearing procedures include management accountability for clearing, demarcation of areas to be cleared in the field, and awareness by supervisors and equipment operators as to clearing limits and specific environmental sensitivities (i.e. the presence of hyperaccumulator species) beyond the limit.

Salvaging of species, through the removal of seeds, seedlings, and soils known to contain seed stocks of hyperaccumulator species, from areas designated for clearing, provides valuable source material for propagation. Integrating the identification and collection of seedlings and parent trees of known hyperaccumulator species as part of the land clearing procedure, through the application of pre-clearing biodiversity surveys, is an efficient method for boosting stocks of seedlings. Where an abundance of one or more hyperaccumulator species is found in an area to be cleared, it may be practical to delineate the area and remove the soil, as part of standard topsoil removal procedures, for direct return to areas to be trialled.

## 4.4 Conservation Actions: Offsetting

In the event that avoidance and minimization have not adequately maintained viable populations of hyperaccumulator species with known agromining potential, project owners may commit to developing an offset either based on hyperaccumulator species richness, or as part of a broader strategy for mitigating biodiversity losses and reinforcing existing populations and *ex situ* conservation. As the last step in the mitigation hierarchy, offsets are a risky strategy and are likely to only be applied where there is a regulatory and/or financial investment requirement to do so. However, offsets do present a significant opportunity to maintain genetic diversity of hyperaccumulator species, if a ‘like-for-like’ approach is taken in the selection of offset sites. Offsite conservation of hyperaccumulator species is particularly important for projects that propose agromining as a post-mining land use and where the size of mines requires large areas to be rehabilitated.

## 5 Propagation

The propagation stage provides further refinement of the prioritization process and delivers the optimum basis from which recovery of the target mineral can take place. In this sense, propagation can be seen as the point in the agromining process in which the potential of the hyperaccumulator species can begin to be realized. A key outcome of the *Propagation* stage is that an accurate economic model can be developed for the feasibility of agromining for metals or ecocatalyst production.

### 5.1 Trials

Trials are a critical part of demonstrating that hyperaccumulator species are capable of growing in the modified environmental conditions following mining. The development of future rehabilitation planning depends upon accurate estimates of germination rates, propagation potential, growth rates, canopy spread, site capture, and root spread (for erosion protection). Field trials need to clear any barriers to successful implementation of agromining production, by delivering critical insights into the suitable combinations of soils, amendments, and plants in order to obtain optimal growth conditions. Relevant parameters should be recorded and monitored, e.g. growth, biomass production by unit surface, metal concentration, etc. For each type of trial conducted, a full balance would need to be made (biomass production × Ni phytoextraction yield).

### 5.2 Economic Model

Scenarios of economic yield can be built on the data obtained from the field-based trials and be used as decision-making tools for implementing agromining at full scale. Objectives for agromining, i.e. as a post-mining land use and/or recovery of minerals from uneconomic or technically constrained ore deposits (see Recovery section), will play an important role in assigning the assumptions upon which the economic model is based. Results of grade control drilling to determine available minerals at the base of mined-out pits or in uneconomic ore bodies can contribute important data (Robinson et al. 1999), to support the development of an economic model for agromining as part of a mine plan.

## 6 Recovery and Restoration

Recovery and restoration is the final stage in the agromining cycle and, if well implemented, can last beyond mine closure. Recovery involves the extraction of the target mineral from the source plant material and conversion to a saleable product. Restoration entails the integration of the use of hyperaccumulator species in the mine rehabilitation programme. Although these two processes are inter-related, they are not necessarily sequential.

Where results from floristic surveys (either through the biodiversity baseline development or during pre-clearing surveys) indicate an abundance of hyperaccumulator species at a particular location, and test work on these plants suggests concentrations of the target mineral (e.g. Ni) suitable for extraction, the Project Owner may choose to recover this nickel. Alternatively, where ore grades are found to be uneconomic for mining in a conventional sense, or where mineral deposits lie in an area outside the constraints set for safe development of a mine pit, such as in steep topography or near watercourses, agromining presents an opportunity for additional mineral recovery. In the latter case, special attention should be paid to environmental impacts and safety risks that may arise. Broad-scale removal of vegetation from slopes carries a significant risk of erosion, and mitigation may prove costly. A phased approach in which hyperaccumulator species are initially planted in the form of an ‘enrichment planting’ among existing native vegetation may ensure the stability of soils, and eliminate the need for heavy equipment usage. As the initial plantings begin to stabilize/mature, further infill planting can be conducted.

**The Halo**

Agromining could be implemented both on the large 'halo' of mineralization surrounding the mine leases, and as part of the progressive rehabilitation process after conventional resource extraction. A full demonstration of agromining at the mine site is required in order to provide evidence of real-life performance of economic viability and to evaluate the fundamental parameters (i.e. success of the crop, Ni yield per hectare).

## 7 Planning

The incorporation of hyperaccumulator species into mine rehabilitation and closure plans will help ensure that these species are managed during the life of the operation. Appropriate soil and plant management practices, based on insights from laboratory or field tests, need to be undertaken in order to produce high yields of the selected 'metal crop.' Optimizing nutrition in hyperaccumulator species is a pre-requisite to successful large-scale agromining. Although opportunities exist in tropical regions for agromining operations, few studies have been conducted in such regions to ascertain the type and quantity of fertilizer that enhances biomass production while maintaining or improving annual mineral yields. It is possible that N + P + K fertilization will increase biomass production, yet cause a 'dilution' of the target mineral in the biomass of tropical hyperaccumulator plants.

Agriculture developed on ultramafic soils often has low productivity due to the lack of Ca, K, and P and excess Mg and Ni. Over time, agromining can will improve fertility and significantly decrease Ni availability; this can be regarded as a significant agronomic service.

### 7.1 Case Study 1: Halmahera Island, Indonesia

Prior to screening conducted at the Weda Bay project owned by Eramet, no hyperaccumulator plants were known from Halmahera Island, and only five were documented in all of Indonesia. The first stage in the research project was the identification of native hyperaccumulator plants occurring on the mine lease. During the fieldwork, a total of 21 non-permanent plots were made in which 824 herbarium vouchers and associated foliar samples, in addition to soil samples (totalling 90 samples), were collected for laboratory analysis. Plants were screened in the field (>1000 specimens) for Ni hyperaccumulation using dimethylglyoxime, and after positive reaction a detailed sample suite was collected for these hyperaccumulator plants. Further screening of collected plants was conducted using both handheld XRF and elemental analysis using traditional wet chemical methods. This screening included samples of the rhizosphere soil, root, wood, branches, and leaves. In total 18 Ni hyperaccumulators species and one Mn hyperaccumulator species were discovered (Fig. 2).

### 7.2 Case Study 2: New Caledonia

Ultramafic rocks cover one third of New Caledonia and possess substrate properties that virtually exclude agriculture and pastoral grasses, thus minimizing human impact. Lateritic soils developed on such rocks provide rich sources of, Ni, Cr and Fe ores and have been a focus of intensive mining activity for over a century (L'Huillier et al. 2010). Early revegetation trials in the 1970s focused on fast-growing exotic nurse trees, grasses, and native N-fixing *Casuarina collina* trees and *Acacia spirorbis* shrubs, in order to stabilize slopes and generate a vegetation cover. These N-fixing species were planted to stimulate succession on mine overburden from surrounding native communities, by improving the nutrient content of the soils

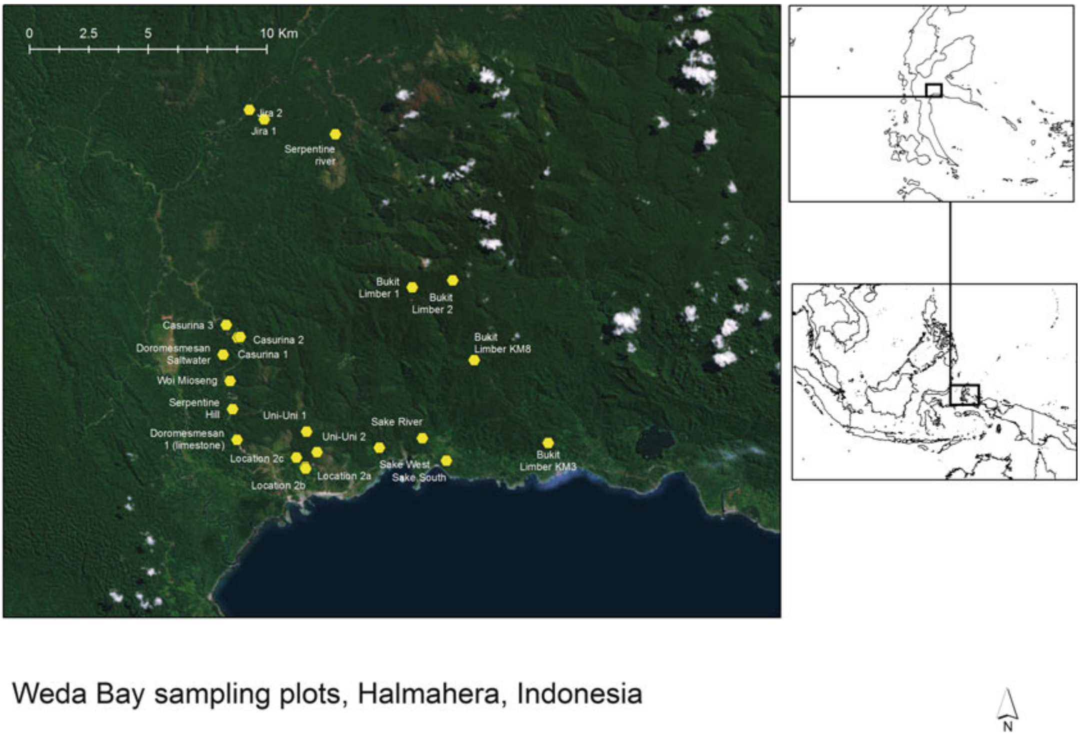

**Fig. 2** Location of hyperaccumulators at Halmahera Island discovered after two weeks of field reconnaissance

(Cherrier 1990; Sarraih 2002; L'Huillier et al. 2010). However, most of these nurse species failed to generate these colonizing conditions at mine sites on mountain tops, as they were not adapted to high altitude environments. Certain ultramafic heathland species (genera *Maytenus*, *Grevillea*, *Geissois*) were identified as Ni or Mn hyperaccumulators in the 1970s and 1980s, and were trialled on plantations without full knowledge of their potential in mine restoration (Jaffré and Latham 1976; Jaffré 1980). The first trial in New Caledonia on mine sites having hyperaccumulators included *Grevillea exul* (Fig. 3) and *Grevillea gillivrayi* (Fig. 4) in 1974 (Jaffré and Latham 1976). Later experiments were conducted with *Grevillea exul* (ssp. *exul* and ssp. *rubiginosa*), *G. gillivrayi*, *G. meisneri* (Proteaceae), *Geissois pruinosa* (Cunoniaceae), or *Cloezia artensis* (Myrtaceae). These were considered key revegetation species because of their good growth and ability to adapt to different conditions (Jaffré et al. 1993; Rigault et al. 1996; Sarrailh 2002). Results of such pioneering studies were expanded in the 1990s in order to evaluate the diverse endemic flora of these ultramafic massifs, and to identify species adaptations tolerant to lateritic substrates on mine sites in New Caledonia (Jaffré and Rigault 1991; Jaffré and Pelletier 1992; Jaffré et al. 1994, 1997, 2009; Luçon et al. 1997; L'Huillier et al. 2010). The objective of using this diverse species pool is to provide a range of rehabilitation solutions depending on the mine location and its final land use. Species screened included certain Ni or Mn hyperaccumulators (Jaffré 1980; Jaffré et al. 2013; Losfeld et al. 2014b) from maquis heathlands around mine sites (Jaffré and Pelletier 1992; L'Huillier et al. 2010) that have potential in agromining/phytomining on mine dumps and abandoned mine sites, as they comply with survival and harvest criteria (Table 1).

More recently, mining companies such as Société Le Nickel (SLN), Vale NC, and Koniambo Nickel SAS (KNS) in collaboration with research institutions such as L'Institut de Recherche pour le Développement (IRD),

**Fig. 3** Wild growing *Grevillea exul* ssp. *exul* in New Caledonia (Copyright IAC)

**Fig. 4** *Grevillea gilivrayi* found in New Caledonia has reasonable growth rates and is a noted Mn accumulator (8200 μg $g^{-1}$) (Copyright IAC)

Institut Agronomique néo-Calédonien (IAC), Centre de coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), and the University of New Caledonia (UNC) have expanded the knowledge base of heathland species in mine site revegetation to identify a suite of pioneer species that are easy to propagate and are adapted to exposed mine site environments. Vale NC has an extensive propagation program that involves the annual production of 120 endemic species from the Goro area for revegetation of its mine lease and surrounding areas in southern New Caledonia. Started in 1996, the revegetation programme also includes several Mn hyperaccumulators (*Grevillea exul* ssp. *rubiginosa*, *Grevillea gillivrayi*, *Polyscias pancheri*, *Pancheria billardierei*, *Denhamia fournieri*, *Peripterygia marginata*) and Ni hyperaccumulators (*Geissois pruinosa*, *Codia spatulata*, *Normandia neocaledonica*, *Cloezia artensis*) commonly found on laterites of southern New Caledonia (Jaffré 1980; McCoy et al. 1999). Most species re-sprout after fires, which provides indirect

**Table 1** Nickel or manganese hyperaccumulators from New Caledonia maquis heathlands around mine sites with potential for mine rehabilitation [(1) Germination (level or time)/end survival in the nursery (%), (2) Growth in nursery]

| Family | Species | Leaf Ni (μg $g^{-1}$) range | Max. leaf Mn (μg $g^{-1}$) | Reference | Ecological distribution | Ecological role | Phenology/ dispersal mode | Seed production/ storage | Propagation history | Germination/ survival % (1) | Nursery seedling ht. year (1) | Nursery seedling ht. year (2) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Araliaceae | *Polyscias pancheri* | – | 14.000 | Losfeld et al. (2014a) | Localised common in south | Maquis Shrub | Irregular, Zoochore | Low, orthodox | Experimental scale germination—(<100 seedlings) | <12 months, 10% | 5 cm | 7 cm |
| Celastraceae | *Denhamia fournieri* ssp. *fournieri* | 68–1575 | 33.000 | Jaffré (1977) | Widespread west coast massifs serpentinite | Small maquis shrub | Irregular, Anemochore | Low, orthodox | Small scale germination & cuttings (<1000 seedlings pa.) | 6 months, 10% | 10 cm | 15 cm |
| | *Peripterygia marginata* | 23–1800 | – | Jaffré (1980), Jaffré et al. (2013) | Widespread ultramafic | Small maquis shrub | Annual, Anemochore | Low, orthodox | Small scale germination & cuttings (<1000 seedlings pa.) | Low, 10% | 4 cm | 10 cm |
| Cunoniaceae | *Pancheria billardierei* | – | 6.500 | Jaffré (1980) | Widespread main island | Small maquis shrub | Annual, Anemochore | Moderate, orthodox | Small scale germination (<1000 seedlings) | <2 months, 20% | 12 cm | 20 cm |
| | *Codia spatulata* | 27–1420 | – | Jaffré et al. (2013) | West & south coast massifs | Small maquis tree | Irregular, Anemochore | Moderate, orthodox | Moderate scale germination (20,000 seedling pa.) | low, 20% | 10 cm | 18 cm |
| | *Geissois pruinosa* (all varieties) | 3000–15,106 | – | Jaffré et al. (1979, 2013) | Southern & west massifs | Small maquis tree | Annual, Anemochore | Moderate, orthodox | Moderate scale germination (20,000 seedling pa.) | low, 10% | 7 cm | 20 cm |
| Mytaceae | *Cloezia artensis* ssp. *artensis* | 35–1729 | – | Jaffré et al. (2013) | Southern massif | Small maquis shrub | Annual, Anemochore | Moderate, orthodox | Small scale Germination & Cuttings (<1000 seedlings pa.) | Low, 20% | 3 cm | 10 cm |

(continued)

**Table 1** (continued)

| Family | Species | Leaf Ni (μg $g^{-1}$) range | Max. leaf Mn (μg $g^{-1}$) | Reference | Ecological distribution | Ecological role | Phenology/ dispersal mode | Seed production/ storage | Propagation history | Germination/ survival % (1) | Nursery seedling ht. year (1) | Nursery seedling ht. year (2) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Proteaceae | *Grevillea exul* ssp. *rubiginosa* | – | 6.200 | Jaffré (1979) | Widespread common in south | Small maquis tree | Annual, Anemochore | Moderate, orthodox | Large scale germination/ seeding (20,000 seedlings pa.) | <2 months 70% | 8 cm | 18 cm |
|  | *Grevillea gillivrayi* | – | 8.200 | Jaffré (1979) | Widespread common in south | Small maquis tree | Irregular, Anemochore | Low, orthodox | Moderate scale germination (5000 seedlings pa.) | <2 months 50% | 10 cm | 20 cm |
|  | *Grevillea meisneri* | – | 11.000 | Losfeld et al. (2014a) | Northern massifs | Small maquis tree | Annual, Anemochore | Moderate, orthodox | Small scale germination (<1000 seedlings) | <2 months 50% | 8 cm | 18 cm |
|  | *Grevillea exul* ssp. *exul* | – | 3.900 | Jaffré (1979) | Widespread common in south | Small maquis tree | Annual, Anemochore | Moderate, orthodox | Moderate scale germination (5000 seedlings pa.) | <2 months 70% | 10 cm | 20 cm |
|  | *Stenocarpus milnei* | – | 3.900 | Jaffré (1979) | Widespread ultramafic | Small maquis shrub | Annual, Anemochore | Moderate, orthodox | Small scale germination (<1000 seedlings) | <2 months 70% | 8 cm | 18 cm |
| Rubiaceae | *Normandia neocaledonica* | 23–1000 | – | Jaffré et al. (2013) | Southern & central massifs altitude | Small maquis shrub | Annual, Anemochore | Moderate, orthodox | Small scale cuttings (1000 seedlings pa.) | 60% survival cuttings | 3 cm | 10 cm |
| Sapindaceae | *Guioa glauca* | – | 4.500 | Jaffré (1980) | Widespread | Small maquis shrub | Annual Zoochore | Low, orthodox | Small scale germination (1000 seedlings pa.) | <3 months 10% | 3 cm | 10 cm |

evidence of regular aerial shoot production that is necessary for harvesting (McCoy et al. 1999). However, each species presents its own challenges. The most extensively produced species on an annual basis, with more than 20,000 seedlings per year, is *Grevillea exul* ssp. *rubiginosa*. This pioneer species, which can attain more than 20 cm of new growth per year, is readily planted with other maquis species (*Gymnostoma deplancheanum*, *Costularia comosa*, *Stenocarpus umbelliferus*, *Myodocarpus fraxinifolius*) to rehabilitate mine dumps and degraded laterites covering 10–20 ha. Since 2010, annual seedling production of pioneer shrub species such as *Grevillea exul* ssp. *rubiginosa* is subcontracted by Vale NC to satellite nurseries (Fig. 5), with coastal villages at Yaté showing the potential socio-economic gains of such ventures with agromining projects. *Grevillea gillivrayi*, *Geissois pruinosa Codia spatulata*, and *Pancheria billardierei* also have good growth rates similar to that of *G. exul* ssp. *rubiginosa* but seed production in these species is irregular, with the result that seedlings are produced only when seeds are available. *Denhamia fournieri*, *Normandia neocaledonica*, and *Cloezia artensis* are produced from cuttings, because seedlings are small or seed production is low. *Polyscias pancheri* produces few seeds that germinate after six months, suggesting dormancy issues. No harvest technology has been applied, thus the next stage of screening for agromining/phtyomining candidates would be to evaluate the growth and foliar contents of metals after harvesting on amended plots.

**Fig. 5** *Grevillea exul rubiginosa* production at satellite nurseries at a satellite nursery in New Caledonia (Copyright Vale NC)

In spite of the renown of metallophyte biodiversity in New Caledonia (Jaffré 1993), agromining there was never considered until recently (Losfeld et al. 2012). With the development of ecocatalysis approaches that permit the preparation of ecocatalysts from metal trace elements extracted from hyperaccumulator or accumulator plants (Losfeld et al. 2012), several New Caledonian endemic species were identified as candidates for plantations on degraded areas. The first trials, done in 2012 by IAC and Chimeco-CNRS in collaboration with SLN, consisted of experiments on two mine spoils at Camp des Sapins mine, Thio (500 m asl.). The aim of the experiment was to assess the growth response of *Geissois pruinosa* and *Grevillea exul* ssp. *exul* or ssp. *rubiginosa* (1-year-old young plants produced from seeds), on mine spoils or on mine spoils with addition of topsoil. Effects of the addition of several fertilizers were also evaluated (Losfeld et al. 2014b). A total surface of 3.5 ha of mine spoil was planted (1250 plant/ha) with *Grevillea* and *Geissois*. A plantation of *Geissois pruinosa* (≈1.5 ha) was established later in the year (Figs. 6 and 7) to investigate the effects of topsoil and fertilisers, together with those of the endemic, N-fixing plants *Gymnostoma deplancheanum* (Casuarinaceae), *Serianthes calycina*, and *Storckiella pancheri* (Fabaceae). A similar protocol was applied in 2014 at the Kouaoua mine on an area of 2800 m$^2$ using different accompanying species (*Gymnostoma intermedium*, *Serianthes calycina* et *Storckiella pancheri*) in relation to both *Grevillea exul* subspecies. Other projects are currently underway

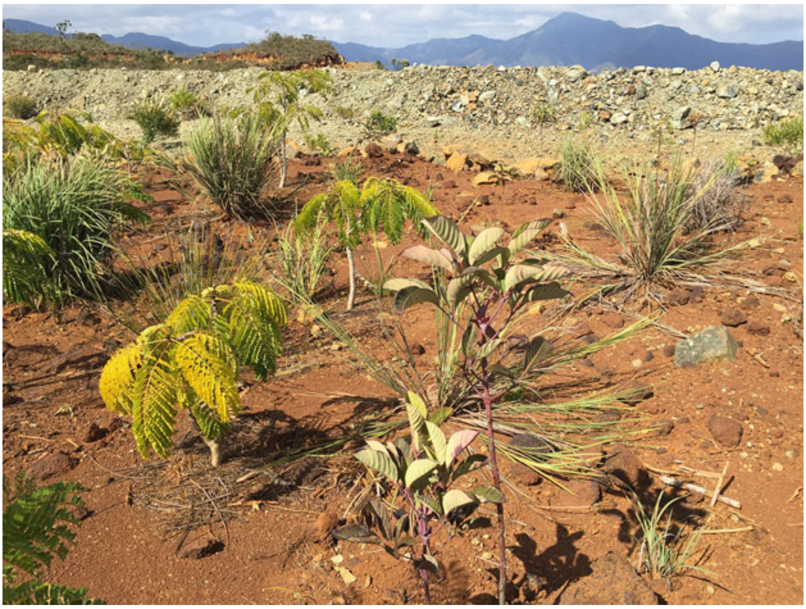

**Fig. 6** A plantation of *Geissois pruinosa* developed to investigate topsoil, fertilisers and the effect of endemic nitrogen-fixing plants

**Fig. 7** A plantation of *Geissois pruinosa* developed to investigate topsoil, fertilisers and the effect of endemic nitrogen-fixing plants

near the massif of Tiebaghi, using the Mn hyperaccumulator *Grevillea meisneri* (Losfeld et al. 2014a) with different amendments of P. More recently, trials have been developed by KNS on the Koniambo massif in the northern province of New Caledonia. For all of the experiments, mortality, growth, and metal accumulation in young and old leaves have been evaluated annually. These data have helped guide the planting of metallophytes on mine spoils for reclamation, and also for providing estimates of revenue from the ecocatalysis biomass production.

## 8 Conclusions

Successful mines and agromining projects require extensive planning and careful design before they begin operations. Similarly, rehabilitation planning that includes hyperaccumulator plants needs to be detailed early in the mine planning process and integrated with the mining operation. There is a natural tendency for rehabilitation plans to reflect whatever appears to be the simplest land-use for a given area, such as grazing or plantations, rather than an attempt to use hyperaccumulator plants for which propagation and agronomic techniques are less well known. While this predisposition is not surprising, the opportunity to maximize the biological resources of a site by discovering and utilizing these unusual plant species, for either their local adaptations to difficult growing conditions or potential for agromining, is an opportunity that should be taken so that unique biodiversity can become a valuable part of the mining process. Ultimately, this type of approach can also facilitate multiple additional benefits including increased metal productivity per unit of biodiversity loss, improved soil fertility in mined-out soils hence availability for a wider range of metal-sensitive plants, reduced erosion on mined-out soils and wastes, and a more productive and viable post-mining use of lands.

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# Phytoextraction of Cadmium: Feasibility in Field Applications and Potential Use of Harvested Biomass

Thibault Sterckeman and Markus Puschenreiter

## Abstract

Cadmium is one of the most threatening soil contaminants because of its high toxicity and widespread anthropogenic distribution. Therefore, remediation of Cd-polluted soils is urgently required. Cropping Cd-accumulating plants appears to be the most relevant approach for removing this pollutant from large soil surfaces. Several field studies have shown the potential of phytoextraction to clean-up moderately Cd-contaminated sites, but this process still has important limitations. Hyperaccumulating plants such as *Noccaea caerulescens* and *Sedum plumbizincicola* show considerable Cd removal rates due to their extraordinary accumulation capacities, but commonly suffer from strongly limited biomass production, especially *N. caerulescens*. Interesting results were also found for other plants, e.g. some cultivars of 'indica' rice *Oryza sativa* or *Solanum nigrum*, but in these cases, further studies are required for confirmation. Some fast-growing willow clones, cultivated in short rotation coppice, offer a possibility to produce energy biomass on contaminated soils more than by decontaminating them, because of their low Cd extraction rate. Relatively little is known about the use of harvested biomass produced through phytoextraction. A few studies showed that combustion is a feasible option, because Cd is retained mainly in the fly ash, whereas the bottom ash contains relatively low amounts of Cd and could even be used as a fertilizer. Further investment is required to evaluate the possibility of producing high-performance cultivars of the best Cd hyperaccumulators. If this approach yields positive results, the complete process chain will need to be tested on a large scale, including the valorisation of biomass.

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## 1 Introduction

Cadmium toxicity was first recognized in the middle of the nineteenth century and several cases of acute poisoning by the metal, resulting from intense exposure of workers, were described in the first half of the twentieth century, followed by confirmation of its toxicological effects through animal experiments. Cadmium and its compounds were subsequently classified as carcinogenic to humans (Nordberg 2009). Furthermore, Cd was demonstrated to be strongly retained in the human body, particularly in kidneys (Järup and Åkesson 2009). This Cd retention makes low-level chronic exposure a serious threat for human health, through accumulation of the metal that can cause kidney failure and increase the risk of cancer (EFSA 2012). As environmental regulations have reduced atmospheric Cd emissions, food is now the primary source of human exposure in the non-smoking population. The 'Itai-Itai' disease, described in Japan in the 1950s, was the first case of acute Cd intoxication of humans via food intake. This painful bone disease was caused by the consumption of Cd-contaminated rice. A mine had released the metal in the Jinzu River from which water was used to irrigate the paddy fields (Nordberg 2009). Subsequently, various epidemiological studies were carried out, revealing that numerous people were affected by Cd exposure (Nawrot et al. 2010). The consequence of these investigations is that the U.S. Agency for Toxic Substances and Disease Registry (ATSDR) (ATSDR 2012) and the European Food Safety Authority (EFSA) (EFSA 2011) set recommendations for provisional safe intake limits for Cd. If the EFSA limit is considered (2.5 μg $g^{-1}$ Cd of body weight per week), the populations of Europe, USA, and China are on average just at the limit, whereas those of Japan and much of the remaining world are above (according to FAO/WHO). Referring to the US limit (0.7 μg $g^{-1}$ Cd of body weight per week), the health of nearly all populations in the world would be threatened by Cd in food (Clemens et al. 2013). In Europe, EFSA estimated that grains and grain products (26.9%), vegetables and vegetable products (16.0%), and starchy roots and tubers (13.2%) had the greatest impact on dietary exposure to Cd (EFSA 2012). The Cd contamination of food is due to widespread low-level contamination of soils and to transfer of the metal towards the harvested crop through root absorption and subsequent translocation. Soil contamination originates from human activities that release Cd into the environment, as the metal is an impurity in various raw materials (fuels, phosphate ores, lead and zinc ores, etc.), or is used in products such as batteries or pigments (Clemens et al. 2013). Despite its toxicity, industrial Cd usage in the world has increased steadily from 18,400 tonnes in 2003 to 20,400 tonnes in 2007 (Moulis and Thévenod 2010). In agricultural soils, Cd inputs are mainly derived from atmospheric deposition, phosphate fertilizers, sewage sludge, farm manure, and amendments (Six and Smolders 2014). In Europe, the median Cd content in top soils is 0.145 μg $g^{-1}$; 90% of the top soils contain <0.48 μg $g^{-1}$, although concentrations up to 14.1 μg $g^{-1}$ have been measured (FOREGS 2005), whilst much higher concentrations of several tenths to several hundred-μg $g^{-1}$ can be found in some industrial or mining regions, as well as naturally high Cd in soils that overlie Zn-Pb metal deposits.

In the densely populated and industrialized Nord-Pas de Calais region (France), Bourennane et al. (2010) found that the Cd concentration in agricultural topsoil was up to 10 times higher than that in the pedogeochemical background in unaffected rural areas, and 30 times or greater the background surrounding industrial or urban areas. Although the use of Cd tends to be increasingly restricted, for instance in Europe by the REACH regulation, the Cd contained in polluted soil will not significantly disappear because it is strongly retained by soil constituents, particularly organic matter. Moreover, the increasing need for phosphate fertilizers, in the face of a limited phosphate resource (Gilbert 2009), will likely enhance the use of low-grade phosphate ores as well as recycling of urban waste composts or sewage sludge, all containing Cd as an impurity. The cultivated soil surfaces affected by Cd contamination are vast and two solutions seem currently possible to reduce the concentrations in plant production: selecting low Cd-accumulating cultivars and cultivating plants that extract the soil metal (Clemens et al. 2013). The feasibility of the latter approach, i.e. Cd phytoextraction (which does not exclude the first) is examined in this chapter.

The ability of some plants—the hyperaccumulators—to accumulate high concentrations of metallic elements in their above-ground biomass has been known for more than 100 years, but for most of this time period was only considered a botanical curiosity. In the early 1980s, however, it was proposed that hyperaccumulators could be used for phytoextraction (Chaney 1983). The 'metal yield' of this process is the harvested biomass of shoots and related metal concentration. Especially in temperate climates, these plants are normally relatively small, thus the metal yield remains unsatisfactory. As an alternative to the use of hyperaccumulators, two approaches were developed: (i) cultivation of high-biomass and fast-growing plants that moderately accumulate Cd, and (ii) chemically assisted cultivation that introduces acidifying amendments or metal chelants (Vangronsveld et al. 2009). As the second strategy revealed poor efficiencies and threats to water resources through leaching of chelated contaminants (Nowack et al. 2006; Lin et al. 2014), it is not considered further in this chapter.

The phytoextraction approach has been tested for various metal contaminants including Cd, Zn, As, Ni, Pb, and Cu. However, only Cd phytoextraction is considered here because this element is likely the most critical regarding contamination of the food chain, as indicated above, and also because, in the context of soil clean-up, its phytoextraction seems more feasible relative to other contaminants. Indeed, Cd typically occurs in relatively low concentrations in soils, and sufficient Cd extraction rates were reported for several plant species. Therefore, we will evaluate the feasibility of Cd phytoextraction based on results of field trials aiming at soil decontamination. An important issue concerning phytoextraction is the fate of the harvested plant parts, containing elevated amounts of the toxic metal. Several processes of treating this biomass in a way that reduces the cost of the clean-up have been proposed, and are also examined.

## 2 Cd Phytoextraction Using *Noccaea caerulescens*

About 15 field trials that tested *N. caerulescens* (Alpine pennycress, formerly *Thlaspi caerulescens*) cultivation for soil-metal extraction were carried out over the last 25 years at several places in the world. The first trials of this type were conducted from the beginning of the 1990s in Great Britain, at Woburn (Fig. 1), under the supervision of the Rothamsted Experimental Station (McGrath et al. 1993, 2000, 2006; Baker et al. 1994). At that time, the U.S. Department of Agriculture also carried out a field experiment, in Beltsville (Maryland) (Brown et al. 1995). In the UK, another trial was conducted in 2000, in the area of Nottingham (Maxted et al. 2007b). In France, Schwartz (Schwartz 1997; Schwartz et al. 2003) first cultivated the hyperaccumulator in the field over two growth seasons, at a site in the vicinity of Nancy (La Bouzule, Lorraine region) (Fig. 2). His studies were followed 12 years later by Lovy (2012), who grew *N. caerulescens* for three successive cultivations (2008–2011), in large trays under natural climatic conditions (in Vandoeuvre, Lorraine region). Also in Lorraine, at the Homécourt Experimental Station, Rees (2014) cultivated this species in two 2 $m^3$ lysimeters to test the effect of biochar application on Cd and Zn phytoextraction. Several trials were also carried out in Switzerland, from the end of the 1990s to the beginning of 2000, mainly in the Jura region, and particularly in Dornach (Felix 1997; Kayser et al. 2000; Hammer and Keller 2003; Keller et al. 2003; Keller and Hammer 2005). All of these trials were done in a temperate climate, but there was also one attempt to phytoextract Cd with *N. caerulescens* under tropical climatic

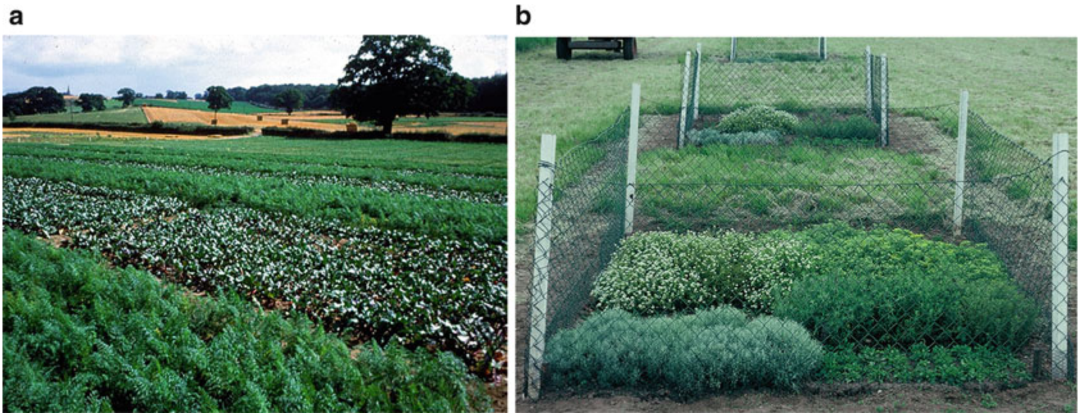

**Fig. 1** A view of the original Woburn site (Rothamsted Research Station, UK) with the early horticultural experiment (**a**) and sub-plots of *Noccaea caerulescens* (*right-hand corner of* **b**) and various metallophytes (photos by Alan JM Baker)

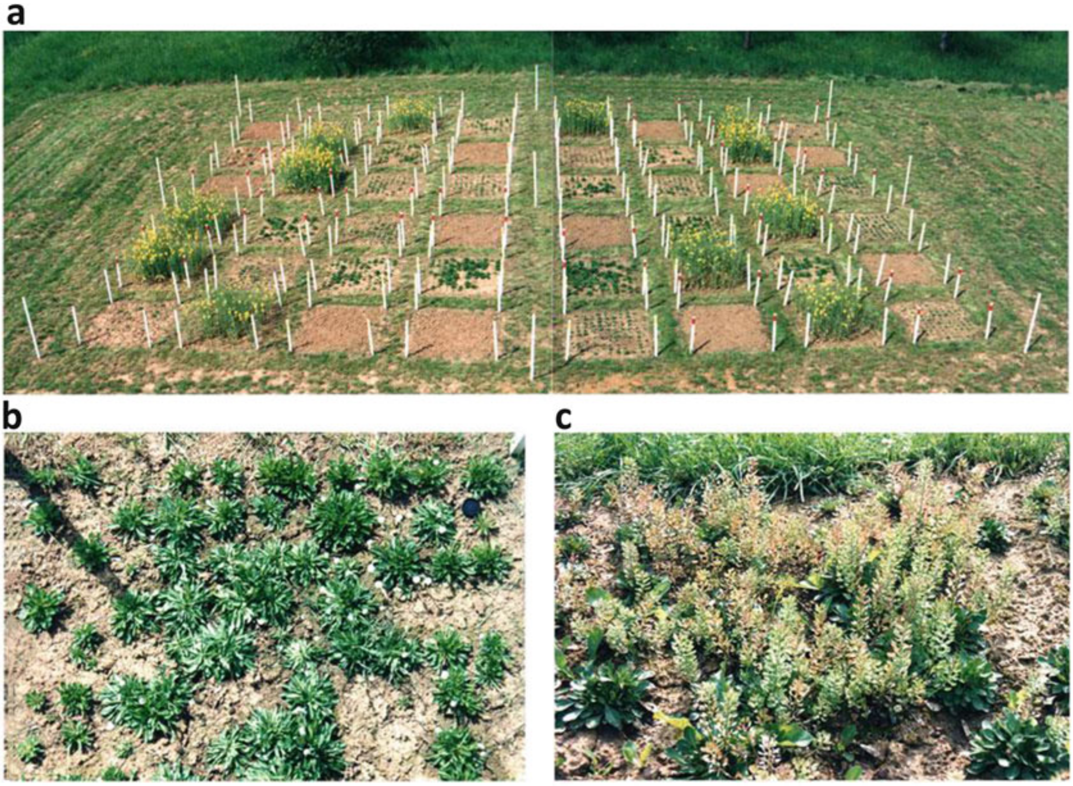

**Fig. 2** A general view of the La Bouzule experiment (ENSAIA, France) with sub-plots planted with *Noccaea caerulescens* and *Brassica napus* (**a**). The hyperaccumulator at the beginning of the flowering stage (**b**) and at the end of the fructification stage (**c**) (photos by Christophe Schwartz)

conditions in Thailand (Mae Sot, Tak Province) (Simmons et al. 2014).

The soils used in the above trials were agricultural and not at urban or industrial sites, and were contaminated from amendments such as sewage sludge, composts, and farmyard manure (Woburn, Nottingham, Beltsville, La Bouzule) or by emissions from metallurgical plants (Dornach, Homécourt) or mining activities (Mae Sot). These soils contained variable amounts of Cd, from ca. 0.2 to 50 μg $g^{-1}$, and typically other metals in excess, such as Zn, Cu, and Pb, with pH values from slightly acid to neutral.

## 2.1 Plant Genotypes Used

The plant accessions (i.e. populations) used in these trials were all wild, and not ameliorated through breeding. The calamine population from Prayon (Belgium), which is now known to only weakly accumulate Cd, was used in the first UK trials at Woburn (McGrath et al. 1993, 2000; Baker et al. 1994). It was also used in the Dornach trial (Hammer and Keller 2003), in Beltsville (Brown et al. 1995), La Bouzule (Schwartz 1997), and probably at Ziefen (Felix 1997). The 'Ganges' population, derived from a mining site in the Cévennes and well known to be among the more Cd-accumulating populations, was used later, in the last Woburn trials (McGrath et al. 2006), at Dornach and Caslano (Hammer and Keller 2003), Nottigham (Maxted et al. 2007b), and Homécourt (Rees 2014).

In the first Woburn trials, the Whitesike population was also used. It comes from a mining site located on Alston Moor (Cumbria, UK) but was no longer used after the trials of Baker et al. (1994). The Viviez population was used in the trials at La Bouzule (Schwartz 1997), at Vandoeuvre (Lovy 2012), and at Mae Sot (Simmons et al. 2014). In this Thai trial, other Cévennes populations were tested for the first and only time in the field: at Saint-Félix-de-Pallières, Les Malines, and La Sanguinède. The number of populations (ca. 7) tested is rather small compared to those of inventoried populations in Western Europe, on the order of one hundred (Gonneau 2014; Gonneau et al. 2017). The populations used were all from calamine sites but in most trials, generally the older ones, were not those that strongly accumulate Cd (Prayon, Viviez, and Whitesike).

## 2.2 Planting and Harvesting

As far as can be ascertained from published technical details, in most cases the crops were planted as seedlings 4–12 weeks old, at densities of 16–200 plants $m^{-2}$, more often as 100 plants $m^{-2}$. The great majority of the crops started cultivation in April or May and were harvested 2–4 months later. Most crops were not vernalized so they probably did not flower. However, variable cultivation calendars were also used to conduct the crop trials. Hammer and Keller (2003) harvested the shoots in November, 7 months after sowing, whereas Baker et al. (1994) harvested in July or August those plants that had been transplanted 6 months earlier (in January or February). Only Lovy (2012) grew *N. caerulescens* for 10 months, based on the natural cycle of the species, i.e. sowing by the end of August and harvesting the following spring or beginning of the summer.

Hammer and Keller (2003) carried out three successive croppings of about 2 months each, between April and November, with transplanting each time, or two crops of about 3 months, after sowing. Keller et al. (2003) transplanted *N. caerulescens* in late July for harvest in mid-October. McGrath et al. (2000) observed a regrowth of the plants after harvest of the rosette, from crowns left in the soil during winter. McGrath et al. (2006) also conducted a lengthy cultivation (8 months) with vernalization, the planted done in October for harvest in June of the following year, when the plants fruited.

## 2.3 Biomass Production

In the field trials, the dry biomass production varied from 0.28 to 7.8 t $ha^{-1}$. It is difficult to explain this wide range, because factors such as population, soil type, and cropping practice can strongly influence plant growth. Many crops produced less than 1 t $ha^{-1}$. A yield of 2 t $ha^{-1}$ can be considered good and 4 t $ha^{-1}$ exceptionally high. Yields of more than 7 t $ha^{-1}$ were obtained in Woburn by McGrath et al. (2000) in a trial having a duration of *ca.* 10–14 months, for which half the plants had been harvested the previous year and then provided re-growth. These authors obtained a production on the order of 4 t $ha^{-1}$ after 4 months of cultivation at the same site. It should be noted that the production obtained in the same test plot a few years later was more modest, from 0.08 to 3.6 t $ha^{-1}$, with an average of 2.14 t $ha^{-1}$ for a 14-month-old crop (McGrath et al. 2006). However, individual plants of 28 g dw (Prayon) biomass and 31 g (Whitesike) were also achieved in the Woburn trials during 1991–1992 (Baker et al. 1994; McGrath et al. 2000). Planted at a density of 35 plants $m^{-2}$, such individual plants would enable a biomass production of ca. 10 t $ha^{-1}$. Crops grown in tropical soils (Mae Sot, Thailand) provided amongst the lowest yields, at 0.28–0.65 t $ha^{-1}$, likely due to incompatible climatic conditions and severe pest attacks.

It does not seem that increasing the duration of cropping can enhance yield. Copper toxicity (>500 μg $g^{-1}$ Cu in soils), but also the more clayey texture, could explain the low yields obtained in the Dornach trials. Schwartz (1997) lost plants sown in the autumn because of the winter cold and insufficient Zn supply that could also have caused the low plant growth he observed.

## 2.4 Effectiveness of Phytoextraction

In the field trials cited above, Cd concentrations in the shoot varied three orders of magnitude (1–1000 μg $g^{-1}$), as well as quantities harvested (in g $ha^{-1}$), although the latter have not always been published by other workers. Again, it is difficult to explain this variability. The Cd concentrations in the plants depend in particular on those in the soil, its properties (especially pH), and on the plant population. Additionally, differences in dry matter yield contribute to variability of the exported quantities. Maxted et al. (2007b) clearly demonstrated the great variability of Cd concentrations that accumulated among individuals from the same population of *N. caerulescens* ('Ganges') grown on a single plot, with a factor of 10 evident between the greatest and least concentrations. However, concentrations in the shoots have been shown to be generally much lower for plants of the Prayon population. The Cd concentrations in plants from Viviez and Whitesike were also relatively low, whereas those found in the 'Ganges' population can be extremely high, such as reported in the Homécourt trial where over 3000 μg $g^{-1}$ Cd was achieved. The effectiveness of phytoextraction was not always assessed, and when it was, it was revealed to be quite variable. The amounts of exported Cd were in the range of 10–700 g $ha^{-1}$ (McGrath et al. 2000, 2006; Keller et al. 2003; Keller and Hammer 2005). Unfortunately, no estimate of the exported Cd quantity or of the biomass, was given in the Beltsville trial (Brown et al. 1995). Concentrations of Cd in the *N. caerulescens* plants (Prayon) were very low (2–30 μg $g^{-1}$) and it is likely that the remediation potential is therefore very low.

Clean-up of the Dornach site (achieving soil Cd concentration of 0.8 μg $g^{-1}$) would require 33–49 years depending on whether *N. caerulescens* was transplanted or sown (Hammer and Keller 2003). For the Caslano soil, the remediation time was somewhat shorter, on the order of 9–26 years. It should be noted that these authors considered a constant Cd uptake by each successive crop, which assumes a constant availability of soil Cd during phytoextraction. Significantly, this assumption is incorrect, because the extractable amount decreases as the clean-up progresses (the Law of Diminishing Returns). It seems likely that transplanting to enable a greater number of short-duration crops and a higher biomass production would be more favourable than sowing. Kayser et al. (2000) did not report any time for the clean-up involved at the Dornach site using the Prayon *N. caerulescens* population. However, they estimated that a plant containing 45 μg $g^{-1}$ Cd and producing 10 t $ha^{-1}$ biomass could remove half of the soil Cd within 10 years.

No estimate for the proposed clean-up time was given from results of the Woburn trials. McGrath et al. (2000) calculated that a crop producing 10 t DM $ha^{-1}$ containing 100 μg $g^{-1}$ Cd would decrease Cd soil content from 1 to 0.2 μg $g^{-1}$ in 2 years, and from 10 to 3 μg $g^{-1}$ in 18 years. These time periods would be brought to 5 years and 46 years, respectively, if the plant contained only 40 μg $g^{-1}$ Cd. A 14-month-old crop extracted, on average, 8.7% of the Cd from variously contaminated soils; in one of these, containing 7.5 μg $g^{-1}$ Cd, one crop of the 'Ganges' population extracted 21.6% of the metal from the top 20 cm layer of soil. This extraction record corresponds to the most concentrated plants that produce 2.69 t dw $ha^{-1}$ (McGrath et al. 2006). The Cd concentration in the plants correlates only weakly with that of the soil ($r^2 = 0.46$); the change in biomass strongly relates to the differences in phytoextraction performance. Plants from the Viviez population cultivated in Vandoeuvre extracted 5–10% of the total Cd present in the upper 10 cm layer that contained 5 μg $g^{-1}$ Cd (Lovy 2012). The clean-up duration was estimated to be 20 years, assuming a constant uptake of Cd each year.

The aim of the Maxted et al. (2007b) trials was to evaluate the frequency of a phytoextraction crop to extract the Cd provided by sewage sludge regularly spread on agricultural plots. Thus, a *N. caerulescens* crop producing 4 t DM $ha^{-1}$ every 14 years would be sufficient to eliminate an annual application of 10 tonnes of sludge containing 4.0 μg $g^{-1}$ Cd. These values are reasonable based upon realistic contents and modelling, and bioavailability of the soil Cd and its concentration in the plant. The La Bouzule cultivations (Schwartz et al. 2003) allowed the Viviez plants to extract only 4–9% of the Cd provided by the sludge and compost amendments. This trial, which showed the highest Cd phytoextraction efficiency, is that of Homécourt. On both lysimeters, one a 'Ganges' crop of 4.2 months, extracted 25% of the metal from the upper 30 cm layer that contained 17 μg $g^{-1}$ Cd. This performance contrasts with that of the other trials, and can be explained by use of the 'Ganges' population.

## 2.5 Appraisal of the Cd Phytoextraction Efficiency When Using *N. caerulescens*

Results of most field trials using *N. caerulescens* in terms of efficiency or duration of phytoextraction are very disappointing. The extraction rates were low, the estimated clean-up duration was long, often exceeding one or several decades, and usually also was underestimated because the authors considered a constant Cd concentration in the aerial tissues of the successive crops. The main reason for the low efficiency was the reduced production of biomass and the insufficient Cd concentration in the shoots. Whilst several authors agree on the need to produce at least 10 t of dry matter per hectare, the trials have often led to yields 5–10 times lower. Moreover, in many trials, low-accumulating Cd populations were used (as Prayon and Viviez) or when a more effective population such as the 'Ganges' was used, it suffered from soil toxicity as in Dornach (Cu toxicity). Despite this, the field trials suggest opportunities to improve the efficiency of phytoextraction of Cd. Firstly, the Homécourt trial and one of the Woburn trials, both using the 'Ganges' population, showed extraction rates of more than 20% after one cropping in a highly Cd-contaminated soil. In addition, the Nottingham trial offered a reasonable period (14 years) for a *N. caerulescens* crop to remove the Cd provided by annual amendments of sewage sludge. These results therefore provide hope for the feasibility of Cd phytoextraction to decontaminate soil or to maintain an acceptable balance of the pollutant brought by agricultural inputs. In addition, some results show the large variability of Cd contents in plants and the existence of very high concentrations in some populations and individuals. Similarly, plants having a significantly higher biomass than the others were observed; this indicates a genetic variability that could serve as the basis for improving biomass production and tissue concentrations. Suitable agricultural measures may further improve the plant yield (Kidd et al. 2015).

# 3 Cadmium Phytoextraction Using Other Hyperaccumulator Plants

Among Zn and Cd hyperaccumulators, *Sedum alfredii* (Yang et al. 2004) and *S. plumbizincicola* (Wu et al. 2013) were recently discovered at mining sites of China. These Crassulaceae are adapted to subtropical climates. Although their hyperaccumulation characteristics and physiology have now been studied extensively at the laboratory scale, there are relatively few published data on their performances for Cd phytoextraction at field scale.

Wu et al. (2007) cultivated *S. alfredii* in comparison with, and co-cropped with, maize on a paddy field soil containing 2.0 μg $g^{-1}$ Cd. After 6 months of cultivation the hyperaccumulator extracted about 5% of the total soil Cd (ca. 40 g Cd $ha^{-1}$), similar to that extracted by a maize crop, whereas co-cropping the two plants enabled removal of ca. 20% of the metal. Another experiment was carried out in the same area by Zhuang et al. (2007), comparing *S. alfredii* to seven other species on a soil containing 7.2 μg $g^{-1}$ Cd. This hyperaccumulator extracted 0.25% of the soil Cd after 3 months of cultivation, producing 5.5 t $ha^{-1}$ of dry matter and about 50 g Cd $ha^{-1}$. This extraction rate was the lowest after that of *Dianthus chinensis* (0.10%). *S. alfredii* extracted less Cd than the hyperaccumulating *Viola baoshanensis* (0.88%) and the metal-tolerant *Rumex crispus* (0.86%), *Rumex* 'K1' (0.34%), and *Vetiveria zizanioides* (0.50%), all of which produced much higher biomass.

*Sedum plumbizincicola* (Crassulaceae) (Fig. 3) was tested for the phytoextraction of Cd (and Zn) in a long-term field experiment over 8 years by Deng et al. (2016). The soil initially contained 3.0–4.6 μg $g^{-1}$ Cd in the upper 15 cm. The total soil Cd content decreased by 85.5% after 7 years of *Sedum*/maize intercropping, and by 87.8% after *Sedum* mono-cropping. The peak removal rate was observed in the fourth year, at 1.03 kg Cd $ha^{-1}$ $y^{-1}$. The Cd concentrations present in the grains of maize and sorghum intercropped with *S. plumbizincicola* did not decrease together with phytoextraction but remained below the Chinese National Food Quality Standard (0.2 μg $g^{-1}$ Cd).

*Solanum nigrum* (Solanaceae) is also a recently discovered Cd hyperaccumulator (Wei et al. 2005). Niu et al. (2015) grew it for 3 months, before the cultivation of Chinese cabbage, in a soil containing 0.53–0.97 μg $g^{-1}$ Cd. They observed a decrease of 10.7% of soil Cd and also in the cabbage shoot, which was 0.15 μg $g^{-1}$ without previous phytoextraction and 0.06 μg $g^{-1}$ after remediation, i.e. below the Chinese National Food Quality Standard. Growing *S. nigrum* for 3 months also, alone or together with Welsh onion, Wang et al. (2015) observed a decrease of nearly 8% in soil Cd concentration, which initially ranged from 0.45 to 0.62 μg $g^{-1}$ Cd. However, the Cd phytoextraction had no effect on Cd content of the onion, which was uniformly below 0.1 μg $g^{-1}$. Ji et al. (2011) applied *S. nigrum* in a field experiment conducted on a moderately polluted arable soil in China. The maximum Cd concentrations reached 9.9 μg $g^{-1}$ on a site containing 1.96 μg $g^{-1}$ Cd in the topsoil (0–10 cm). In this work, different agronomic practices were tested in order to increase phytoextraction efficiency, including fertilization, variable plant density, double cropping, and double harvesting. Double cropping at a planting density of 11 plants $m^{-2}$ (30 × 30 cm) was the most successful approach, resulting in a total biomass of 42.1 t $ha^{-1}$ $y^{-1}$ and a Cd removal of 0.4 kg Cd $ha^{-1}$ $y^{-1}$.

# 4 Cadmium Phytoextraction With Non-Hyperaccumulator Species

## 4.1 Trials Using Willow (*Salix* spp.)

Agroforestry practices such as short-rotation coppicing (SRC) for the cropping of *Salix* are well-established, since chipped willow stems have been used for decades as biomass fuel for

**Fig. 3** A field experiment to evaluate the ability of *Sedum plumbizincicola* to remediate a Cd contaminated soil in Xiangtan county, Hu'nan Province, China (**a**). Co-cropping of *S. plumbizincicola* and maize (*Zea mays*) in Zhejiang Province, China (**b**) (photos by Longhua Wu)

energy production (Pulford and Dickinson 2005). Although *Salix* spp. typically have lower Cd concentrations in above-ground tissues (5–70 μg $g^{-1}$) compared to hyperaccumulators, the efficiency of SRC for Cd phytoextraction has been tested because of its large biomass production and fast growth that allow a consistent metal yield together with energy production. This type of evaluation has been made in several field trials on agricultural soils that were slightly or moderately contaminated, in Belgium, the UK, Sweden, and Switzerland.

Eriksson and Ledin (1999) studied the changes in soil Cd concentrations in slightly contaminated soil (maximum concentration *ca.* 0.25 μg $g^{-1}$ Cd in topsoil) in 8- to 30-year-old *S. viminalis* plantations used to produce stem wood at various cutting frequencies. These authors did not find a significant change in total Cd in the soil. However, it seems that growth of the trees decreased the plant-available Cd up to a depth of 65 cm, although this diminution was not always significant. Hammer et al. (2003) cultivated a Swedish clone of *S. viminalis* in two moderately contaminated soils. The total metal uptake was 170 and 197 g Cd ha$^{-1}$ over 5 years in control and S-treated plots, respectively. They estimated that 31 years of cropping would be necessary to decrease the Cd concentration in the upper 20 cm soil layer from 2.3 to 0.8 μg g$^{-1}$, and 142 years if a 60-cm soil depth was considered.

Maxted et al. (2007a) cultivated six willow genotypes at a site near Nottingham, UK, on a sewage sludge-contaminated soil containing 41.6 μg g$^{-1}$ Cd. The bioavailable Cd, determined as isotopically-exchangeable Cd, was 32% of the total amount. Their experiments showed that willows achieved approximately 15–20% of the uptake rate required to extract the bioavailable Cd in 25 years. Willow clearly appeared unsuitable to decontaminate such highly Cd-contaminated soils, even if only the phytoavailable fraction is targeted.

Using eight accessions of different willow species and hybrids (extracting 17–241 g Cd ha$^{-1}$), Van Slycken et al. (2013) estimated 101 to 1303 years as the time needed to reduce soil contamination from 6.5 to 3.0 μg g$^{-1}$ Cd, harvesting the leaves together with the stems, which is not the usual practice for SRC. This is still a high contamination level for the sandy soil from Campine, where the background concentration was originally *ca.* 0.2 μg g$^{-1}$ Cd. This time span is of the same order as that found by Vangronsveld et al. (2009) and Ruttens et al. (2011) in Lommel (Belgium), in order to reach a total soil Cd concentration of 2.0 μg g$^{-1}$, starting from 5.7 μg g$^{-1}$. Removing shoots together with leaves, the amount of extracted metal ranged from 113 to 244 g Cd ha$^{-1}$ (Ruttens et al. 2011).

Greger and Landberg (2015) recently reported the results of Cd phytoextraction using *S. viminalis* in two moderately Cd-contaminated soils, one clayey with 0.32 μg g$^{-1}$ and one sandy containing 0.45 μg g$^{-1}$. In their experiment, the Cd phytoextraction efficiency was not only assessed by reduction of total Cd concentrations in the soil but also by the change in Cd availability for the subsequent crop, wheat. After 4 years of Cd phytoextraction with willow, the total Cd in soil was reduced by up to 27%, but the Cd concentration in wheat grain decreased by up to 33%. However, there was a high deficit in the Cd mass balance of the remediation crop. Within the clayey soil, a decrease of 475 g Cd ha$^{-1}$ was achieved after the 4 years of willow cultivation, whereas the analyses of the plant parts showed a Cd removal of only 22 g Cd ha$^{-1}$. On the basis of these results, the authors suggested that the difference of 453 g Cd ha$^{-1}$ could have 'disappeared' from the system, by (i) dispersion of abscised leaves during the three autumns before harvest, but this is estimated at only 22.6 g Cd ha$^{-1}$; or (ii) leaching below the rooting zone at a rate of 430.4 g Cd ha$^{-1}$. Taken together, it suggests that in this case, phytoextraction was in fact a 'phyto-leaching' of the contaminant, which would have led to dilution of the metal in a higher volume of substrate and to potential contamination of ground waters. Moreover, the authors calculated that reducing the concentration of Cd at the measured concentration by actual plant phytoextraction would take 87 croppings, harvesting both shoots and roots each time.

In summary, it seems that for willow, clean-up times calculated based on the reduction of total Cd concentrations in soil still indicate that several decades are required for achieving the remediation target. Thus, Cd phytoextraction with *Salix* spp. should preferably be combined with other options for biomass use such as energy production. This approach has been suggested as an effective phyto-management strategy for a contaminated site, rather than being primarily a remediation option (Robinson et al. 2009).

## 4.2 Feasibility of Cd Phytoextraction with Other Non-Hyperaccumulating Species

The Lommel trial in Belgium mentioned above was also used to test other high-biomass and fast-growing species such as poplar, maize (*Zea mays*), rapeseed (*Brassica napus*), sunflower (*Helianthus annuus*), and tobacco (*Nicotiana tabacum*) (Vangronsveld et al. 2009). The time period needed to decrease the total soil Cd concentration from *ca.* 5.0 to 2.0 µg $g^{-1}$ ranged from 58 to 255 years. The lowest time span was obtained using tobacco, all the other plants requiring a clean-up time of greater than a century. Time spans on the order of one to several centuries were also found by Felix (1997) with the same species being tested in a Jura soil containing 6.6 µg $g^{-1}$ Cd and involving required remediation to the Swiss regulatory limit (0.8 µg $g^{-1}$). In this situation, phytoextracting of Cd with Indian mustard (*B. juncea*) would take 214 years. Maize, sunflower, Indian mustard, and tobacco were also used in the Dornach site trials, and compared to those using *N. caerulescens* (Kayser et al. 2000). Here the hyperaccumulator only produced 0.49 t DM $ha^{-1}$ because of soil compaction and heat, but also probably because of very high concentrations of Cu in the soils. Cadmium concentrations measured in tobacco were below those of the hyperaccumulator, but clearly above those of Indian mustard, sunflower, and maize. The amount extracted by the different species ranged from two to 30 g Cd $ha^{-1}$. As in the Lommel trial, tobacco appeared as the crop having the highest Cd content in its above-ground biomass, which was even more than in *N. caerulescens*, because of its high biomass production. The authors did not report values for the time span of the clean-up, but they estimated that even the highest metal removal rates achieved in their study were still far from what would be required to make phytoextraction practical for remediation of the Dornach site down to 2.5 µg $g^{-1}$ Cd.

Interesting results were obtained by Murakami et al. (2009) for the phytoextraction of Cd from contaminated paddy fields. They cultivated the 'indica' rice cv. Chokoukoku for 2 years without irrigation in soil containing 1.6 µg $g^{-1}$ Cd. This experiment extracted 883 g Cd $ha^{-1}$, i.e. 38% of the total soil Cd, and reduced by 47% the Cd content of the subsequently grown 'japonica' food rice, without decreasing its yield. The above results suggest that adapted cultivars of tobacco and 'indica' rice could be used to decontaminate moderately Cd polluted soils, mainly agricultural soils. More research is needed to select the most appropriate cultivars and test these in the field, in order to evaluate their impact on the amount of bioavailable and total soil Cd and on metal concentrations remaining in subsequent food crops.

## 4.3 Biomass Valorisation Options for Cd Phytoextraction Crops

The biomass obtained from harvesting phytoextraction plants could be used for various purposes similar to other agricultural non-food crops, including combustion or gasification for energy production or the production of fibre, oil, or biofuels. Owing to a high concentration of metals and/or metalloids, the biomass could also serve as a source for recovery of these elements, either directly from the biomass or from the ash after incineration. However, very little information is available concerning the potential options for the use of biomass. A survey conducted by Bert et al. (2016) has revealed that people working on combustion technology or anaerobic digestion in different European countries showed that there is still little awareness of phytotechnologies in general and phytoextraction in particular, but these people also responded that biomass obtained from phytoremediation sites could be used under certain conditions. However, biomass obtained from phytoextraction plots is considered riskier than that produced from phytostabilization approaches.

### 4.3.1 Hyperaccumulator Plants

Keller et al. (2005) reported on potential use of harvested *N. caerulescens* leaves obtained from a phytoextraction field trial, where it was shown that gasification under reducing conditions (pyrolysis) is more suitable for recovery of Cd from fly ash and a concomitant use of the bottom ash as fertilizer. The fate of metals during the combustion process was investigated for *S. plumbizincicola* by Zhong et al. (2015), who showed that the metal distribution between bottom and fly ash was largely temperature dependent and that the percentage of metals recovered from the fly ash increased with increasing temperature. The use of kaolin or activated carbon can further reduce the emission of Cd and other potential pollutants (e.g. PAHs, NOx, CO) (Wu et al. 2013). Recovery of Cd (and Zn) from the harvested biomass of *N. caerulescens* has recently been investigated and seems promising using a cementation process (Hazotte et al. 2016).

### 4.3.2 Willow

*Salix* spp. and *Populus* spp. have been grown for decades in short rotation coppice (SRC), using the harvested biomass for energy production (Pulford and Dickinson 2005). Consequently, this option has also been discussed and tested for use of these woody species as phytoextraction crops (Keller et al. 2005; Delplanque et al. 2013). For SRC phytoextraction plants, work on harvested biomass has focused on either leaves or stems. Keller et al. (2005) investigated the combustion of *Salix* leaves and found, in general, the same results as for incineration of foliar *N. caerulescens* biomass (see above). They reported that *S. viminalis* leaves from a Cd- and Zn-phytoextraction field trial required treatment with gasification under reducing conditions (pyrolysis) for greater potential recovery of Cd, and a concomitant use of the bottom ash as a fertilizer. Similarly, Delplanque et al. (2013) reported that the highest concentrations of Zn and Cd were obtained in the fly ash, which may therefore serve as a potential medium for Cd recovery.

## 5 Conclusions

Cadmium is one of most threatening soil contaminant as nearly all populations in the world are, or are about to be, overexposed to this metal through food consumption. The metal arrives in the diet through root absorption by crops, Cd contamination being at relatively low concentrations in most cultivated soils. Reducing the Cd contamination of soil is a direct method to improve food quality, but this approach requires the decontamination of vast surface areas of cultivated land. In principle, phytoextraction is the most appropriate technique to reduce soil Cd concentrations in such a situation, allowing preservation of the soil properties. Plants are known that can concentrate Cd within harvestable parts and cropping them at reasonable expense would not require a special ‘know-how’ or tools very different from those used for other specialist crops. The main problems remaining to be solved in order to develop successful Cd phytoextraction are the creation of high-metal yielding cultivars and efficient processes for treatment of the contaminated harvested biomass.

The field trials discussed in this chapter typically have yielded disappointing results because the phytoextraction rate is generally low, and hence the time needed to reach an acceptable Cd concentration in soils is too long, regardless of the species tested. This limitation is the consequence of an insufficient metal yield, the plant with a high biomass production being often weakly concentrated in Cd, and *vice versa*. However, there are still a few options that could lead to success, as some plant species have potential for attaining a high metal yield. This is the case for *N. caerulescens*, which showed very high decontamination rates in some experiments; this plant also has high phenotypic variability both in terms of biomass and Cd accumulation, neither of which has yet been exploited to select high yielding varieties. An investment in plant breeding should be made in coming years to create a cultivar of *N. caerulescens* with a regular biomass production of ca. 10 t DM $ha^{-1}$, together with a Cd concentration factor at least equal to that of the ‘Ganges’ population. Use of such a cultivar would enable the decontamination of soil containing up to 10 μg $g^{-1}$ total Cd.

Our review indicates that in areas of (sub) tropical climate *S. plumbizincicola* is a species having performance close to that of *N. caerulescens*. However, as yet there are few trials with this Crassulaceae and more field experiments should be carried out to confirm its ability for Cd phytoextraction. *Sedum plumbizincicola* should also be investigated with the aim of cultivar creation and multiplication.

For cultivated soils containing $<1$ μg g$^{-1}$ Cd, several plant species could be used. *Noccaea caerulescens* is one of these. Because this plant has one of the highest abilities to concentrate the metal, a relatively low biomass production of *ca.* 5 t DM ha$^{-1}$ would be sufficient to reach high extraction rates. However, this promising hyperaccumulator should be tested more extensively in agricultural soils containing low amounts of Cd, and of other metals such Zn, which could be necessary for normal growth. Trials of this species in mildly contaminated soils are needed. An 'indica' rice cultivar has demonstrated its ability to efficiently decontaminate moderately polluted paddy fields. However, there are few results published on this possibility as a technique for Cd phytoextraction, and further field trials are required for confirmation. This is also the case for *Solanum nigrum*, which showed promising results suggesting that this species could be helpful in decontaminating some contaminated soils used for food production.

The amounts of Cd extracted in the context of soil clean-up are on the order of few 100 s g ha$^{-1}$, making metal recovery from the biomass poorly profitable. Moreover, valorization of the harvested biomass from Cd phytoextraction crops has been insufficiently investigated. A few studies have shown that combustion is a feasible option that would potentially allow the use of bottom ash as a fertilizer, since most of the Cd is retained in the fly ash.

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# Element Case Studies: Nickel

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## Abstract

Initial experiments using Mediterranean Ni-hyperaccumulator plants for the purpose of phytomining were carried out in the 1990s. In order to meet commercial phytoextraction requirements, a technology has been developed using hyperaccumulator species with adapted intensive agronomic practices on natural Ni-rich soils. Ultramafic soils in the Balkans display a great variability in Ni concentrations and available Ni levels, both in Albania and the Pindus Mountains of Greece. In Albania, Vertisols are currently being used for low-productivity agriculture (pasture or arable land) on which phytomining could be included in cropping practices. *Alyssum murale* occurs widely on these ultramafic Vertisols and is a spontaneous weed that grows among other crops. This review chapter presents the different steps that were investigated during the study of soil suitability, and selection of plants up to optimization of agronomic practices, at field scale, as recently developed to reach the implementation stage of Ni agromining in Albania. During a 7-year study we addressed the following questions: (i) what are the optimal soils for Ni agromining in terms of fertility and Ni availability? (ii) what is the phytoextraction potential of local populations of Ni hyperaccumulator species? (iii) what should be the agronomical practices used to optimize the cropping of *A. murale* for extensive phytomining adapted to a Balkan agricultural setting?

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# 1 Introduction

Ultramafic soils cover large areas in the Balkans, more than in any other part of Europe. Therefore, this region is a potential target for agromining activities (van der Ent et al. 2015). However, no woody and perennial species such as *Rinorea bengalensis* or *Phyllanthus securinegioides* from Southeast Asia can be cropped under Mediterranean conditions (van der Ent et al. 2015; Nkrumah et al. 2016). Moreover, the Balkans also has the highest diversity in Ni hyperaccumulator plants in Europe and is home to the widespread plant *A. murale*, one of the most studied species worldwide for phytomining (e.g. Nkrumah et al. 2016). Initial experiments using Mediterranean Ni-hyperaccumulators for the purpose of phytomining were carried out in the 1990s. In order to meet commercial phytoextraction requirements, technology has been developed using hyperaccumulator plant species with adapted intensive agronomic practices on natural Ni-rich soils. In a first stage, several studies have been carried out in order to identify the best soil conditions to establish field trials. Then the native species were evaluated for their potential in phytomining; finally, trials were set to optimize cropping conditions in the Balkan context.

# 2 Agromining Nickel in The Balkans

## 2.1 Properties of Ultramafic Soils the Balkans and Suitability for Agromining

Outcrops of ultramafic rocks in the Balkans (Dilek and Furnes 2009) extend towards central Bosnia, western and central Serbia, southern Bulgaria, and north-central and southeastern Albania to the ultramafic formations of Epirus and Thessalia in Greece (Fig. 1; Stevanovič et al. 2003). The number of Balkan endemics growing on ultramafic-derived soils comprises approximately 335 taxa, of which 123 are obligate. The most floristically rich areas are situated in northwestern Greece (Epirus), the island of Evvia, and northern Albania, together with southwestern Serbia and northern Greece (Mount Vourinos). The Pindus Mountains are located in northwestern Greece and extend about 160 km from the southern border of Albania. Ultramafic terranes in Albania and the Pindus Mountains constitute the largest blocks of ultramafic outcrops in the Balkans. The ouctcrops are also part of similar floristic assemblages: northeastern and southeastern Albania to northwestern Greece (Reeves et al. 1983; Shallari et al. 1998; Tan et al. 1999; Stevanovič et al. 2003), located in similar climate zones (a Mediterranean climate with montane influences). The ultramafic soils of Albania and the Pindus Mountains (Greece) contain elevated levels of metals such as Ni, Cr, Co and Fe. Most soil cover on these ultramafic materials falls into the Cambisol type (Echevarria, Chap. 8 in this book). Surface horizons of the soils are characterized by extremely high Mg/Ca quotients of up to 30 in Albania and up to 17.7 in Greece (Bani et al. 2009, 2010), typical of ultramafic soils worldwide (Reeves et al. 1997). Concentrations of Co in these soils are relatively high, also typical of Mediterranean ultramafic soils (Reeves et al. 1997; Wenzel and Jockwer 1999). Cobalt concentrations vary between 93 μg $g^{-1}$ (Malakasi-Soil 2) and 280 μg $g^{-1}$ (Pojska Soil 2); Cr concentrations in soils range from 667 μg $g^{-1}$ (Pojska-Soil 1) to 3250 μg $g^{-1}$ (Pishkash). The soils with the highest Ni contents come from eastern Albania and have *ca.* 3000 μg $g^{-1}$. The Ni concentrations in soil samples from the Gjegjan area, in northern Albania, vary from 1070 to 2580 μg $g^{-1}$. In the Greek soils, Ni is also lower than in eastern Albania: between 1280 to 2660 μg $g^{-1}$. The soils of both areas have a similar geological setting, varying from partly serpentinized peridotite (harzburgite) to serpentinite (Bani et al. 2014; Estrade et al. 2015).

The nature of Ni-bearing phases and the overall soil mineralogy strongly influence Ni availability. Because soil mineralogy depends on pedogenetic properties, one can say that Ni availability depends on soil genesis. As observed for

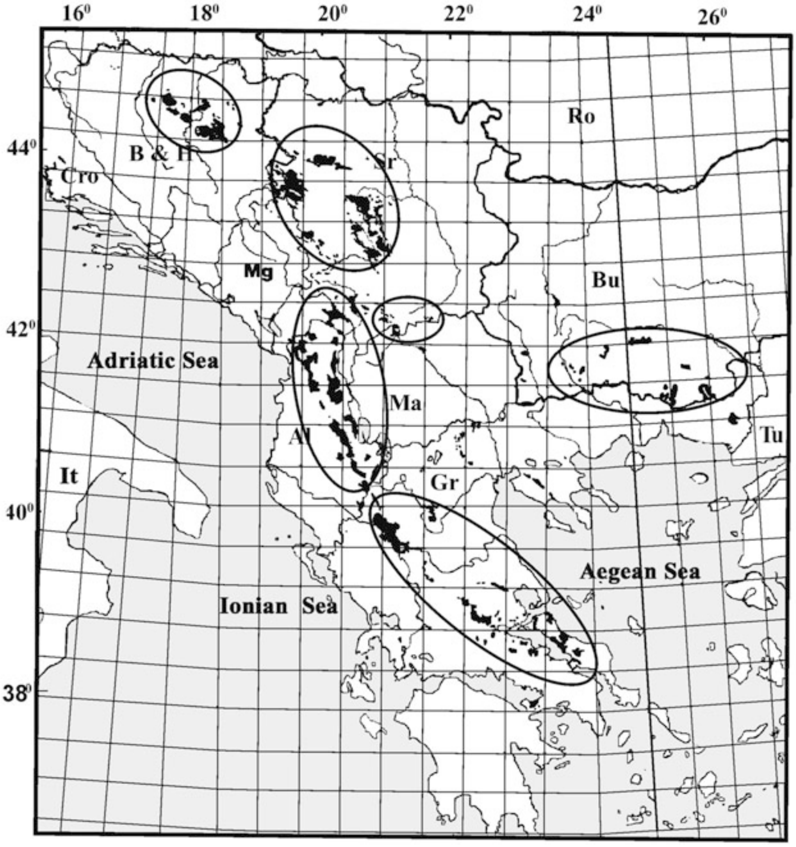

**Fig. 1** Ultramafic sites of Balkans (Stevanovič et al. 2003)

other soils, the fate of Ni is strongly related to the 'free' status of Fe in the soils (Massoura et al. 2006; Chardot et al. 2007; Cheng et al. 2011; Raous et al. 2013); 'free' Ni partition is strongly related to the content of amorphous Fe-oxides in the soils. In Albanian ultramafic soils, DTPA-Ni ranges from 81 to 285 μg $g^{-1}$. In the Pindus Mountain Cambisols, DTPA-extracted Ni in soils varies from 48 to 126 μg $g^{-1}$ (Bani et al. 2009). The pH values of the Greek ultramafic soils (6.3–7.0) are in general lower than those in the Albanian soils (6.7–8.2), probably because of the higher altitudes of the former (Bani et al. 2009). In this case, there is a direct effect of pH on DTPA-Ni extractability. However, Ni partitioning among mineral phases and its speciation play the most important roles within these ultramafic soils (Massoura et al. 2006). In particular, the association of Ni with amorphous Fe-oxides (Cambisols and other Cambic soils) and with secondary high-exchange clays (Vertisols and Saprolites) are the main factors determining Ni phytoavailability in these ultramafic soils (Massoura et al. 2006; Bani et al. 2014). In our study, soils from the Pindus are shown to be the least weathered from the point of view of Fe speciation. Nickel is clearly associated with unweathered clay minerals and amorphous Fe-oxides in the Cambisols of the entire region. The soil having the highest amount of Ni associated with the amorphous Fe-oxides is unsurprisingly the soil with the highest chemical availability of Ni (Pojska, Albania). Vertisols were selected among all candidates as the target soils for agromining in these regions, because of high Ni phytoavailability, and position in the landscape (downslope soils and alluvial valleys) that conditions water availability to crops and easy access to agricultural machinery. In Albania, ultramafic outcrops cover 10% of the surface, and the Mg-rich arable Vertisols have been estimated to cover about 10,000 ha of the 700,000 ha of the total agricultural land available in the country (Zdruli 1997). These Vertisols are currently being used for low-productivity agriculture on which agromining could be included in management practices (Bani et al. 2007).

## 2.2 Nickel Hyperaccumulation by Plant Species of the Balkans

Ultramafic flora and vegetation have attracted much interest from botanists in those parts of the world where significant ultramafic terranes occur. The largest number of Ni-hyperaccumulators is found in the Brassicaceae family in temperate climates, especially Mediterranean Europe and Turkey (Reeves and Adıgüzel 2008). Hyperaccumulation of Ni is defined as accumulation in aerial parts up to concentrations of >1000 μg $g^{-1}$ on a dry-matter (DM) basis (Brooks et al. 1977; Reeves 1992; van der Ent et al. 2013). This natural process was first discovered in *Alyssum bertolonii* (Brassicaceae) in Italy (Minguzzi and Vergnano 1948). Subsequently, similar reports have been given for *A. murale* in Armenia (Doksopulo 1961), and *A. serpyllifolium* ssp. *lusitanicum* in Portugal (Menezes de Sequeira 1969). Later investigations revealed that Ni hyperaccumulation is widespread among *Alyssum* species on ultramafics of Mediterranean Europe, Turkey, and adjacent countries (Brooks et al. 1979; Reeves and Adıgüzel 2008). Nearly 50 taxa of *Alyssum*, all in Sect. *Odontarrhena* (now accepted as a separate genus by Španiel et al. 2015), act as Ni hyperaccumulators (Brooks 1987; Baker and Brooks 1989, Reeves and Adıgüzel 2008). Nickel hyperaccumulation in the Brassicaceae of the Balkan countries occurs not only in *Alyssum* species, but also in species of the genera *Thlaspi s.l.* (*Noccaea*), *Bornmuellera*, and *Leptoplax* (*Peltaria*). Two additional Ni hyperaccumulators are reported from other families: the Asteraceae (*Centaurea thracica* and the Violaceae (*Viola vourinensis*) (Psaras and Constantinidis 2009).

Thirteen *Alyssum* hyperaccumulator taxa are distributed on the Balkan Peninsula (Bani et al. 2010, 2013). This number is a significant proportion of *Alyssum* hyperaccumulators recorded in Europe (Cecchi et al. 2010), suggesting that the Balkan Peninsula is a 'hotspot' for ultramafic flora of the continent. Although this number of Ni hyperaccumulators will be reduced after new molecular evidence and accepted taxonomy of the genus (Cecchi et al. 2010; Španiel et al. 2015), it is higher than in other parts of Europe. The majority of Ni hyperaccumulators distributed in the Balkans are ultramafic endemics; Greece has the largest number of local endemics. The number of facultative hyperaccumulator species, i.e. hyperaccumulator species that occur naturally on both metalliferous and non-metalliferous soils (Pollard et al. 2014) in the Balkans is small. They occur on a variety of substrates having widely varying Ni concentrations, and show a large range of plant Ni concentrations as a result (Reeves and Adıgüzel 2008). Data for Ni accumulation carried out over the last 30 years on a wide variety of herbarium and field-collected *Alyssum* species on the Balkan Peninsula are listed in Table 1. The highest Ni concentrations are recorded in *A. murale* and *A. heldreichii* (Bani et al. 2010, 2013). The taxonomically complex genus *Thlaspi s.l.* (divided into many separate genera, *Noccaea* being the largest) has numerous Ni hyperaccumulator species. The highest Ni concentrations are measured in *N. ochroleuca* (15–23,400 μg $g^{-1}$ according to Bani et al. 2010). Facultative Ni-hyperaccumulators (*N. ochroleuca*, *N. praecox* and *N. kovatsii* have Ni-accumulating potential only where growing on ultramafic soils. Measured Ni concentrations in the leaves of the above species from the Balkans are approximately four times higher than those reported for Turkey (Reeves and Adigüzel 2008). Local species endemic to ultramafic soils in Greece are *N. epirota*, *N. graeca* and *N. tymphaea*. *Noccaea* species are known to hyperaccumulate more than one metal (Cd, Ni, Pb, Zn). *Noccaea ochroleuca* grown on various substrates shows a wide range of concentrations of both Ni and Zn, each element exceeding 1000 μg $g^{-1}$ (Reeves and Adigüzel 2008).

**Table 1** Data for Ni accumulation in *Alyssum* species according to Bani et al. (2010, 2013)

| Plant taxa | Origin | Ni in leaves (μg $g^{-1}$) |
|---|---|---|
| *A. baldaccii* Vierh. ex Nyar. | GR (Crete) | 1430–17,670 |
| *A. bertolonii* Desv. ssp. *scutarinum* Nyar. | AL | 6320–10,200 |
| *A. chalcidicum* Janka | GR | 4800–11,680 |
| *A. chlorocarpum* Hausskn. | GR | 4110 |
| *A. euboeum* Halácsy | GR (Euboea) | 26–4550 |
| *A. heldreichii* Hausskn. | GR | 1440–32,040 |
| *A. lesbiacum* Candargy (Rech. f.) | GR (Lesbos) | 2900–22,400 |
| *A. markgrafii* O.E. Schulz | AL | <3–19,100 |
| *A. murale* Waldst. & Kit. ssp. *murale* | AL, BG, GR | 7–34,690 |
| *A. murale* Waldst. & Kit. ssp. *pichleri* (Velen.) Stoj. and Stef. | BG | 10–4120 |
| *A. smolikanum* Nyar. | GR | 1700–6600 |
| *A. tenium* Halácsy | GR (Tinos) | 730–9460 |
| *A. saxatile* L. | AL | 4125 |

The genus *Bornmuellera* is restricted to Greece, Kosovo, Serbia, S. Albania, and Turkey (Marhold 2011). Some of its species are ultramafic-endemics. The highest Ni concentration is reported for *Bornmuellera tymphaea* from 1590 to 31,200 μg $g^{-1}$ (Bani et al. 2010). Recently, in the light of available molecular data (Rešetnik et al. 2013) and a re-evaluation of morphological characters, the monotypic genus *Leptoplax* (previously considered closely related to and even included in *Peltaria*) has been merged with *Bornmuellera* (Rešetnik et al. 2014). A local ultramafic-endemic species, *L. emarginata*, occurring mainly in northern Greece and on the island of Evvia, are reported to have varying Ni concentrations of 2040 to 34,400 μg $g^{-1}$ (Bani et al. 2010). This species typically forms hybrids with *B. baldaccii* and *B. tymphaea*. Psaras and Constantinidis (2009) described two more hyperaccumulator species from the flora of Greece, *Centaurea thracica* and *Viola vourinensis*, with 3830 μg $g^{-1}$ and 1023 μg $g^{-1}$ Ni, respectively. The leaves remain the most important plant part to harvest for phytomining purposes, although stems have intermediate Ni concentrations and potential for high biomass production (Zhang et al. 2014).

The percentage of leaves in the biomass of different hyperaccumulator species is highly variable, even within a given species, thus being crucial for defining the potential phytoextraction yield. In an Albanian Vertisols site, the mean height of *A. murale* plants at flowering stage varied from 45 to 93 cm, and for one plant, the percentage of leaves in the whole biomass ranged from 11.6 to −17.3% by weight (Bani et al. 2015b). The highest Ni contents in plants were recorded for the leaves of *L. emarginata* with more than 30,000 μg $g^{-1}$ Ni (Zhang et al. 2014). *Alyssum murale* with more than 20,000 μg $g^{-1}$ Ni in leaves (Bani et al. 2014) is the predominant species on all ultramafic sites in Albania and Greece (and many other Balkan countries). The highest Ni concentrations recorded in *A. murale* leaves collected from ultramafic sites of Bulgaria (Rhodope massif) ranged from 5000 μg $g^{-1}$ (Kardzali) to 1.5 μg $g^{-1}$ (Kazak) (Bani et al. 2010), whereas for ultramafic sites in Serbia the leaf-hosted Ni contents were 700 to 13,000 μg $g^{-1}$ (Tumi et al. 2012). No relevant effect of pH on Ni accumulation in *Alyssum* could be deduced from our results in ultramafic sites of the Balkans (Bani et al. 2009, 2010). The Ni concentration of this species is highly dependent on the site of collection, and greatest in the area of Prrenjas and Pojska in Albania where shoots contain >10,000 μg $g^{-1}$ (Bani et al. 2010, 2013, 2015a, b). Other *Alyssum* species have lower Ni concentrations (Bani et al. 2009; Zhang et al. 2014). The concentration of Ni in tissues of *A. markgrafii* collected at 10 sites in ultramafics of Kosovo was moderately high, ranging from 1586 μg $g^{-1}$ to 7564 μg $g^{-1}$ (Salihaj et al. 2016). Distribution of Ni among the different organs of hyperaccumulator plants depends on the biology of the species and can be influenced by edaphic factors such as climate, soil pH, *etc*. It is clear that the endemic *A. markgrafii*, several efficient populations of *A. murale*, *L. emarginata*, and *B. tymphaea* (Greece), displayed the best Ni-efficiency for use in phytomining (Chardot et al. 2005; Bani et al. 2009; Zhang et al. 2014).

## 3 Case Study in Albania: 7-Year Agromining Field Experiments with *Alyssum murale*

*Alyssum murale* occurs widely on ultramafic Vertisols (Bani et al. 2009) and is a spontaneous weed to other crops. We consequently started investigating the performance of a phytoextraction system with some agronomic practices to evaluate whether semi-extensive phytoextraction of Ni is feasible on Mg-rich Vertisols. *A. murale* was the easiest crop to plant, especially because of its high rate of seeding and high frequency of occurrence in ultramafic agricultural lands of Albania. Experiments were undertaken from 2005–2009 and from 2012–2014 on *in situ* experimental plots. Field experiments (Bani et al. 2015a, b) were aimed at studying (i) the distribution of Ni and other elements in plant organs, (ii) plant nutrition and fertilization, (iii) weed control, (iv) the crop establishment technique (natural cover *vs.* sown crop), and (v) plant density. Firstly, the cropping of native stands of *A. murale* was studied for 3 years. After this period, the *A. murale* cover was renewed by sowing native seeds without density control. Finally, in the last set of experiments, the effect of density was studied from data on 1 to 6 plants per m$^2$. The final aim was to propose a cropping system for *A. murale* that would optimize agromining in the Balkans.

### 3.1 Fertilization Regime and Nutrient Requirements

At first, native stands of *A. murale* were cropped in Pojska from 2005 until 2007 (Bani et al. 2015a). In March 2005, six 36 m$^2$ plots were designed, three of which were fertilized in April with 120 kg ha$^{-1}$ of N, P and K and 77 kg ha$^{-1}$ of Ca (ammonium nitrate, potassium sulfate, and calcium phosphate). The three other plots were not fertilized (Bani et al. 2007). In 2006, we used the same fertilization protocol as above, but also treated the three plots with anti-monocot herbicide (FocusTM ultra 33 mL applied in 3 L water sprayed onto 108 m$^2$) (FH); a further three were not treated (NFNH). The optimal fertilization pattern for *A. murale* had been carefully studied on the same soil in glasshouse pot experiments (Bani et al. 2007; Nkrumah et al. 2016). Plants were harvested for the first and second year at the end of June (before studies on Ni distribution in plant parts according to the phenological stage). In 2007, the fertilization protocol involved 100 kg ha$^{-1}$ P and K, and for N there was a split application of 120 kg ha$^{-1}$. In early April, 60 kg ha$^{-1}$ were brought into early vegetation and a further 60 kg ha$^{-1}$ were fertilized at the end of April, during the stem elongation stage. Following phenological studies (Bani et al. 2015a; Estrade et al. 2015), plants were harvested in mid-June for the last 3 years of the experiment. The overall vegetation responded dramatically to fertilization, by doubling the biomass and phytoextraction yield. For the first year (2005) in the fertilized plots we obtained a total biomass for all species of 6.3 t ha$^{-1}$ (dry weight) and a Ni phytoextraction yield of 22.6 kg Ni ha$^{-1}$; in unfertilized plots, it was only 3.2 t ha$^{-1}$ (Figs. 2 and 4) with a Ni phytoextraction yield of 1.7 kg Ni ha$^{-1}$. These data thus show a highly significant difference ($p < 0.01$) between fertilized and unfertilized plots (Fig. 4). In total phytoextraction yield, the main contributory species was *A. murale*. In the third year (2007) after we had changed the fertilization protocol and the date of harvest, the biomass yield in the fertilized plots was 6.0 t ha$^{-1}$, whereas in unfertilized plots, it was only 0.5 t ha$^{-1}$ (Fig. 2) (Bani et al. 2015a).

### 3.2 Sowing *Alyssum murale*

In 2007–2008, the soils of the six initial 36 m$^2$ plots were tilled to prepare appropriate seedbeds (Bani et al. 2015a). Seeds had previously been collected from the immediate vicinity of the experimental plots. All plots were hand sown in September 2007. Fertilized plots received 100 kg

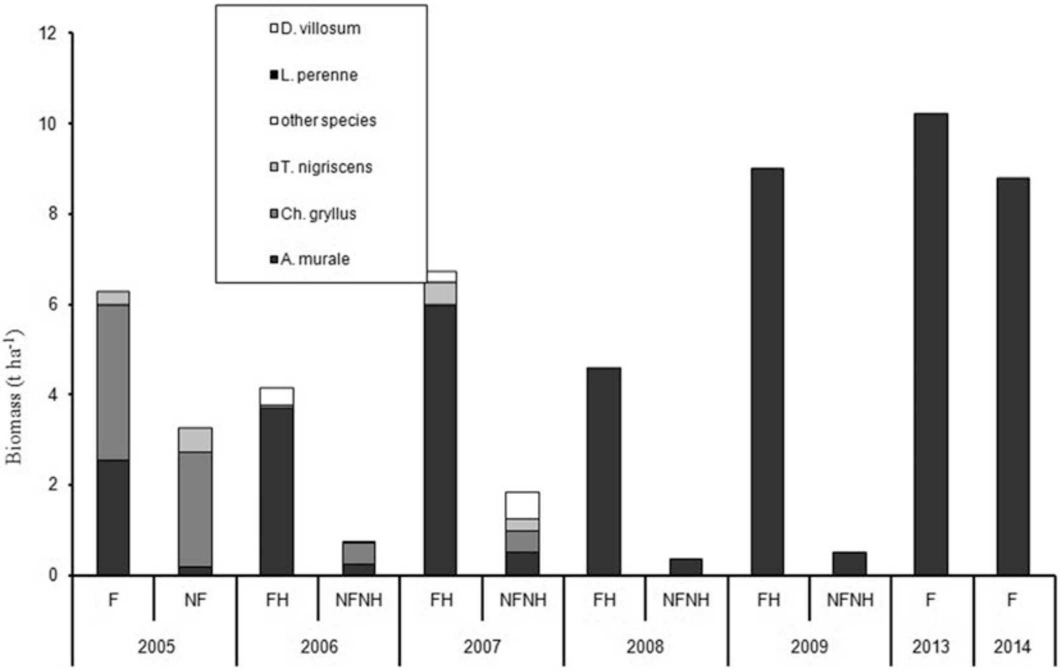

**Fig. 2** Evolution of biomass-production of *Alyssum murale* during 7 years of field-study in Pojska, Pogradec, Albania

ha$^{-1}$ P and K, 65 kg ha$^{-1}$Ca, and 50 kg ha$^{-1}$ N in April, and were treated with anti-monocot herbicide. A second N fertilization was performed (50 kg ha$^{-1}$) 2 weeks later. The N addition was lowered in order to take into account the mineralization of plant residues. The other three plots were not treated (neither fertilized nor herbicide-treated). During the growth period the treated experimental plots were irrigated by water from a natural stream in the area. The harvesting date was early July 2008, when the biomass was considered high enough (little flowering had occurred in the newly planted plots). In 2009, the protocol of fertilization and irrigation was repeated exactly as carried out in 2008, and the plots were harvested on June 12 at the mid-flowering stage. In 2008, with split-N fertilization, irrigation, and the herbicide treatment, the biomass production was 4.6 t ha$^{-1}$ in the fertilized and herbicide-treated plots; the Ni phytoextraction yield was 55 kg ha$^{-1}$ (Figs. 2 and 4), in contrast to only 0.4 t and 4.3 kg Ni ha$^{-1}$, respectively, in the unfertilized plots with a significant difference ($p < 0.05$). In 2009, in the fertilized plots the biomass production was 9.0 t ha$^{-1}$ and Ni phytoextraction yield was 105 kg ha$^{-1}$, which subsequently proved to be optimal in such edaphic conditions (Bani et al. 2015a, b).

## 3.3 Competition with Other Species and Density Effects

When enhancing natural vegetation stands by fertilization (Bani et al. 2015a), *A. murale*, *Chrysopogon gryllus* (Poaceae), and *Trifolium nigrescens* (Fabaceae) were the most abundant species identified on this site in the first year (2005). Other species were reported on the plots, although their contribution to total biomass production was negligible (Fig. 2). Because *C. gryllus* was identified as the most competitive species and *T. nigrescens* tended to disappear with fertilization, a good weed control was obtained on the fields by addition of 120-kg ha$^{-1}$ N fertilization and a single spraying with an anti-monocot herbicide at the end of April of 2005 (Bani et al. 2015a).

**Fig. 3** Photo of *Alyssum murale* in Albanian ultramafic sites. (**a**) Pojska site 2013, (**b**) Pojska site 2014, (**c**) *A. murale* 1 plant for $m^2$, (**d**) Harvesting of *A. murale* in Pojska site (June 2016)

During 2012–2014 we evaluated the influence of plant density on Ni phytoextraction yield in the case of sown/planted crops of *A. murale* on two representative ultramafic Vertisol bodies at Pojska (POJ) in the municipality of Pogradec (Fig. 3), and Domosdova (DOM) in the municipality of Prrenjas (Bani et al. 2015b). Three different densities were studied: (i) at the Domosdova site, *A. murale* was either transplanted without density constraints (*ca.* 1.5 plant $m^{-2}$) (DOM-1) or a density of 6 plants $m^{-2}$ on an adjacent plot (DOM-6). It was grown at a density of 4 plants $m^{-2}$ in Pojska (POJ-4). In 2014, the Domosdova field was inadvertently ploughed, and was therefore unavailable for more cropping, hence only data for 2013 were available for this site.

The biomass of DOM-1 (10.6 t $ha^{-1}$) was much higher, nearly twice as much as for DOM-6 (4.8 t $ha^{-1}$). Nickel yield followed the same trend: DOM-1 (77 kg $ha^{-1}$) was higher than DOM-6 (41 kg $ha^{-1}$) (Table 2). With a density of 4 plants $m^{-2}$ (POJ-4), biomass was the highest (10.2 t $ha^{-1}$ in 2013 and 8.8 t $ha^{-1}$ in 2014); in

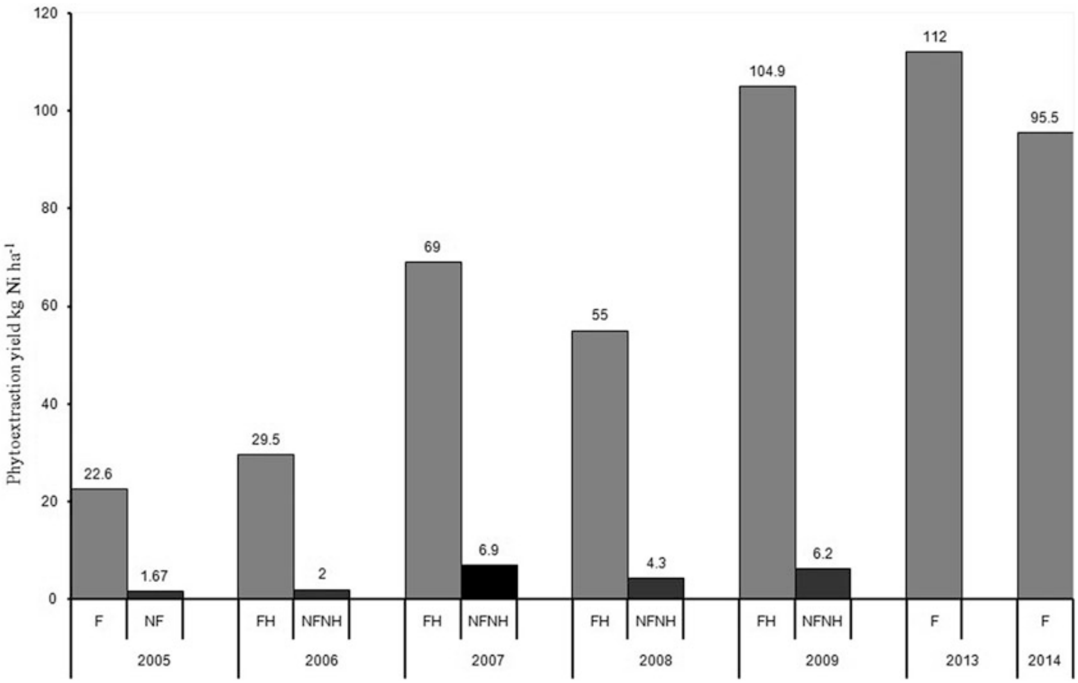

**Fig. 4** Evolution of phytoextraction yield kg Ni ha$^{-1}$ of *Alyssum murale* during 7 years of field-study in Pojska, Pogradec, Albania

**Table 2** Nickel production by *Alyssum murale* for two consecutive years from the plots sown in Pojska and Domosdova site (data from Bani et al. 2015b)

| Site | Years | Plant density | Average plant mass (g plant$^{-1}$) | Ni concentration in plants (g kg$^{-1}$) | Biomass yield (t ha$^{-1}$) | Ni yield (kg ha$^{-1}$) |
|---|---|---|---|---|---|---|
| POJ4 | 2013 | 4 plant m$^{-2}$ | 257 | 11.0 | 10.2 | 112 |
| POJ4 | 2014 | 4 plant m$^{-2}$ + recruits | 220 | 10.9 | 8.8 | 95.9 |
| DOM-1 | 2013 | 1 plant m$^{-2}$ | 1079 | 7.1 | 10.8 | 76.6 |
| DOM-6 | 2013 | 6 plant m$^{-2}$ | 74.5 | 8.9 | 4.84 | 41.0 |

2014, *A. murale* was in competition with its own spontaneous recruits. The Ni yields in the biomass of *A. murale* for 2013 (112 kg per ha$^{-1}$) were higher than in 2014 (95.9 kg ha$^{-1}$) (Table 2 and Figs. 2 and 4). According to the process for producing ammonium nickel sulfate hexahydrate (ANSH) from *A. murale* ash (Barbaroux et al. 2012; Zhang et al. 2014), it is crucial to evaluate the ash-producing yield and the percent recovery of Ni at 550 °C (Zhang et al. 2014). The total mass of ash that could be recovered was 1.3 t per ha for DOM-1 and 0.4 t per ha for DOM-6. Consequently, the total Ni mass in the ash of *A. murale* in the DOM-1 treatment was 76 kg ha$^{-1}$ with almost no loss of Ni during the combustion process. The quantity of ash of *A. murale* produced in the DOM-6 treatment was only 41 kg ha$^{-1}$, also without significant loss of Ni during ashing. Using the optimized process to produce ANSH from *A. murale* ash (Barbaroux et al. 2012), at least 45 g of ANSH (close to 100% purity) was obtained from one plant of *A. murale*, which represents 450 kg ANSH ha$^{-1}$ in the DOM-1 plot.

Nickel phytoavailability is thought to have a stronger influence on uptake of this metal in shoots than does plant density (Bani et al. 2015b). We also stress the fact that the biomass yield of each plant does not impair Ni uptake, and that no strong dilution effect is observed in cropped *A. murale* (Bani et al. 2015a). Therefore, on a given site, it is important to maximize the yield of plant biomass. Biomass yield at harvest under these edaphic conditions (i.e. ultramafic Vertisols of southeastern Albania) seems to reach a maximum of ca. 10 t $ha^{-1}$ when using seeds of native populations (Bani et al. 2015a, b). Only genetic selection can further improve biomass yields (i.e. 20 t $ha^{-1}$), through more production of performing cultivars (Li et al. 2003), which are keys to increased Ni phytoextraction yield (Bani et al. 2015b).

### 3.4 Elemental Balance in Fields Cropped with *A. murale*

Calcium and potassium are strongly exported by *Alyssum* crops (Bani et al. 2015a, b). It seems that the Ca/Ni quotient varies between soils (Zhang et al. 2014) and hence the soils from Pojska produce biomass of better quality for Ni recovery (Zhang et al. 2014; Bani et al. 2015a, b) as they show lower Ca contents than at Domosdova. Based on biomass yields and on the Ca and K contents of *A. murale* at Pojska (*Alyssum* 6 in Zhang et al. 2014), Ca and K exports can reach approximately 135 and 100 kg $ha^{-1}$ $y^{-1}$, respectively, when assuming a biomass yield of 9 t $ha^{-1}$. These results are somewhat balanced by the fertilization pattern that was validated in previous pot (Bani et al. 2007) and field (Bani et al. 2015a, b) experiments. Potassium uptake is fully compensated by fertilization, but Ca losses could appear over time due to the imbalance (77 kg added only). Calcium is not as essential as K, and this may possibly lead to regulation by *A. murale* and thus a decrease in Ca uptake. It is crucial that the phosphate fertilizer used in Ni agromining not be the common Di-ammonium phosphate (DAP), widely in use worldwide, because maintaining Ca fertility in soils is imperative over the long term. However, as a consequence of a lower Ca uptake, Ni uptake could be increased in *A. murale* crops, as suggested by our field data for these specific soils and native populations (Bani et al. 2009). Nevertheless, other studies have pointed out that Ca concentrations enhance Ni uptake under controlled conditions (Chaney et al. 2008). In any case, when agromined soils are returned for cultivation of other 'normal' crops, and therefore Ca fertility will be of great importance.

## 4 Conclusion and Perspectives

Our experimental data should help in the development of an improved growing system for Ni agromining in the Balkans. Results obtained so far demonstrate that it is necessary to sow or transplant *A. murale* at a density of 4 plants $m^2$ on well-structured (ploughed) soil under conditions of non-limiting Ni availability on ultramafic Vertisols (e.g. Pojska). The fertilization pattern is now efficiently set for these soils, at 120 kg N $ha^{-1}$ with a split application and additional P, K, S, and Ca fertilization ($K_2SO_4$, and $Ca(H_2PO_4)_2$) at 100 kg $ha^{-1}$ for K and P, and 77 kg $ha^{-1}$ for Ca. Crops are likely to export ca. 100 kg of Ni and K and more than 135 kg of Ca annually. Calcium and K should be returned by fertilization over the years to keep fertility of the soil suitable for agromining. Nickel availability had not changed after 3 years of cropping (Bani et al. 2015a), but probably would decrease eventually. Weeding is crucial and targets competing species as well as young recruits of *A. murale* to maintain an optimum density. Harvest can be carried out at the mid-flowering stage, which maximizes the recovery of Ni. *Alyssum murale* is harvested by hand and allowed to dry on the field as a hay crop. It can then be baled, ready for combustion. The technology can thus be applied to all ultramafic terranes of the Balkans, where agromining has the potential to become an economically justifiable agricultural cropping system and hence enter traditional crop rotations. Other suitable edaphic situations (including Cambisols) should also be tested under field conditions in order to potentially widen the applications of agromining. Other nickel products (e.g. ANSH) with high purity can easily be obtained from *A. murale* shoots, some of which may have a much higher value than gross nickel metal (more than five times for a given Ni yield). In this case, the profit from the crop would be substantially higher (Barbaroux et al. 2012; Zhang et al. 2016).

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# Element Case Studies: Cobalt and Copper

Michel-Pierre Faucon, Olivier Pourret, and Bastien Lange

**Abstract**

Cobalt is economically considered a critical metal for a variety of technologies. Globally, the most important Co ore deposits occur in the Katangan Copperbelt (Democratic Republic of Congo) where a richness of Cu-Co-tolerant and accumulator plants have developed naturally. Cobalt mining there has resulted in the dissemination of large quantities of waste in the environment and is a major environmental issue. Reduction of environmental risks and Co dispersion can be performed by phytoremediation and/or agromining, using trace-element-tolerant and putative hyperaccumulator plants that originated from the biodiversity of natural Co and Cu-rich habitats. Accumulation of foliar Co to $>300\ \mu g\ g^{-1}$ is exceptionally rare globally, being known principally from the Copperbelt of Central Africa. This chapter highlights advances in our knowledge of Co accumulation in plants, examines potential for use of a Co-accumulator in agromining, and defines perspectives for Co agromining by designing multi-functions and services of agroecosystems.

## 1 Introduction

Cobalt (Co) is one of the elements defined as being a critical metal over the next 5–15 years (Pourret and Faucon 2016). Demand for Co increased considerably after World War II, driven mainly by the need for high-purity Co in jet engines and gas turbines. Cobalt demand and corresponding production have further accelerated over the past 30 years. Indeed, this reflects the increased use of Co as an essential constituent of materials in high-technology industries including rechargeable Li batteries, super alloys, and catalysts (Crundwell et al. 2011; Slack et al. 2017). The most economically important land-based Co ore deposits are found in the Katangan Copperbelt (Democratic Republic of Congo) where approximately 48% of known world reserves are identified. The Katangan Copperbelt acts as the pre-eminent global producer of cobalt (Fig. 1), accounting

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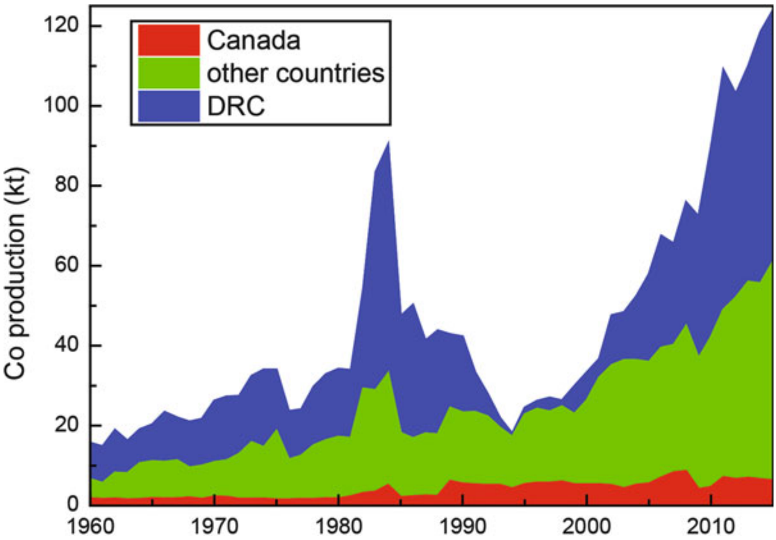

**Fig. 1** Production of cobalt (data collected from mineral commodity summaries yearly published by USGS)

for 50.8% of world production in 2015 (USGS 2016). Significant land-based resources of Co also occur in lateritic and sulphide Ni deposits (Mudd et al. 2013; Decrée et al. 2015; Slack et al. 2017). The average cobalt price has ranged from 30,000 to 50,000 US\$ $t^{-1}$ over the last several years (London Metal Exchange).

For over a century, mining activities have disseminated large quantities of waste in the environment and as a result generated major environmental issues in many areas (Pourret et al. 2016). As a consequence, these activities have created secondary metalliferous substrates having elevated concentrations of trace elements including Co (more than 1000 times greater than the concentration in normal soils) (Faucon et al. 2011, 2012). In some cases, such exploitation has resulted in very large occupations of soil (over 30,000 $km^2$) and may include soil degradation (Dupin et al. 2013). In southeastern Central Africa, contamination by trace metals constitutes a major environmental issue, related to low pH of Ferralsol that increases trace element mobility, intensity of erosion by rainfall in the rainy season, and aerial dispersal of metal particles by wind in the dry season (Pourret et al. 2016). Moreover, Co is highly toxic and directly impacts human heath, given that there is an elevated exposure to Co in the general population of southern DR Congo (Banza et al. 2009; Cheyns et al. 2014; Squadrone et al. 2016).

Reduction of environmental risks and Co dispersion can be achieved with phytoremediation and agromining, using putative hyperaccumulator plants that are tolerant to trace elements based on the plant biodiversity of natural habitats enriched in Co and Cu (Ilunga et al. 2015; Boisson et al. 2015; Faucon et al. 2016). Agromining is an alternative treatment for contaminated soils and a potential application of phytotechnologies to exploit secondary metal resources. Agromining was studied at field scale (Chaney et al. 2007; van der Ent et al. 2015; Bani et al. 2015), but to date has been limited to Ni. Cobalt agromining may, therefore, be considered for Co-contaminated lands because 30 putative Co hyperaccumulator plants have been identified in southeastern DR Congo (Brooks et al. 1980; Reeves and Baker 2000; Faucon et al. 2007), and because of possible attainable yields and the high product value of the metal.

Cobalt accumulators present a high variation of foliar Co concentration that can be explained by genetic variation and chemical soil factors involved in Co availability (Lange et al. 2017). In addition, Co-accumulators display high functional diversity (i.e. in life cycles, lateral spreading capacity, type and depth of underground system, growth phenology, N symbiotic fixation, etc.) (Ilunga et al. 2015). This diversity has an influence on ecosystem processes such as biomass productivity, Co mobility, and transfer (Faucon 2015). Cobalt agromining should consider intra- and inter-specific variation of leaf Co accumulation, and functional plant diversity, in order to optimize the multi-functional design and services of these new cropping systems.

In this chapter, we focus on putative Co hyperaccumulator plants and their applications in Co agromining. We review recent advances in understanding processes of Co accumulation, examine the potential of a Co-accumulator in agromining, and define perspectives for Co agromining by designing multi-functions and services for agroecosystems.

## 2 Variations in Cobalt Accumulation

Cobalt-tolerant populations of plants have been reported in the literature in several dozen species collected from nature (i.e. Cu- and Co-steppic savanna). However, the capacity to grow these species under elevated Co concentrations without toxicity symptoms or growth inhibition has been only partially demonstrated experimentally (Lange et al. 2017). Variations in Co accumulation have been documented among three closely related *Silene* taxa (Baker et al. 1983). Cobalt accumulation exists in *Anisopappus chinensis* (Asteraceae), as foliar Co concentration increases in relation to Co soil concentration without a decrease of biomass or development of toxicity symptoms (Lange 2016).

High inter- and intra-specific variation of Co accumulation exists (Faucon et al. 2007, 2009; Lange et al. 2014). This high-phenotypic variation of Co accumulation can be explained by differences in total Co concentration in soils and by chemical soil factors that influence Co mobility and availability (Faucon et al. 2009). The natural Cu-Co ore outcrops have high total soil Co concentrations of up to 3451 μg $g^{-1}$, from which a fraction is mobile (39–2146 μg $g^{-1}$) (Pourret et al. 2016). Existence of this mobile fraction permits high Co accumulation by *A. chinensis* (up to 2822 μg $g^{-1}$ foliar Co). Overall, the strong affinity of Mn oxides for Co may explain the lower Co mobility that characterizes Mn-rich soils (Collins and Kinsela 2011; Lange et al. 2014). Cobalt accumulation, such as in *Crepidorhopalon perennis* (Linderniaceae) and *A. chinensis*), was strongly influenced by free Co and by Co adsorbed onto organic matter and Fe-oxide fractions (Lange et al. 2014).

The most tolerant taxon of *Silene* is the least accumulating for a given soil Co concentration. Intra-specific variation of Co accumulation by metallophytes from natural Co-rich soils has not yet been tested. For the facultative metallophyte *A. chinensis*, the strong variation in the degree of Co tolerance among populations suggest an intra-specific variation of accumulation (Lange 2016).

## 3 Potential of Cobalt Agromining

Cobalt agromining was proposed in over 25 years ago as a possible means of recovering Co using Ni-hyperaccumulator species grown on ultramafic soils (Homer et al. 1991). However, Ni limits the uptake of Co in most Ni-hyperaccumulator plants growing on ultramafic soils (Malik et al. 2000) and so negatively influences the performance of Co agromining. The best candidate species for Co agromining are plants having elevated extracting yields (i.e. biomass × Co shoot concentration), and plants that are both tolerant to Cu and Co given that most Co-tolerant plants in southeastern DR Congo grow in Cu- and Co-rich soils.

Lange (2016) evaluated the potential for Co agromining using metallicolous populations of *A. chinensis* from Co-enriched soils that present naturally elevated Co concentrations in leafs (30–2822 μg $g^{-1}$). The mean shoot biomass of individuals of *A. chinensis* ($n = 20$) was 0.8 g (range 0.14–3.79 g). Biomass productivity (Ybio) was estimated at 16 g $m^2$ $y^{-1}$ or 160 kg $ha^{-1}$ $y^{-1}$ (range 20–320 kg $ha^{-1}$ $y^{-1}$). Total phytoextracted Co (YCo = FCo.Ybio) from a contaminated soil using plants of *A. chinensis* from the Fungurume V mine area was 176 g $ha^{-1}$ $y^{-1}$ (max 864 g $ha^{-1}$)

(Lange 2016). Due to great variation of its ability to extract Co from soil, agromining using *A. chinensis* should be improved by careful plant selection and genotyping (Chaney et al. 2007). Potentially, Co agromining could be improved by performing a multispecies system that introduces other Co-tolerant and accumulator species. Legume species such as *Crotalaria cornetii* (Fabaceae), which grows in natural and secondary metalliferous habitats (Leteinturier 2002), can improve the facilitation process and increase biomass productivity and potentially Co recovery. Such an innovative system for Co-contaminated soils could lead to increased phytoextraction yield as some species, such as *Haumaniastrum robertii*, are known to accumulate Co in their shoots more effectively than *A. chinensis* (Lamiaceae, Co leaf concentration 4000 μg $g^{-1}$) (Morrison et al. 1981). This first evaluation on the phytoextraction of soils contaminated by Co in tropical Central Africa has shown limited achievable yields of Co by phytoextraction. Future perspectives would be tests at field scale using a multi-species system that introduces functional diversity of the Co-accumulator plants (species and populations) (Fig. 2).

## 4 Functional Plant Diversity of Cobalt Agromining Systems

In the last decade, considerable progress in ecology has been achieved due to elaboration of the concept of functional traits. Functional traits are

**Fig. 2** Several putative Co accumulator plants. (**a**) *Anisopappus chinensis* (Asteraceae) photo by Lange B, (**b**) *Haumaniastrum robertii* (Lamiaceae) photo by Faucon M-P, (**c**) *Triumfetta welwitschii* (Malvaceae). (**d**) *Crepidorhopalon perennis* (Linderniaceae) photo by Faucon M-P, (**e**) *Buchnera henriquesii* (Orobanchaceae) photo by Séleck M, (**f**) *Acalypha cupricola* (Euphorbiaceae) photo by Lebrun J

defined as "morpho-physio-phenological traits which impact fitness indirectly via their effects on growth, reproduction and survival" (Violle et al. 2007). Relationships among traits associated with the response of organisms to environmental factors such as resources and disturbances (response traits), and among traits that determine the effects of organisms on ecosystem functions, have been studied recently in a few processes and ecosystem services (Garnier et al. 2016). Phytoremediation and agromining generally attempt to study the effects of species or species mixtures on ecological processes, in order to restore and design ecosystems independently of a functional trait approach (Faucon et al. 2017). Agromining based on taxonomical description does not allow quantifying the effects of plant species on ecosystem processes and services. The challenge is to link results of recent studies on below- and above-ground functional traits of plants and ecosystem processes such as trace element mobility, water transfer and sediment transport, for improving ecological engineering and associated services, especially agromining yield, landscape restoration, reduction of trace element transfer and water contamination, carbon sequestration, etc.

## 5 Plant Functional Diversity for Improving Co Agromining

In Co accumulators, functional divergence of root traits should be considered in order to improve the biomass productivity and yield of agromining for cobalt. From 30 putative Co-accumulators that present high variability of morphology, life cycle and physiology (Ilunga et al. 2015; Malaisse et al. 2016), several traits can be selected to develop a multifunctional Co agromining system. Effectively, the intercropping with functional diversity of crop species corresponds to temporal and spatial niche differentiation in the resource acquisition for these species, linked to an increase in productivity compared to a monoculture (Li et al. 2014; Faucon et al. 2015). Spatial niche differentiation associated with root morphology and the capability to exploit nutrients in soils can also explain how species colonize the soil profile and the total soil volume occupied by the crop (Lynch 2011). Physiological root traits should also be considered for improving the yield of Co agromining, especially root-induced processes such as proton and/or carboxylate releasing that would directly affect Co availability in the rhizosphere (Lange et al. 2017). In contrast to functional diversity (FD) of root traits, FD of leaf traits and plant heights can present a negative effect on biomass productivity, explained by an increased competition for light.

Traits of perennial cycle, high-lateral spreading capacity and growth phenology in the dry season, will be prioritized in order to develop a dense cover for restoring the landscape, reducing runoff and soil erosion, and thus, Co transfer (Egoh et al. 2011). Functional diversity should be considered to improve these ecosystem services, such as FD of deep underground systems in order to improve root density and hence soil cohesion and stability (Gyssels et al. 2005), of growth phenology (both dry and rainy seasons) (Ilunga et al. 2015).

## 6 Conclusions

The application of Co accumulator plants in agromining is limited because their accumulation characteristics mean that only in (very) Co-rich soils can plants yield sufficient metal accumulation in the shoots to permit this technology. However, cobalt agromining may be feasible for strongly Co-contaminated soils and tailings, because Co is economically considered a critical metal (Gunn 2014; Slack et al. 2017). Perspectives are needed in order to better understand the relationships between plant functional traits and ecosystem processes/services, and to design multi-functional and multi-service agromining systems.

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# Element Case Studies: Selenium

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**Abstract**

Selenium hyperaccumulator plants such as *Stanleya pinnata*, *Astragalus bisulcatus*, and the newly discovered *Cardamine hupingshanensis* may play an important role in the Se cycle from soil to plant to human, especially in China. Se-hyperaccumulators can be used for agromining or for phytoremediation of Se, as well as for applications to Se-deficient soils in Se-biofortification strategies.

## 1 Introduction

Biofortification has yielded valuable methods for enriching edible crop tissues. These methods include applying agricultural practices or manipulating genetic activities to increase accumulation of essential elements such as iron, zinc, and/or selenium within crops (e.g. high Fe cassava) (Global Panel 2015; Malagoli et al. 2015), as well as nutraceutical compounds (e.g. antioxidants, carotenes, phenolics) in edible tissues (Brummell et al. 2011; Malagoli et al. 2015). The success of biofortification relies not only on promoting the accumulation of essential nutrients, but also on a more efficient bioavailability and assimilation of the nutrients by humans or animals. The agricultural-based strategy focuses on adding a source, e.g. inorganic Se, to grow Se-enriched food crops that will contribute to a greater Se intake in otherwise Se-deficient communities (Global Panel 2015; Malagoli et al. 2015; Winkel et al. 2015). An alternative source for inorganic Se as a biofortification strategy is by application of plant tissue from a Se-hyperaccumulator (after harvest and milling), for use as a green Se-biofertilizer (Bañuelos et al. 2015). This chapter focuses mainly on three Se-hyperaccumulator plant species and their potential use in agromining, and their use as a green Se-biofertilizer within a Se biofortification strategy, as a concept for addressing human health concerns related to naturally low Se intake.

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## 2 Ideal Candidates for Agromining

*Stanleya pinnata*, *Astragalus bisulcatus*, and *Cardamine hupingshanensis* are suggested plant species for agromining of high-Se soils due to their ability to extract excessive amounts of Se from soil, and accumulate Se to high concentrations in shoots that do not lead to toxicity. Prince's plume (*Stanleya pinnata*) is a perennial member of the Brassicaceae family. This plant is very well adapted to the western area of North America with high biomass production, growing as much as 1.5 m in height and 2 m wide. These growth characteristics are ideal for use as a Se 'agromined crop' (Feist and Parker 2001; Guerinot and Salt 2001; Freeman et al. 2006, 2010; Freeman and Bañuelos 2011). Several studies have shown that this primary Se-hyperaccumulator accumulates between 2000 to 5000 μg $g^{-1}$ Se DW on seleniferous soils (i.e. 2–10 μg $g^{-1}$ Se) without suffering toxicity, whereas a non Se-accumulator plant will accumulate two orders of magnitude less Se (e.g. 25 μg $g^{-1}$) (Feist and Parker 2001; Parker et al. 2003; Freeman et al. 2006, 2010; El Mehdawi et al. 2011, 2012, 2015; Freeman and Bañuelos 2011). Leaves, stems, and roots of *S. pinnata* contain high concentrations of Se up to 3500, 1500, and 2300 μg $g^{-1}$ Se, respectively, in spring; in contrast, concentrations of Se decline sharply in old leaves (<200 μg $g^{-1}$) in autumn. In reproductive organs (flower and seed), concentrations can range from 1800 to 3300 μg $g^{-1}$ Se. These observations indicate a peak accumulation of Se in young tissues during spring, and then Se mobilization into reproductive tissues in summer, falling back during autumn (Galeas et al. 2007).

*Astragalus bisulcatus*, commonly known as two-grooved milkvetch or silver-leafed milkvetch, is a member of the Fabaceae family and a primary Se-hyperaccumulator (Parker et al. 2003). This species is a perennial plant native to North America, and in contrast to *S. pinnata*, *A. bisulcatus* exhibits slow growth and produces less biomass (Feist and Parker 2001; Parker et al. 2003). However, this Se-hyperaccumulator can accumulate more than 10,000 μg $g^{-1}$ Se, predominantly as methylselenocysteine (MeSeCys) in young leaves and reproductive organs (Freeman et al. 2006, 2010; Statwick et al. 2016). Similar to that observed in *S. pinnata*, leaves of *A. bisulcatus* experience seasonal fluctuations in Se concentrations, with the highest accumulation detected in young leaves during spring (up to 12,700 μg $g^{-1}$ DW) and the lowest concentrations in autumn (600 μg $g^{-1}$) (Galeas et al. 2007). Speciation of the total Se showed that the predominant organic Se forms were MeSeCys (53%) and γ-glutamyl-Se-methylselenocysteine (γ-GMeSeCys) (47%) in the young leaf tissues. The methylated organic form (MeSeCys) was detected in both *A. bisulcatus* and *S. pinnata* (Freeman et al. 2006), whereas selenocystine ($SeCys_2$) was the predominant form identified in *C. hupingshanensis* (Yuan et al. 2013). In mature leaves of *A. bisulcatus*, Se (VI) was found as the predominant inorganic form of Se (Pickering et al. 2003). During reproductive development, flowers and seeds of *A. bisulcatus* accumulated 9000 and 6500 μg $g^{-1}$ Se DW, respectively (Galeas et al. 2007). Overall, 10- to 14-fold higher total Se concentrations were observed in the young leaves of *A. bisulcatus* compared to mature leaves (Freeman et al. 2006). The Se fluctuations found within foliar tissues in Se-hyperaccumulators is a distinctive characteristic of Se remobilization that may be helpful in optimizing the harvest time of these plants.

The recently discovered plant species *C. hupingshanensis* (Fig. 1) was identified in Enshi in western Hubei, China (Bai et al. 2008; Yuan et al. 2013). This plant belongs to the Brassicaceae family and has been designated as a secondary Se-accumulator owing to its moderate ability to absorb and volatilize Se. However, *C. hupingshanensis* is more like a Se-hyperaccumulator than other secondary Se-accumulators, e.g. *Brassica juncea* (Terry et al. 2000), because *C. hupingshanensis* is able to accumulate 1965 μg $g^{-1}$ Se DW in shoots and

**Fig. 1** *Cardamine hupingshanensis* in Suzhou, China

4414 µg $g^{-1}$ Se in roots, while growing in a Se-laden soil (10–70 µg $g^{-1}$ Se) in China (Yuan et al. 2013). Recent findings suggest one advantage in using the perennial herb *C. hupingshanensis* for phytoremediation of Se in Se-contaminated areas of China is the use of the plant tissue after harvest as an organic Se-biofertilizer in Se-deficient soils (Yuan et al. 2013; Shao et al. 2014; Wu et al. 2015). In comparison to the data available on *A. bisulcatus and S. pinnata*, more detailed information is needed on *C. hupingshanensis* for its use as a biofertilizer.

The use of these three Se-hyperaccumulator plants makes them clear candidates for agromining due to their ability to extract high concentrations of Se. However, only a few field studies have provided detailed descriptions of total and extractable Se concentrations in soil, and resulting Se concentrations in plant tissues. Most studies that used these Se-hyperaccumulators were performed in glasshouses, hydroponics, and under laboratory controlled conditions. As a result, these results cannot be used to accurately predict Se accumulation behaviour in the field.

## 3 Enhanced Se/S Quotients, Bioconcentration Factors, and Discriminant Coefficients

A higher Se/S quotient (White et al. 2007) is a distinctive characteristic of Se-hyperaccumulators, such as *A. bisulcatus* and *S. pinnata* (Parker et al. 2003; Pickering et al. 2003). This enhanced quotient allows them to be more suitable for colonizing alkaline and well-oxidized soils, predominantly having high sulphate levels (Bañuelos 2001, 2006). In contrast to hyperaccumulators, high sulphate concentrations in soils compete with selenate in non-Se-hyperaccumulators owing to Se uptake occurring primarily via sulphate transporters (Shibagaki et al. 2002; White et al. 2004; Hesketh 2008; Chao et al. 2014; Schiavon et al. 2015). Within Se-hyperaccumulators, there is a higher transcript expression of sulphur transporters (e.g. Sultr1, 2) in roots and shoots (Cabannes et al. 2011; Schiavon et al. 2015), as well as a higher expression of ATP-dependent sulphurylases (APS) (Pilon-Smits et al. 1999; Schiavon et al. 2015), which are suggested to be responsible for the high Se/S quotients in

Se-hyperaccumulator plants. These metabolic sulphur genes are predominantly overexpressed in Se-hyperaccumulators and not in secondary Se-accumulators, if exposed to competition assays with selenate/sulphate (Pilon-Smits et al. 1999; Terry et al. 2000; Feist and Parker 2001; Pickering et al. 2003; Galeas et al. 2007; Freeman et al. 2010; Schiavon et al. 2015). These findings suggest that because of the physical and chemical similarity of sulphate and selenate, and the elevated expression of high-affinity sulphate transporters (HASTS) in Se-hyperaccumulators relative to non-Se-hyperaccumulators, hyperaccumulators may be especially suitable for the agromining of Se (Pilon-Smits et al. 1999; White et al. 2007; Schiavon et al. 2015).

Nonetheless, Se accumulation varies even within Se-hyperaccumulator populations. For example, variation in Se-accumulation ability was found in 15 different populations of *S. pinnata*, reflecting the importance of selecting the appropriate population for agromining field purposes (Feist and Parker 2001). The results showed that population CO4 from Colorado showed the highest accumulation of Se in shoot tissue under the highest treatment (80 μM selenate) with 950 μg $g^{-1}$ Se compared to 200 μg $g^{-1}$ Se in population UT5 from Utah. For this reason, genotype CO4 was suggested to be the most promising population for phytoremediation purposes under those test conditions (Feist and Parker 2001; Parker et al. 2003).

Two parameters are used to indicate how much of an element, such as Se, is taken up by the plant roots and transported to shoots. The first is termed the ‘Bioconcentration Factor’ (BCF) (Table 1). For Se, the BCF is the quotient of Se concentration in dry shoots/total concentration of Se in soil. This quotient is very low in non-accumulators relative to the BCF ratios for *A. bisulcatus* and *S. pinnata* (both >100), and 10.7 in the stems of *C. hupingshanensis* (Yuan et al. 2013). Once the BCF reaches its plateau indicating that homeostasis has been reached, there will be no further increase in Se uptake regardless of how much Se is available in the rhizosphere. The BCF can be complemented with another parameter known as the Discrimination Coefficient (DC), as proposed by Bell et al. (1992). This is the quotient of [(Se/S concentrations in plant tissue) to ($SeO_4$/$SO_4$ concentrations in soil)], which is useful for detecting preferences in uptake between these two analogues. Bell et al. (1992) indicated that *A. bisulcatus* exhibited a DC from 2.4 to 9.0 compared to *Medicago sativa* L at 0.4 to 0.7. The DC indicates that *A. bisulcatus* selectively preferred selenate over sulphate (Bell et al. 1992), whereas DC values observed for *S. pinnata* were 1.2 to 4.3 (as expected for a Se-hyperaccumulator) among 15 populations (Bell et al. 1992; Feist and Parker 2001). Both BCF and DC are useful parameters in selecting for plants growing in soils enriched in S and Se.

## 4 Continuous Accumulation of Se Without Phytotoxicity

Selenium hyperaccumulating plants are equipped with a plethora of mechanisms that allow survival without experiencing Se-phytotoxicity under continuous exposure to a high-Se environment. Within these plant species, selenate is absorbed, metabolized, and assimilated via reduction into selenoamino acids (e.g. selenocysteine, selenomethionine, selenocystathionine, Se-methylselenocysteine and dipeptide γ-glutamyl-Se-methylselenocysteine) (Terry et al. 2000; Shibagaki et al. 2002; Chao et al. 2014; Schiavon et al. 2015). Many of these amino acids occur in methylated forms (Neuhierl et al. 1999; Terry et al. 2000), in order to prevent misincorporation during protein translation (Hesketh 2008; Terry et al. 2000). Selenium hyperaccumulators have increased methylation of these selenoamino acids by a higher activity of methyltransferase (MT) enzymes, which incorporate a methyl group in the SeCys (Ellis et al. 2004; LeDuc et al. 2004; Neuhierl et al. 1999). A chloroplastic selenocysteine methyl transferase (SMT) was first characterized from *A. bisulcatus* (Neuhierl et al. 1999; Sors et al. 2009), and its over-expression in non-Se-hyperaccumulators (*Arabidopsis thaliana*, *B. juncea*) enhanced Se tolerance and Se accumulation (Ellis et al. 2004; LeDuc et al. 2004). In

**Table 1** Selenium accumulation and bioconcentration factor (BCF) in primary Se-hyperaccumulators, secondary Se-accumulators and non-accumulators grown in Se-laden soils

| | Growing season | Total Se in soil (μg $g^{-1}$)[c] | Se in shoots (μg $g^{-1}$)[c] | Bioconcentration factor | |
|---|---|---|---|---|---|
| Plant species | Days | DW | DW | (BCF)[a] | References |
| Primary Se-hyperaccumulators | | | | | |
| Two-grooved milkvetch (*Astragalus bisulcatus*) | 248 | 7.2 | 595 (80) | 83 | Retana et al. (1993) |
| Prince's plume (*Stanleya pinnata*) | 180 | 9.0 (3.8) | 1620 (600) | 180 | Freeman and Bañuelos (2011) |
| Secondary Se-accumulators | | | | | |
| Indian mustard (*Brassica juncea*) | 180 | 9.0 (3.8) | 37 (9) | 4 | Freeman and Bañuelos (2011) |
| Lamb's cress (*Cardamine hupingshanensis*) | 100 | 46.0 (4.6) | 539 (117) | 12 | Shao et al. (2014) |
| | 100 | 25.4 (14.2) | 432 (471) | 17 | Yuan et al. (2013) |
| Non-accumulators | | | | | |
| Tall Wheatgrass Grass (*Thinopyrum intermedium*) | 231 | 20.5 | 12.0 (0.9) | 0.58 | Retana et al. (1993) |
| Lettuce (*Lactuca sativa*)[b] | 70 | 0.27 | 0.1 | 0.37 | Temmerman et al. (2014) |
| Spinach (*Spinacia oleraceae*)[b] | 50 | 0.3 | 0.2 | 0.7 | Temmerman et al. (2014) |
| Leek (*Allium ampeloprasum*)[b] | 150 | 0.32 | 0.1 | 0.3 | Temmerman et al. (2014) |
| Celery (*Apium graveolens*)[b] | 130 | 0.31 | 0.1 | 0.34 | Temmerman et al. (2014) |
| Sugar cane (*Saccharum officinarum*) | 240 | 1.4 (0.67) | 27 (18) | 19 | Dhillon and Dhillon (1991) |
| Oat (*Avena sativa*) | 60 | 2.5 | 47 | 19 | Dhillon and Dhillon (2009) |

[a] Bioconcentration factor (BCF) as the quotient of Se concentration in dry shoots/total DW concentration of Se in soil
[b] Growing season was estimated accordingly to growing seasons in Belgium. Data presented from composited samples
[c] Values represent the mean followed by the standard deviation in parentheses

*A. bisulcatus*, SMT activity is not induced by exposure to selenate or selenite, but its expression is constitutive (Pickering et al. 2003). A highly-elevated activity of SMT in *S. pinnata* is suggested to be partially responsible for successful Se-hyperaccumulation and tolerance in this plant (Freeman et al. 2010). Within *C. hupingshanensis*, 78% $SeCys_2$ and 12.9% MeSeCys were determined to be the most abundant organic forms of Se in leaves and roots (Yuan et al. 2013). These findings indicate the existence of an alternative pathway for Se detoxification in this species; however, the activity of SMT in *C. hupingshanensis* is still unknown (Yuan et al. 2013).

The methylated organic forms of Se found in hyperaccumulators not only prevent Se toxicity, but their presence makes the plant tissue potentially ideal for pharmaceutical uses as some Se compounds in leaf tissue may possess anti-carcinogenic properties (Domínguez-Álvarez et al. 2016). Some organic forms such as MeSeCys and γ-glutamyl-Se-methylselenocysteine (GGSeMeSeCys) have shown natural anti-cancer properties and may play an important role in interfering with the growth of various types of cancer cells (Dong et al. 2001; Brummell et al. 2011; Domínguez-Álvarez et al. 2016). Speciation studies on these Se compounds showed that in young leaves of

*S. pinnata*, 88% of the total Se occurs as MeSeCys and 12% as selenocystathionine (SeCysth) in leaf margins and tips (Freeman et al. 2006). Within *A. bisulcatus*, the organic forms of Se are localized predominantly in trichomes: 53% as MeSeCys and 47% as γ-glutamyl-Se-methylselenocysteine (γ-GMeSeCys); no SeCys or $SeCys_2$ species were detected (Freeman et al. 2006).

In summary, the high enzymatic activity of SMT in Se-hyperaccumulator plants, and the beneficial organic selenocompounds that accumulate in shoots, make these Se-hyperaccumulators ideal candidates for agromining and also for Se biofortification strategies, as described by Bañuelos et al. (2015).

## 5 Expression of Higher Pest-Defense Proteins and Protection Against Herbivory

Concentration of Se may provide protection against pathogens and/or herbivores to Se-hyperaccumulator plants (Martens and Boyd 2002; Palomino et al. 2007; Freeman et al. 2009). As a result, growers will not need to expend financial resources on additional measures to prevent herbivory (i.e. wire fences, barriers, pesticides). For example, Freeman et al. (2009) observed in a 2-year field experiment that pre-exposure of *S. pinnata* to 40 μM selenate before planting reduced herbivory by black-tailed prairie dog (*Cynomys ludovicianus*), relative to the control group that was pre-treated with 2.0 μM selenate, and a negative relationship between plant Se accumulation and herbivory. The results suggested that accumulation of Se within the plant tissues (~50–750 μg $g^{-1}$ Se) protected the plants against mammalian herbivory in their natural environment (Freeman et al. 2009). Similarly, Se accumulation by plants appears to be an effective means to control many insect pest attacks, e.g. moth and butterfly larvae, aphids, grasshoppers, thrips and spider mites (Hanson et al. 2004; Quinn et al. 2011). Thus, considering that Se-hyperaccumulators can reach Se concentrations greater than 1000 μg $g^{-1}$ DW, these plants can exert self pest control. Indeed, a field survey of *A. bisulcatus* and *S. pinnata* growing naturally in a seleniferous habitat showed fewer arthropod loads and less herbivore damage compared to non-hyperaccumulators (Galeas et al. 2008). These authors detected a higher number of arthropods in plants containing Se concentrations below 600 μg $g^{-1}$ DW. As Se increased in plant tissues (up to 8000 μg $g^{-1}$ DW), fewer arthropods fed on them, suggesting that Se acts as an elemental defence against these insects. The authors also detected low bioaccumulation of Se within the arthropods feeding on the plants; their Se levels were at least one order of magnitude less than in the plant tissue, indicating that if these Se-hyperaccumulator plants are used in agromining or as an organic Se-biofertilizer to biofortify food crops, there is minimal risk for bioaccumulation of Se into the trophic chain under these conditions (Galeas et al. 2008).

Selenium hyperaccumulators not only possess physiological mechanisms for survival in Se-rich soils, but reportedly they also can promote Se uptake into neighbouring non-Se-accumulators by plant interaction, as well as provide protection against herbivory and attacks on adjacent plants (El Mehdawi et al. 2011). These results were observed in *Artemisia ludoviciana* and *Symphyotrichum ericoides* growing naturally in proximity to *A. bisulcatus* and *S. pinnata* (El Mehdawi et al. 2011). Where growing adjacent to Se-hyperaccumulators, *A. ludoviciana* and *S. ericoides* showed increased size, increased leaf Se concentration (up to 20-fold), and S concentration (+25%), as well as 40% reduced damage by herbivory (El Mehdawi et al. 2011). These findings suggest the possibility of co-cultivating or intercropping Se-hyperaccumulators to promote Se uptake into edible crops. The authors further suggested that the presence of a Se-hyperaccumulator may change availability of certain nutrients in the rhizosphere, for example S, as well as influence microbial communities due to increases in Se concentrations in the rhizosphere of the Se-hyperaccumulators (El Mehdawi et al. 2011). For more information on important associations among Se-hyperaccumulators and microbial-fungal communities, relevant studies are discussed in detail by Di Gregorio et al. (2005), Wangeline et al. (2011); Valdez-Barillas et al. (2012), Alford et al. (2012, 2014), Lindblom et al. (2013), Tong et al. (2014), and Sura-de Jong et al. (2013).

## 6 Better Performance and Growth Under High Osmotic Stress

Several studies have shown that Se-hyperaccumulators *S. pinnata* and *A. bisulcatus* exhibit the ability to tolerate highly saline and B-rich soil conditions, while also hyperaccumulating Se (Retana et al. 1993; Freeman et al. 2010; Freeman and Bañuelos 2011). Under salinity conditions (electrical conductivity (EC) of 4.8–8 dS $m^{-1}$), *S. pinnata* was able to remove 30% of the total soil Se between 0–30 cm soil depth, during two growing seasons (Freeman and Bañuelos 2011). This hyperaccumulator exhibited tolerance to high osmotic stress, as observed from a better performance by some ecotypes of the species that were grown in highly saline soils contaminated with B (Parker et al. 2003). In addition, Retana et al. (1993) evaluated Se uptake by the Se-hyperaccumulators *A. bisulcatus* and *A. racemosus* grown in a highly saline and B-rich soil (Se 0.1–1.6 μg $g^{-1}$; B 7.8–17 μg $g^{-1}$ and EC from 5.2–14 dS $m^{-1}$) under glasshouse conditions. The authors observed that *Astragalus* spp. removed 2 to 3.5 kg Se $ha^{-1}$ from a total of 92 kg Se $ha^{-1}$ soil, between 0–90 cm soil depth, in one growing season; in contrast, non-accumulator grasses removed only 100 g Se $ha^{-1}$ under the same B and high salinity conditions (Retana et al. 1993). These observations suggest that Se-hyperaccumulators are well suited for accumulating Se under highly saline and B-rich soil conditions.

## 7 Economics of Se Phytomining

Economically speaking, the market price for certain phytomined elements will depend on their annual yield per unit area (amount of the element/biomass produced), as well as on the distribution and concentration of the element in a particular geographic area. The economics related to Se-hyperaccumulators for ‘agromining’ of Se showed that Se price per metric tonne (annual yield per unit area) is relatively high (US$ 52,000) (van der Ent et al. 2015). Planting the Se-(hyper) accumulators *S. pinnata*, *A. bisulcatus* and *C. hupingshanensis* in Se-laden soils and utilizing the harvested biomass may provide advantages and profit opportunities for growers. For example, the Se-enriched tissues can be carefully used as a blend in cattle feed or as biofertilizers in Se-biofortification strategies (Bañuelos 2001; Bañuelos et al. 2015, 2015; Freeman and Bañuelos 2011). Another important feature that promotes the use of agromining of Se is that hyperaccumulating plants are grown in Se-laden soils, which generally are of poor quality and would not support food crops. A drawback from using plants to agromine Se is that the percentage of Se removed seasonally will not be constant or predictable, owing to variable soil and environmental conditions, and consequently, Se concentrations in plant tissues will likely vary (Galeas et al. 2007). Hence, the economic value of plant products based upon tissue Se concentrations may also be difficult to predict.

## 8 Applicability of Se-Hyperaccumulating Plants

Applying a plant-based agronomic technology (agromining) on soils having high concentrations of Se will produce a highly Se-enriched biomass after harvest. There are some hot spots known with naturally Se-rich soils, for instance in western China (Zhu and Zheng 2001; Wu et al. 2015). These types of soils are necessary for Se agromining, in order to produce Se-enriched plant material that can be used as a Se-biofertilizer in Se-deficient soils. Rates of biofertilizer application can only be determined after soil analysis for Se has been performed. Depending on the Se concentration found in the harvested plant material, the Se-enriched meal can be added to soil where edible crops are grown. In this regard, Bañuelos et al. (2015) first established this experimental approach by adding different rates of *S. pinnata* as an organic Se-biofertilizer to soil for growing broccoli and carrots under field conditions. The *S. pinnata* meal was enriched with organic forms of Se, consisting of 50% CysSeSeCys (selenocystine), 44% C-Se-C forms, 3% SeOMet (oxidized form of SeMethionine), and 3% inorganic $SeO_3^{2-}$. The application of this organic Se-biofertilizer produced Se-enriched carrots, with Se organic forms present as 17% SeMet, 33.4% CysSeSeCys, 7.6% MeSeCys, and 9.5% $SeO_4^{2-}$ and Se-enriched broccoli florets with 18% SeOMet, 23% CysSeSeCys, 55% (SeMet + MeSeCys), and 4% $SeO_4$ (Bañuelos et al. 2015). The rate of application did not significantly affect speciation of Se within the plant tissue. It was unclear whether the application of *S. pinnata* influenced organic forms of Se, e.g. MeSeCys, within the carrots and broccoli. Future studies should evaluate Se-biofortified foods for their MeSeCys content, as influenced by the source of Se applied to soil (inorganic *vs.* organic), because MeSeCys has been suggested to play a role in the prevention of cancer in humans (Dong et al. 2001; Brummell et al. 2011; Domínguez-Álvarez et al. 2016). In addition, there are very few field studies that can provide data related to Se absorption by human consumption of Se-enriched crops produced by the application of organic Se-biofertilizers. Therefore, it is imperative to encourage this type of experimental work, which can lead to better understanding the effects of Se intake by humans and animals where hyperaccumulators are used as Se-biofertilizers. Studies using plant-based Se-biofertilizers are limited, and no data are available on their use in long-term field studies, although long-term studies are currently in progress (Bañuelos et al. 2016).

A biofortification strategy for Se-deficient soils will require the application of Se-biofertilizers or inorganic Se fertilizers for supplying additional Se to crops (Kápolna et al. 2009; Wu et al. 2015). Meta-analyses on Se-biofortification has shown that inorganic Se forms as foliar applications are the most common alternatives in the mitigation of Se-deficient soils (Ros et al. 2016). In contrast to inorganic Se fertilizers, growers using Se-biofertilizers will need to find feasible, accessible, practical, and economically viable strategies to incorporate vast quantities of Se-enriched plant materials into Se-deficient soils in order to biofortify staple crops.

## 9 Conclusions

All three species of Se-hyperaccumulators *S. pinnata*, *A. bisulcatus*, and *C. hupingshanensis* exhibit major advantages for agromining, including high capacities to discriminate between Se and S and accumulate selenocompounds, and better adaptability to grow in highly saline and high-B soil conditions. Also, owing to high Se concentrations in their tissues, Se-hyperaccumulators are more pest-tolerant and deter herbivory when planted alone or with food crops. *Stanleya pinnata* plant meal has already been demonstrated to be effective as a Se-biofertilizer for broccoli and carrot crops, but more research is needed for *A. bisulcatus* and *C. hupingshanensis* in their potential for use as Se-biofertilizers. Assuming Se-rich soils are available, selecting, growing, and utilizing a Se-hyperaccumulator species should be part of agronomic strategies to provide growers with an additional organic source of Se, as a Se-biofertilizer for Se biofortification.

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# Element Case Studies: Thallium and Noble Metals

Brett Robinson and Chris Anderson

## Abstract

Thallium is a highly toxic and valuable element for which there are known fast-growing hyperaccumulator plants that have some of the greatest bioaccumulation coefficients (plant/soil concentration quotients) of any non-essential element. As with other elements, many hyperaccumulators discovered to date are in the Brassicaceae family. In contrast, hyperaccumulation of the precious metals Au, Pd, and Pt is not recorded for any plant species. To achieve uptake of these precious (noble) metals, chemicals must be added to the soil in order to induce metal solubilisation; and for these particular metals, cyanide has proven time and again to be the most effective agent to promote uptake. However, cyanide does not specifically target the noble metals. Increased solubility and uptake of more toxic Cu and Ag can limit the uptake efficiency of a phytomining or agromining crop (a co-metallic effect). Worldwide, numerous soils are known that have a high Tl burden ($>1.5$ μg $g^{-1}$) and hence are unsuitable for safe food production, of low value, thus being ideal for agromining. Among all elements that could potentially be agromined, Tl has perhaps the greatest potential to be economically successful. Despite this promising technique, Tl has received relatively little attention. In contrast, the geographical scope for noble metal uptake is much more limited. Research is warranted for discovering new hyperaccumulators, the economics of recovering Tl and noble metals from biomass, and quantification of areas where agromining for valuable metals may be feasible.

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# 1 Introduction

Thallium and the noble metals are distinct from other potentially agro-minable elements because of their extraordinary high commercial and industrial values (Anderson et al. 1999; Scheckel et al. 2004). Thus, the threshold concentrations of these metals in crops for successful agromining are significantly lower than those for other elements. However, in all other respects thallium and the noble metals are also distinct. Thallium is a chalcophile (S-loving) element, which in soils has a small but significant proportion of the total concentration occurring in the soil solution. In contrast, the noble metals, of which the most important are Au, Pt and Pd, are siderophiles (‘iron-loving’) and are characterized by an inordinate lack of chemical reactivity—they were historically described as ‘noble’ because these metals do not take part in ‘common’ chemical reactions. Therefore, the noble metals occur at exceedingly low concentrations in soil solution, and unlike Tl, have negligible interactions with plants unless lixiviants are employed to force noble metals into solution. Given these differences, this chapter investigates the agromining potential of Tl and the noble metals separately.

**Fig. 1** *Iberis Intemedia*, growing near St Laurent le Minier, Southern France. Photograph by CWN Anderson

# 2 Thallium

Interest in Tl agromining (phytomining) increased following the findings of Leblanc et al. (1999), who reported that *Iberis intermedia* from southern France (Fig. 1) accumulated >500 μg g$^{-1}$ (DM) (dry mass) in aerial tissues and that a crop grown on contaminated soil could return a net profit of more than \$US 1000 ha$^{-1}$ y$^{-1}$. Owing to high toxicity, Tl has traditionally found use as a pesticide, but in recent years newly discovered properties of this metal have made it valuable in a range of electronics applications (USGS 2017). Consequently, the value of Tl has increased greatly with time, from US\$300 kg$^{-1}$ in 1999 (Anderson et al. 1999) to US\$6000 kg$^{-1}$ in 2011 (Guberman 2012). This price increase has significantly changed the prospect of economically viable agromining for Tl. In 1999, Anderson et al. (1999) concluded that a net return of US\$1200 ha$^{-1}$ y$^{-1}$ could be achieved with a 10 t ha$^{-1}$ y$^{-1}$ crop containing 800 μg g$^{-1}$ Tl. Today, assuming production costs from small-scale Tl agromining are US\$1500 ha$^{-1}$ y$^{-1}$, twice that of wheat (Gloy 2016), that same crop would give a net profit of US\$46500 ha$^{-1}$ y$^{-1}$. Herein, we aim to determine the current potential for Tl agromining, critical success factors, and fertile areas for future research.

## 2.1 Thallium in Soil

Globally, the background concentration of Tl in soil is <1 μg g$^{-1}$ (Anderson et al. 1999). Tremel et al. (1997b) reported a median value of just 0.29 μg g$^{-1}$ Tl in French soils, but with some soils containing geogenic Tl concentrations up to 55 μg $g^{-1}$. In some areas, such as western Siberia, geogenic Tl can occur in soils at concentrations of 1.5 to 3.0 μg $g^{-1}$ (Il'in and Konarbaeva 2000). Being a chalcophile element, Tl is commonly associated with pyritic mineral deposits. In PR China, natural soils associated with sulphide ores contain 1.5–6.9 μg $g^{-1}$ compared to <0.5 μg $g^{-1}$ in background soils (Xiao et al. 2004). Thallium is a co-contaminant in mined ores and slags, as well as in products of coal combustion (Bunzl et al. 2001). Lis et al. (2003) reported that mining and processing of Zn-Pb ore increased soil Tl concentrations by up to two orders of magnitude, with concentrations reaching 35 μg $g^{-1}$ in affected areas. Mine-affected soils near Keban, Turkey, contain 3.0 to 27.6 μg $g^{-1}$ Tl. In Poland, soil associated with historic mining of Ag, Pb, and Zn contain an average of 20.8 μg $g^{-1}$ Tl (Woch et al. 2013).

Elevated Tl concentrations have been reported near tungsten smelters (Cheng et al. 2013) as well as cement plants, with concentrations of 1.2 to 19.9 μg $g^{-1}$ Tl (Lee et al. 2015). Earlier, Li et al. (2010) reported that continual irrigation of a cotton plantation with sewage increased soil Tl levels to ca. 0.42 μg $g^{-1}$. In soil, Tl is bound to quartz and aluminosilicate minerals, with lesser amounts associated with the organic fraction (Jacobson et al. 2005a; Gomez-Gonzalez et al. 2015). Vanek et al. (2010) reported that manganese (Mn III, IV) oxides, illite, and probably amorphous silicates are the main phases responsible for Tl retention in soils. Being typically monovalent and of similar ionic size, Tl may substitute for K in soil constituents (Tremel et al. 1997b).

## 2.2 Thallium in Plants

Of all non-essential trace elements, Tl has some of the highest bioaccumulation coefficients (BC, plant/soil concentration quotients) among plants, with especially high values reported in members of the family Brassicaceae (Tremel et al. 1997a). Cabbage (*Brassica oleracea* var. *capitata*), may have BC values of 1 to 11 (Jia et al. 2013) and achieve concentrations surpassing 500 μg $g^{-1}$ Tl (Xiao et al. 2004). Another common vegetable crop, kale (*Brassica oleracea* var. *acephala*), is reported as having a bioaccumulation coefficient of 80 in spiked soils, achieving a maximum of 326 μg $g^{-1}$ Tl (Pavlickova et al. 2006). In natural soils, the brassicaceous Tl hyperaccumulators *Biscutella laevigata* and *I. intermedia* have bioaccumulation coefficients of *ca.* 20.2 and 29.4, respectively (LaCoste et al. 1999), although Wierzbicka et al. (2004) reported negligible amounts of Tl in tissues of *B. laevigata*. In anthropogenically contaminated soil, *I. intermedia* can have a bioaccumulation coefficient of >100 (LaCoste et al. 2001). Among plants outside the Brassicaceae, Wierzbicka et al. (2004) reported that *Plantago lanceolata* (Plantaginaceae) accumulated an average of 65 μg $g^{-1}$ Tl in a mine spoil containing an average of 43 μg $g^{-1}$ Tl. In contrast, *Silene vulgaris* (Caryophyllaceae) accumulated only 6.5 μg $g^{-1}$ Tl.

Plants access anthropogenic Tl in preference to geogenic Tl that is poorly available for plant uptake (Al-Najar et al. 2005). *Iberis intermedia* significantly depletes the easily available fraction, which is then gradually replaced by residual fractions (Al-Najar et al. 2003). High concentrations of Mo, Cu, Pb, Zn, Ag and As inhibit Tl uptake by plants (Sasmaz et al. 2007). Mn-oxides can reduce plant uptake of Tl, even in hyperaccumulating species (Grosslova et al. 2015), because they sorb significant amounts of Tl (Jacobson et al. 2005b). Thallium is taken up and stored by plants primarily as Tl(I) - (Krasnodebska-Ostrega et al. 2012), although Sadowska et al. (2016) found traces of Tl(III) in *Sinapis alba*. Thallium appears to be transported primarily in the xylem with relatively little phloem mobility. The highest Tl concentrations are found in the aqueous sinks such as vascular tissue (Jia et al. 2013).

## 2.3 Feasibility of Thallium Agromining

At current prices, achieving a net profit of US \$1500 $ha^{-1}$ $y^{-1}$ for Tl agromining would require the phytoextraction of 0.5 $ha^{-1}$ $y^{-1}$, assuming that there is no monetary return from combustion of the biomass. Given a crop of *I. intermedia* that produces 10 t $ha^{-1}$ $y^{-1}$, a plant concentration of just 50 μg $g^{-1}$ Tl would be required. Assuming a bioaccumulation coefficient of 29.4 for geogenic Tl (LaCoste et al. 1999), *I. intermedia* could achieve this concentration on a soil containing just 1.7 μg $g^{-1}$. Although there has been no comprehensive soil survey to date, the reports in the literature indicate that such areas should be widespread. However, given the environmental and economic constraints of repeated croppings for phytoextraction (Robinson et al. 2015), viable agromining of Tl is more likely to occur on anthropogenically contaminated areas that are unfit for other agricultural systems. The limited size of these areas may increase production costs, because of reduced economics of scale. Therefore, higher plant Tl concentrations would be required, but these should be easily achievable with a hyperaccumulator species such as *I. intermedia*. Whereas other common plants take up inordinate concentrations of Tl, especially members of the Brassicaceae, these are unlikely to be useful for agromining because plant Tl toxicity appears at concentrations of *ca.* 221 μg $g^{-1}$ (Babic et al. 2009). Therefore, it is imperative to search for more Tl-hyperaccumulating species. Further research is also warranted on processes by which Tl could be recovered from biomass, and the possibility of producing valuable plant compounds containing Tl. To date, there have been no flow sheets described for recovering or utilising Tl in biomass,

## 3 Noble Metals

In contrast to Tl, there are no known hyperaccumulators of Au and the platinum-group metals (PGM; otherwise known as precious or noble metals). This absence is because noble metals are considered 'non-essential' in plant metabolism (Lambers et al. 2008), and have very low abundance and solubility in normal soils (Babula et al. 2008). Plants simply have not evolved to be able to hyperaccumulate precious metals. The concentrations of noble metals in normal plants growing in mineralized soil are generally on the order of 10 ng $g^{-1}$ (Anderson et al. 1998; Lintern et al. 2013). Considerably more data are available on the concentration of Au in plants relative to the PGM. Very few phytoextraction studies have focused on the PGMs, and these have been restricted mainly to Pd. There is, however, compelling evidence for an elevated concentration of noble metals in plants where favourable geochemical conditions increase their solubility. Elevated soil solution concentrations of chloride and thiosulphate ions derived through weathering processes, and cyanogenic compounds derived from biological pathways, are able to increase the solubility of Au (and PGM) leading to concentrations of these metals in soil that are in excess of 'normal' levels. For example, Lintern et al. (2013) reported Au concentrations in *Eucalyptus* spp. leaves as high as 60 ng $g^{-1}$ DW.

Better understanding of the conditions under which the noble metals can be made soluble has led to mechanisms for promoting the solubility of these metals in the soil solution. This result has created the potential for noble metal phytomining or agromining, based on principles of induced hyperaccumulation (Anderson 2005). Gold phytoextraction is extensively reported in the literature, with recent reviews published by Wilson-Corral et al. (2012) and Sheoran et al. (2013). However, published data on Pd phytomining are rare. Several MSc theses conducted at Massey University in New Zealand have focused on this subject (Walton 2002; Aquan 2015), the latter being conducted as part of a G8 Materials Efficiency funded collaboration between York University, U.K., The University of British Columbia, Canada, and Yale University, U.S.A. (PHYTOCAT project; www.phytocat.org). This work sought to accumulate Pd in plants such that the biomass could be used as a catalyst in chemical reactions (Clark et al. 2013). Palladium uptake was effected using both chemical (induced hyperaccumulation) and genetic mechanisms. Taylor et al. (2014) proposed that Au uptake was mediated by cation transports in *Arabidopsis* sp. with similar observations apparent for Pd. This result indicates that a potential exists to target noble metal uptake through manipulation of the genetic pathways.

## 3.1 Lessons Learned from Agromining Studies: Effect of 'Co-metals' on Uptake of Noble Metals

The concentration of a noble metal that can be induced into plants is proportional to the bioavailable metal concentration in the growth medium. The PHYTOCAT project, to the best of our knowledge, is the only example of a comprehensive study of PGM uptake by plants. The target Pd concentration in plants for this work was 1000 μg $g^{-1}$ (DW), which necessitated a concentration of metal in soil that was significantly higher than that found in mine wastes (generally <0.1 μg $g^{-1}$). After extensive global exploration for a suitable growth medium, pot trials were conducted using gossan rock collected from the Broken Hill district in New South Wales, Australia, about 20 km northeast of the main Pb-Zn-Ag ore body (Table 1).

Magmatic PGM deposits are generally enriched in sulphide minerals (BGS 2009), and this has a significant impact on the pH of any ore or waste material that might be used for phytomining (generally a very low pH). In the Broken Hill area gossan, extensive weathering of the original sulphide ultramafic ore body (weathering is required to form a gossan) has converted acid-generating sulphides in the rock into Fe-oxides. As a consequence, the gossan has become PGM-rich material (e.g. 48 mg $kg^{-1}$ Pd; Aquan 2015) that is suitable for phytomining. The gossan was hand-mined 100 years ago by miners looking for Cu. This early mining left fragments of gossan lying on the surface over a small area (Fig. 2). The total volume of the gossan is low; the available mass is <1000 tonnes, thus making the deposit uneconomic for a conventional mining operation.

Phytomining pot trials were undertaken at Massey University in 2015 (Aquan 2015). Initial attempts to grow *Brassica juncea* on the Broken Hill gossan failed (owing to high Cu concentration; Table 1). However, it was found that hemp (*Cannabis sativa*) was able to grow on this gossan. Uptake of metals was induced by applying a dilute solution of sodium cyanide to the surface of the pots 33 days after germination. The final metal concentration in the dry biomass was as follows: Cu 6730 μg $g^{-1}$, Ni 184 μg $g^{-1}$, Fe 1290 μg $g^{-1}$, Au 7.6 μg $g^{-1}$, Pd 62.4 μg $g^{-1}$. The Pd concentration was significantly lower than anticipated, based on the total metal concentration in the gossan and considerably lower than any likely economic threshold for phytomining (Wilson-Corral 2012). The most likely reason is the Cu concentration in the plant. Cyanide

**Table 1** Selected metal concentrations in the Broken Hill Gossan

| Element | Concentration (μg $g^{-1}$) | Analytical method |
|---|---|---|
| Gold | 2.5 | INAA |
| Iridium | 7.1 | INAA |
| Osmium | 3.8 | INAA |
| Palladium | 48 | INAA |
| Platinum | 15 | INAA |
| Rhodium | 4 | INAA |
| Ruthenium | 3 | INAA |
| Copper | 13,900 | AAS |
| Nickel | 6350 | AAS |

*INAA* Instrumental Neutron Activation Analysis, *AAS* Atomic Absorption Spectroscopy

**Fig. 2** The landscape at Broken Hill (**a**) showing a pit mined 100 years ago for Cu minerals (**b**). The dark coloured pieces of rock are the heavily mineralised gossan. Photographs by CWN Anderson

induced a considerable increase in the concentration of Cu in plants and this effectively killed the plants before they could accumulate Pd (and Au). This result has been described by Anderson et al. (2013) as a 'co-metallic effect,' whereby more toxic metals that are also present in noble metal-rich soils (e.g. Cu, Ag) induce necrosis in plants used for phytomining. In media to be used for Au and PGM phytomining, it is likely that Cu will also be present, and the toxicity of Cu that is made soluble by the same treatments used to promote noble-metal solubility seems to be a key limitation on the phytomining of precious metals.

## 3.2 Lessons Learned from Agromining Studies: Need for a Viable Biomass Processing Technology

Anderson (2013) and Robinson et al. (2015) described how a lack of any proven engineering systems to recover precious metals from biomass is a major impediment to the large-scale development of noble metal (or Tl) agromining or phytomining. Proposed processing techniques are based on the ashing of dry biomass and then wet chemistry (acid dissolution and solvent extraction) used to recover target metals from the ash. Such techniques are, however, costly and environmentally challenging. For example, the production of a waste stream of acid will require further processing before disposal. Recent trials in Indonesia have sought to assess how Au phytomining in developing countries might be relevant to the sustainable management of artisanal and small-scale Au mine waste (Krisnayanti et al. 2012, 2016).

Krisnayanti et al. (2016) described the use of a simple smelting technique called the 'borax method' to recover precious metals from plant ash using Ag as a collector metal. The principle of smelting is to melt the metal constituents of a given material. However, the energy required to melt Au is considerable. A flux is therefore used to reduce the melting point of metals, and in the borax method the flux is borax ($Na_2B_4O_7 \cdot 10H_2O$). This technique is today being used extensively by artisanal and small-scale Au miners in the Philippines to recover Au from concentrates without the use of mercury; Krisnayanti et al. (2016) were the first to apply this technique to process biomass. The borax method relies on a high concentration of Au in the ore to be efficient (generally a concentrate with *ca.* 25% Au is smelted). To make the borax method effective for plants, Ag can be added to the smelt to act as a collector. The Ag melts and then 'collects' valuable metals within the smelt (Cu, Au and Ag) to form a bullion product that can be sold for subsequent refining.

Krisnayanti et al. (2016) demonstrated the borax technique to an artisanal mining community during a trial conducted in 2015 on the island of Lombok, Indonesia. Biomass was smelted using Ag as a collector. Tobacco was grown on mine waste with 1 $\mu g\ g^{-1}$ Au, treated with NaCN to induce uptake, harvested, sun-dried, ashed, and then the ash was smelted (Fig. 3). The trial failed to induce significant Au uptake (final Au concentration was 1.2 $\mu g\ g^{-1}$) and this was attributed to the high concentration of Ag induced into the plants (54 $\mu g\ g^{-1}$); however, the mass of Ag added (31 g) increased to 39 g at the end of the smelting. This increase in mass was assessed to be predominantly iron and Ag derived from the plant ash, indicating the

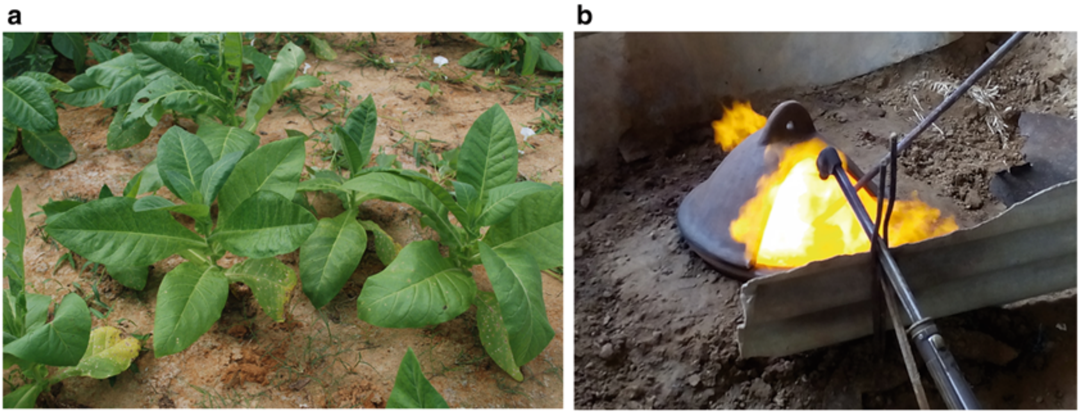

**Fig. 3** Tobacco plants growing on cyanidation artisanal Au mine tailings in Lombok Island, Indonesia (**a**); borax smelting of the plant ash using an air-acetylene torch (**b**). Photographs by BD Krisnayanti

efficacy of the technique to recover metals from the biomass.

The results from this work were by no means conclusive, but indicated that simple techniques do exist that are acceptable to artisanal mining communities and use existing equipment with which miners are familiar. This program creates new opportunities for first-stage processing options in agromining or phytomining crops that can operate in remote field areas. The product from such options would not be a final pure metal, but value could still be attributed, leading to a financial reward for operating farmers/miners that might create sufficient incentive to apply the technology. Refining of the bullion from a borax smelt could be readily done using existing facilities around the world.

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# Element Case Studies: Manganese

Chuan Wu and Shengguo Xue

**Abstract**

Manganese (Mn), one of the important trace element in different concentrations in living tissues, is also widely used in the metal industry. It is an essential micronutrient for plants, taken up under the +2 oxidation state, which is crucial in the reactions of some enzymes (malic dehydrogenase, oxalosuccinate decarboxylase, superoxide dismutase), and is an activator of those involved in the tricarboxylic acid cycle. In soils, Mn is commonly in the form of +4 and +3 valency states oxides, which could be reduced to the +2 form by some of the ways, such as acidizing soil solution, waterlogging soil, heating and drying, and by the activity of anaerobic and aerobic micro-organisms. Mn can be taken up by plants growing in base-rich soils in high concentrations. Mn hyperaccumulation plants have been defined by a threshold foliar concentration of over 10,000 μg $g^{-1}$ dry weight (DW). Mn toxicity could cause stunting, chlorosis, curled leaves, brown lesions, as well as inhibition of photosynthesis and respiration in plants. Several elements such as P and Ca are reported to have important impacts on the uptake and accumulation of Mn in plants. Mn can be stored in vacuoles, cell walls, golgi apparatus, chloroplast lamellae structure, and to form black agglomerations in plant cells.

## 1 Introduction

Mining, particularly extracting metal, is a major source of Mn contamination in soils (Singh et al. 2005). Pollution associated with mining operations and related tailings may cause both direct and indirect economic losses each year (Liu and Shu 2003). Extractable Mn concentrations reported for non-polluted agricultural soils worldwide can reach 30.6 μg $g^{-1}$ (Wang et al. 2008), 37.6–38.5 μg $g^{-1}$ (Peris et al. 2007), or 58.5 μg $g^{-1}$ (González and González-Chávez 2006); all of these values are

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considered to fall within the normal range (<100 μg $g^{-1}$; Baker et al. 2000). In contrast, studies of contaminated sites have reported that known concentrations of Mn reach 226 μg $g^{-1}$ at a soil-ore crushing area and 550 μg $g^{-1}$ in soil tailings at an abandoned mine site (Archer and Caldwell 2004), 179,524 μg $g^{-1}$ in tailings of a manganese mine in Guangxi, China, and 289 to 1356 μg $g^{-1}$ in heavily contaminated tailings (Li et al. 2007; Rivera-Becerril et al. 2013). Moreover, Boudissa et al. (2006) showed that in the wings of a near Mn alloy production plant, mean concentrations of Mn in the soils are up to 283,000 μg $g^{-1}$, because of direct deposition on the solid Mn-rich residue and atmospheric erosion of particles. Normal concentrations of Mn in plant tissues range from 15 to 150 μg $g^{-1}$ (Reimann and Caritat 1998; Nagajyoti et al. 2010). Accumulation of high concentrations of Mn leads to a decrease of the photosynthetic rates in leaves, brown spotting (also petioles and stems) over time, and spots can increase in number and size, necrotic lesions and even death of the plant, with roots commonly becoming brown (Nagajyoti et al. 2010).

There is ample literature on the rehabilitation of Mn-contaminated mine lands (Xue 2002; Zhang et al. 2004; Yang et al. 2006). Paschke et al. (2005) provided Mn toxicity threshold for grasses commonly applied in restoration, including redtop, slender wheatgrass, tufted hairgrass, big bluegrass, basin wildrye, and common wheat. It is necessary and important for the establishment of vegetation to stabilize the bare area and reduce the environmental pollution (Krämer et al. 2000; Wong 2003). Phytostabilization for stabilizing toxic mine spoils and phytoextraction for the removal of toxic metals from the spoils are commonly used in the remediation, and some soil amendments should be also added to aid stabilizing mine spoils or to enhance metal uptake (Wong 2003). Despite poor physical structure and nutrient deficiencies in the local soil, the Mn mine waste site at Molango in Hidalgo State, Mexico, has been colonized by at least five plant species, where tissues of these species accumulate Mn at normal levels (Fernando and Lynch 2015). Moreover, in Guangxi Province of China, Li et al. (2007) used the local pioneer species *Digitaria sanguinalis*, *Imperata cylindrica* var. major, and *Erigeron canadensis* to colonize the Mn mine wasteland, combining the fast growing indigenous species *Castanea henryi*, *Pinus massoniana*, and *Phytolacca acinosa*, which could remedy and restore some functional uses of the wasteland (Li et al. 2007).

## 2 Manganese Hyperaccumulator Plants

Baker and Brooks (1989) defined manganese hyperaccumulator plants by the threshold foliar concentration over 10,000 μg $g^{-1}$ DW. Currently there are less than 30 manganese tolerance Mn-hyperaccumulation plants reported including nine species listed by Reeves and Baker (Baker et al. 2000). The first local Mn-hyperaccumulator, *Phytolacca americana* L. (Phytolaccaceae) from PR China, was showed by Xue (Figs. 1 and 2; Xue et al. 2003, 2008). Other native Mn hyperaccumulating plants have been discovered from other Asia-Pacific regions, such as *Polygonum pubescens* (Polygonaceae) (Hua et al. 2009), *Polygonum hydropiper* (Polygonaceae) (Ren et al. 2007), *Chengiopanax sciadophylloides* Franch. et Sav. (Aralicaceae) (Mizuno et al. 2013). These include seven endemic species from New Caledonia reported nearly 3 decades ago (Jaffré 1977, 1980) but there have been limited following-up studies. What's more, *Gossia bidwillii* (Myrtaceae) and *Phytolacca acinosa* (Phytolaccaceae) were reported to be Mn hyperaccumulators from Australia and China respectively (Bidwell et al. 2002; Xue et al. 2004). Most recently, Xue et al. (2016) found that Mn in leaves of *Chenopodium ambrosioides* L. can reach the highest concentration of 26,070 mg $kg^{-1}$ when the Mn treatment concentration increased to 8000 μmol $L^{-1}$. In addition, Mn concentrations in leaves of *Polygonum perfoliatum* L. (Xue et al. 2017) reached 13,138 mg $kg^{-1}$ grown in 500 μmol $L^{-1}$ Mn, while Mn content reached its maximum 41,400 mg $kg^{-1}$ in leaves in 10,000 μmol $L^{-1}$ Mn treatment. And the concentrations in leaves

**Fig. 1** *Phytolacca americana* growing at Xiangtan manganese tailings (China)

**Fig. 2** *Phytolacca americana* growing in South China

of *C. ambrosioides* and *P. perfoliatum* were both higher than roots. The heterogeneity trait of Mn hyperaccumulation were studied in an herbaceous species *P. americana*, under the green house condition (Xue et al. 2005), and in the tree *G. bidwillii* under natural conditions (Fernando and Batianoff 2007), showing that the trace is constitutive in the former, whereas the latter was highly heterogeneous when growing on a variety of substrates.

The Mn-hyperaccumulator *C. sciadophylloides* inhabits temperate forested regions of Japan and can accumulate Mn concentrations up to

23,000 mg kg$^{-1}$ DW in its shoots (Mizuno et al. 2013). These studies also showed that half Mn in the leaves of *C. sciadophylloides* could be easily extracted with water, and almost all of the Mn could be extracted with hydrochloric acid (HCl). When the pH was adjusted to 8–10, the highly purified Mn compound was precipitated from leaf ash solutions. Thus, this study shows that the collection of Mn from *C. sciadophylloides* is straightforward, making the plant a candidate for manganese phytomining (Mizuno et al. 2013).

The perennial herb *P. acinosa* that occurs in southern China was found to be a new Mn hyperaccumulator by means of field surveys on Mn-rich soils and by glasshouse experiments (Xue et al. 2004, 2008). The maximum Mn concentration in the leaf dry matter of plants living in Xiangtan manganese tailings was 19,300 μg g$^{-1}$, and the reported average concentration was 14,480 μg g$^{-1}$ (Xue et al. 2004, 2008). Under the condition of nutrient solution culture, *P. americana* could grow normally with Mn supplied at a concentration of 800 mol L$^{-1}$, although with less biomass than control; the Mn concentration in the leaves can reach its highest value (36,380 μg g$^{-1}$) at an Mn supply concentration of 12,000 mol L$^{-1}$. This result gives a plant resource for exploring the Mn hyperaccumulation mechanism, and has the potential for application in phytoextraction of Mn-contaminated soils, and hence phytomining Mn (Xue et al. 2004, 2008). The absorption and accumulation of manganese in the two comparative populations were studied, including a population (MP) of Xiangtan manganese tailings and another (NMP) of a *Magnolia grandiflora* plantation with lower Mn status. All of these results indicate that the high tolerance and hyperaccumulation of Mn in *P. americana* are constitutive properties (Xue et al. 2005, 2008).

So far, most of the manganese hyperaccumulator plants have been found in New Caledonia, and little is known about ecological physiology. Fernando et al. (2008) reported on natural populations of one such species, the endemic shrub *Maytenus fournieri*. The average foliar Mn concentrations of the two populations grown on ultramafic substrates with different soil pH values were very different from the average foliar Mn concentrations. Energy dispersive spectroscopy and scanning electron microscopy (SEM-EDS) analysis showed that Mn mainly occurred within dermal tissues. This discovery, most foliar Mn highly concentrated in non-photosynthetic tissues, was similar with other woody plants research results (Fernando et al. 2008).

*Phytolacca americana* (pokeweed) has been found to be a new Mn-hyperaccumulator plant based on field surveys of Mn-rich soils and by hydroponic experiments. The maximum Mn concentration in leaf dry matter was 8000 μg g$^{-1}$ on Xiangtan manganese mine tailings, showing a high transfer factor (>10.8); under hydroponic conditions, Mn concentrations were increasing with the increasing Mn supply, reaching a maximum concentration of Mn in leaves of 47.1 μg g$^{-1}$ at 50 mol L$^{-1}$ external Mn supply (Yuan et al. 2007). Moreover, EDTA treatment significantly inhibited root and shoot biomass and chlorophyll production of *P. hydropiper* in Soybean, whereas chlorophyll production of *P. americana* was opposite, clearly promoted by the treatment of EDTA (Yang et al. 2013). Within or without EDTA treatment, the concentration of shoots in the two plants was significantly lower than the nominal threshold, indicating that the two plants had their hyperaccumulating ability in the studied soils.

It was found that *Schima superba* (Theaceae), a subtropical tree species occurring in the wasteland of manganese mine, was found to contain abnormally high concentrations of Mn in leaf tissue (Yang et al. 2008). A pot growth experiment showed that Mn in tissues increased significantly with increasing Mn supply, and peaked (62,410 μg g$^{-1}$) in stems at treatment of 150 mol L$^{-1}$. Manganese concentrations of above ground parts in all treatments were higher than those in roots. Most of Mn was taken from the substrate to the upper part, such as

86% in the upper end of the 150 mol $L^{-1}$ treatment, which suggests that *S. superba* is a Mn hyperaccumulator.

## 3 Manganese Toxicity in Plants

In general, excessive manganese has direct cytotoxic effects, such as inhibition of the absorption and activity of Ca, Fe, Mg, induction of oxidative stress, reducing chlorophyll contents, therefore destroying chloroplast ultrastructure and reducing photosynthetic rate, and even causing plant death (Xue et al. 2017). Manganese is involved in some key plant physiological functions like photosynthesis, redox processes and mitigation of damage from reactive oxygen (ROS) (Graham et al. 1988). However, extravagant cellular concentrations of Mn could cause oxidative destruction, and Mn uptake, transport and distribution within the plant must be strongly controlled (Ducic and Polle 2005). Elevated $Mn^{2+}$ concentrations in the tissue also drive toxic effects on photosynthesis and antioxidant enzyme activity and Mn toxicity is often the result of competitive interference with phosphorus (P), calcium (Ca), and ferrum (Fe) (González et al. 1998; St Clair and Lynch 2004; St Clair et al. 2005). Excessive manganese can cause phytotoxicity in plants and high concentrations will decrease plant growth and interrupt the metabolic processes (Shi et al. 2006). Weng et al. (2013) also showed that manganese toxicity led stunted growth, chlorosis, crinkled leaves, brown lesions (speckles), as well as inhibiting the photosynthesis and respiration. The main effects of Mn toxicity on plants is induced by oxidative stress, which is due to the direct electron transfer in single electron reaction, reactive oxygen species (ROS) formation leading to the increase of metabolic disorders, and the deactivation and down-regulation of the enzymatic antioxidant defence system (Shi et al. 2006).

Fernando and Lynch (2015) indicated that there are two main conflicting theories on the mechanisms of Mn phytotoxicity and tolerance. One theory is that Mn toxicity occurs at leaf apoplast, and the other theory is that Mn toxicity develops through the disruption of photosynthetic electron flow at chloroplasts. The first theory showed that the black spots on leaf is the most important indicator of Mn phytotoxicity and Mn phytotoxicity is primarily mediated at apoplast (Horst and Marschner 1978; Wissemeier and Horst 1992; Fecht-Christoffers and Horst 2006). However, the experiments were most conducted in the low light greenhouse not in the real environment and there is lack of symplastic components analysis. Another theory was employed in a series of investigations under the ‘real’ climatic conditions (González et al. 1998; González and Lynch 1999; St Clair and Lynch 2004, 2005; St Clair et al. 2005). It was showed that Mn phytotoxicity is mediated via the effects of antioxidative enzymes, like ascorbate peroxidase and glutathione reductase (González et al. 1998; St Clair and Lynch 2004; St Clair et al. 2005), and chlorosis was recognized by Horiguchi (1988) as a consistent and notable symptom of its toxicity (González et al. 1998). These two conflicting results may be due to the different light regimes, with the latter studies being carried out under near natural light as the main mechanism of the oxidative stress (Fernando and Lynch 2015). As well as exacerbating Mn toxicity by photo-oxidative stress, sunlight can also enhance foliar Mn accumulation (Fernando et al. 2009) as well as acid rain, which could change soil chemistry and raise soil Mn bioavailability (St Clair and Lynch 2005). In addition, the absorption and utilization of Mn in two distinct populations of *P. acinosa* were studied, and the results showed that the high tolerance and super accumulation ability of Mn in *P. acinosa* are constitutive properties (Xue et al. 2005). The effects of light, temperature, precipitation and other climatic variables on the availability and toxicity of manganese were discussed (Fernando and Lynch 2015).

In addition, in response to Mn toxicity, plants have developed a variety of mechanisms, such as separation, chelation, avoidance and exclusion, antioxidant and ionic interactions. For example, a plant can remove metal ions from the cell or bind it to the inner cell compartment (Kim et al.

2004). The plant chelated Mn and then accumulated Mn in leaves and stems, thereby reducing its toxicity (Fernando et al. 2013), such as the chelation in *P. perfoliatum* as a Mn tolerant plant (Xue et al. 2017). In addition, some plants can also seep into organic acids, which helps detoxify Mn (internal and external) (Xue et al. 2017). For example, during relatively lower Mn addition, *Phytolacca acinosa* could exudate and transport organic acid which enhanced the tissue tolerance to Mn (Xue et al. 2004). The fourier transform infrared (FTIR) spectroscopy analysis showed that the absorption bands of *P. americana* differ greatly in carbohydrate and protein, mainly due to the exudation and transport of organic matter (Ren et al. 2008). It is consistence with the more recently research by Xue et al. (2017), which showing that low concentrations of Mn stimulated plants to produce organic acids and other exudates to overcome Mn toxicity. The release of $Mn^{2+}$ from soil is important for the formation of Mn oxides, and to evaluate the bioavailability of $Mn^{2+}$ in acidic soils. The ability of organic acids to promote $Mn^{2+}$ release follows the following sequence: citric acid > pyrite > tartaric acid > malic acid > lactic acid (Yang et al. 2011). In addition, the conversion of Mn to a metabolically inactive clathrate by the Mn-oxalate complex was a key detoxification mechanism (Dou et al. 2009); Mn can also be separated into intracellular compartments, which is one of the main mechanisms of Mn tolerance (Xu et al. 2015). For example, Xue et al. (2017) showed that the oversupply of Mn increased soil cation exchange capacity by demethylation of pectin in cell wall, and increased Mn tolerance.

And another tolerance mechanism of Mn toxicity was its reactions with some elements, especially phosphorus (P), calcium (Ca), and ferrum (Fe) (Esteban et al. 2013). Dučić et al. (2012) showed that the accumulation of Mn in epidermal cells indicates that the root endodermis hinders the transport of Mn and protects the normal physiological process of cells of *P. amelioration*. Moreover, Xue et al. (2017) indicated that P content was high in acicular substances (Xue et al. 2017), showing that P concentration and its precipitation with Mn may reduce Mn biological activity, which is the main mechanism recognized by heavy metal phytoremediation studies (Hauck et al. 2003; Kochian et al. 2004).

## 4 Chemical Forms of Manganese in Plants and Its Interaction with Other Metals

The additional Mn supply can also impose stress by the result of competitive interference of P, Ca, Mg, K and Fe (St Clair and Lynch 2005; St Clair et al. 2005). Researchers have demonstrated that elements including Ca, Si, Fe, and P exert significant effect on the accumulation of Mn in plants. Among these elements, Ca is now the hottest topic (Mukhopadhyay and Sharma 1991; Alam et al. 2006; Juice et al. 2006). Dou et al. (2009) suggested that Ca-oxalate crystals in the Mn-hyperaccumulator plant pokeweed (*Phytolacca americana*) lack direct effects on the detoxification of Mn. Moreover, the presence of P and O in the Mn-containing crystals indicates that excess Mn could be deposited by phosphate, which may contribute to Mn accumulation and detoxification in pokeweed. Xu et al. (2009) have demonstrated sufficient oxalic acid in *P. acinosa* exists for complex Mn. X-ray absorption spectroscopy data result verified the existence of bivalent Mn in plant leaves and large portion (90%) of which is Mn-oxalate. In addition, oxalate biosynthesis was not affected by Mn concentration because the density of oxalate in the leaves of *P. acinosa* was unaltered when they increased the amount of Mn in the solution. Moreover, phosphate also exerts a significant impact on the tolerance plants to heavy metals (Kochian et al. 2004). Much research has been devoted to the role that phosphate plays on Mn and other heavy metal fixation and precipitation reactions (Hauck et al. 2003; Shao and Sun 2007). Using energy dispersive X-ray (EDX) analysis, the density of phosphate concentrations in acicular substances was determined to be 7.92% and 11.55% (Xue et al. 2016), which may be caused by the settlement and precipitation of Mn with phosphate.

It was shown that oxidation caused by Mn can make changes at cellular level in the plants (Najeeb et al. 2009), including the variation of chloroplast swelling, cell size and shape, disruption of thylakoid membrane, and plastoglobuli augment. While high degree of endurance to the toxicity of Mn was found in these plants, which illuminated a large number of Mn removed from the media. Moreover, EDTA can effectively promote the assimilation and translocation of Mn, and plant growth can be stimulated by citric acid.

Moreover, Shi and Zhu (2008) studied the influence of salicylic acid (SA) on the toxicity of Mn in cucumber plants (for example, *Cucumis sativus*). Excess Mn caused serious growth inhibitions, effectively promoted the Mn accumulation in shoots and roots of plants, and in addition hindered the assimilation of Ca, Mg, and Zn. However, additional amount of SA could reduce Mn translocating from roots to shoots, and ease the assimilating inhibition effects of Ca, Mg and Zn.

## 5 Localization of Manganese in Plants

Compartmentation, exclusion, and detoxification mechanisms may all be involved in plant tolerance to excess Mn (Fernando and Lynch 2015). Compartmentation of heavy metals can reduce the damage to plant components such as cells, tissues, and organelles, making localization of heavy metals and plant ultrastructure analysis important for understanding the mechanisms (Weng et al. 2013; Rizwan et al. 2015; Wu et al. 2016, 2017). When plants are under stress due to exposure to toxic metals, the changes in the plant ultrastructure are the causes of irregular plant physiological effects (Xue et al. 2004; Keller et al. 2015). The cytoplasm is acknowledged as the site for cell metabolism. Damage of cytoplasm makes it shrinks and disappears, producing vacuoles, then metabolism ceases leading to cellular death (Xue et al. 2004; Fernando et al. 2006a, b).

Manganese is an important micronutrient for the chloroplasts, as well as evolving in the structural construction of various photosynthetic enzymes. However, excess Mn can damage chloroplasts heavily (Fernando et al. 2006a; Xue et al. 2016, 2017). Fernando et al. (2006a, b) showed that foliar Mn in *G. bidwillii* was primary localization in photosynthetic tissues, while in other studies the highest foliar Mn localized in the non-photosynthetic tissues including *P. acinosa* (Robinson and McGrath 2003; Bidwell et al. 2004; Xu et al. 2006). Excess Mn may reduce the amount of Fe and Mg, and thus reduce the chlorophyll density in plants because Fe plays an important role in chlorophyll biosynthesis (Shi et al. 2006). Moreover, excess Mn can also damage chloroplast structure, leading to reduction chlorophyll biosynthesis with decreasing photosynthetic rates (Shi et al. 2006). For example, manganese concentrations >3000 μmol $L^{-1}$ induced damage of the chloroplasts of pea plants, and the change in shape of thylakoids (twisted) (Doncheva et al. 2005). In addition, Dou et al. (2009) found the deposits observed around vacuole, showing high Mn density in *P. americana* significantly caused damage to leaf cell chloroplasts (Dou et al. 2009). It was also showed that lower concentrations of Mn did not break ultrastructure of *P. americana*, when the chloroplasts exposed to heavy Mn stress (12,000 μmol $L^{-1}$), they would shrink, their outer membrane disrupted, the thylakoids swollen, therefore, the chloroplasts became heavily hollowed (Liang et al. 2011).

Doncheva et al. (2005) claimed that when increased the amount of Mn, Pea (*Pisum sativum* cv. 'Citrine') chloroplasts would be damaged, thylakoids would twist, and the starch grains would largely increase in terms of number and size. Moreover, this also leads to the reduction of osmiophilic globules (Xue et al. 2016). However, a contrasting study showed that with augment of Mn concentrations, the number of *P. americana* starch grains reduced and they also became smaller, while the number of osmiophilic globules increased (Liang et al. 2011). The difference of plant species may cause this effect, that *C. ambrosioides* behaved different with *P. americana*, lacking the detoxification mechanism.

The Mn localization study of *Gossia bidwillii* (Myrtaceae) found that different parts of the plant had different Mn concentration, with the largest Mn density located in the upper-layer palisade mesophyll and the lowest in the mesophyll (Fernando et al. 2006b). According to Xu et al. (2006), Mn prefers to stay in the marginal part of the leaf of the hyperaccumulator plant species *P. acinosa*. Moreover, based on cross-sectional study of the leaf, the epidermis had higher Mn concentration than that in the mesophyll. This distribution was adjusted by the the transpiration rate. Mn cannot move anymore after it transported from the roots to shoots of *P. acinosa*. Recently, newly discovered Mn-hyperaccumulators have led to renewed interest in the New Caledonian species, leading to Mn localization studies on Virotia neurophylla (Proteaceae), an endemic Mn-hyperaccumuating species. As in G. bidwillii, studies found unusual spatial distributions of foliar Mn (Fernando et al. 2006b), with primary sequestration of Mn occurring within the palisade mesophyll cells.

Plant can evolve mechanisms to prevent the toxicity from metal, including efflux of metal ions from cells and sequestration into internal cellular compartments (Kim et al. 2004). Mn could store in vacuoles, chloroplast lamellae structures, Golgi apparatus, and cell walls (Ren et al. 2007; Sytar et al. 2013). It was found that the Mn accumulation in cell wall and vacuole of leaf cells in *Polygonum hydropiper* might be one of the Mn tolerance and hyperaccumulation mechanism (Wang et al. 2008). In addition, Xue et al. (2016) showed the structural variation of *C. ambrosioides* leaf cell, such as forming black agglomerations. Other researchers also proposed the black agglomerations in plant cells (Papadakis et al. 2007; Dou et al. 2009), and those found in leaf cells of *P. americana* were actually Mn-oxides (Dou et al. 2009). Compared with the controls, black agglomerations were also discovered in the *Polygonum perfoliatum* L. cells with the Mn concentration of 1000 and 10,000 μmol $L^{-1}$ treatment (Xue et al. 2017). The additional Mn oxides would result in brown spots and chlorosis on leaves (Liu et al. 2010). Chlorosis was found in the leaves of *C. ambrosioides*, which may due to high concentration of Mn, and the black agglomerations identified in this research may be Mn-oxides (Xue et al. 2016). Moreover, at 10,000 μmol $L^{-1}$, Mn was found to stay as acicular structures in the leaf of cells and intercellular spaces in *C. ambrosioides* and *P. perfoliatum*. The energy spectrum study revealed that in acicular crystals, the concentration of Mn is much higher than the amount of Mn in other locations, showing the tolerance and accumulation of Mn (Xue et al. 2016, 2017).

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# Element Case Studies: Arsenic

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**Abstract**

Arsenic contaminated soil is a major issue in PR China. The discovery of an As hyperaccumulator fern, *Pteris vittata* opens a door for phytoextraction of As-contaminated soils. *In situ* phytoextraction projects using *P. vittata* have been established that achieved high removal rates of As. The first phytoextraction project in the world was established in Chenzhou, Hunan Province. Subsequently, more phytoextraction projects were established in Guangxi Zhuang Autonomous Region, Yunnan Province, Henan Province and Beijing. During these field-based projects, the safe disposal and re-utilization of *P. vittata* biomass were considered essential processes. Incineration technologies for *P. vittata* biomass are well developed. Safe landfilling has been applied for the disposal of the burned ash of *P. vittata* when the amount of that ash is small. When the ash amount is large, a recycling method has to be applied. Agromining of Ni has been successfully achieved, but agromining of As is at present only an idea, owing to the low commercial value of As. Nevertheless, production of a biofuel resulting from the incineration process, together with the recycling of As, could be a potential opportunity for agromining of this metalloid.

## 1 Introduction

Contamination by heavy metals/metalloids is a major issue in the world (Zucchi et al. 2005; Zimmer et al. 2011). Arsenic is a toxic metalloid, existing ubiquitously in the environment as a result of natural and anthropogenic activities. Chronic exposure to As through drinking water and/or staple foods is a major health concern globally (Eisler 1985; Sturchio et al. 2013). Considering the wide distribution and high toxicity of As, many efforts are devoted to investigating suitable remediation schemes. Phytoextraction has increasingly attracted attention, since it is

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environmental friendly, simple in operation, and economically efficient (Chen et al. 2002). In situ phytoextraction projects using the As hyperaccumulator *Pteris vittata* have been established on farmlands and residential areas in PR China and the USA, with high As removal rates recorded (Huang et al. 2007; Ebbs et al. 2010; Wan et al. 2016). The disposal of As-rich biomass harvested by a hyperaccumulator plant has received increasing attention. The potential economic value of heavy metals recovered in the harvests is the basis for the concept of phytomining.

Phytomining employs hyperaccumulator plants to take up metals from the growth medium into harvestable plant biomass. Harvesting, drying, and incineration of the biomass generates a high-grade bio-ore (van der Ent et al. 2015). Agromining could provide local subsistence-level farming communities with an alternative type of agriculture on degraded lands: farming not only for food crops, but also for metals such as Ni. This technology also enables us to extract metals from secondary resources (e.g. metal-enriched and contaminated soils, mineral wastes) that are unsuitable for economic mining, and thus manufacture high-value products (Zhang et al. 2016a).

Several ferns have been identified as hyperaccumulators. *Pteris vittata* is a fern that has been widely applied to As-contaminated agricultural soils. Owing to serious As contamination in parts of China, several phytoextraction projects with a total area of 200 ha using *P. vittata* have been established (Wan et al. 2016). However, the low commercial value of As is one of the main reasons for restricting As phytomining as a viable technology. Recently, the production of nano-material from the ash of incinerated *P. vittata* biomass and the recovery of biofuel have provided novel directions for further research.

## 2 *Pteris vittata* and Its As Accumulation Characteristics

The As hyperaccumulator *P. vittata* was discovered in 2001 by two groups, one from China and the from the USA (Ma et al. 2001; Chen et al. 2002). *Pteris vittata* has been demonstrated to have extreme tolerance to As. This fern is able to grow on soils having As concentrations as high as 4000 μg $g^{-1}$, and even on mine tailings with 23,400 μg $g^{-1}$ As (Chen et al. 2002). It also shows an extreme As-accumulating ability. In a pot trial, the above-ground As concentration of *P. vittata* accumulated up to 4383 μg $g^{-1}$, with a bioaccumulation factor of 77.6. The fern can accumulate As from different As compounds, including NaMMA, CaMMA, $K_2HAsO_4$, $Na_2HAsO_4$, and $Ca_3(AsO_4)_2$ (Tu and Ma 2002). The distribution of As in *P. vittata* is in the order pinnae > stems > roots; As contents in the pinnae are significantly higher than those in the rest of the tissues (Chen et al. 2002). This property favours the collection of harvests and concentrated As disposal. Arsenic in *P. vittata* resides mainly in the form of inorganic As(V) or As(III), together with small amounts of $FeAsO_4$, $AlAsO_4$, and $Ca_3(AsO_4)_2$ (Tu and Ma 2002; Zhang 2002). The extremely high concentration of As that may be reached in *P. vittata* and its remarkable biomass produced under cropping conditions suggests that this fern is an appropriate plant for effective phytoextraction and potential agromining of As.

## 3 Case Studies of As Phytoextraction

*In situ* phytoextraction projects using *P. vittata* have been established on 10 sites in China. The first phytoextraction project in the world was established in Chenzhou, Hunan Province (Fig. 1). During this project, the effect of fertilizer and harvesting frequency on extraction efficiency was studied. $CaH_2PO_4$ was found to be the most efficient fertilizer, aiming to increase As removal from the soil. By comparing different harvest frequencies, three harvests a year was found to be the optimum. After *P. vittata* had been cultivated for 7 months, the total As concentration in the soil decreased by 5.0 μg $g^{-1}$, with a remediation efficiency reaching 7.84% in the most efficient treatment (Liao et al. 2004). Based on the first successful phytoextraction project, additional phytoextraction projects

**Fig. 1** Case study of As phytoextraction in Chenzhou, Hunan Province

were established in Guangxi Zhuang Autonomous Region, and in Yunnan and Henan Provinces, and in the city of Beijing (Table 1).

The phytoextraction project established in Huanjiang, Guangxi Province, was the largest phytoextraction project in the world (Fig. 2); phytoextraction, intercropping, and phyto-barrier technologies were all used. The phytoextraction technology using *P. vittata* resulted in an overall annual As removal rate of 10%. After 4 years, the As concentration in the soil met the national standard for farmland soil. The phyto-barrier technology significantly decreased As concentration in agricultural products, at <5% the above-standard rate. The intercropping technology also reached an above-standard rate of As at <5%. As a result of the Huanjiang project, a new model for the "Government guidance, scientific and technological support, enterprise participation, and the implementation of farmers" was established and promoted to be adopted at other sites.

The phytoextraction project in Jiyuan (Henan Province) was the first established in the monsoon climate region of medium latitudes. Previous projects of this type were all carried out in the subtropical monsoon climate zone. *Pteris vittata* is naturally distributed only in this subtropical monsoon region. To overcome this limitation, a series of measures was established to assist *P. vittata* survival during cold winters; with a suitable insulating cover material, the survival rate of *P. vittata* can reach as much as 45% (Zhang et al. 2016b). After a 2-year remediation, the removal rate of As from soil reached 16.6%, validating the possibility of utilizing arsenic hyperaccumulation in northern China.

Scientists from the University of Florida found an even higher As extraction ratio. After *P. vittata* had been cultivated for 2 years, the average concentration of As decreased from 190 $\mu g\ g^{-1}$ to 150 $\mu g\ g^{-1}$, indicating a removal rate of 26.3% (Kertulis-Tartar et al. 2006). Such

**Table 1** Phytoextraction projects of arsenic contaminated soil in PR China

| Place | Contaminant | Technology | Remediation efficiency |
|---|---|---|---|
| Chenzhou, Hunan Province | As | Phytoextraction | Through 3–5 years remediation, soil As content decreased from 50 μg $g^{-1}$ to <30 μg $g^{-1}$, reaching the second national soil quality standard (GB15618–1995). |
| Shimen, Hunan Province | As | Phytoextraction and intercropping | Phytoextration technology removed 13% As from soil each year.<br>In the intercropped system, agricultural products from intercropped cash crops meet the national standard. |
| Huanjiang, Guangxi Zhuang Autonomous Region | As, Cd and Pb | Phytoextraction, intercropping and phytobarrier | Phytoextration technology removed 10.5% Cd and 28.6% As from soil after 2-year remediation.<br>In the intercropped system, the yield of maize, rice and sugarcane increased by 154%, 29.6% and 105%, respectively; and the As, Cd and Pb concentration in corn kernel decreased by 39%, 4.1 and 4.9%, respectively.<br>In the phytobarrier system, the over standard rate of heavy metals in agricultural products was less than 5%. |
| Gejiu, Yunnan Province | As and Pb | Phytoextraction | Phytoextration technology removed 18% As and 14% Pb from soil each year. |
| Huize, Yunnan Province | As | Phytoextraction | Phytoextration technology removed 12% As from soil each year. |
| Jiyuan, Henan Province | As, Cd and Pb | Phytoextraction | Phytoextration technology removed 13.9% As, 0.5% Pb and 16.1% Cd from soil each year.<br>After 2-year remediation, 338.5 g As, 36.2 g Cd and 104.5 g Pb were removed from each Mu soil. |
| Fangshan, Beijing City | As | Phytoextraction | Phytoextration technology removed 17.2% As from soil each year. |
| Dabaoshan, Guangdong Province | As, Cd, Cu, Pb and Zn | Intercropping and phytobarrier | Products of cash crops met the national standards |

**Fig. 2** Case study of As phytoextraction in Huanjiang, Guangxi Zhuang Autonomous Region

a difference may result from different pollution sources and pollution extents. The As contamination in soil reported by the Kertulis-Tartar et al. study resulted from the use of chromated copper arsenate (CCA), involving very high concentrations of As, whereas projects carried out by our group dealt with farmland soil contaminated by mining and smelting activities, with the average concentration of As in soil being 30–50 μg $g^{-1}$.

During the establishment and duration of these case studies, several funding and manpower enabling steps received focused public attention and publicity.

## 4 Fast Reproduction of *P. vittata* Sporelings

Obtaining an adequate supply of *P. vittata* sporelings is the first vital step in a successful phytoextraction project for clean-up of As-contaminated soil. As a pteridophyte, *P. vittata* is from an ancient division of the plant kingdom, and as such requires spores to propagate. The minute spores are only tens of microns in size and therefore can store only a very limited amount of nutrients. After germination of the spores, a sexual fertilization process is necessary for the formation of diploid sporophores. These processes have special requirements for both light and moisture. Through a series of studies on germination and fertilization processes, optimized parameters were proposed. Fast reproduction technologies for *P. vittata* sporelings were established and implemented in all of our large-scale projects (Lei M et al. unpublished data).

## 5 Harvesting and Incineration of the Hyperaccumulator Biomass

*Pteris vittata* is a perennial fern. With the roots kept in soil, the above-ground parts can be harvested several times a year. The effects of harvesting on As accumulation and phytoextraction efficiency of *P. vittata* were studied. Our results showed that repeated harvesting increases both parameters. Through this field practice, 16 kg As, 8.5 kg Pb, and 9.2 kg Zn can be removed from one hectare soil each year by harvesting twice per year (Xie et al. 2010).

The disposal of hyperaccumulator plants enriched with toxic As is an essential step for an As phytoextraction project. Direct disposal to hazardous waste landfill, incineration/ashing, and liquid extraction are possible methods for the disposal of hyperaccumulator biomass. Among these, incineration is here proposed as the most feasible, being an economically acceptable and environmentally sound method (Sas-Nowosielska et al. 2004). Incineration can decrease the volume and weight of the hyperaccumulator biomass by ~90%. During incineration, the risk caused by emission of As to the atmosphere deserves special attention. Data obtained on this process by Yan et al. (2008) have shown that 24% of the total As accumulated by *P. vittata*, which contained a high concentration of As (1170 $\mu g\ g^{-1}$), was emitted at 800 °C, and that 62.5% of emitted As (0–800 °C) is volatilized below 400 °C. Based on such empirical characteristics, reagents such as CaO that can immobilize As during the incineration process can be added, in order to reduce the emission of As to the atmosphere.

## 6 Reusing the Biomass of As Hyperaccumulator as Resources

In the early days of the development of phytoextraction, safe disposal of hyperaccumulator biomass was considered unsustainable. However, current thinking requires the reuse of harvested biomass as a resource, not a waste product. Agromining is proposed as a method to reuse the biomass of hyperaccumulator plants, and it has been successfully applied to Ni hyperaccumulators (Echevarria et al. 2015; Kidd et al. 2015; Rosenkranz et al. 2015, and Chap x this volume). Nonetheless, the potential for As phytomining is not as promising as that for Ni, due to the low commercial value of As and its compounds. Recently, some studies have explored a hydrothermal method to convert biomass into biomass fuels with high caloric value (Srokol et al. 2004). In this case, not only valuable Ni was recycled from plants, but also a biofuel was obtained. Carrier et al. (2011) applied supercritical conditions to safely dispose the biomass of

*P. vittata*, transferring the biomass to liquid fuel. The preparation of nano-metallic materials from hyperaccumulators is another approach to potential resource utilization. This constitutes a new insight for the recycling of hyperaccumulators and provides a novel route for further development of green nanostructure syntheses (Qu et al. 2012). At present, scientists from the Chinese Academy of Sciences and Cranfield University, UK, are attempting to enhance the commercial value of harvested *P. vittata* biomass. In addition, some scientists in PR China are trying to reuse the biomass of this fern for potential medicinal properties. Hopefully, the recovered post-process energy and extraction of active ingredients from biomass can significantly increase the financial viability of phytoextraction projects and reduce the environment impacts of contaminated biomass disposal (Jiang et al. 2015).

## 7 Future Prospects

China is a country having the highest geological As reservoir in the world. However, the grades of unmined As ore in this country are quite low as a result of long-term exploitation of high-grade ores. Concurrently, problems that have been recognized in As-contaminated soils have enabled the advancement of phytoextraction technology for this metalloid. Agromining can remove As from contaminated soil and at the same time provide high-grade raw materials for mining (incinerating ash of hyperaccumulator biomass). However, the low commercial value of As has hindered the development and viability of As agromining. Recently, a move in this positive direction has progressed because of the proposed use of As biomass for the production of biofuel and nano-metal materials. Due to the 2016 enactment in PR China of the Soil Pollution Prevention and Control Action Plan, it could be expected that many more soil remediation projects will be established in PR China. Further development of phytoextraction technology and a concomitant increase in the amount of *P. vittata* biomass, demands an appropriate recycling method for this species. With the collaboration of Cranfield University and the Institute of Geographic Sciences and Natural Resources Research, the Chinese Academy of Sciences is now conducting research on commercial utilization of the biofuel sourced from the incineration of *P. vittata* biomass. Hopefully, as a result of this research, a completed industrial-scale chain of phytoextraction and agromining for As-contaminated soil will be established in the near future.

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# Element Case Studies: Cadmium and Zinc

Longhua Wu, Pengjie Hu, Zhu Li, Tong Zhou, Daoxu Zhong, and Yongming Luo

**Abstract**

*Sedum plumbizincicola* (Crassulaceae), a new *Sedum* species, was originally discovered in 2005 in Zhejiang Province, eastern PR China. It was identified as a Cd-Zn hyperaccumulator in 2007. During the past decade, great efforts have been made to understand its metal-accumulating capacities, physiological mechanisms for metal hypertolerance and hyperaccumulation, enhancing measures of phytoextraction, field application phytoremediation practice, and disposal of harvested biomass. This chapter provides a brief review of the progress on phytoremediation of Cd- and Zn-contaminated soils using this species. Agronomic measures to enhance Cd and Zn phytoextraction efficiency by *S. plumbizincicola* were studied, including cultivation management, intercropping with other plant species, and nutrient management. Changes in soil and plant metal uptake were investigated during long-term and repeated phytoextraction of Cd- and Zn-contaminated soils using *S. plumbizincicola*. Field assessment results confirm that phytoextraction using *S. plumbizincicola* is a promising technique for the remediation of slightly Cd-polluted soils without the need to halt normal agricultural production.

## 1 Introduction

Heavy metals such as Zn and Cd are common pollutants that are derived mainly from anthropogenic activities such as mining and the application of phosphatic fertilizers. Zinc is an essential element to plants but can be highly toxic at high concentrations. Cadmium is a non-essential element and also is highly toxic to both plants and animals, even at low concentrations. Among the various remediation techniques available, phytoextraction—the use

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of metal hyperaccumulator plants to remove pollutants from soils—is considered to be a cost-efficient and environmentally friendly method, although it still faces many challenges (McGrath et al. 2006).

The success of metal phytoextraction relies on several aspects. Firstly, an ideal hyperaccumulator produces a large biomass, grows rapidly and is easily cultivated. Secondly, sound agronomic measures such as optimum moisture, nutrition, planting density, and harvest strategies are required. Intercropping with other crop species, for example, might be a feasible method of remediating contaminated soils without halting routine agricultural production. Thirdly, the harvested biomass must be safely treated. *Sedum plumbizincicola* (Crassulaceae), a newly described *Sedum* species, was originally discovered in 2005 in a Zn-Pb mine area of Zhejiang Province in eastern PR China. This species was identified as a Cd-Zn hyperaccumulator in 2007. Subsequently, much effort has been made to understand its metal-accumulating capacity, physiological mechanisms for metal hypertolerance and hyperaccumulation, enhancing measures of phytoextraction, field application practice for phytoremediation and disposal of harvested biomass. This chapter provides a brief review of the progress made in the phytoremediation of Cd- and Zn-contaminated soils using this remediation plant species.

## 1.1 Characteristics of *Sedum plumbizincicola*

*Sedum plumbizincicola* (Crassulaceae) was originally discovered in 2005 when our research team undertook extensive field reconnaissance to search for metal-hyperaccumulating plant species in Zhejiang Province, eastern PR China (Wu et al. 2006). Some unusual and isolated populations that superficially resembled *S. alfredii*, but which produced four-merous flowers, were found in Chun’an and Lin’an counties, together with four other species, *S. alfredii*, *S. emarginatum*, *S. hangzhouense* and *S. bailey* (Fig. 1). Based on geographic distribution, growth habit, phenology, macromorphological characters, stem and leaf anatomical features, seed micromorphology, and nrDNA internal transcribed spacers (ITS) sequence data, formal description of the new species *S. plumbizincicola* and clarification of the affinities between *S. plumbizincicola* and closely-related taxa were conducted (Wu et al. 2013a).

*Sedum plumbizincicola* is a perennial, light green or yellowish-green herb and is known only from the type locality, Zitong town (Zn-Pb mining areas), northwest of Hangzhou city in west Zhejiang Province, eastern PR China. Annual rainfall here varies from 980 to 2000 mm and occurs mainly in the summer, with a mean annual temperature of 15–18 °C. Soils in this area are typically sandy, acidic, highly leached, and commonly thin. This species has a strong ability to hyperaccumulate Zn and Cd, and is promising for the restoration of metal-polluted soils by phytoremediation. The specific epithet ‘*plumbizincicola*’ refers to the distribution of the species in the Pb and Zn mining areas of west Zhejiang Province.

## 1.2 Hyperaccumulation Ability for Cadmium and Zinc

The potential ability of *S. plumbizincicola* to extract Cd and Zn from contaminated soils has been demonstrated in glasshouse and field experiments. A field survey was conducted at a mining area in Chun’an county, Zhejiang Province, where the soils were strongly enriched with Cd (36–157 $\mu g\ g^{-1}$), Zn (1930–7250 $\mu g\ g^{-1}$), copper (Cu) (530–8340 $\mu g\ g^{-1}$) and Pb (71–6940 $\mu g\ g^{-1}$) due to pollution from mine tailings and processing of wastewater (Hu et al. 2015). *Sedum plumbizincicola* was the predominant species at this area and the shoot Cd and Zn concentrations ranged from 574 to 1470 $\mu g\ g^{-1}$ and from 9020 to 14,600 $\mu g\ g^{-1}$, respectively. The metal concentrations in plant shoots were $>100\ \mu g\ g^{-1}$ for Cd and nearly/over 10,000 $\mu g\ g^{-1}$ for Zn, these levels defining Cd- and Zn-hyperaccumulation

**Fig. 1** *Sedum plumbizincicola* in its (**a**) vegetative and (**b**) flowering states in the original habitat

ability according to the criteria of van der Ent et al. (2013). Moreover, Cd concentrations in the leaves were approximately twice as high as in the stems. Zinc concentrations in the leaves were somewhat higher than, or equal to, those in the stems. In a hydroponic experiment, Cao et al. (2014) recorded the Cd and Zn concentrations in the shoots (7010 and 18,400 μg $g^{-1}$, respectively) were about seven-fold and five-fold higher than those in the roots (840 and 3000 μg $g^{-1}$, respectively) after exposure to 100 μM $CdSO_4$ and 600 μM $ZnSO_4$, respectively.

## 1.3 Spatial Distribution of Elements in *Sedum plumbizincicola*

Heavy-metal sequestration in less-metabolically active parts of plants such as the epidermis and trichomes is one possible mechanisms of heavy-metal detoxification in hyperaccumulators. The spatial distribution of Cd, Zn, and other nutrient elements in frozen-hydrated sections of *S. plumbizincicola* from a mine area were quantitatively determined using cryo-micro-PIXE (Proton-Induced X-ray Emission) (Hu et al. 2015). In the roots, Zn and Cd were concentrated in the cortex but was low in the central stele. In the stems, taking tissue area (or weight) into account, 22.1, 46.2 and 31.8% of total Zn mass and 17.1, 71.6 and 11.3% of total Cd mass were partitioned in the epidermis, cortex, and central cylinder (including the pith), respectively. In the leaves, high concentrations of Zn were noted in the upper and lower epidermis. Cadmium was distributed more or less homogeneously in the entire leaf section, somewhat enriched in the vascular bundle, and slightly depleted in the spongy mesophyll. Taking tissue area into account, 23.8, 31.4, 23.8, 20.2 and 0.9% of total Zn mass was distributed in the upper epidermis, palisade mesophyll, spongy mesophyll, lower epidermis, and vascular bundles, respectively. However, 52.2 and 29.1% of total Cd mass was distributed in the palisade and spongy mesophyll, respectively. These data indicate that the preferential sequestration of Zn in stem and leaf (and to a lesser extent, Cd in the stem) into the epidermis is an important mechanism for hyperaccumulation and detoxification in *S. plumbizincicola*. Nevertheless, the parenchyma cells, e.g. mesophyll in the leaf, cortex, and pith in the stem, play more important roles in storage and detoxification of Cd than Zn within *S. plumbizincicola*. This result also indicates that different storage and detoxification strategies may exist for Cd and Zn in the shoots of *S. plumbizincicola*.

Additionally, in hydroponically cultured *S. plumbizincicola* supplied with Cd and Zn (Cao et al. 2014), micro-PIXE analysis and tissue fractionation show that young and mature leaves of *S. plumbizincicola* exhibit different accumulation and distribution characteristics for metals. The concentrations of Cd and Zn in young leaves (4330 and 9820 μg $g^{-1}$, respectively) were about six-fold and two-fold higher than those in mature leaves (636 and 2620 μg $g^{-1}$), respectively. Zinc was predominantly localized in epidermal cells in both young and mature leaves, but large amounts of Zn were also found in the mesophyll cells of young leaves.

## 1.4 Agronomic Measures to Increase Cd and Zn Phytoextraction

*Sedum plumbizincicola* is suitable for clonal reproduction. The shoot branches are usually selectively picked for reproduction. A tissue culture method has been established to provide large amounts of plant material (Wu et al. 2010). For field application, appropriate plant density, soil moisture, light, and harvest strategy have been optimized for plant biomass and Cd and Zn uptake and minimal time for the phytoremediation period. A field plot experiment was established to study the effects of planting density on growth and heavy metal uptake by *S. plumbizincicola* (Liu et al. 2009). The results show that when the planting density was 15 × 15 cm (4.4 × $10^5$ plants $ha^{-1}$), *S. plumbizincicola* had the highest yield as well as Cd and Zn removals. Neither lower nor higher densities were preferable. Humid-dry conditions (~70% of water-holding capacity) are preferable for plant growth and metal accumulation to extremely dry or flooded conditions (Cui et al. 2009). *Sedum plumbizincicola* is a shade species, and Zn and Cd accumulation did not decline when irradiance decreased to 50% of the control level (Li et al. 2010). *Sedum plumbizincicola* is a perennial and can be harvested two or three times each year, for example before a hot summer and cold winter. During cutting, retaining 3–5 cm of stubble will promote multiplication vegetatively (Li et al. 2009).

## 1.5 Intercropping

Much effort has been devoted in the past few years to exploring the feasibility of simultaneous safe grain production and phytoremediation of heavy metal-contaminated soils using *S. plumbizincicola* in major food crop areas of China. Shade tolerance ensures that *S. plumbizincicola* can be employed to intercrop with tall straw plants such as maize, sorghum,

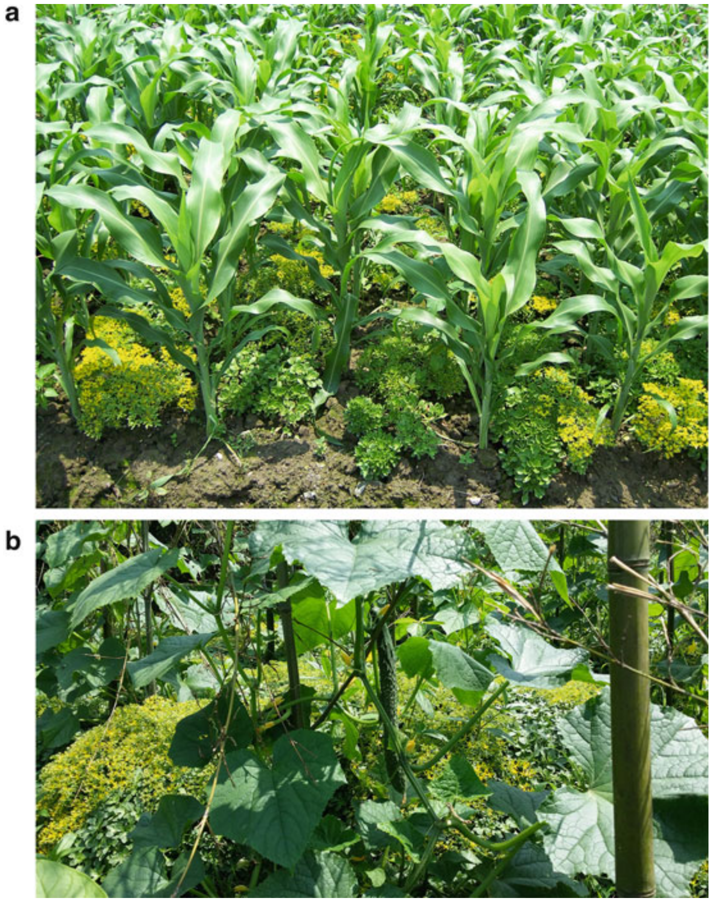

**Fig. 2** Field practice of intercropping of *S. plumbizincicola* with maize and cucumber

sugarcane and wheat (Fig. 2). *Sedum plumbizincicola* is also suitable for rotation with rice in southern PR China, e.g. growing *S. plumbizincicola* from October to June and rotating rice from July to October.

Nevertheless, the roots of *S. plumbizincicola* may activate soil Cd and increase Cd availability, which may increase the heavy-metal risk in intercropping or rotation plant. For example, Zhao et al. (2011) found that compared with mono-cultured wheat, intercropping with *S. plumbizincicola* significantly increased the soil $NaNO_3$-extractable Zn and Cd, which increased metal uptake by wheat. Shen et al. (2010) also recorded that Zn and Cd concentrations in rotated rice after *S. plumbizincicola* were higher than the control, while the amendment of phosphate fertilizer (particularly calcium magnesium phosphate) in rice season decreased the accumulation of metals in rice and promoted rice production as well. The activation mechanism of soil metals by roots of *S. plumbizincicola* is not clear up to now. It seems soil type and plant species dependent in intercropping and rotating system (Ju et al. 2015). Therefore, such intercropping should be done carefully.

## 1.6 Nutritional Management

The effects of nutritional status on plant growth and metal accumulation by *S. plumbizincicola* were investigated (Hu et al. 2013; Arnamwong et al. 2015). Hydroponic experiments were designed to evaluate whether and how the form of N ($NO_3^-$ *vs*. $NH_4^+$) influences Cd uptake, and translocation and subsequent Cd phytoextraction by *S. plumbizincicola* (Hu et al. 2013). The results from a 21-day hydroponic culture showed that shoot biomass and Cd concentrations were 1.51 and 2.63 times higher in the $NO_3^-$-fed than in the $NH_4^+$-fed plants. Besides, short-term (36 h) $^{107}$Cd uptake and translocation experiment using positron-emitting tracer imaging system showed that the rates of Cd uptake by roots and transport to the shoots in the $NO_3^-$ treatment were higher than in the $NH_4^+$ treatment. Moreover, $NO_3^-$ treatment increased Cd, Ca, and K concentrations but inhibited Fe and P in the xylem sap. Therefore, *S. plumbizincicola* clearly prefers $NO_3^-$ to $NH_4^+$ nutrition for Cd phytoextraction in hydroponic condition. The effects of different N fertilizers on shoot yield and Cd and Zn accumulation by *S. plumbizincicola* were also investigated using pot experiments (Arnamwong et al. 2015). The soil was contaminated with 0.99 μg g$^{-1}$ Cd and 241 μg g$^{-1}$ Zn. The results suggested that treatment by urea + DCD (dicyandiamide, a nitrification inhibitor) was a benefit for Cd phytoextration by *S. plumbizincicola*. Apart from N, low P and high K levels were recommended for metal phytoextraction by *S. plumbizincicola* (Shen et al. 2011).

## 1.7 Other Measures

Organic materials having different functional groups can be used to enhance metal bioavailability. It was found that amendment of the soil with ground rice straw or ground clover increased by 1.92 and 1.71 times the Cd concentrations in shoot of *S. plumbizincicola* compared to the control soil. Therefore, these traditional organic materials can be effective and environmentally friendly in enhancing the phytoremediation efficiency of Cd-contaminated soil (Wu et al. 2012). On the other hand, treatment with EDDS increased metals concentrations in shoots of *S. plumbizincicola*, however, EDDS also resulted in high soil concentrations of soluble metals and consequently a high risk of groundwater contamination (Wu et al. 2012).

*Sedum plumbizincicola* usually adapts to acidic soil. But for strongly or extremely acidic soil, the application of proper dosage of lime was thought to be benefit for the growth of *S. plumbizincicola* and metal uptake as well (Han et al. 2013). It has also been suggested that an appropriate application of sulphur fertilizer promotes the growth of *S. plumbizincicola*, and that the addition of calcium magnesium phosphate (CaMgP) fertilizer reduces the concentration of active heavy metals in the soil solution and the contamination risk for subsequently grown vegetables (Ren et al. 2013).

# 2 Repeated Phyto-extraction of Cd-Zn-Contaminated Soils Using *Sedum plumbizincicola*

Phyto-extraction using hyperaccumulator plants requires long periods of remediation time and successive crops to remove excessive metals from contaminated soils to achieve the permissible levels (Koopmans et al. 2008). Short-term remediation cannot give accurate prediction the plant metal uptake and soil metal changes during the phytoextraction process. Thus plant growth, metal uptake, and soil metal changes were investigated during repeated phytoextraction of Cd-Zn-contaminated soils using the hyperaccumulator *S. plumbizincicola*.

## 2.1 Plant Metal Uptake and Soil Metal Changes in Soils During Repeated Phytoextraction

Two levels of Zn- and Cd-polluted soil (0–15 cm) were collected from an agricultural field, namely lightly polluted soil (S1) collected 150 m from the pollution source (a Cu smelter) and highly polluted soil (S4) collected only 30 m from the smelter. These two polluted soils were then mixed in proportions of 1:2 and 2:1 by weight, to gain another two contamination levels (S2 and S3). These four soils were then

**Table 1** Chemical properties of four contaminated soils with different pollution levels (Li et al. 2014b)

| Soils | pH<br>($H_2O$) | Total C<br>(g $kg^{-1}$) | Total N | Total P | Total K | CEC<br>(cmol $kg^{-1}$) | Total Zn<br>(μg $g^{-1}$) | Total Cd |
|---|---|---|---|---|---|---|---|---|
| S1 | 6.47 | 42.2 | 3.75 | 0.24 | 21.6 | 16.1 | 321 | 1.11 |
| S2 | 6.95 | 37.6 | 3.19 | 0.22 | 22.3 | 16.6 | 2367 | 5.82 |
| S3 | 7.13 | 33.7 | 2.84 | 0.24 | 24.4 | 14.4 | 4343 | 10.6 |
| S4 | 7.24 | 29.1 | 2.21 | 0.22 | 22.9 | 11.9 | 6499 | 15.3 |

*S1* lightly polluted soil, *S2* and *S3* intermediate polluted soils, *S4* highly polluted soil

remediated by successive planting with *S. plumbizincicola* in a pot experiment (Li et al. 2014b). The main properties of S1, S2, S3 and S4 are listed in Table 1. *S. plumbizincicola* grew well during repeated phytoextraction and the plants did not have visible toxicity symptoms by metal. Cadmium concentrations in plant shoot decreased at early crops of the repeated phytoextraction process, but for later crops plant Cd kept relative constant, especially in soils S1 and S4. Plant Zn decreased in the first three crops in S1, but increased in the first four crops of S2 and S3, and then remained stable at later several crops for S1, S2 and S3. During the repeated phytoextraction S4 had similar plant Zn concentrations for all crops.

Changes in total Cd and Zn in the four contaminated soils with and without phytoextraction were investigated. Compared to the unplanted controls, after nine repeated phytoextractions over about 4 years, both total Cd and Zn decreased greatly; soil Cd and Zn decreased by 89.1, 96.8, 90.1 and 80.1% and 64.2, 70.1, 32.0 and 24.8% in S1, S2, S3 and S4 respectively. The removal efficiencies of metal were also high at relatively low levels of soil contamination, but in the more highly polluted soils the removal efficiencies were low, especially for Zn. This pattern indicates successful phytoremediation of the metal-contaminated soil by the hyperaccumulator plant.

Metal fractions extracted by the BCR (Community Bureau of Reference) method were used to discriminate among different degrees of metal availability during repeated phytoextraction. For the unplanted soils, over 90% soil Cd and Zn was mainly partitioned into the acid-soluble and reducible fractions, but Zn in S1 was partitioned in the acid-soluble fraction, and then the residual, oxidizable, and reducible fractions. Phytoextraction induced the largest decrease in the acid-soluble fraction, followed by the reducible fraction, and had no significant effects on the oxidizable and residual fractions. This trend indicates that acid-soluble fraction represented the most plant-available fractions, and then the reducible fraction; *S. plumbizincicola* can remove these two fractions of Cd and Zn, but the oxidizable and residual fractions were non-available to *S. plumbizincicola*. Measures should be taken to release the acid-soluble Cd and Zn or to redistribute the reducible fractions to enhance remediation efficiency.

## 2.2 Plant Metal Uptake and Metal Changes in Soils During Repeated Phytoextraction

Samples of four metal-contaminated soil types were collected from agricultural fields in different parts of China. Two acidic soils were collected from Huludao in Liaoning Province, north PR China, and Dabaoshan in Guangdong Province, southern PR China, and are named to here as HLD and DBS, respectively. The calcareous soils were collected from Shuanglingdong and Zhujiawu in Zhejiang Province, eastern PR China, and are referred as SLD and ZJW, respectively. Selected chemical properties of the contaminated soils are shown in Table 2. These four soils were remediated by successive plantings of *S. plumbizincicola* in a pot experiment (Li et al. 2014a).

**Table 2** Chemical properties of four contaminated soils without and/or with long-term phytoextraction (Li et al. 2014a)

| | pH | | TOC (g kg$^{-1}$) | | CEC (cmol kg$^{-1}$) | Cd (μg g$^{-1}$) | Zn (μg g$^{-1}$) |
|---|---|---|---|---|---|---|---|
| Soil | NP* | P | NP | P | NP | NP | NP |
| DBS | 3.71 | 3.87 | 24.3 | 23.7 | 8.31 | 0.42 | 291 |
| HLD | 4.77 | 4.71 | 15.1 | 13.2 | 15.4 | 8.68 | 476 |
| SLD | 7.74 | 7.71 | 28.4 | 25.7 | 18.9 | 16.9 | 1308 |
| ZJW | 7.14 | 7.72 | 20.6 | 19.5 | 11.2 | 2.28 | 1201 |

NP, without phytoextraction; P, the soil was repeatedly phytoextracted by the hyperaccumulator *Sedum plumbizincicola*

Shoot Zn and Cd concentrations in each crop were determined in order to investigate the changes in plant metal uptake during repeated phytoextraction. Shoot Zn concentrations in the calcareous soils (ZJW and SLD) were much lower than in the acidic soils (DBS, and HLD). As the crops of phytoextraction increased, plant shoot Zn concentrations in DBS and HLD and shoot Cd concentrations in HLD decreased markedly. However, there was no obvious decreasing trend in shoot Zn or Cd during repeated phytoextraction of ZJW and SLD, or in shoot Cd in DBS. Besides, a slight Cd increase in the later crops was found compared to the first several crops. This indicates that plant metal uptake under low available metal conditions depends on both soil and plant metal processes such as rhizosphere activity.

Both soil total Zn and soil Cd decreased markedly after repeated phytoextraction seven times. The highest decrease was found for the acidic soil HLD having high concentrations of Cd; after repeated phytoextraction, soil total Zn and Cd were decreased from 476 to 229 and 8.68 to 0.93 μg g$^{-1}$, respectively. Compared to the unplanted control soil, the soil total Cd and Zn in P7 soils (seven phytoextractions) from HLD, DBS, ZJW and SLD declined by 89, 64, 38 and 37%, and 52, 37, 19 and 12%, respectively. In addition, after phytoextraction with seven successive croppings, available Zn and Cd declined markedly in all four soils. Compared to the soils that were not exposed to phytoextraction, after seven remediation croppings $NH_4OAc$-extractable Zn decreased 98, 97, 58 and 30% in HLD, DBS, ZJW and SLD, respectively; $NH_4OAc$-extractable Cd decreased by 98, 95, 41and 57%, respectively. The equivalent values Zn extracted by $CaCl_2$ were 94, 92, 27 and 24% for HLD, DBS, ZJW and, SLD and for Cd were 96, 87, 60 and 68%.

The total or available metals extracted by chemical agents are based on equilibrium and cannot reflect metal re-supply processes usually occurring in the rhizosphere. The metal resupply capacity from the soil solid phase can be evaluated by the response time (the characteristic time taken by the system to approach equilibrium, Tc), and by the metal desorption rate constant ($k_{-1}$) measured by the diffusive gradients in thin-films technique (DGT). Effects of phytoextraction on these two parameters were studied further by Li et al. (2016).

In the unplanted soils, Tc values were much lower in the calcareous soils (SLD and ZJW) than in the acidic soils (DBS and HLD), suggesting a fast metal supply from the solid phase to solution in the calcareous soils. It is reasonable for a longer response time for acidic soils than calcareous soils. Acidic soils have high soil solution metal but small metal pool sizes in soil solid phase, which leads to a large equilibrium shift induced by DGT metals removal. After phytoextraction, the Tc values of Zn and Cd had marked decreases in the acidic soils, but increased in the calcareous soils. During repeated phytoextraction the $k_{-1}$ value of Cd remained constant in DBS, suggesting that the same mechanism regulates metal desorption from the solid phase to soil solution. However, $k_{-1}$ values of Cd and Zn in all of other soils declined greatly after phytoextraction. This result suggests that different metal pools regulates metal releases to soil solution during repeated phytoextraction, and that the residual metal in soil after phytoextraction might have stronger combining forces to soil solid phase.

## 2.3 Field Application of Phytoextraction with *S. plumbizincicola*

*Sedum plumbizincicola* was employed for field phytoextraction of heavy metal-contaminated soils beginning in 2006. To date, research and demonstration bases have been established in several provinces in PR China, namely Zhejiang, Hu'nan, He'nan, Jiangxi, Jiangsu, Guangdong, Guizhou, and others. This species uniformly exhibits extremely strong adaptation under diverse climatic, edaphic, and topographic conditions. *Sedum plumbizincicola* has shown much greater efficiency in the removal of Cd from slightly to moderately polluted acidic soils than from neutral soils.

A large-scale (~1 ha) field assessment of Cd phytoextraction by *S. plumbizincicola* was initiated in 2012 in Xiangtan county, Hu'nan Province (Fig. 3) with a soil pH of 4.7 and soil total Cd concentration in the arable layer of 0.49–0.71 μg $g^{-1}$. Successive phytoextractions using *S. plumbizincicola* were conducted from 2012 to 2015. The plants were transplanted in October and harvested in June of the following year. After phytoextraction, different rice cultivars were grown in order to assess accumulation of Cd in the grain. The results suggest that successive (repeated) phytoextraction of Cd using *S. plumbizincicola* is a promising technique for the remediation of slightly Cd-contaminated soils, without the need to halt normal agricultural production. For slightly to moderately contaminated acidic soils, the time required might be 2–5 years to reduce soil total Cd below the official safety level (0.3 μg $g^{-1}$). Low-Cd-accumulating cultivars are recommended for growth on phytoextracted soils, and some form of metal stabilization is necessary if ordinary rice cultivars are grown.

## 3 Treatment of *Sedum plumbizincicola* Biomass

The safe and economic disposal of the harvested hyperaccumulator plants for avoiding secondary environmental pollution is a challenging problem. Comparing with the other general disposal methods of composting, disposal to hazardous waste landfills, or compaction and ashing, the incineration and pyrolysis of harvested biomass are generally recommended for contaminated biomass owing to issues of high-volume reduction rates and energy-reuse efficiency (Sas-Nowosielska et al. 2004; Demirbas 2010).

**Fig. 3** Demonstration base of Cd phytoextration with *S. plumbizincicola* in polluted acid soil in Xiangtan, Hu'nan Province

Goals of the incineration and pyrolysis of metal-contaminated materials are to prevent the release of heavy metals and other pollutants into the environment, and accumulation of the heavy metals in the char/ash residue (Lievens et al. 2009; Wu et al. 2013b). Zhong et al. (2015, 2016) studied the incineration and pyrolysis of harvested biomass of *S. plumbizincicola* and showed changes in heavy metals, PAHs, and some regular pollutants in the incineration and pyrolysis products of *S. plumbizincicola* and characteristics of pollutant emissions and migrations in the products.

## 3.1 Incineration Products of *Sedum plumbizincicola*

*Sedum plumbizincicola* biomass sampled from different polluted fields was treated by incineration processes. The experimental facility comprised a flow control valve and meter, a flue gas absorption device, a horizontal quartz tube with a surrounding electrically heated furnace, and two high-pressure gas sources ($O_2$, $N_2$). Experiments were performed at temperatures from 350 to 950 °C and air flow rates of 0.5, 0.75, 1.0 and 1.2 L $\text{min}^{-1}$. The Thermal Gravity (TG) analysis curves show that the mass percentages of bottom ash to original biomass after incineration generally decreased with increasing temperature in the horizontal tube furnace, and that the weight losses of *S. plumbizincicola* were 6.17–10.8, 49.0–50.4 and 82.7–89.4% at three temperature steps of <185 °C, 185–400 °C and 400–697 °C, respectively (Zhong et al. 2015).

The distribution of heavy metals in flue gas is an important basis for using the gas cleaning system for disposal of *S. plumbizincicola*. With total Cd and Zn concentrations of 152 and 7480 μg $\text{g}^{-1}$ occurring in *S. plumbizincicola*, the data show concentrations of these metals in control flue gas were 0.101 and 46.4 mg $\text{m}^{-3}$, respectively, during the incineration process (Wu et al. 2013b). The additives aluminium oxide ($Al_2O_3$), CaO, kaolin, and activated carbon decreased the Cd concentration by 33.0, 45.2, 91.2 and 97.6% in gas, thus meeting the Chinese emission standard. About 21.6, 50.8, 88.1 and 99.1% of the Zn in the gas was removed after use of these adsorbents. By comparison, activated carbon was more effective than the other three additives for removal of Cd and Zn from the flue gas. In the control, the total recoveries of Cd and Zn were only 11.2 and 23.8% at the end, respectively. The additives $Al_2O_3$, CaO, and kaolin increased the recovery of Cd from the bottom ash and fly ash, and of Zn from the fly ash. During incineration of *S. plumbizincicola*, the recoveries of Zn, Cd and Pb largely decreased with the elevated temperatures in the bottom ash and increased in the fly ash, thus following the volatilization behaviour of heavy metals (Zhong et al. 2015). Nearly 80.0% of Cd was present in the fly ash. The major proportions of Pb (>95%) and Zn (>90%) remained in the bottom ash at temperatures of 350–650 °C. When the temperature was above 650 °C, most of the Zn and Pb were found in the fly ash. Recoveries of Cd, Zn and Pb from flue gas were lower than 0.05%. The melting point was the key factor determining the recoveries of these metals. Elemental sulphur and sulphide increased the retention of Cd and Zn on the bottom ash through the formation of sulphides under the local reducing environment in the furnace. In contrast, the presence of sulphur in the forms of $Na_2SO_3$ and $Na_2SO_4$ had little effect on heavy metal volatilization. Chlorine compounds did not increase this volatilization or partitioning on the fly ash during incineration, because most of the chlorine was present as KCl. The Cd and Zn were found to occur as pure metals, oxides, and carbonates; silicate compounds developed under low-temperature conditions during the incineration process.

Heavy-metal recovery rates with increasing airflow rate from fly ash were higher than from bottom ash, which indicates that at higher temperatures both the oxidizing atmosphere and reducing conditions favoured the transfer of Cd, Zn and Pb to the fly ash phase (Zhong et al. 2015). However, more Pb and Cd were found in the bottom ash under reducing conditions (air flow rate < 0.75 L $min^{-1}$) than under oxidizing conditions, whereas more Zn was found in the bottom ash under oxidizing conditions at 850 °C. Results from the equilibrium distributions of heavy metals when there was an air excess ranged from 0.2 to 2 at a temperature of 850 °C, showing that sulphur compounds are most important in the vapour of heavy metals under reducing conditions, relative to oxidizing conditions. Zinc is found as Zn, ZnO and ZnS when the air excess quotient is <0.6, whereas only ZnO was produced when the air excess quotient is >0.6. Cadmium and CdS are found under reducing conditions (the air excess quotient is <1), whereas CdO and Cd are found when the air excess quotient is >0.2.

## 3.2 Characteristics of Organic Pollutant Emissions

As shown in Table 3, incineration of *S. plumbizincicola* biomass produced high total PAH concentrations (35,420 μg $kg^{-1}$) in a laboratory scale, entrained flow-tube furnace of the control treatment, including 86.0% of PAHs found as 3-ring and 13.9% as 4-ring congeners (Wu et al. 2013b). Most 3-ring PAHs were present in the gas phase emissions whereas the PAHs with higher ring numbers were associated with fly ash. Total PAH emissions decreased with increasing temperature during incineration of *S. plumbizincicola*, especially in the bottom ash. With increasing temperature, the toxic equivalent quantity (TEQ) of total PAHs showed increasing trends in both the flue gas and fly ash, and decreasing trends in the bottom ash. There were high concentrations of PAHs in flue gas. Over 99% of total PAHs were removed from the flue gas when activated carbon was used as the adsorbent. When silica was used as the bed material, the 4-ring PAHs decreased and the 3-ring PAHs increased.

The concentrations of polychlorinated dibenzofurans (PCDFs) in flue gas were 131 pg $m^{-3}$ and 107 TEQ pg $m^{-3}$. These values were lower than the Chinese emission standard (500 TEQ pg $m^{-3}$) but higher than the emission limit (92.0 pg TEQ $m^{-3}$) set for waste incinerators in many countries (Wu et al. 2013b). The OCDD was the highest concentration in PCDD/F, which was present at 25.4 pg $m^{-3}$.

## 3.3 Characteristics of Conventional Pollutant Emissions

During the incineration of *S. plumbizincicola* the concentration of CO (>1000 mg $m^{-3}$) in flue gas was >10 times the Chinese Emission Standard (GB18484–2001) and the $NO_x$ emission was about 500 mg $m^{-3}$ (Table 4). With increasing temperature, the CO emission decreased but the $NO_x$ emission increased. Additives can change the release of CO and $NO_x$. The additive CaO significantly increased emissions of CO and $NO_x$. When the temperature was >850 °C, the concentrations of CO and $NO_x$ in flue gas were higher than the Chinese Emission Standard (GB18484–2001). Using kaolin as an additive gave the lowest concentration of $NO_x$, 461 mg $m^{-3}$, which is lower than 500 mg $m^{-3}$ (the Chinese Emission Standard). Using $Al_2O_3$

**Table 3** PAH concentrations measured in flue gas [μg $m^{-3}$ at 11% $O_2$] (Wu et al. 2013b)

| Treatment | Control | Activated carbon | $SiO_2$ + Activated carbon |
|---|---|---|---|
| Naphthalene | 4918 | 52.8 | 58.6 |
| ∑PAHs with 2 rings | 4918 | 52.8 | 58.6 |
| ∑PAHs with 3 rings | 30,451 | 90.8 | 289 |
| ∑PAHs with 4 rings | 92.6 | 8.34 | 2.48 |
| ∑PAHs with 5 rings | ND | ND | ND |
| ∑PAHs with 6 rings | ND | ND | ND |
| ∑PAHs | 35,420 | 152 | 350 |
| TEQ concentration | 35.8 | 0.40 | 0.39 |

**Table 4** $NO_x$, $SO_2$, CO, and HCl concentrations measured in flue gas (mg $m^{-3}$ at 11% $O_2$) (Wu et al. 2013b)

| Treatment | CO | $SO_2$ | NO | $NO_2$ | $N_2O$ | $NO_x$ | $NH_3$ | HCl | HCN |
|---|---|---|---|---|---|---|---|---|---|
| Control | 1009 | 6.99 | 536 | 17.6 | 0.003 | 553 | 15.1 | 1.62 | 3.95 |
| Kaolin | 2444 | 0.08 | 426 | 34.2 | 1.43 | 461 | 38.2 | 0 | 5.16 |
| CaO | 2578 | 0.03 | 677 | 25.1 | 0.12 | 702 | 35.9 | 2.54 | 4.64 |
| $Al_2O_3$ | 1854 | 0.66 | 493 | 19.3 | 0.06 | 512 | 31.5 | 2.19 | 5.68 |

also decreased $NO_x$ in flue gas. $SO_2$ and HCl in flue gas were much lower than their respective Emission Standards (GB18484–2001).

## 3.4 Pyrolysis Products of *Sedum plumbizincicola*

The Thermal Gravity (TG) Analysis curves also show that the mass percentages of bottom ash to original biomass after incineration generally decreased with increasing temperature in the horizontal tube furnace (Zhong et al. 2015). Weight losses of *S. plumbizincicola* were 3.56–9.51, 49.8–50.8 and 73.9–81.2% at three temperature steps of <170 °C, 170–400 °C and 400–800 °C during the pyrolysis process.

During the pyrolysis of *S. plumbizincicola* the tar yield increased to 6.34 wt% at 650 °C and decreased to 3.19 wt% at 750 °C. The bio-oil yield increased from 22.0 wt% at 450 °C to 31.7% at 650 °C and decreased to 15.9% at 750 °C. Char yield decreased from 32.4 wt% at 450 °C to 22.5 wt% at 750 °C. The gas yield remained nearly constant up to 650 °C, then increased significantly at 750 °C. The main components of the pyrolysis oils were acids (at 450 °C) that decreased with increasing pyrolysis temperature. Pyrolysis at 650 °C led to the highest yield of alkanes with low-oxygen compounds being found in the bio-oil.

During the pyrolysis process of *S. plumbizincicola*, the kinetic analysis shows that the value of activation energy changed from 146.4 to 232.4 kJ $mol^{-1}$ and the value of frequency factor changed greatly from $1.34 \times 10^{11}$ to $8.99 \times 10^{15}$ $s^{-1}$ (Zhong et al. 2016). Heavy metals in the gas were not detected. At temperatures of 450–750 °C more than 43.6% of Zn remained in the char, whereas 54.4% of Zn was found in bio-oil at 750 °C. More than 87.6% of Cd was vaporized during pyrolysis and was detected in the bio-oil. By comparison, mass balances of heavy metals under pyrolysis conditions were higher than those under incineration conditions (Lu et al. 2012). The Zn content in flue gas increased with increasing temperature but the Cu and Cd contents fluctuated in an entrained flow tube furnace. In a horizontal tube furnace, incineration favoured the volatilization of Cd in contrast to pyrolysis in flue gas. The percentages of heavy metals in bottom ash after pyrolysis were 3.5, 2.7 and 2.3 times higher than those after incineration, especially for Cd, Pb and Zn, which indicates that the oxidizing atmosphere favours the transfer of these metals to the gaseous phase.

## 4 Conclusion

*Sedum plumbizincicola*, originally discovered in Zhejiang Province, east China, is a new *Sedum* species and identified as a Cd-Zn hyperaccumulator. In an area of Pb-Zn mining, the shoots of *S. plumbizincicola* accumulated up to 1470 μg $g^{-1}$ Cd and 14,600 μg $g^{-1}$ Zn. In hydroponic experiments the shoot accumulated 7010 μg $g^{-1}$ Cd and 18,400 μg $g^{-1}$ Zn. Spatial distribution analysis showed that the preferential sequestration of Zn in stem and leaf (and to a lesser extent, Cd in stem) into the epidermis, is an important mechanism for hyperaccumulation and detoxification in *S. plumbizincicola*. Nevertheless, the parenchyma cells, e.g. mesophyll in the leaf, cortex, and pith in the stem, play more important roles in the storage and detoxification of Cd than does Zn in *S. plumbizincicola*. Suitable agronomic measures including cultivation management, intercropping with other plant species, and nutrient management can enhance Cd and Zn phytoextraction efficiency by *S. plumbizincicola*. Intercropping and a rotating system of *S. plumbizincicola* with other crops may realize the combined benefits of phytoremediation of metal-polluted soil and simultaneously safe production of food, but activation of soil Cd by *S. plumbizincicola* roots and following effects on crops should be studied further. Repeated pot phytoextraction results indicated that soil properties, such as pH and metal concentrations, affect growth and metal removal efficiency. The removal efficiencies of Cd and Zn were high for relatively lightly and acidic polluted soils. During repeated phytoextraction, different available metal pools controlled metal desorption, and acid-soluble, reducible, and oxidizable fractions can be removed by the hyperaccumulator. Field assessment results confirm that phytoextraction using *S. plumbizincicola* is a promising technique for the remediation of slightly Cd-polluted soils without halting normal agricultural production. Finally, incineration and pyrolysis can be employed for the disposal of harvested *S. plumbizincicola* biomass.

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# Element Case Studies: Rare Earth Elements

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## Abstract

The growing demand of strategic resources, e.g. rare earth elements (REEs), for development of modern technologies has spurred an increase in mining activities and consequently a release of REEs into the environment, posing a potential threat to human health. Phytoremediation, regarded as an *in situ* and low-cost means to remediate polluted soils, uses the growth and harvest of hyperaccumulator plants that take up high concentrations of metals in their shoots, allowing metal removal from contaminated soil (phytoextraction) or commercial production of high-value metals (phytomining). In this chapter, we review the discovery of REE hyperaccumulators worldwide, particularly focusing on the fern species *Dicranopteris dichotoma* that preferentially takes up light REEs. Though less understood, mechanisms of REE uptake, translocation, and distribution in hyperaccumulator plants are also discussed. Finally, taking *D. dichotoma* as an example, we estimate the phytomining potential for REEs using this species, based on its biomass production, REE concentrations in the ash, and current market prices of REEs.

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## 1 Introduction

Rare earth elements (REEs), which include 15 lanthanides and yttrium and have similar chemical and geochemical properties, make up a special group of heavy metals in the research fields of environmental toxicology and plant nutrition. In recent decades, rapid industrialization and urbanization have spurred increased use of REEs in modern green technologies, such as high-strength magnets, electric vehicles, and medical devices (e.g. Long et al. 2010). In addition, REE micro-fertilizers have been widely used in agricultural activities in China since 1990, because the application of REE at low levels is thought to benefit both yield and quality of crops (Diatloff et al. 1999; Hong et al. 2000; Redling 2006). The growing demand for these elements has triggered an increase in mining activities and a subsequent release of REE residues into the environment, posing a potential threat to human health (Haley 1991; Hirano and Suzuki 1996). Although REEs are not as toxic as some other metals and metalloids such as Cd or As, they have long-term adverse effects on human health. It has been reported that the IQ of children exposed to high REE concentrations was significantly lower than that of children in control areas (Zhu et al. 1996). Long-term exposure to REE has been found to cause damage to the human circulatory and immunologic systems (Zhang et al. 2000). The ingestion of REE can also decrease the conduction velocity of the human nervous system (Zhu et al. 1997a) and increase the incidence of arteriosclerosis (Zhu et al. 1997b). Consequently, control and remediation of REE-polluted water and soil systems have become a serious environmental concern, and REE as a group have been classified as one of the main chemical pollutants in China since the 1990s (NNSFC 1996).

Phytoremediation is considered a cost-effective and environmentally acceptable process that employs plants to achieve remediation of polluted soils (e.g. Chaney et al. 1997). Among several phytoremediation techniques, phytoextraction or phytomining uses the growth and harvesting of hyperaccumulator plants that take up high concentrations of metals in shoots, allowing their removal from a contaminated site or commercial production of high-value metals (Chaney et al. 1997; van der Ent et al. 2015). The effectiveness of phytomining depends ultimately on the amount of metals that accumulate in the harvestable biomass per year; and some plants (e.g. *Alyssum murale*, a Ni-hyperaccumulator) meet both the requirements of high biomass yield and metal hyperaccumulation. In addition, a prerequisite of feasible phytoextraction or phytomining is gaining a comprehensive understanding of how targeted metals are assimilated and translocated to the aboveground part of hyperaccumulator plants. To date, the hyperaccumulators of heavy metals such as Ni, Zn or Cd have been intensively studied, but less attention has been paid to studying the mechanisms of REE hyperaccumulation and potential for REE phytomining.

## 2 REE Pollution in Soil and Their Bioavailability

The average concentrations of REEs (sum concentration of the various REEs) in Chinese soil and the Earth's crust are 177 μg $g^{-1}$ (Liang et al. 2005) and 189 μg $g^{-1}$ (Wei et al. 1991), respectively. No significant difference exists among them, but some agricultural soils in China (e.g. Changding of Fujian Province) have relatively higher REE concentrations (243 μg $g^{-1}$; Li et al. 2013a). It is reported that in Jiangxi Province, the REE tailings from mined ion-absorption clays that contain high quantities of REEs reached 31.4 Gt (billion tonnes) in the year 2000 (Guo et al. 2014). Because of high mobility within the tailings, REE readily disperse to the surrounding agricultural soil through water flow and wind. As a consequence, the agricultural soil near the mine tailings has much higher concentrations of total REE (870–1100 μg $g^{-1}$) than those distal from the mining area (243 μg $g^{-1}$; Zhu et al. 2002; Li et al. 2013a). In addition, the concentrations of La and Ce in the soil near the

REE tailings dam, in Baotou city, are as high as 11,100 μg $g^{-1}$ and 23,600 μg $g^{-1}$, respectively, which are 340 and 481 times higher than the average REE soil concentrations in Inner Mongolia, PR China (Guo et al. 2013). Soil contaminated by REE has become a serious environmental concern in many countries, especially PR China.

The bioavailability of REE in soil is a critical factor that determines the amounts of these metals that can be accumulated by plants. In a preliminary trial for remediating ionic REE tailings at Ganzhou, Jiangxi Province, we found that tailings contained a total REE concentration of 450–620 μg $g^{-1}$, the bioavailable fraction (extracted with 0.1 mM $CaCl_2$) of which accounted for 11 to 13% (Qiu et al. unpublished data). These proportions are similar to that measured in tailings (14.9%, using the Tessier extraction procedure) collected in Xinfeng county, southern Jiangxi Province (Wen et al. 2013). Because of the large volume of REE tailings that exist in Jiangxi, as mentioned above (Guo et al. 2014), the relatively high bioavailability of REE in tailings suggests potential for agromining using REE hyperaccumulator plants. However, in general the actual bioavailability is strongly affected by site-specific properties such as pH, and contents of adsorbing phases including clay, organic matter, and oxides (Groenenberg et al. 2010). Thus, one should evaluate the change of REE speciation and availability before practical agromining, because amendments with organic matter or secondary minerals are normally required to tackle the limiting factors (e.g. high bulk density, lack of organic matter, nutrients and clays) for successful plant colonization on tailings (Liu et al. 2015).

## 3 REE Hyperaccumulator Plants

The threshold concentration for REE hyperaccumulators is notionally defined as 1000 μg $g^{-1}$ in the dry biomass of the aerial parts (Wei et al. 2006). This criterion is the same as used for other trace metals (Co, Cu, Ni, Pb, Sb), metalloids (As), and nonmetals (Se), which is typically two or three orders of magnitude higher than that present in normal plants (e.g. van der Ent et al. 2013). Moreover, the bioaccumulation factor (BF), which is the quotient of REE concentration in shoots to that in soil, is generally required to be greater than one, being $>1$, indicative of a high ability of soil-to-plant REE transfer (Krzciuk and Galuszka 2015). To date, approximately 22 plant species have been reported to accumulate or hyperaccumulate REE through field investigations; these species occur across 11 families and 11 genera, with the greatest numbers being within ferns (Table 1).

The discovery of high REE accumulation in plants can be traced to 1930s. In studies on mutual dependence of the soil-plant composition, Robinson and Scribner (1938) and Robinson (1943) found, quite by accident, that hickory (*Carya cathayensis*) leaves contained detectable REE. The REE concentrations in the hickory leaves ranged from 3 to 2296 μg $g^{-1}$ dry weight (DW), with the highest level found in hickory that had grown on soils derived from granite and gneiss. Moreover, the ash of these leaves contained more than 2.5% REE. Later, high concentrations of REE in dry leaves (e.g. $>1000$ μg $g^{-1}$) were successively reported in a number of plant species, including *Carya tomentosa* (1350 μg $g^{-1}$ in ash; Thomas 2011), *Dicranopteris dicthotoma* (3358 μg $g^{-1}$; Wang et al. 1997; Fig. 1a and b), *Blechnum orientale* (1022 μg $g^{-1}$; Xiao et al. 2003), *Pronephrium simplex* (1234 μg $g^{-1}$; Lai et al. 2005), and *Pronephrium triphyllum* (1027 μg $g^{-1}$; Xue 2009). Despite less the lower capacity for REE accumulation, *Phytolacca americana*, a high-biomass plant that is naturalized worldwide (Fig. 1c), accumulated REEs up to 623 μg $g^{-1}$ in dry leaves, although plant REE concentrations varied substantially among sampling sites (Ichihashi et al. 1992). Recently, our field survey observed a high occurrence of *P. americana* within an ionic REE mine site in south Jiangxi Province, where the highest concentration of REEs reached 1012 μg $g^{-1}$ in leaves. Furthermore, *P. americana* exhibited a preferential accumulation of light REEs (LREEs) during soil-to-

**Table 1** Rare earth element (REE) hyperaccumulator and potential hyperaccumulator species reported

| Family | Genus | Species | Accumulation characteristics in foliage | Distribution | Reference |
|---|---|---|---|---|---|
| Juglandaceae | *Carya* | *Carya tomentosa* | ∑REE in foliage: 1350 μg $g^{-1}$ (in ash) | America | Thomas (2011) |
| Juglandaceae | *Carya* | *Carya cathayensis* | ∑REE in foliage: 2296 μg $g^{-1}$ | America | Robinson (1943) |
| Gleicheniaceae | *Dicranopteris* | *Dicranopteris dicthotoma* | ∑REE in foliage: 3358 μg $g^{-1}$ | China | Wang et al. (1997) |
| Blechnaceae | *Blechnum* | *Blechnum orientale* | ∑REE in foliage: 1022 μg $g^{-1}$ | China | Xiao et al. (2003) |
| Thelypteridaceae | *Pronephrium* | *Pronephrium simplex* | ∑REE in foliage: 1234 μg $g^{-1}$ | China | Lai et al. (2005) |
| Thelypteridaceae | *Pronephrium* | *Pronephrium triphyllum* | ∑REE in foliage: 1027 μg $g^{-1}$ | China | Xue (2009) |
| Euphorbiaceae | *Glochidion* | *Glochidion triandrum* | – | – | Koyama et al. (1987) |
| Phytolaccaceae | *Phytolacca* | *Phytolacca americana* | ∑REE in foliage: 623 μg $g^{-1}$ | Japan | Ichihashi et al. (1992) |
| Athyriaceae | *Athyrium* | *Athyrium yokoscence* | ∑REE in foliage: 202 μg $g^{-1}$ | Japan | Ichihashi et al. (1992) |
| Lindsaeaceae | *Stenoloma* | *Stenoloma chusana* | ∑REE in foliage: 725 μg $g^{-1}$ | China | Xiao et al. (2003) |
| Blechnaceae | *Woodwardia* | *Woodwardia japonica* | ∑REE in foliage: 367 μg $g^{-1}$ | China | Xiao et al. (2003) |
| Dryopteridaceae | *Dryopteris* | *Dryopteris erythrosora* | BF of La $>1$ | Japan | Ozaki et al. (2000) |
| Dryopteridaceae | *Dryopteris* | *Dryopteris fuscipes* | BF of La $>1$ | Japan | Ozaki et al. (2000) |
| Aspleniaceae | *Asplenium* | *Asplenium filipes* | BF of La $>1$ | Japan | Ozaki et al. (2000) |
| Aspleniaceae | *Asplenium* | *Asplenium hondoense* | BF of La $>1$ | Japan | Ozaki et al. (2000) |
| Aspleniaceae | *Asplenium* | *Asplenium ruprechtii* | BF of La $>1$ | Japan | Ozaki et al. (2000) |
| Aspleniaceae | *Asplenium* | *Asplenium ritoense* | BF of La $>1$ | Japan | Ozaki et al. (2000) |
| Aspleniaceae | *Asplenium* | *Asplenium subnomale* | BF of La $>1$ | Japan | Ozaki et al. (2000) |
| Aspleniaceae | *Asplenium* | *Asplenium trichomanes* | BF of La $>1$ | Japan | Ozaki et al. (2000) |
| Parkeriaceae | *Adiantum* | *Adiantum monochlamys* | BF of La $>1$ | Japan | Ozaki et al. (2000) |
| Blechnaceae | *Blechnum* | *Blechnum subnomale* | BF of La $>1$ | Japan | Ozaki et al. (2000) |
| Gleicheniaceae | *Dicranopteris* | *Dicranopteris strigose* | BF of La $>1$ | Japan | Ozaki et al. (2000) |

∑REE: the sum of 16 rare earth elements; BF (bio-concentration factor): the quotient of the concentration in leaf/frond of a plant to the concentration in the soil

root assimilation, and a preferential accumulation of heavy REEs (HREEs) during stem-to-leaf translocation (Qiu et al. unpublished data; Ming et al. 2017). Moreover, other potential hyperaccumulator species have been reported (Table 1), all of which are ferns. For example, Ozaki et al. (2000) reported that many pteridophyte species enriched in La and Ce belong to

**Fig. 1** (**a**) *Dicranopteris dichotoma*, a natural perennial fern species widely distributed in southern China, is a LREE hyperaccumulator plant around REE mining sites of Ganzhou, Jiangxi province; (**b**) *D*. *dichotoma* is able to propagate asexually and disperse its population horizontally via stolon in the REE-rich soil; (**c**) *Phytolacca americana*, a high-biomass herbaceous plant which prefers to accumulate high HREE, could colonize and complete its life history on REE mine tailings of Ganzhou, Jiangxi province. (Image credit Ming Yuan)

genera such as *Polystichum* and *Dryopetris* in the Dryopteridaceae, *Diplazium* in the Woodsiaceae, and *Asplenium* in the Aspleniaceae. The high concentrations of lanthanide elements within these ferns is suggested to render them better at adapting to changing environments.

Among reported REE hyperaccumulator plants (Table 1), the majority of studies has focused on *D. dicthotoma*, a fern species that is widely distributed in sub-tropical climatic zones (Fig. 1a and b). The concentration and fractionation parameters of REEs in samples of *D. dichotoma* from different sampling sites are summarized in Table 2. The total REE concentrations of *D. dichotoma* fronds differed greatly among sites, ranging from 225 to 2649 µg $g^{-1}$; BFs of all plant samples were mostly greater than one (2–75), indicating its great ability to accumulate and transport REEs, especially LREEs. Furthermore, strong negative Eu anomalies were observed in all *D. dichotoma* samples. Positive Ce anomalies were obvious in *D. dichotoma* plants that grew on the background sites and unmined area, whereas negative Ce anomalies occurred in plants from Hetai goldfield and Zn-Pb mining areas. Cerium in *D. dichotoma* from background sites and the unmined area showed the greatest ability for transportation from the underground to aboveground biomass (Wei et al. 2001; Wang et al. 2005; Miao et al. 2010; Li et al. 2013b).

## 4 Mechanisms of REE (Hyper) Accumulation

### 4.1 REE Uptake

Previous studies have reported that REE uptake is related to the original REE concentration pattern (e.g. the ratio of different REEs) in the soil. In the naturally grown REE hyperaccumulator *D. dichotoma*, Wei et al. (2001) found that the concentration pattern of soluble REEs in the soil A layer was very similar to that in roots; the Ce anomalies in *D. dichotoma* organs were similar to those of soluble REEs in the soil A layer. The negative Ce anomaly present in this plant was more obvious than that in the soil. Rare earth elements in soils generally occur in trivalent state except Eu and Ce. Cerium is typically present in tetravalent form ($Ce^{4+}$) that is more likely to precipitate by a hydrolytic reaction, making Ce difficult to accumulate in plants. Therefore, this phenomenon shows that the distribution characteristics of soil-soluble REEs may directly influence REE absorption by plants.

Because of the similar ionic radii and physiochemical properties of $La^{3+}$ and $Ca^{2+}$, the $Ca^{2+}$ channel is considered a specific pathway for the uptake of lanthanides. It was demonstrated that $La^{3+}$ might enter into root cells via the membrane and intracellular $Ca^{2+}$ channels; La also participated in signal transduction networks via Calmodulin (CaM) (Liu et al. 2012). However, the region in the mesophyll where REEs were concentrated was not the same as that for Ca. In mesophyll cells, REEs were observed mainly in chloroplasts (Ozaki et al. 2000). These results showed that REEs do not behave just as Ca, as was suggested for some plants, but that these metals are transferred to chloroplasts by special processes.

In addition, low-molecular amino acids and organic acids enhance the uptake and sequestration of LREEs in *D. dichotoma*, although the enhancement mechanism of each acid is different. In the soil-root system, organic acids (e.g. citric and malic acids) increased LREE desorption from soil, and thus enhanced the uptake of LREE into the soil solution by fern roots; histidine promoted the sequestration of LREEs in cells by forming complexes with LREEs (Shan et al. 2003).

### 4.2 REE Translocation

Through electron microscopic and X-ray microanalyses of *D. dichotoma*, LREE deposits were observed in the cell wall, intercellular space, plasmalemma, vesicles, and vacuoles of the root endodermis and stele cells but not in the Casparian strip of the adventitious root. Moreover, LREE deposits were observed in the phloem and xylem of the rhizome (Shan et al. 2003). These results suggest that in *D. dichotoma*, both symplastic and apoplastic

**Table 2** Information of sampling sites and concentration and characteristic parameters of REE in samples of *Dicranopteris dichotoma*

| Location | Study area | Soil type | ∑REE in leaf | ∑REE in soil | EFs | LREE/HREE | δCe | δEu | Reference |
|---|---|---|---|---|---|---|---|---|---|
| N 23°17′–23°20′<br>E 112°15′–112°22′ | Hetai goldfiel | Gold deposit | 1964 | 277 | 7 | 159 | 0.67 | 0.49 | Miao et al. (2010) |
| N 23°21′–E 112°14′ | Yunlougang | Background area | 424 | 159 | 3 | 77.8 | 5.98 | 0.43 | |
| N 23°22′<br>E 112°19′ | Wucun | Background area | 225 | 93.6 | 2 | 109 | 11 | 0.52 | |
| N 24°57′<br>E 115°29′ | Shipai village Wenfeng township Xunwu county | LREE-enriched mining area | 1660 | 1031 | 2 | 18.41 | 0.14 | 0.23 | Wei et al. (2001) |
| N 24°42′<br>E 115°0′ | Shitou village Chengjiao township Dingnan county | LREE-enriched mining area | 2271 | 1224 | 2 | 9.47 | 0.54 | 0.23 | |
| N 25°30′<br>E 115°6′ | Shuikou village Hanfang township Gan county | Mining area where both LREE and HREE were enriched | 1412 | 342 | 4 | 5.01 | 0.57 | 0.32 | |
| N 25°48′<br>E 115°6′ | Shibawan village Datian township Gan county | HREE-enriched mining area | 977 | 195 | 5 | 2.20 | 0.13 | 0.13 | |
| N 25°50′<br>E 114°50′ | Shahe township Gan city | Non-mining area | 1121 | 15 | 75 | 40.83 | 4.20 | 0.25 | |
| – | Guangxi Village Longnan County | LREE mine | 2649 | 331 | 8 | 6.78 | 0.26 | 0.40 | Wang et al. (2005) |
| – | Zudong Village Longnan County | HREE mine | 2090 | 207 | 10 | 6.90 | 0.24 | 0.05 | |
| – | Non-mining area in Longnan County | Non-mining Area | 1495 | – | – | 24.28 | 0.51 | 0.40 | |
| N 25°18′–26°02′<br>E 116°00′–116°39′ | Changding County | Abandoned REE mine | 2190 | 202 | 11 | – | – | – | Li et al. (2013a) |
| N 25°18′–26°02′<br>E 116°00′–116°39′ | Changding County | Non-mining | 351 | 499 | 0.7 | – | – | – | |

∑REE: the sum of 16 rare earth elements; BFs (bio-concentration factor): the quotient of the concentration in leaf of a plant to the concentration in the soil; LREE/HREE: the quotients of the sum concentrations of light rare earth elements (LREE, La to Eu) to heavy rare earth elements (HREE, Gd to Lu); δCe: $\delta Ce = Ce/(0.5Pr + 0.5La)$, the value of Ce, La and Pr are chondrite-normalized; δEu: $\delta Eu = Eu/(0.5Sm + 0.5Gd)$, the value of Eu, Sm and Gd are chondrite-normalized transport mechanisms for REEs co-exist (Shan et al. 2003). Wei et al. (2001) compared the accumulation coefficient of the soil-plant system and found that REE transport was easier from soluble REEs in soil A-layer to root, and from petiole to lamina, but was more difficult from stem to petiole. The most conspicuous fractionation of LREEs from HREEs appeared during the translocation of REEs from stem to petiole. In addition, Shan et al. (2003) found a modest increase of LREE in fern fronds amended with histidine, and proposed that free histidine is involved in the uptake and translocation of LREEs from roots to fronds, and in the sequestration of LREEs in fern fronds. In another study using tomato, it reported that aspartic acid, asparagine, histidine, and glutamic acid may be related to xylem long-distance transport of La and Y in tomato (Wu et al. 2009). Extraneous additions of aspartic acid, asparagine, histidine, and glutamic acid in a hydroponic solution enhanced La absorption in tomato, compared with La alone. Further work compared tomato with *P. americana* and showed that the La contents of the tomato increased by 449 μg and 139 μg in the presence of aspartic acid and asparagine, relative to *P. americana* (1801 μg), and were 1.25 times and 1.08 times the La content of *P. americana* (Wu et al. 2013). Therefore, aspartic acid and asparagine could potentially promote the remediation of La contamination in soil when used as a chelator (Wu et al. 2013). However, whether these compounds can have the same effect on other REEs or other plant species still needs investigation. Similarly, in wheat, HREE enrichment was found in leaves, being ascribed to the formation of stronger organic complexes of HREEs than LREEs within the xylem solution, thus suggesting that organic acids may be involved in the long-distance transport of REE, especially HREE (Ding et al. 2007).

## 4.3 REE Distribution

The distribution of REEs among the main organs of vascular plants differs considerably. However, roots typically have higher concentrations than other plant organs. Numerous studies have shown the decrease of REE concentrations in the order root > leaf > stem, in a variety of crops such as wheat and maize (Xu et al. 2002; Ding et al. 2007). Conversely, REE hyperaccumulators exhibited decreasing REE concentrations in the order leaf > stem > root (Wei et al. 2001). Furthermore, the total amount of REEs within different subcellular fractions of the *D. dichotoma* leaf were cell walls > organelles > the ‘soluble’ fraction (i.e. cytosol and vacuoles) > cell membranes (Wei et al. 2005). In the leaf of *D. dichotoma*, more than 50% of REE deposits were observed in the cell wall, which may be one of the mechanisms involved in REE hyperaccumulation. Within *D. dichotoma*, high concentrations of histidine in leaf cells also indicate that histidine could facilitate plant-cell loading of LREEs, which may promote the sequestration of LREEs by forming complexes with LREEs in the cells (Shan et al. 2003).

Although REEs have little phytotoxicity to plants at low concentrations, these metals may influence the physiological activities of plants. In naturally grown *D. dichotoma*, chlorophyll La and chlorophyll Ce were found (Zhao et al. 1999; Wei et al. 2005); chlorophyll REE could replace or partly replace chlorophyll Mg (Wei et al. 2004). Chlorophyll La is suggested to favour the photosynthesis process of plants and promote photosynthetic reaction II. Moreover, REE detoxification by proteins is likely one of the mechanisms against physiology toxicity by REE (Wang et al. 2003). Significantly, REE-bound DNA may play an important role in heredity (Wang et al. 1999).

## 5 Potential for Agromining of REEs

Phytomining or agromining is a technology that uses hyperaccumulator plants to take up heavy metals and recover them from the biomass for resource cycling. Phytomining technology has been applied in Ni phytoextraction by using the Ni-hyperaccumulator *Alyssum murale* (Zhang et al. 2014; van der Ent et al. 2015). Economic feasibility of phytomining depends on market price for the element, annual yield per unit area (biomass produced and contained amount of target element), and availability of surface areas enriched in the element (van der Ent et al. 2015). Current (2016) prices, in US dollars per tonne, are high to very high for $Pr_2O_3$ ($47,000), $Nd_2O_3$ ($38,500), $Eu_2O_3$ ($85,000), $Gd_2O_3$ ($15,000), $Tb_2O_3$ ($450,000), $Dy_2O_3$ ($185,000), $Ho_2O_3$ ($35,000), $Er_2O_3$ ($26,000), $Yb_2O_3$ ($28,000), $Lu_2O_3$ ($800,000), and $Sc_2O_3$ ($3000,000), but relatively low for $La_2O_3$ ($1800), $Ce_2O_3$ ($1500), $Sm_2O_3$ ($1800), and $Y_2O_3$ ($4000). These prices are from the website of the Association of China Rare Earth Industry (http://www.ac-rei.org.cn/portal.php) and were converted from renminbi (RMB) into US$ as of 19 September 2016.

*Dicranopteris dichotoma,* the hyperaccumulator plant for REEs, is considered appropriate for REE phytoextraction. Based on our field survey with *D. dichotoma* collected at a mining tailings site in Ganzhou, Jiangxi Province, we could expect to harvest 12 to 15 t of dry biomass of shoots per ha containing 0.2 wt% REE, yielding 240 to 300 kg REE ha$^{-1}$ (Qiu et al. unpublished data). In the same way, as for Ni phytomining, several methods can be explored to recover REE from ferns. The first stage is combustion, required to remove organic matter and obtain a concentrated bio-ore. Alternatively, the REE contained in the plant can be recovered by direct biomass leaching. Processes should, however, be adapted to the specificity of the hyperaccumulating plant(s), and especially to their high contents of Si and Al. The REE concentrations in the biomass and the ash of *D. dichotoma* obtained at 550 after 2 h are shown in Table 3. The quotient (mass of biomass)/(mass of ash) was 8.5, and the average recovery rate of REE was 93%. Ashes contained up to 1.6 wt% total REE, and for individual elements such as La, Ce, Pr, Nd, Sm, Gd, Dy and Y, the concentrations were up to 0.76 wt%, 0.14 wt%, 0.13 wt%, 0.41 wt%, 0.05 wt%, 0.03 wt%, 0.01 wt %, 01% and 0.04 wt%, respectively (Qiu et al. unpublished data). However, the ashes mainly contain amorphous phases, which make an effective acidic leaching nearly impossible (Laubie et al. 2016). The presence of Si leads to the formation of a glass, stabilizing REEs. Other processes are currently in development, e.g. direct extraction (Laubie et al. 2016). Several complexing molecules (i.e. citric and glutamic acids) have been tested to assess the extraction

**Table 3** REE concentration of *Dicranopteris dichotoma* collected from Ganzhou, Jiangxi Province

| | REE concentration in dry plant | REE concentration in ash (500 °C, 2 h) | REE concentration in ash is converted into that in dry plant | Recovery rate (%) |
|---|---|---|---|---|
| La | 983 | 7575 | 893 | 91 |
| Ce | 180 | 1421 | 167 | 93 |
| Pr | 167 | 1310 | 154 | 92 |
| Nd | 504 | 4069 | 480 | 95 |
| Sm | 65.9 | 527 | 62.1 | 94 |
| Eu | 9.21 | 72.4 | 8.52 | 93 |
| Gd | 43.5 | 348 | 41.0 | 94 |
| Tb | 4.73 | 36.4 | 4.29 | 91 |
| Dy | 17.7 | 134 | 15.7 | 89 |
| Ho | 2.49 | 18.7 | 2.20 | 89 |
| Er | 4.98 | 40.7 | 4.80 | 96 |
| Tm | 0.50 | 4.80 | 0.57 | 114 |
| Yb | 3.23 | 23.5 | 2.77 | 86 |
| Lu | 0.50 | 4.05 | 0.48 | 96 |
| Y | 45.8 | 371 | 43.7 | 95 |
| Cs | 0 | 2.02 | 0.24 | – |
| ∑REE | 2032 | 15,956 | 1881 | 93 |

yield in the aqueous phase. EDTA seems to be a good candidate, yielding more than 80% extraction. Downstream steps such as calcination or selective precipitation should now be designed to recover Al and at least pure REE mixtures.

## 6 Conclusions

The widespread use of REEs in modern technologies and fertilizer have in some areas resulted in REE pollution in soils and caused serious problems for human health. Phytoremediation is a feasible means to decrease the harmful effects of metals in soil, and thus has great potential as a method for in-situ, low-cost remediation. Some plants like *D. dichotoma* have been proved to have a high accumulation ability for REE, and hence potential for REE phytomining. REEs can be taken up by plants through ion channels, especially $Ca^{2+}$. REEs cannot only store at cell walls but also in chloroplast, and thus increase photosynthetic activity of the plant. Low molecular weight organic acids likely have a major contribution to REE translocation. The above factors can also result in different fractionation characteristics among different organs. However, further studies are needed using a combination of plant physiological, molecular biological and geochemical tools in order to better understand specific mechanisms of REE fractionation and accumulation in plants.

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# Conclusions and Outlook for Agromining

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The development of agromining technology has created a new era in the recovery of strategic metals from natural or secondary resources, including subeconomic ores and industrial wastes. During the last decades, hyperaccumulator plants have changed recognition from merely a botanical curiosity to a prospect having tangible socio-economic and environmental applications. The knowledge base for this group of plant species, with unique properties capable of surviving and thriving in toxic and stressful environments, has increased greatly during the last 20 years, thanks to thorough investigations at various scales involving several disciplines, including botany, ecology, ecophysiology, microbiology, soil science and agronomy. The processes and mechanisms that preside over the hyperaccumulation of toxic metals and metalloids in plants are now better understood. For example, the fate and biopathways of elements in plants is actively being investigated using powerful new explanatory techniques, such as synchrotron and microprobe analysis. In addition, new species with exceptional ability to accumulate metals continue to be discovered, thereby considerably increasing the number of known hyperaccumulators. In parallel, work conducted by agronomists and soil scientists has allowed the domestication of selected hyperaccumulator species and hence enabling large-scale implementation of agromining. This approach promotes a new form of agriculture, which could generate income for communities in developing countries that live on agriculturally mediocre lands, such as those derived from ultramafic bedrock. Finally, the implication of scientific knowledge in the chain, such as chemistry and chemical engineering, and the stimulation of pluri-disciplinary research programs, bring hope to the feasibility

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of manufacturing specialist products of high industrial interest from agromined bio-ore.

Phytoextraction is a branch of phytoremediation that specifically aims to remove toxic metals and metalloids from polluted media (e.g. soils, sediments, wastes) using hyperaccumulator plants. The biomass derived from phytoextraction operations is considered contaminated waste, but in phytomining it is a source of valuable metals—a true bio-ore. Agromining encompasses the entire chain of processes that leads to the production of compounds of industrial and economic interest, in which agricultural systems are applied to metalliferous resources. Indeed, agromining is a combination of technologies, that has spawned a new sector aimed at producing strategic and valuable elements from agriculture and agroforestry. However, agromining should not be considered a substitute for conventional mining processes. It will not replace current mining technologies for satisfying demands by the global market for metallic elements, if only by the economy of scale. Agromining can, however, complement the range of ways of gaining additional value from natural ore deposits (with a very low environmental impact), whilst allowing for the utilization of resources that are otherwise uneconomic for metallic elements, especially in wastes.

Apart from areas that host metallic deposits, or areas that are polluted (e.g. sites impacted by smelting or mining), there are numerous sources of secondary materials derived from industrial activities that may be used as matrices for agromining. As such, agromining adds to the circular economy and is another means for effective recovery of valuable elements from (industrial) wastes. Although Ni agromining has been successfully demonstrated at different scales (including at the field scale) on ultramafic soils, agromining is not confined to Ni as many other elements may be potentially agromined. These include elements of industrial and economic interest such as Cd, Co, platinum group elements (PGEs), rare earth elements (REE) and even Au, which are present at low concentrations in certain soils or mine tailings. Many of these elements can be recovered provided that the market and its fluctuations do not impair the economic value of the chain. Recovery of metallic elements of industrial interest, as well as those whose natural sources are increasingly depleted (e.g. P), are major challenges that agromining should carefully consider in the long term.

The performance of agromining is closely related to available resources of the target elements, and to the ability of plants to grow and extract these elements from the resources. In particular, the extent of bioavailability of the target elements in soils is a major factor that determines the potential of a site for agromining. This knowledge on bioavailability is of high value for developing a means of manipulating soil metal availability (e.g. in the rhizosphere), and for promoting practices that ensure maximum transfer of elements to and within plants. However, plants remain the key ‘tool’ in agromining, and improvement of yields relies on knowledge of their physiology and ecophysiology, and on our capacity to produce efficient cultivars. Agromining is still at an early stage of development wherein seeds used are rarely derived from an appropriate selection of the best cultivars, and plant breeding is certainly one of the main remaining challenges for future development and profitability. Therefore, attention should be drawn to both quantitative and qualitative performance of ‘metal crops.’ Quantitatively, the doubling or even tripling of the quantities of extracted metal yields with selected plants and appropriate agronomy can be forecast. Qualitatively, the limited number of elements of interest that can be recovered could be increased, and selection strategies should be oriented towards the most relevant elements, considering their intrinsic value as well as the value of the land where these elements can be potentially mined (e.g. susceptible and fragile ecosystems and organisms that are negatively impacted by conventional mining activities). Despite the substantial research effort demonstrated in this book, there is still insufficient knowledge about the botanical reservoir, physiology, ecophysiology, and genetics of hyperaccumulation by plants. Molecular approaches for genomics, transcriptomics, and metabolomics should be encouraged in order to better understand the mechanisms of the hyperaccumulation phenomenon in plants, and to make this unique property a means of sustainable mineral-resource management.

Ultimately, agromining should be subjected to the same rules as conventional production systems such as food crops (e.g. wheat, rice, soybean). In food agriculture, production is driven by the market, which controls the demand in quantity and quality of products to be traded. This situation is similar to that for agromining, and decisions made by all actors along the chain of production (e.g. plant breeders, agronomists, farmers) are dependent on market needs. In addition, with growing awareness of the fragility of ecosystems, raised by the Millennium Ecosystem Assessment, and subsequent requested sustainable management, agromining should be equated with ecosystem conservation goals and hence follow the general trend of agricultural evolution. Indeed, agriculture is changing from intensive systems, often mono-specific with high inputs (i.e. fertilizers, pesticides, energy, water), to systems that rely heavily on soil biological functions. Within those agroecology and agroforestry systems, multi-functionality is sought in order to provide and optimize a wide range of ecosystem services. As a result, agromining could generate a wider range of services than solely the provision of particular metals, including regulating services that lack negative impact on biodiversity, and positive outcomes for carbon neutrality, contamination attenuation, and erosion control. Rehabilitation of mine sites and cultivation of ultramafic soils by agromining are suitable applications that promote this approach.

We believe that the ultramafic outcrops in many equatorial regions, especially South America, Southeast and the Pacific Region have tremendous potential for developing agromining. In these regions, agromining has the potential to provide opportunities for improving degraded land to make it suitable for other uses, by offering local communities with a new source of income and hence contributing to sustainable livelihoods. Agromining removes most of the nickel from the soil during the 10–30 years of operation and as a result improves soil fertility making it suitable for other future land uses. Unlike the competition between food crops and biofuels on fertile soils, agromining does not replace food crop production, but is a temporal activity that improves soil quality sufficiently to allow food crop production after the metal resource has been extracted.

A mature and sustainable agromining system will fit both industrial and market demand for bio-sourced compounds, and contribute to ecosystem services (e.g. no negative impact on biodiversity, reduction of soil run-off, carbon neutrality, etc.). It is rare in agricultural history to have such a new production system emerging almost *ex nihilo* and within such a short period of time. If research has made sufficient progress to ensure the transfer from concept to practical application, the concept must not mask the sophistication of the subject and the numerous questions that remain to be addressed in order to make this technology effective in the long term. The danger of too rapid communication without sufficient proof of concept, thus raising false expectations, is often the main reason of failure when launching a new technology. The establishment of large-scale agromining must be based on sound scientific information, as with more traditional agricultural production systems. Application of agromining at field scale, while respecting the concepts of agroecology and agroforestry, appears to be a feasible model of development both for disadvantaged regions and those areas that have suffered from over-exploitation of natural resources (e.g. over-logging, land clearance). Finally, it is important to be aware that any chain of production has not only technical implications, but is mainly controlled by social and economic constraints that are major drivers of the chain. Overall, we have a positive outlook on seeing the development of agromining for several metals, at scale, in various regions during the next decade.

Recently, there have been many indications that agromining is gaining momentum. In Europe, a milestone has been reached with the creation of an active network of academics collaborating to implement nickel agromining at the demonstration scale. This includes two large EU-funded projects started in 2016 that aim to promote the agro-ecological revolution of agromining and the implementation of pilot-scale agromining demonstrations. The Agronickel Project (ERA-NET FACCE Surplus), which involves scientists and companies from seven countries will create field demonstrations in Albania, Austria, Greece and Spain. The project encompasses the full chain from producing ‘metal crops’ to metallurgical processing of bio-ores, and ends with a Life Cycle Assessment. The LIFE Agromine Project (EU LIFE Programme) aims at establishing full-scale demonstration field plots for the production of bio-ore from ultramafic soils and from Technosols that contain Ni-rich wastes. These plots are located in five European countries and are linked to chemical engineering pilots to recover energy and produce speciality products from the bio-ore. Finally, several Ni mining companies have shown interest in using agromining to develop a new approach of managing mine rehabilitation. This technology could help improving the quality of reconstructed ecosystems by using hyperaccumulator plants as a pioneer vegetation cover while producing additional nickel from low-grade ores and mined land. On the other side of the world, in New Caledonia and Malaysia, recent herbarium X-ray Fluorescence (XRF) scanning of thousands of herbarium specimens has led to the discovery of hundreds of new nickel-hyperaccumulator species. Only a very small proportion of all hyperaccumulator species have a favourable combination of characteristics to be used as ‘metal crops’ in agromining, such as high target metal accumulation), fast growth rate and adaptability to grow in exposed conditions on poor soils. The recent discoveries add to the global inventory of known hyperaccumulators to draw from for selecting suitable ‘metal crops’. We anticipate that extending herbarium XRF scanning to other regions of the world (especially in extremely resource- and plant species-rich countries such as Brazil, Cuba, South Africa, etc.) will undoubtedly yield a ‘treasure trove’ of potential ‘metal crop’ candidate species, not only for nickel, but also for other elements such as cobalt, manganese and zinc.