Chapter 5

General Discussion
Hyperaccumulation of nickel (Ni), zinc (Zn), cadmium (Cd), manganese (Mn), arsenic (As), selenium (Se) (reviewed in Van der Ent et al., 2013), and thallium (Tl) (Pošćić et al., 2013) have been confirmed experimentally in one or more plant species. Lead (Pb) hyperaccumulation, however, has not yet been confirmed beyond doubt in any species. Natural foliar Pb concentrations exceeding or approaching the proposed 1000-µg/g nominal threshold criterion for Pb hyperaccumulation (Reeves and Brookes, 1983) have been reported (e.g., Johnston and Proctor, 1977; Reeves and Brooks, 1983; Assunção et al., 2003; Rotkittikhun et al., 2006), but all the reported cases are for lead mine tailings with soil Pb concentrations usually far above those of the foliage of the supposed Pb hyperaccumulators among the sampled plant species. This leaves open the possibility that apparent Pb hyperaccumulation in nature may in fact result from deposition of airborne contamination on the leaves, rather than from uptake via the roots and subsequent translocation to the leaves. Airborne contamination of plant samples may lead to erroneous claims of hyperaccumulation, such as demonstrated for several supposed south-central African Cu hyperaccumulators (Faucon et al., 2007). Therefore, claims of hyperaccumulation require validation through reproduction of the phenomenon under controlled, ecologically realistic conditions, while excluding potential airborne contamination (Van der Ent et al., 2013).

In chapter 2 we aimed to check the reproducibility of natural Pb hyperaccumulation from low concentrations in hydroponics. To this end we compared Pb accumulation and translocation in metallicolous and non-metallicolous accessions of the non-hyperaccumulator Silene vulgaris, the hyperaccumulator Noccaea caerulescens, and a novel putative Pb hyperaccumulator from Iran, Matthiola flavida. The natural variation in Pb accumulation appeared to be reproduced in N. caerulescens, in that one naturally Pb-hyperaccumulating accession also hyperaccumulated Pb from hydroponics (>1000 µg/g DW in leaves), already at a non-toxic 1-µM exposure level, whereas three naturally non-Pb-hyperaccumulating accessions did not. Although the shoot to root Pb concentration ratio in the Pb-hyperaccumulating accession remained below unity (± 0.6), it was much higher than in the non-Pb-hyperaccumulating ones, which further supports its Pb hyperaccumulator status. Its Pb tolerance, estimated from the root growth response, was not
significantly different from another calamine, but non-Pb-hyperaccumulating, accession, which suggests that Pb tolerance correlates with the Pb availability level of the soil at the site of origin, rather than the plants’ Pb hyperaccumulation capacity, such as previously demonstrated for Zn and Cd (Assunção et al., 2003). On the other hand, natural Pb hyperaccumulation was not reproducible in hydroponics in *Matthiola flavida*. Although a calamine accession of this species accumulated up to 7,800 µg/g in leaves in nature, while maintaining a shoot to root Pb concentration ratio above unity, it did not show any capacity to hyperaccumulate Pb from hydroponics. Other remarkable findings were that there was no difference in Pb tolerance between the metallicolous and the non-metallicolous accessions of this species, and that both accessions were even more Pb-sensitive than the non-metallicolous accession of the facultative non-hyperaccumulator metallophyte, *Silene vulgaris*, the latter being in turn considerably more Pb-sensitive than the conspecific calamine accession. A possible reason for the absence of Pb hypertolerance in the metallicolous *M. flavida* accession is the calcareous nature of its natural substrate, such as previously suggested for calamine accessions of other facultative metallophytes (Simon, 1978; Ernst 1982; Brown and Brinkmann, 1992). To test this hypothesis, the effect of calcium (Ca) on Pb tolerance and Pb accumulation and translocation was checked in metallicolous *M. flavida*, and it appeared that Ca strongly counteracted Pb accumulation and toxicity, indeed. Apart from this, high Ca appeared to enhance Pb root-to-shoot translocation, albeit only under low or moderate Pb exposure. However, the shoot to root Pb concentration ratio remained far below unity (up to 0.06).

The above results suggest that natural Pb hyperaccumulation was correctly reproduced in hydroponics experiments with low Pb concentrations in *N. caerulescens*, but not in *M. flavida*. This could be taken to suggest that Pb hyperaccumulation is ‘real’ in the concerned accession of *N. caerulescens*, but reflects an artifact, produced by airborne contamination, in *M. flavida*, the more so since the latter species possesses a dense foliar indumentum, which might promote the trapping of airborne contamination (Faucon et al, 2007). In addition, the natural substrate of the supposed Pb-hyperaccumulating *M. flavida* population is an extremely fine clay, which easily forms dust clouds under dry, windy
conditions. On the other hand, it cannot be excluded that inter-specific comparisons might be biased, owing to species x environment interactions regarding soil versus nutrient solution. Regarding Pb tolerance, the results are suggestive of such interactions indeed, particularly since in hydroponics the non-metallicolous *S. vulgaris* accession appeared to be more Pb-tolerant than the most Pb-tolerant metallicolous *N. caerulescens* accession, which is highly unrealistic. Likewise, it is possible that there are comparable species x environment interactions regarding Pb accumulation and translocation, as suggested by the hyperaccumulator-like foliar Pb concentrations under sub-lethal exposure in non-metallicolous *S. vulgaris*.

In chapter 3 the ability to hyperaccumulate from soil was checked in a controlled climate room experiment, using the same accessions. Plants were grown in two fertilized metalliferous substrates, one being a coarse-textured smelter slag (pH 5.9, Ca 1 mg/g), collected near Plombières, Belgium, which is the natural substrate of the metallicolous *S. vulgaris* accession and one of the metallicolous *N. caerulescens* accessions under study, the other being a fine-textured milled mine waste (pH 7.1, Ca 8 mg/g) from the Irankouh mining area, Iran, which is the natural substrate of the metallicolous *M. flavida* accession (Chapter 3). In the Plombières substrate only the native local accession of *S. vulgaris*, as well as a southern-french, calamine *N. caerulescens* accession, were able to maintain growth until the end of the experiment (40 d), whereas growth was completely arrested after a couple of days to weeks in the other *N. caerulescens* accessions. The non-metallicolous *S. vulgaris* accession as well as the metallicolous and non-metallicolous *M. flavida* accessions died shortly after germination. On the other hand, all accessions of all the three species were able to grow in Irankouh substrate, although the total acid-extractable Zn, Cd and Pb concentrations were very similar to those in Plombières substrate. Moreover, the non-metallicolous and metallicolous accessions of all the species performed equally well in the Irankouh substrate, apart from the *N. caerulescens* accession from serpentine soil, which performed less than the others, most probably owing to its hypersensitivity to Cd (Assunção et al., 2003). Thus, with this exception, the Irankouh substrate seemed to be barely metal-toxic to all the accessions tested. This is in agreement with the absence of detectable hypertolerance in hydroponics to Cd, Zn, or Pb in the local
metallicolous *M. flavida* accession, in comparison with the non-metallicolous one. The Plombières slag, on the other hand, was toxic for all the accessions. Even the ones that were able to maintain at least some growth until the end of the experiment (40 d) showed severe stunting (the calamine *N. caerulescens* accession from South-East France), and, in case of the local *S. vulgaris* accession, spotted necrosis of the leaves, which occurs also *in situ*. It was remarkable that the local *N. caerulescens* accession was not able to grow in its native substrate. This might relate to the fact that at the Plombières site *N. caerulescens* often grows in the center of old tussocks of *Festuca halleri*, rather than the bare slag, suggesting that it is partly protected from metal toxicity in its natural environment, possibly through organic matter accumulation in the upper soil layer. It is difficult to identify the specific metals that limit the performance of the accessions in Plombières slag. From the fact that the local *S. vulgaris* and *N. caerulescens* accessions are hypertolerant to Zn, Cd, and Pb (Schat et al., 1996; Mohtadi et al., 2012), and that these hypertolerances are under independent genetic control, at least largely (Schat et al., 1996), it can be safely assumed that all these metals are present at toxic levels for non-metallicolous accessions. The apparent low level of Pb toxicity of the Irankouh substrate, in comparison with the Plombières substrate, is probably attributable to the dominance of Ca over Pb in the exchangeable soil cation fraction (Simon, 1978; Brown and Brinkmann, 1992). It seems not unlikely that the apparent low levels of Zn and Cd toxicity of this substrate are likewise attributable to a high degree of Ca saturation of the cation exchange complex.

Considering the question of whether controlled experiments with soil can correctly predict hyperaccumulation in nature, it is interesting that all the *N. caerulescens* accessions hyperaccumulated Zn in their leaves (>10,000 µg/g) from the Plombières slag, regardless of whether they were able to maintain growth or not, whereas Cd hyperaccumulation (>100 µg/g) and a tendency towards Pb hyperaccumulation (500 – 900 µg/g) were confined to the south-eastern French population, which is in agreement with the natural accumulation patterns (Lombi et al., 2000; Reeves et al., 2001; Zhao et al., 2002; Assunção et al., 2003). On the other hand, none of the *N. caerulescens* accessions were able to hyperaccumulate Zn from the Irankouh substrate, while Cd hyperaccumulation (± 225 µg/g), and a tendency towards Pb hyperaccumulation (± 750 µg/g) were reproduced,
again exclusively by the southern-french accession. The most remarkable result was that the metallicolous *M. flavida* accession clearly hyperaccumulated Pb from its natural substrate (± 2200 µg/g), and approached closely the 100-µg/g threshold for Cd hyperaccumulation (97 µg/g). The non-metallicolous accession also accumulated Pb and Cd at relatively high concentrations in its leaves (± 700 and ± 90 µg/g, respectively). The foliar Zn concentrations were also relatively high in both accessions, that is, far below the hyperaccumulation threshold, but comparable with those of *N. caerulescens*. In conclusion, natural Pb hyperaccumulation in the metallicolous *M. flavida* accession is reproducible when it is grown in its native soil, but in the absence of airborne contamination. This demonstrates unambiguously that it is capable of Pb hyperaccumulation via root uptake and root-to-shoot translocation.

The reason for the extremely contrasting accumulation phenotypes of *M. flavida* in hydroponics (retention in roots) versus soil (hyperaccumulation in leaves) is elusive. Reported discrepancies between soil and hydroponics experiments usually displayed the opposite pattern, that is, hyperaccumulation from hydroponics, but not from soil, probably due to the use of unrealistic free metal concentrations in hydroponics (Van der Ent et al., 2013). It may be argued that *M. flavida* requires a particular microbial community for hyperaccumulation, which might not be present in hydroponics. However, it seems unlikely that any microbial community can turn a shoot excluder into a hyperaccumulator. As mentioned previously, it is conceivable that Ca might play some role here, because it promotes the root-to-shoot translocation of Pb. On the other hand, Ca counteracts Pb root uptake, and high Ca supply did not produce hyperaccumulator-like foliar Pb concentrations and shoot-to-root concentration ratios, at least not in hydroponics (Chapter 2). Another possibility is that Pb is accumulated inadvertently, instead of some other essential cation nutrient. If so, then Fe seems to be a good candidate for such a nutrient. When grown in Irankouh substrate, both *M. flavida* accessions, but particularly the metallicolous one, starts to develop light-green to yellow leaves within a few weeks after establishment. Since this chlorosis is particularly manifest in the youngest leaves, it might well represent Fe deficiency. If so, then Pb hyperaccumulation may be triggered by the Fe deficiency response, which includes rhizosphere
acidification and induction of IRT1/2-type transporters, which seem to have low degrees of metal-specificity and, therefore, capable to transport other cations when they are present in excess (Korshunova et al., 1999; Nakanishi et al., 2006; Nishida et al., 2011), although there are no data for Pb yet. However, although such a scenario may potentially explain a high rate of Pb root uptake, it does not necessarily explain why Pb accumulation takes place in the roots in hydroponics, but mainly in the leaves when plants are grown in Irankouh substrate. The latter would require a non-metal-specific Fe deficiency-induced translocation system, which has not been described thus far. In any case, the hypothesis that Pb hyperaccumulation in M. flavida is triggered by Fe deficiency is easy to test. First, it should be checked whether Fe-deficient plants can hyperaccumulate Pb from hydroponics. Second, it should be checked whether Pb hyperaccumulation from soil is suppressible by foliar Fe application.

Speaking in general, Pb transport in plants is very poorly studied, in comparison with, for example, Zn, Fe, Cu, or Cd transport. The transporters involved are barely known. It is generally assumed that divalent cation transporting P-1b-type ATPases, in particular HMA2, -3, and -4, can also transport Pb (Mills et al., 2003), although there is little evidence that they do so in planta (Gravot et al., 2004). Anyway, these transporters are either directly involved in xylem loading (HMA2/4), or vacuolar sequestration (HMA3), rather than root uptake. Pb uptake has been suggested to occur via Ca channels (Arazi et al., 2000), at least to some degree, in agreement with the results described in chapter 2 of this thesis. In any case, a basic understanding of Pb hyperaccumulation presupposes a much more profound fundamental knowledge of the molecular physiology of Pb uptake and plant-internal transport.

In Chapter 4 the possibilities to induce chelator-assisted Pb hyperaccumulation from hydroponics in M. flavida were investigated, using the natural chelator citrate, and the synthetic but biodegradable chelator EDDS. The results clearly showed that M. flavida does not possess high-affinity systems for uptake of either Pb-citrate or Pb-EDDS. In fact, when supplied at concentrations expected to bind virtually all of the Pb in the nutrient solution (1 µM), both chelators inhibited Pb uptake almost completely. However, at high Pb concentrations in the nutrient
solution (100 µM, 800 µM, 990 µM, each with 1 mM EDDS), Pb was accumulated at high rates, particularly in the shoot, even though the Pb in the nutrient solution must have been completely chelated. The latter clearly shows that Pb-EDDS can be taken up, though only when it is present at high concentrations. The uptake mechanism is elusive, but it seems that Pb-EDDS induces its own uptake, when it is present at concentrations above some threshold value. It is not clear whether this is also true for Pb-citrate, because the citrate concentrations needed to chelate 100 µM Pb more or less completely led to heavy bacterial infestation of the nutrient solution. Anyway, using EDDS as a chelator, foliar hyperaccumulation was evident when Pb was supplied at 800 or 990 µM (±1200 or ±6000 µg/g, respectively). However, these treatments completely inhibited root growth and killed the plants within a short period, apparently owing to toxicity of Pb-EDDS, rather than free EDDS, which was not detectably toxic. One hundred µM Pb-EDDS was not detectably toxic, but insufficient to induce foliar hyperaccumulation (±400 µg/g). It cannot be excluded that there is a narrow range of concentrations that allow foliar hyperaccumulation without killing the plant.

Finally, the results in this thesis clearly demonstrate that Pb hyperaccumulation through root uptake and root-to-shoot translocation does exist in nature. The question of whether natural Pb hyperaccumulators can be used to decontaminate soils through phyto-extraction remains unanswered. The Pb hyperaccumulators that were confirmed or identified in this study, *N. caerulescens* from south-eastern France and *M. flavida* from Iran, like all the other potential Pb hyperaccumulators reported in the literature, are both from extremely Pb-enriched substrates, where phyto-extraction is not a viable strategy for decontamination. It remains to be seen whether natural Pb hyperaccumulators can still hyperaccumulate Pb from slightly or moderately contaminated soils. If not, then chelator-assisted Pb hyperaccumulation is still an option. However, even though EDDS is biodegradable, it seems that it can only effectively induce Pb hyperaccumulation when the Pb-EDDS concentrations in the soil solution are at least close to lethal, which further enhances the risk of contaminant spread via the ground water.
References


Pošćić F, Marchiol L, Schat H, (2013) Hyperaccumulation of thallium is population specific and uncorrelated with caesium accumulation in the thallium hyperaccumulator, Biscutella laevigata. Plant and Soil 365, 81-91

Reeves RD, Brooks RR, (1983) Hyperaccumulation of lead and zinc by two metallophytes from a mining area in Central Europe. Environal Pollution 31, 277-288


Schat H, Vooijs R, Kuiper E, (1996) Identical major gene loci for heavy metal tolerances that have independently evolved in different local populations and subspecies of *Silene vulgaris*. Evolution 50, 1888-1895

Simon E, (1978) Heavy metals in soils, vegetation development and heavy metal tolerance in plant populations from metalliferous areas. New Phytologist 81, 175-188
