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# Arbuscular mycorrhizal fungi alter Hg root uptake and ligand environment as studied by X-ray absorption fine structure

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ABSTRACT

Mercury (Hg) – plant – fungal interactions are only poorly studied. Hg speciation and ligand environment in maize roots inoculated with arbuscular mycorrhizal (AM) fungi were investigated in order to better understand the role of AM in Hg soil to root transfer.

The maize plants were grown in Hg polluted substrate (50 $\mu g\,g^{-1}$ as dissolved $HgCl_2$) and inoculated with AM fungi originating from: a) highly Hg polluted environment of a former Hg smelting site in Idrija, Slovenia, (*Glomus* sp. – sample AmI), and b) non-polluted environment (commercial AM inoculum Symbivit® – sample AmC). Hg speciation and ligand environment in maize roots was studied by Hg-$L_3$ XANES and EXAFS with emphasis on XAS methodology – modelling and fitting the XAFS spectra to extract in a reliable way as much information on Hg coordination as possible.

The AmI plants developed more arbuscules and less vesicles than the AmC plants, and also accumulated more Hg in the roots. A clear difference in Hg coordination between the AM (AmC & AmI) and the control (ConC & ConI) plants is recognized in Hg $L_3$-edge EXAFS analysis: in the ConC & ConI maize roots 73–80% of Hg is attached between two sulphur atoms at the distance of 2.34 Å. The remaining ligand is nitrogen at 2.04 Å. In AmI & AmC roots another Hg-S attachment encompassing four thiol groups at the S-distance of ~2.50 Å are identified, accounting for 21–26%. AM fungi can modify Hg ligand environment in plant roots, thus playing an important role in biogeochemical cycling of Hg in terrestrial ecosystems.

## 1. Introduction

Mercury (Hg) is one of the most toxic and hazardous metals found in environment. For its mobility and persistence it is considered a global pollutant. It accumulates in food webs with biomagnification pattern posing a threat to human and animal health (Adriano, 2001). The occurrence of Hg polluted soils is associated either with natural deposition or anthropogenic dispersion from mining and smelting activities as in the case of Idrija in Slovenia or Almaden in Spain (Carrasco-Gil et al., 2013; Miklavčič et al., 2013; Morel et al., 1998).

Hg can be found in environment as a metal ($Hg^0$), in ionic form ($Hg^{2+}$), or as a constituent in metal ores (*e.g.* cinnabar, HgS). Its methylated forms ($CH_3Hg^+$, $(CH_3)_2Hg$), exert the highest toxic effect in animals and humans (Clarkson, 1972). Hg transformations, generally through reduction or methylation and demethylation, strongly depend on environmental conditions and affect its toxicity and distribution in the environment (Clarkson, 1972).

Phytostabilization is an *in situ* technology involving soil amendment and metal-tolerant plants to establish a ground cover that can reduce migration of metals to air, surface and ground

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*Abbreviations:* AM, arbuscular mycorrhiza; EXAFS, extended X-ray absorption fine structure; XANES, X-ray absorption near edge structure; AmI, maize plants inoculated with AM fungi from Hg polluted Idrija site; AmC, maize plants inoculated with commercial AM fungal mixture; ConI, maize plants inoculated with soil bacteria extracted from Hg polluted Idrija site; ConC, maize plants inoculated with soil bacteria extracted from commercial AM inoculum; Con, maize plants grown in sterile soil.

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water and reduce soil toxicity. The roots of plants with excluder metal tolerance strategy provide binding sites for metals that are either sequestred in the vacuoles of root cortical cells or bound to the cell wall components (Barceló and Poschenrieder, 2003). It has been shown that a properly developed mutual symbiosis between suitable fungal endophytes and plants (mycorrhiza) improving nutrient and water acquisition and providing protection from metal toxicity (Audet and Charest, 2007; Rajkumar et al., 2012) may be crucial for the survival of plants in metal polluted areas. Among fungal endophytes, mycorrhizal fungi form the most intimate relationship with plants. Owing to their metal tolerance they have been found in great abundance in the roots of the plants from metal-enriched sites. In addition to mycorrhiza, dark septate endophytes may also assist in conferring tolerance to the plants in metal polluted environments (Likar and Regvar, 2013). Mycorrhizal fungi play an important role in biogeochemical cycling of metals in terrestrial ecosystems (Rhee et al., 2012). Introducing suitable plant-fungal systems to Hg- contaminated sites may therefore help to retain Hg in the soil or in the root system and lower Hg transfer from the soil to the above-ground plant parts as well as from the soil directly to the atmosphere via volatilization.

Knowledge about distribution and speciation of toxic metals in plants is of fundamental importance for understanding the accumulation and transport mechanisms and in turn for optimization of plant performance in phytostabilization technologies. The existing synchrotron radiation facilities offer optimal possibilities to get insight into metal distribution and speciation in plants via synchrotron X-ray fluorescence microscopy and X-ray absorption spectroscopy (XAS) (Gräfe et al., 2014; Koren et al., 2013; Vogel-Mikuš et al., 2010). The latter provides a suitable tool, sensitized to a particular target atom by the choice of the energy range of the absorption scan in the vicinity of the target absorption edge. With fluorescence detection mode the method can be extended to trace concentrations of heavy metal pollutants. The absorption signal, in adjacent spectral regions of XANES and EXAFS, carries the fingerprints of the immediate chemical surroundings of the target atom, with its ligands directly recognizable. In XANES method, the measured profile of the absorption edge is compared to the corresponding segments in the absorption spectra of standards – candidate chemical compounds of the metal.

The EXAFS signal is an interference pattern impressed onto a typically ~1000 eV track of the absorption spectrum above the edge. The signal is compared to a synthetic spectrum built from a hypothetic arrangement of the neighbour atoms to the target metal atom.

Hg-$L_3$ edge EXAFS and XANES have already been proven useful tools for determination of Hg speciation and ligand environment in different plants grown under natural and controlled conditions (Carrasco-Gil et al., 2013; Patty et al., 2009; Rajan et al., 2008). Under natural conditions Hg accumulates mainly in the roots (Carrasco-Gil et al., 2013; Debeljak et al., 2013). In *Marrubium vulgare* collected at the Hg polluted area in Almaden, Spain, Hg was found localized in outer root layers, namely rhizodermis, mainly in the form of HgS minerals, which were also the prevailing Hg species found in the surrounding soils (Carrasco-Gil et al., 2013). HgS formation was supposedly facilitated by sulphate reducing bacteria, or was attributed to the root surface contamination by soil particles (Carrasco-Gil et al., 2013). In hydroponics, however, Hg was accumulated in roots mainly in the form of thiol compounds (C-S-Hg-S-C coordination) (Carrasco-Gil et al., 2013) and mainly localized in the cell walls. It is probably bound to the structural proteins such as expansins and extensins, which contain characteristic cystein-rich regions and play an important role in maintaining cell wall structure, integrity and normal functioning (Carrasco-Gil et al., 2013). Binding of Hg to these proteins is also in line with the observed root deformation caused by Hg. In a XANES study of Hg methylation in shredded *Eichornia crassipes*, the technique of linear combination fit (LCF) of XANES spectra with spectra of methylated and (in)organic Hg(II) model compounds revealed that in plants grown in aerobic conditions 5 ± 3% of Hg was in methylated form, while in the shredded plants in anaerobic conditions the percentage of methyl-Hg increased to 22 ± 6%. The rest of Hg was bound to thiol (-SH) ligands (Rajan et al., 2008). In hydroponically grown *Spartina* cordgrass Hg was mainly bound as Hg(II)-cysteine, as methyl-mercury acetate and, interestingly, as HgS (cinnabar and meta-cinnabar) (Patty et al., 2009). The authors claim that HgS formation by sulphate reducing bacteria in the plant rhizosphere is well established and has a potential for phytostabilization of Hg, since Hg complexation into the highly insoluble HgS would reduce leaching as well as Hg volatilization (Patty et al., 2009). Although plants as well as fungi possess enzymatic machinery for sulphate reduction, and sulphide ion ($S^{2-}$) or $H_2S$ is one of the intermediates in transformation of inorganic sulphur compounds, there is only little direct evidence on actual HgS formation in plants or fungi.

In the present study, using Hg $L_3$-edge EXAFS and XANES, the speciation and ligand environment of the metal in plants inoculated with a mixture of AM fungal strains (*Glomus* sp.) originating from highly Hg polluted environment of a former Hg smelting site in Idrija, Slovenia, and non-polluted environment (commercial AM inoculum Symbivit®) is correlated to the Hg uptake and the level of mycorrhization. In the aim to estimate the effects of mycorrhization on Hg soil-plant mobility, we hypothesized from the literature data (Audet and Charest, 2007) that AM fungi may at the soil Hg concentrations of 50 mg kg $^{-1}$ help to retain Hg in the soil by extramatrical mycelium, possibly as HgS complex (Carrasco-Gil et al., 2013), and suppress the transfer to the roots. The experiment showed a rather different picture. With this in mind we spent additional efforts on XAS methodology – modelling and fitting the spectra to extract in a reliable way as much information on Hg coordination as possible. The problem in application of the XAS method to the present study is the wide choice of candidate ligand atoms to Hg: C, N, O, S, P – to remain just within normal biochemistry. It means that there is no fixed, well-defined set of standards for XANES, and that a large number of candidate atomic neighbourhood need to be tested in EXAFS analysis. Moreover, in a sample from a living plant, a number of different ligands should be expected, their fingerprints separable only *a posteriori* in the analysis of the measured signal.

The task is somewhat simplified in the combination of the two methods. XANES method can be efficiently used to sort out the ligands not present in the sample. The method is not molecule-sensitive, just ligand-sensitive. It means, for example, that any monodentate Hg salt of a carboxylic acid and any complex to a –OH rich organic molecule will show the O- ligand fingerprint, with the rest of the molecule contributing only minor variations. It also means that presence of a molecular species in a sample cannot be proved with XANES alone, in spite of such claims in the literature (Arai et al., 2004; Patty et al., 2009; Rajan et al., 2008).

After weeding out with XANES, model Hg neighbourhoods for EXAFS analysis are constructed with the remaining collection of ligands, with preference to molecules known as Hg immobilisers. In the present case, sulphur is the best candidate, followed by nitrogen and oxygen. The ligand carbon, in methyl-Hg derivates, is well documented for its toxicity (Clarkson, 1972), but is supposed to be below the detection level (<5%) in the present case, with the plants grown in well aerated substrate. The testing of model neighbourhoods in the EXAFS analysis has been largely inspired by the excellent study of Hg-DNA interaction by B. Ravel et al. (Ravel et al., 2009).