VĚDECKÉ SPISY VYSOKÉHO UČENÍ TECHNICKÉHO V BRNĚ

Edice Habilitační a inaugurační spisy, sv. 602
ISSN 1213-418X

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INTERACTIONS OF TOXIC METALS AND METALLOIDS WITH FUNGI

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INTERACTIONS OF TOXIC METALS AND METALLOIDS WITH FUNGI

INTERAKCIE HÚB S TOXICKÝMI KOVMI A POLOKOVMI

SHORT VERSION OF HABILITATION THESIS

VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ
NAKLADATELSTVÍ VUTIUM

BRNO 2018

KEYWORDS
Filamentous fungi, biosorption, bioaccumulation, bioleaching, remediation, heavy metals

KĽÚČOVÉ SLOVÁ
Vláknité huby, biosorpcia, bioakumulácia, biolúhovanie, remediácia, ťažké kovy

DEPOSITION OF THESIS:
The original work is deposited at Faculty of Chemistry, Brno University of Technology

ISBN 978-80-214-5657-0
ISSN 1213-418X

ACKNOWLEDGEMENTS

This work was supported by the Scientific Grant Agency of the Slovak Republic Ministry of Education and the Slovak Academy of Sciences under VEGA contract No. 1/0146/18.

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1 INTRODUCTION

Filamentous fungi are ubiquitous, evolutionarily successful group of eukaryotic organisms that play various vital roles in Earth's ecosystem (Smith and Read, 2008). They are efficient and essential decomposers of complex organic matter (especially that of plant origin), including cellulose, lignin, chitin and keratin. They are involved in processes of nutrient recycling and humification, thereby affecting biogeochemical cycles of various elements (Gamauf et al., 2007; Klein and Paschke, 2004). Their importance in terrestrial habitats is also highlighted by their significance in formation and stabilization of soil aggregates. There is also an aspect of various types of mutualistic symbioses that makes fungi essential for wide range of organisms (Christensen, 1989).

Diverse in morphology, physiology and ecology, fungi have also negative impact on human welfare as agents of plant diseases, biodeterioration or as animal and human pathogens (Arzanlou et al., 2015). However, they have been also harnessed for their metabolic activity in commercial applications. They are useful in production of proteins or some simple organic compounds (McNeil and Harvey, 2006); and fermented foods and beverages have been part of human diet for millennia (Bennett, 1998).

All ecosystem-level functions carried out by filamentous fungi will be influenced in contaminated areas, where metal and metalloid pollutants can negatively affect their survival, fitness, and physiology, such as hyphal growth and the ability to produce extracellular enzymes or secondary metabolites (Dighton, 2016). To minimize the adverse effects of exposure to critical concentrations of toxic metals and metalloids, fungi execute various morphological, physiological and molecular changes to maintain the cell homeostasis. Various extracellular and intracellular mechanisms increase metal(loid) tolerance in fungi by avoiding entry of metal(loid)s and reducing their burden in cytosol (Bellion et al., 2006). However, these natural "detoxification" processes can be also exploited for (i) remediation of contaminated areas or (ii) recovery of metals and metalloids from secondary ore and waste materials. This includes mostly processes of biosorption, bioaccumulation , biovolatilization and bioleaching which are shortly discussed in this work.

2 FUNGAL CELL WALL AND ITS SIGNIFICANCE FOR TOXIC METAL(LOID)S IMMOBILIZATION

The fungal hyphae consists of chains of elongated cells which expand from the top apex of the cell (Brand and Gow, 2009). Its growth is accompanied by secretion of exoenzymes involved in lysis of substrates and cell wall synthesis (Archer and Wood, 1995). Cell wall of filamentous fungi consists mainly of cross-linked polysaccharides. This dynamic structure protects cells against changes in osmotic pressure and various environmental stressors. Cell wall's major chemical components are chitin, glucans, mannans, and glycoproteins (Bowman and Free, 2006).

Fungal cell wall is rigid, but flexible "exoskeleton". The flexibility of this structure is essential for rapid and effective response to external and internal stimuli. Microscopic filamentous fungi dynamically change their internal pressure, in accordance with the effect of (exo)enzymes, in order to control the hyphae growth in direction of nutrient gradient or chemoatractors (e.g. hormones), and to avoid unsuitable habitats (Steinberg, 2007).

Fungal cell wall is a very complex structure. The components of the fungal cell wall are divided into: (i) structural components, in particular chitin, β(1-6) -glucan and the β(1-4) -glucan; (ii) intra-structural component known as a matrix, formed mainly of mannoproteins, galacto-oligosaccharides, xylo-mannoproteins, glucuronate-mannoproteins and α(1-3)-glucan. Other major chemical components are various amino acids covalently or non-covalently linked to polysaccharides, and lipids and their derivatives (Feofilova, 2010). The fibrils of chitin (poly-*N*-acetyl-D-glucosamine) in complex with glucans form the basic structure of the cell wall.

The cell wall's biopolymers provide a wide range of functional groups which interact with the dissolved ions of potentially toxic elements in the environment. These are in particular carbonyl, carboxyl, hydroxyl, thiol, and amino groups. If deprotonated, these Lewis bases interact with metal cations on cell wall surface to create internal or external surface complexes. Ions and molecules, however, may interact with the cell wall in terms of a three-dimensional structure and can be incorporated into its matrix (Gadd, 2009). Thus, fungal biomass provides structurally diverse functional groups that can effectively bind and immobilize potentially toxic components from the environment via mechanism of *biosorption*. The chemical composition and structure of the fungal cell wall is therefore essential for its use in the remediation processes. Any change in chemical composition or structure may significantly affect biosorption efficiency. Our experimental results, depicted on **Fig. 1**, show that treatment of *Aspergillus niger* and *Neosartorya fischeri* biomass surface with 0.1 mol.L^{-1} HCl resulted in decrease of biosorption capacity for As(V). This was not unexpected, as similar results were reported by Sathishkumar et al. (2008) who studied As(V) uptake by native and pre-treated biomass of *Aspergillus fumigatus* in column mode adsorption study where acid treated fungal biomass showed minimum As(V) removal. In our batch experiment, acid treatment had more pronounced effect on *N. fischeri* strain's biomass sorption capacity compared to that of *A. niger*. Such significant difference in sorption performance of studied fungal strains after acid treatment points to potentially distinct composition of their cell walls. Besides other chemical modifications of cell wall's biopolymers, hydrochloric acid hydrolyses intra-chain glycosidic linkages and glycosidic linkages of branching points (Jelsma and Kreger, 1975) which affects distribution of available sorption loci and, thus, the overall biosorption performance.

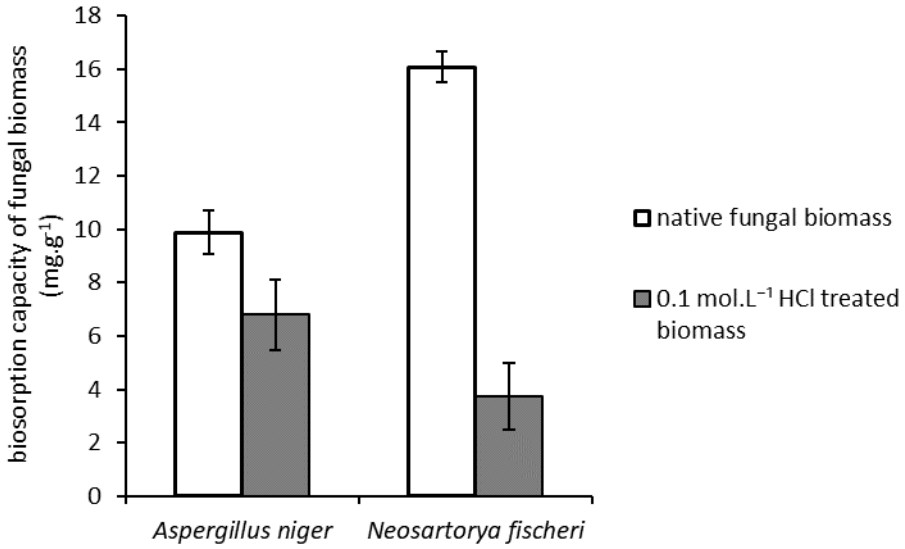

Fig. 1: Biosorption of As(V) by native and chemically modified fungal biomass with 1.0 mg.L^{-1} initial As(V) concentration (Littera et al., 2011).

Structure and properties of the cell wall and cell surface are also affected by the presence and availability of potentially toxic elements in the fungal environment. They mostly affect (i) morphology and structure of the mycelium, (ii) physiological functions of membrane and the cell wall, (ii) activity of exoenzymes and (iv) synthesis and the secretion of secondary metabolites and building blocks of cell wall's polymers (Mishra and Malik, 2013). This results from the general ability of potentially toxic metals and metalloids to form strong coordination bonds and thereby (i) block the essential functional groups in macromolecules, (ii) substitute essential metals in biologically important molecules, (iii) alter their conformation and (iv) undermine the integrity of the cell membrane (Kiss and Osipenko, 1994; Ochiai, 1987).

A common manifestation of stress on a macroscopic scale is modification of structure and morphology of mycelium. This relates to (i) distortion of catabolic pathways and ATP synthesis which reduces the growth rate, and (ii) alteration of the growth apex activity and reduction in branching of hyphae which result in the disruption of the mycelium's edge and reduction of its surface area (Lundy et al., 2001). In the latter case, it is a natural phenomenon that manifests in a hostile environment which forces fungus to change its growth strategy. In the presence of Cu(II) the *Trichoderma virens* strain produces long, unbranched hyphae which main purpose is to survey the substrate. At the end of the hyphae, the aggregated mycelial structures are formed. Their formation is probably inspired by efforts of microorganism to reduce the toxic metal exposure (Fomina et al., 2003). However, there is also a local pre-concentration of extracellular detoxifying agents with chelating properties (e.g. organic acids, siderophores), precipitating agents (e.g. oxalate), metal-binding pigments (e.g. melanin) and polysaccharides (Baldrian, 2003; Gadd, 1993). This strategy significantly increases the viability of filamentous fungi in presence of toxic contaminants (Gadd et al., 2012).

Ions of metal(loid)s are also responsible for changes in cell wall's pigments and other secondary metabolite production. Synthesis of pigments, which effectively bind metals (Mani et al., 2015), is intensified in presence of toxic metal(loid)s. Fungal isolate *Penicillium* sp. significantly increases production of yellow pigment in the presence of Zn^{2+} (Ezzouhri et al., 2009). These pigments are important components of the fungal cell wall (Pihet et al., 2009); and because of the presence of various active groups with high affinity to a toxic elements, they also significantly contribute to reduction of fungal sensitivity towards the elevated concentrations of potentially toxic metal cations (Caesar-Tonthat et al., 1995; Fogarty and Tobin, 1996). Similarly, the extracellular surface active compounds reduce the toxic effects of the elements. *Curvularia lunata* produces glycoprotein type compound which contributes to filamentous fungal resistance to Cd^{2+}, Zn^{2+} and Pb^{2+} (Paraszkiewicz et al., 2007).

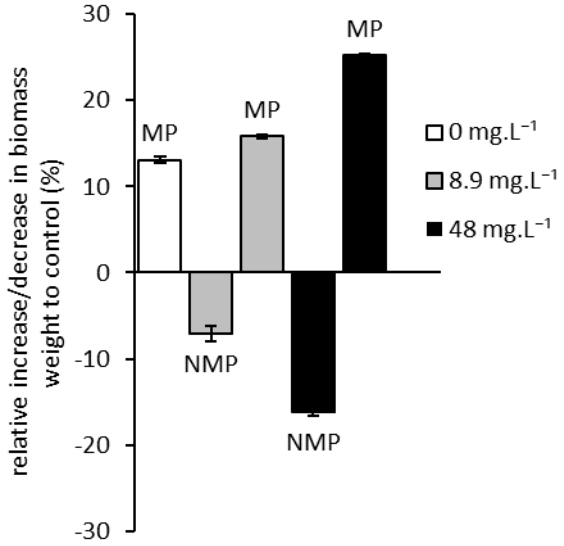

Fig. 2 Relative biomass dry weight increase or decrease compared to Sb(III)- and hausmannite-free control after 14-day cultivation period in hausmannite treatments (MP) and treatments without mineral phase (NMP). Values in legend indicate initial antimony concentrations in culture media (Milová-Žiaková et al., 2016).

Production of all above mentioned metabolites, however, is non-specific response to stress, including exposure to toxic metals. Their production is mostly dependent on the availability of nutrients which are not limited in standard laboratory cultivation media. Laboratory cultivation enables microorganisms to produce secondary metabolites without restriction of nutrient

availability. This factor should therefore be taken into account when extrapolating laboratory based experiments for more limited *in situ* conditions.

We have reported that excess of nutrients eases the toxic metals and metalloids' negative effect on growth parameters. For example, uptake and distribution of manganese is critical for proper function of various manganese-requiring enzymes, including free radical detoxifying enzymes (Whittaker, 2010). Unambiguously, manganese excess enhances fungal growth and disrupts the deleterious effects of Sb(III) (Milová-Žiaková et al., 2016). **Fig. 2** highlights that the biomass dry weight in hausmannite [$(Mn^{2+}Mn^{3+})_2O_4$] presence was significantly higher compared to control, even in Sb(III) treatments. This finding is also explained by (i) excellent sorption properties of applied mineral phase restricting antimony bioavailability in culture media during initial growth phases and (ii) its role as fungal growth enhancing nutrient (Ball and Banik, 2011; Behera et al., 2013).

2.1 BIOSORPTION

Almost all interactions of metals and metalloids with cell wall's components that lead to immobilization of designated toxic chemical species is generally described as process of biosorption. Therefore, biosorption, in context of this work, is defined as a process of pre-concentration of various soluble potentially toxic elements and their species from liquid medium onto/into biological surfaces (Gadd, 1990). In case of filamentous fungi, the biological surface is considered the cell wall and outer part of plasmatic membrane. It is also the first barrier to avoid the entry of substances into the intracellular space. Biosorption, in contrast to *bioaccumulation,* is process which is independent on metabolic energy (Tobin et al., 1994). However, metabolic processes can influence the biosorption efficiency indirectly, e.g. by metabolically induced changes in speciation of given analyte, and by actively regulating the formation, composition and behavior of the cell wall, as indicated previously.

Biosorption is an extremely complex process which involves various physical and chemical interactions on outer surface and within the cell wall. This includes metabolically independent and mechanistically complex processes of complexation/coordination, microprecipitation, adsorption, ion exchange and redox reactions (Tsezos, 2014) which usually take place simultaneously. Fungal *Rhizopus arrhizus* biomass sequesters U(VI) from aqueous media at least by three processes which include coordination and adsorption within the cell-wall chitin structure and microprecipitation of uranylhydroxide (Tsezos and Volesky, 1982). However, the precise binding mechanism is usually unknown and ranges from physical to chemical binding (ionic and covalent) (Brady and Tobin, 1995).

Because biosorption of monovalent and divalent cations (most studied toxicants in biosorption experiments) is usually accompanied by hydrogen cation or Ca(II) or Mg(II) displacement from the biomass, various authors suggest that biosorption occurs mainly via ion exchange (Davis et al., 2003; Naseem Akthar et al., 1995). Thus, biosorption is considered reversible process, except when microprecipitation is involved which may significantly inhibit desorption and shift the equilibrium unpredictably (Naja and Volesky, 2011). This happens by formation of new sorption loci on precipitates and also nonspecific deposition of precipitated substances in cell wall. Furthermore, precipitation may occur independently in bulk solution as a result of secondary metabolite production or leaching of substrate. Dolomite leaching by unknown species of *Mucor* and *Rhizopus* and simultaneous production of oxalate leads to sequential precipitation of Ca-oxalates which engulfs the fungal hyphae and lines the inner cell wall (Kolo and Claeys, 2005). Similarly, our results highlight the formation of new biogenic mineral phase during 14-day cultivation of *A. niger* (Milová-Žiaková et al., 2016). Filamentous fungi naturally produce great amount of oxalic acid (Aung and Ting, 2005; Santhiya and Ting, 2005) which is a suitable substrate for manganese biomineralization. The newly formed manganese containing biomineral

was identified by XRD (**Fig. 3**) as oxalate monohydrate - lindbergite [$Mn(C_2O_4).H_2O$)]. This unique fungal ability of rapid manganese biotransformation indicates the possible role of filamentous fungi as geoactive agent in manganese transformation (Mohanty et al., 2017) which also possesses capability to affect environmental fate of some nutrients and pollutants via sorption processes. Such newly formed biogenic mineral phase may serve both as a sink of heavy metals and natural barrier preventing entering cell interior (Fomina et al., 2005). This is why some authors differentiate between biosorption (as initiation of precipitation) and microprecipitation (Chubar et al., 2013), while others highlight precipitation significance for metal and metalloid biosorption by listing it as one of the biosorption mechanisms (Wang and Chen, 2006).

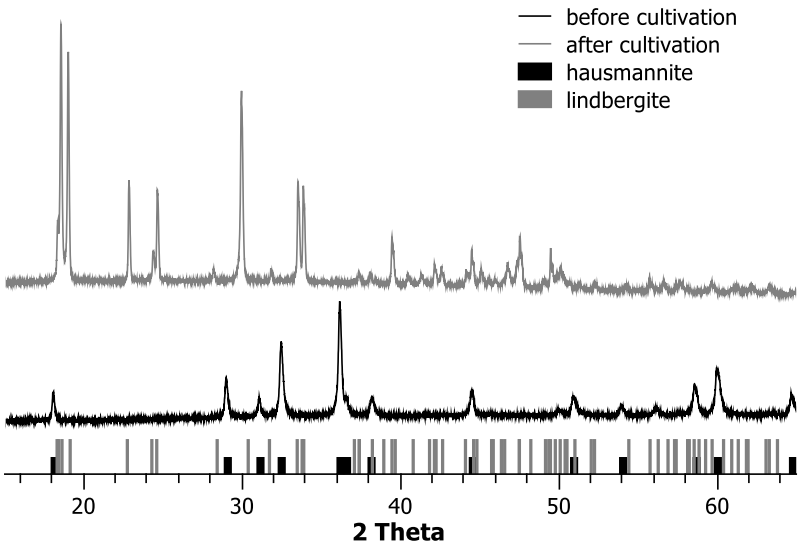

Fig. 3 XRD patterns of synthetically prepared and biotransformed manganese mineral phases highlight fungal transformation of hausmannite [$(Mn^{2+}Mn^{3+})_2O_4$] to lindbergite [$Mn(C_2O_4).H_2O$)] (Milová-Žiaková et al., 2016).

Nevertheless, the sorption experiments using common fungus *Rhizopus arrhizus* (Brady and Tobin, 1995) indicate that ion exchange is neither the sole nor the main biosorption mechanism, and while hard metals exhibit ionic bonding, borderline ions exhibit significant degree of covalent bonding. Therefore, the soft ions (e.g. Pb^{2+} and Ag^{+}) are sorbed preferentially, and biosorption capacity of given biomass positively correlates with the covalent index of metal ions (Chen and Wang, 2007; Kogej and Pavko, 2001). Especially the amino groups on the chitin and chitosan (deacetylated form of chitin) chain serve as efficient chelation sites and can be considered a strong Lewis base. In acidic solutions, these can be easily protonated and cause electrostatic attraction for anionic metals and metalloids (Guibal, 2004). Thus, chitosan and chitin have been applied successfully as efficient sorbents in various studies (Liu et al., 2013; Wang and Chen, 2014). On the other hand, esterification of carboxylic groups in fungal biomass of *Penicillium chrysogenum* and *Trichoderma reesei* significantly decreases the removal efficiency of Zn^{2+} by 55% and 70%, respectively (Fourest et al., 1996). This highlights the significant contribution of other cell wall functional groups in biosorption, and that the biosorption mechanism is mostly affected by chemical and physical properties of the fungal cell wall, analyte chemistry, but also involvement of environmental factors and metabolic processes prevailing in outer and inner cell environment (Bellion et al., 2006; Gadd, 2009). Furthermore, cell wall has some type of individual building structure hierarchy that determines its functionality and variability and also reflects the unique external and internal factors of the organism. It is clear that the presence of specific functional groups is important for biosorption phenomenon; however, it is also necessary to know structural and morphological characteristics of the cell walls. Therefore, studies on the chemical properties and binding force of certain reactive sorption position on cell wall must be also complemented

with information on its structural availability. Some highly reactive functional groups cannot be made available due to the fact that they are blocked within the structure of cell wall's biomacromolecules, or are located in the hydrophobic parts of the cell wall (Tigini et al., 2010; Torkkeli et al., 2002).

2.2 EVALUATION OF UPTAKE KINETICS AND SORPTION PROPERTIES OF FUNGAL BIOMASS

Evaluation of biosorbent's sorption performance in removal of potentially toxic metals and metalloids from aqueous media is generally oversimplified and mostly applies empirical equations of sorption kinetics and isotherm models. However, there is an extensive effort given to understanding of theoretical basis behind these mathematical models with overwhelming scientific discussion (Azizian, 2008; Liu and Liu, 2008). Although these empirical models usually describe the experimental data well, they cannot provide any relevant information on exact mechanism of sorbate binding (Kratochvil and Voleski, 1998). The reason behind is that the theoretical basis of any model is hardly applicable to all types of biosorption mechanisms which act simultaneously. As discussed previously, this involves physical sorption (electrostatic interactions, van der Waals forces), but also the chemisorption (ion exchange, complexation/chelation) (Davis et al., 2003).

Table 1 Most common models of sorption isotherm (modified from their original form) which are applied in biosorption experiments (S_{eq} is the amount of solute sorbed per unit weight of sorbent (mg.g^{-1}); C_{eq} is the equilibrium concentration of solute in the bulk solution (mg.L^{-1}); R is the universal gas constant (8.314 J.mol^{-1}.K^{-1}); T is the absolute temperature (K)).

Isotherm model	Equation	Parameters	Reference
Langmuir	$S_{eq} = \frac{S_{max} bC_{eq}}{1 + bC_{eq}}$	b is the constant related to free energy of sorption (L.mg^{-1}); S_{max} is the maximum sorption capacity (mg.g^{-1})	Langmuir (1918)
Freundlich	$S_{eq} = K_F C^{1/n}$	K_F is a Freundlich constant indicative of the sorption capacity at unitary C_{eq} (L.g^{-1}), n is a sorption site heterogeneity factor	Freundlich (1906)
Temkin	$S_{eq} = \frac{RT}{b_T} \ln\left(A_T C_{eq}\right)$	A_T is a Temkin isotherm equilibrium binding constant (L.g^{-1}); b_T is a Temkin isotherm constant related to heat of sorption (J.mol^{-1})	Temkin (1941)
Dubinin-Radushkevich	$S_{eq} = S_D \exp\left(- B_D \left[RT \ln\left(1 + \frac{1}{C_{eq}} \right) \right]^2 \right)$	S_D is a Dubinin-Radushkevich constant related to sorption capacity; B_D (kJ^2.mol^{-2}) is a constant related to the mean free energy of sorption	Dubinin and Radushkevich (1947)
Redlich-Peterson	$S_{eq} = \frac{K_R C_{eq}}{1 + a_R C_{eq}^{\beta}}$	K_R (L.g^{-1}) and a_R (L.mg^{-1}) are the Redlich-Peterson isotherm constants; β is the exponent, which transform equation into Langmuir isotherm and Henry's law at values of 1 and 0, respectively	Redlich and Peterson (1959)
Sips	$S_{eq} = \frac{S_m \left(k_S C_{eq}\right)^d}{1 + \left(k_S C_{eq}\right)^d}$	S_m is Sips maximum sorption capacity (mg.g^{-1}); k_S is the Sips constant (L.mg-1); d is the exponent of the Sips model	Sips (1948)

In terms of isotherm analysis, these mathematical models, given in **Table 1**, correlate biosorbent's sorption capacity at equilibrium (S_{eq}) and equilibrium concentration of analyte (sorbate) in solution (C_{eq}). Thus, sorption capacity has to be experimentally measured for different initial concentrations of sorbate solution at given pH, temperature and biosorbent/solution ratio (Volesky, 2007).

Generally, the biosorbent's sorption capacity increases with initial concentration of sorbate in the solution up to biosorbent's saturation, while the removal efficiency decreases. This highlights the limited availability of sorption sites provided by biosorbent, and chemical equilibrium basis of

this interaction. This also allows us to calculate maximum sorption capacity (S_{max}) which is often used as a practical indicator of sorbent's sorption properties compared to other sorbents. **Table 2** exemplifies this approach by applying equations of Langmuir and Freundlich isotherms on removal of Bi^{3+} by pelletized biomass of *Aspergillus clavatus*. Calculated S_{max} value approximates 0.35 $mmol.g^{-1}$, thus indicating that bismuth immobilization by *A. clavatus* fungal biomass is more effective than sorption to sorbents based on activated carbon prepared from coconut flakes whose Bi^{3+} S_{max} value was approximately 0.26 $mmol.g^{-1}$ (Sartape et al., 2012), and that it is superior to sorption to polymeric sorbent prepared from polyurethane at almost 0.19 $mmol.g^{-1}$ (El-Shahawi and Al-Mehrezi, 1997).

Table 2 Calculated Langmuir and Freundlich isotherm parameters for Bi^{3+} sorption onto pelletized *A. clavatus* biomass (T = 298.15 K; 130 rpm) (Boriová et al., 2015a), where K_F is a Freundlich constant indicative of the sorption capacity at unitary C_{eq} ($L.g^{-1}$), n is a sorption site heterogeneity factor, b is the constant related to free energy of sorption ($L.mmol^{-1}$); S_{max} is the maximum sorption capacity ($mmol.g^{-1}$); Akaike weight indicates the statistical probability that the model is the best among the whole set of candidate models.

Isotherm	K_F	n	S_{max}	b	R^2	Akaike weight
Freundlich	0.33±0.02	3.7	-	-	0.92	0.58
Langmuir	-	-	0.35±0.04	9.8	0.89	0.42

However, there are some special cases when biosorption performance increases with initial concentration. This is mostly because of new sorption sites formation due to specific properties of sorbate, e.g. precipitation of metals or their salts with subsequent deposition in cell wall (Rearte et al., 2013). Precipitation may occur in presence of redox active metal and metalloid species, such as Se(VI) which can be transformed to zerovalent selenium by microbial exometabolites. Zhang et al. (2003) reported up to 95% reduction efficiency of 2 $mg.L^{-1}$ Se(VI) by bacteria in a 7-day cultivation. However, our results (Urík et al., 2016) indicate that only negligible Se(VI) was reduced to a non-soluble zerovalent selenium residue by filamentous fungus *A. clavatus*. The reduction efficiency was less than 0.4% in case of the initial Se(VI) concentration of 89 $mg.L^{-1}$.

As we mentioned previously, composition of the cell wall has a significant impact on the process of biosorption of potentially toxic metals and metalloids as the distribution, type and spatial availability of functional groups between fungal species may differ significantly. This diversity is to the extent that some methods, including Fourier transform infrared spectroscopy combined with high-throughput liquid micro-cultivation, have been successfully applied for differentiation of fungi on the phylum, genus and species level (Shapaval et al., 2013). The pH of aqueous solutions may then affect fungal biosorption efficiency as each functional group have its specific optimum for metal binding. While at pH below 5 only carboxylic groups contribute significantly to metal removal, in neutral and alkaline solutions the contribution of phosphate, hydroxyl and amino groups to biosorption efficiency increases significantly (Chojnacka et al., 2005). However, since the zero point charge of fungal biomass is generally below pH 4, in highly acidic solutions the positive charge on biomass surface prevails (Aytar et al., 2014; Bairagi et al., 2011; Mukhopadhyay, 2008). This limits binding efficiency of metallic cations onto fungal biomass at low pH due to repulsive Coulombic forces.

Besides sorption site dissociation and protonation, pH also affects the solution chemistry of metals and metalloids such as hydrolysis, complexation with organic or inorganic ligands, redox reactions, and precipitation (Esposito et al., 2002; Yang and Volesky, 1999). This strongly influences speciation and biosorption availability of metals and metalloids in the solution. These differ in their binding efficiencies at given pH with various values of pH optimal for their most efficient immobilization in biomass (Tahir et al., 2017).

Complexity of pH effects on mutual interactions of metal(loid)s and fungal biomass is well documented in our thermodynamic study on *A. niger* and *N. fischeri* strains' sorption properties

(Littera et al., 2011) where unmodified, native fungal biomass of *A. niger* had a higher biosorption capacity at pH 5, whereas *N. fischeri* biomass was more efficient in As(V) removal at pH 7. This reflects the significance of both fungal biomass composition and behaviour of As(V) under different environmental conditions in removal of pollutants. It also highlights the complexity of biosorption process which is influenced by physical and chemical properties of the sorbate (e.g. ionic radii, oxidation state, molecular weight) and biosorbent (e.g. the structure and composition of the cell surface), as well as the conditions under which process is carried out (pH, temperature, concentration of sorbate and biosorbent) (Michalak et al., 2013). Furthermore, efficient immobilization of heavy metals by mycelial surfaces can be significantly altered by the presence of other ions or molecules, including humic acids. Although humic acids adsorption onto mineral phase surfaces or their presence in batch sorption system enhanced the removal efficiency of bivalent heavy metals (Arias et al., 2002; Lai et al., 2002), our study showed that the increasing amount of (pre)adsorbed humic acids onto *A. niger* biomass surfaces had different effect on the fungal sorption capacity for Zn^{2+} (Urík et al., 2014b). Mutual interactions between humic acids and pelletized fungal biomass on Zn^{2+} immobilization indicates that zinc affinity is higher for the fungal surface than for humic acids. These do not provide sufficient active zinc sorption sites, thus resulting in the decreased sorption capacity of mycelial pellets modified with humic acids compared to the unmodified biomass.

Table 3 Most common models of sorption kinetics (modified from their original form) which are applied in biosorption experiments (S_{eq} is the amount of solute sorbed per unit weight of sorbent at equilibrium (mg.g^{-1}); S_t is the instantaneous sorption capacity at time t (mg.g^{-1})).

Kinetics model	Equation	Parameters	Reference
Pseudo-first order	$S_t = S_{eq}\left(1 - \exp\left(-k_1 t\right)\right)$	k_1 is the pseudo-first order kinetic constant (min^{-1})	Lagergren (1898)
Pseudo-second order	$S_t = \frac{S_{eq}^2 k_2 t}{1 + S_{eq} t}$	k_2 is the pseudo-second order kinetic constant (g.mg^{-1}.min^{-1})	Ho and McKay (1998)
Pseudo-nth model	$S_t = S_{eq} - \left[S_{eq}^{1-p} - \left(1-p\right)k_n t\right]^{1/(1-p)}$	k_n is the pseudo-nth order kinetic constant (min^{-1}.(mg.g^{-1})$^{1-p}$) with p indicating reaction order	Özer (2007)

Another important aspect of biosorption is the rate at which the contaminants are removed from the aqueous media. There are numerous kinetic models that are capable of describing the mechanism through which the biosorption process takes place (**Table 3**). Experimentally, biosorption kinetics usually have biphasic character with rapid initial sorption of metal ions to the surface groups of the biomass followed by slow diffusion of metal to internal binding sites during the second phase, referred as intraparticle diffusion (Liu et al., 2006). This is in good agreement with our previous statement on the cell wall's three-dimensional structure significance for pollutant removal.

Biphasic character of biosorption is also highlighted in **Fig. 4** (Boriová et al., 2015a). Sorption of bismuth onto fungal biomass is a relatively fast process due to rapid attachment and the large number of sorption sites available at the commencement of this process. Subsequent slower sorption is attributed to intraparticle diffusion (Sen Gupta and Bhattacharyya, 2011). Our study on kinetics of Se(IV) removal by *A. niger* biomass (Urík et al., 2011) supports rapid selenium biosorption process where dynamic equilibrium was reached after 20 min, although the calculated 1.1 μmol.g^{-1} maximum biosorption capacity is negligible and with no practical perspective in waste-water treatment.

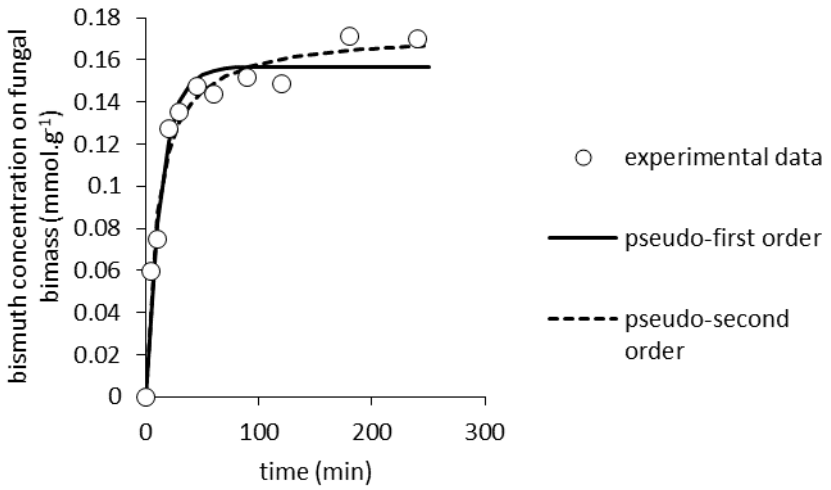

Fig. 4 Bismuth sorption kinetics onto *A. clavatus* biomass with initial Bi^{3+} concentration of 0.39 mmol.L^{-1}, temperature 25°C, 120 rpm (Boriová et al., 2015a).

Table 4 Biosorption performance of fungal biomass. "Sorption capacity" indicates parameter of maximum sorption capacity S_{max} calculated from Langmuir isotherm.

Fungus	Biomass modification	Metal(loid)	Sorption capacity (mmol.g^{-1})	Reference
Aspergillus clavatus	-	Bi^{3+}	0.35	Boriová et al. (2015a)
Aspergillus niger		Cr(VI)	0.10	Vale et al. (2016)
Aspergillus niger	pretreated with NaOH	Cu^{2+}	0.53	Dursun (2006)
Aspergillus niger	-	Ni^{2+}	0.12	Amini et al. (2009)
Aspergillus niger	-	Ni^{2+}	0.16	Shahverdi et al. (2016)
Aspergillus niger	pretreated with NaOH	Pb^{2+}	0.17	Dursun (2006)
Aspergillus niger		Zn^{2+}	0.06	Vale et al. (2016)
Aspergillus ustus	immobilized on nanosilica	Cr(VI)	6.47	Mahmoud et al. (2015)
Aspergillus ustus	immobilized on nanosilica	Cr^{3+}	2.47	Mahmoud et al. (2015)
Fusarium nivale	-	Ag^{+}	3.30	Tyupa et al. (2017)
Fusarium oxysporum	-	Ag^{+}	6.20	Tyupa et al. (2017)
Fusarium sp.	-	Th^{4+}	0.02	Yang et al. (2015)
Fusarium sp.	cetyltrimethyl ammonium bromide	U(VI)	1.50	Hou et al. (2016)
Fusarium verticillioides	immobilized on nanosilica	Cr(VI)	6.40	Mahmoud et al. (2015)
Fusarium verticillioides	immobilized on nanosilica	Cr^{3+}	2.67	Mahmoud et al. (2015)
Mucor racemosus	-	Cr(VI)	0.89	Liu et al. (2007)
Mucor sp.	-	Cd^{2+}	0.71	Xia et al. (2015)
Paecilomyces lilacinus	-	Cd^{2+}	0.69	Xia et al. (2015)
Penicillium canescens	-	As(III)	0.35	Say et al. (2003b)
Penicillium chrysogenum	-	Cd^{2+}	0.89	Xu et al. (2012)
Penicillium chrysogenum	-	Cr^{3+}	0.36	Tan and Cheng (2003)
Penicillium chrysogenum	-	Ni^{2+}	0.22	Tan and Cheng (2003)
Penicillium funiculosum	immobilized on nanosilica	Cr^{3+}	1.87	Mahmoud et al. (2015)
Penicillium glabrum	-	Ag^{+}	1.90	Tyupa et al. (2017)
Penicillium griseofulvum	-	Cr(VI)	1.44	Ambigail et al. (2015)
Penicillium purpurogenum		As(III)	0.67	Say et al. (2003a)
Penicillium purpurogenum		Cd^{2+}	1.11	Say et al. (2003a)
Penicillium purpurogenum		Hg^{2+}	0.40	Say et al. (2003a)
Penicillium purpurogenum		Pb^{2+}	1.38	Say et al. (2003a)
Penicillium simplicissimum	-	Cd^{2+}	0.54	Fan et al. (2008)
Penicillium simplicissimum	-	Pb^{2+}	0.42	Fan et al. (2008)
Penicillium simplicissimum	-	Zn^{2+}	1.19	Fan et al. (2008)
Penicillium sp.	biomass with cross-linked chitosan on fabric	Cu^{2+}	1.01	Zhang et al. (2011)
Rhizopus arrhizus	-	Cr(VI)	1.12	Sağ and Kutsal (1996)
Rhizopus arrhizus	-	Fe^{3+}	0.62	Sağ and Kutsal (1996)

Besides these factors that must be considered for efficient metal and metalloid removal, there are some other conditions that need to be addressed before practical implication of biosorption for treatment of contaminated aqueous media. This includes availability and cost of the sorbent, and

the ease of biosorbent regeneration and modification for various reactor configurations (Bishnoi and Garima, 2004). Furthermore, while biosorbent with strong affinity towards the contaminant is more efficient at low pollutant concentration in large effluent volumes, biosorbent with high uptake capacity is required at high total dissolved pollutant values in small volume of effluents (Hansda et al., 2016). Thus, all fungal sorption capacities given in **Table 4** which characterize fungal biomass performance under specific (mostly optimized) conditions are adequate only for material screening purposes. For the realistic evaluation of equilibrium sorption performance of fungal biomass, the batch experiment needs to be supplemented by process-oriented studies of its kinetics and eventually by dynamic continuous-flow tests (Volesky and Holan, 1995) as biosorption performance decreases with increase in flow rate (Ballester et al., 2017). Thus, the applicability of biosorbent should be also evaluated in a real life situation applying a real wastewater under continuous flow rate (Abdolali et al., 2017).

Other properties of biosorbents commonly addressed in biosorption studies are their chemical stability and mechanical strength. The best option to overcome issues with small particle size, elevated dispensability and its buoyancy and degradability of biomass, is to immobilize or pelletize biomass in supportive material. For such proposes various polymeric substances have been applied, including polyvinyl alcohol and alginate (Cai et al., 2016; Carabajal et al., 2016; Chew and Ting, 2016; Liu et al., 2012) with supplemented substances which help to increase sorption efficiency. This helps significantly to overcome disadvantages when fungal biomass alone is used for metal and metalloid removal from wastewater.

3 ASSESSMENT OF FUNGAL GROWTH INHIBITION IN PRESENCE OF POTENTIALLY TOXIC METALS AND METALLOIDS

To assess the effects of metals and metalloids on fungus, the minimum inhibitory concentration of biomass growth is usually determined under optimized laboratory conditions. It is certainly the easiest way to evaluate the effect of potentially toxic metals and metalloids on microorganism. Thus, most of available experimental studies evaluate growth inhibition using culture medium which is nutritionally very rich and therefore may not reflect the real capability of microorganism to grow in a competitive environments of contaminated soils or other substrates with limited or poorly available nutrients (Iram et al., 2013; Srivastava and Thakur, 2006). Growth inhibition is also influenced by the type of applied media. While the availability of potentially toxic elements in liquid growth media is higher, in agar media the microorganisms appear to be more resistant, most likely due to restricted mobility of these toxic elements (Basu and Paul, 1999).

Valix et al. (2001) introduced the concept of tolerance index which is expressed by the proportion of selected growth parameter (usually a diameter of fungal colony and biomass weight) of toxicant-treated microorganism and toxicant-free control. This index is dependent on the growth stage of the microorganism and reflects the prolonged *lag* phase after exposure to toxic compound. Later on, Valix and Loon (2003) also incorporated time factor to this index (e.g. rate of change during the growth phase) to express fungal adaptive tolerance.

$$y = A\exp\left(-\exp\left[\left(\mu_m e / A\right)\left(\lambda - t\right)+1\right]\right) \qquad (1)$$

In case of time dependent fungal growth assessments of filamentous fungi, it is more appropriate to apply any of the logistic models (Deng et al., 2013). Particularly interesting is modified Gompertz model (**1**) (Zwietering et al., 1990) which includes defined growth parameters: λ - length of the *lag* phase, μ - the specific growth rate, and A - the maximum value of the time dependent growth parameter.

Fungal biomass weight although being strain specific, is considered a direct indicator of biological sensitivity to contamination. Nevertheless, in some instances this parameter is insufficient to reflect the degree of fungal sensitivity towards certain toxicants. In our previous study (Urík et al., 2017) the presence of aluminium oxohydroxide did not significantly change the

calculated maximum biomass weight. However, fungal growth parameters have clearly shown that aluminium slightly prolonged the *lag* phase and decreased the maximum value of growth rate. Thus, other growth parameters also need to be taken into consideration to evaluate adverse effects of metals and metalloids on fungus.

Adaptation of fungi exposed to toxic concentrations of metals and metalloids is quite common phenomenon which facilitates fungal activity even in highly contaminated substrates (**Fig. 5**). Adaptability of microscopic filamentous fungi was exploited in several works, concluding that one can obtain highly tolerant strain if the potentially toxic substance is applied sequentially with increasing concentrations (Anahid et al., 2011). This process allows isolation of resistant mutants, or leads to a rapid physiological adaptation of fungal isolate which, among all other resistance mechanisms, may control the metal(loid) bioaccumulation more efficiently by producing metal-binding metabolites or, in some cases, is capable of biological transformation of toxicant into volatile derivatives via biovolatilization (Dönmez and Aksu, 1999; Fazli et al., 2015; Le et al., 2006; Sazanova et al., 2015). It is less likely that this adaptation relates to increasing fungal sorption capacity (Zafar et al., 2007).

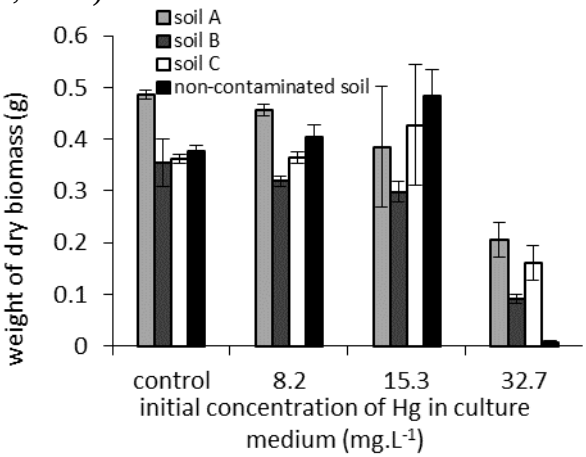

Fig. 5 Biomass dry weight of selected strains of *Cladosporium cladosporioides* evaluated after cultivation at different initial mercury concentration (Urík et al., 2014c). Indicated strains were collected from mercury contaminated soils A, B and C with total mercury concentration of 20.2, 6.9 and 30.9 mg.kg^{-1}, respectively.

Fungal physiological response to potentially toxic metals and metalloids during cultivation can also be indicated by changes in culture medium's pH which indirectly reflects inhibition of fungal metabolism. The growth parameters are often used for evaluation of toxicity (El-Sayed, 2015), while the pH is usually omitted in studies dealing with potentially toxic elements' effects on microorganism. However, the pH differences reflect the fungal struggle to efficiently uptake nutrients because the membrane located ATP-driven proton pump is responsible for maintaining the electrochemical proton gradient necessary for nutrient uptake (Manavathu et al., 1999).

4 EXTRACELLULAR RESISTANCE MECHANISMS OF FILAMENTOUS FUNGI TO TOXIC METALS

As mentioned previously, some isolates of microscopic filamentous fungi are extremely resistant to elevated concentrations of toxic metals and metalloids (Cánovas et al., 2003; Congeevaram et al., 2007). However, various strains of the same species manifest significant differences in responses to toxic metals and metalloids (Cánovas et al., 2004), because the exact mechanism that ensures efficient resistance is not uniform. In general, these mechanisms can be divided according to place where the effect occurs on (i) extracellular and (ii) intracellular resistance mechanisms.

We have already briefly mentioned some of extracellular mechanisms of resistance. This includes (i) binding of metal(loid)s onto the cell wall, and (ii) changes in the fungal growth strategy. Another important general extracellular resistance mechanism relates to (iii) synthesis and secretion of low-molecular weight metabolites capable of binding, or chelating of metal(loid)s.

From the energetic point of view, the least demanding resistance mechanism is passive binding of potentially toxic elements on the cell wall which is negatively charged in slightly acidic and alkali environments (Das et al., 2007; Deng and Ting, 2005). Filamentous fungi may also actively form specific morphological structures where the concentration of cell wall's active (metal(loid) binding) functional groups is increased, and therefore, resistance to potentially toxic substances in the environment is elevated (Fomina et al., 2003). Another strategy is the incorporation of melanin or other substances with high affinity towards metal(loid)s into the cell wall. Besides increasing sorption capacity by providing new functional groups for binding potentially toxic ions (Almeida-Paes et al., 2012), melanin deposition into cell wall also affects zeta potential of the fungal surface (Wargenau et al., 2013). The cell wall is thus a dynamic structure that can be remodelled by fungus according to current stress conditions and to some extent its chemical composition is adjustable. Exposure to toxic concentrations of zinc changes expression of chitin synthetase (Lanfranco et al., 2004). This influences the deposition of chitin in the cell wall and its morphology (Lanfranco et al., 2002). The chitin-rich cell wall enhances sorption capacity and thus also increases immobilization efficiency of toxicants from the cell's environment (Bedioui et al., 2015).

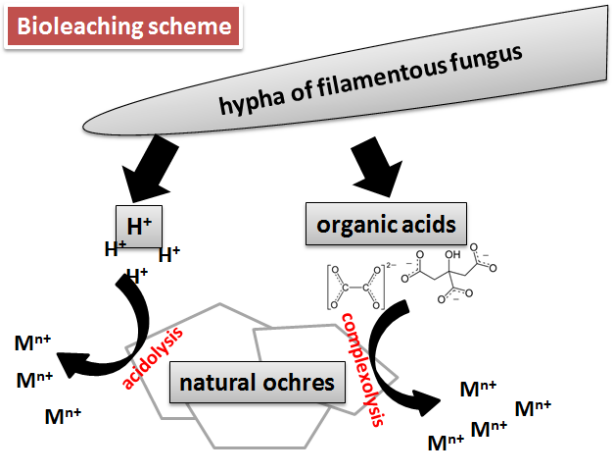

Fig. 6 Schematic description of organic acids and H^+ relevance in metal mobilization form ferric ochres by filamentous fungi.

More energetically demanding is the direct secretion of secondary metabolites in the extracellular environment. The chemical properties of these secreted substances significantly vary. However, in most cases they can be characterized as low-molecular weight organic compounds. Their presence in the environment is essential, as they are involved in the regulation of ecotoxicity of organic and inorganic pollutants, as well as mobility and bioavailability of essential elements, mostly via adsorption and chelation/complexation processes (Xiao and Wu, 2014). The synthesis and excretion of low-molecular weight organic acids by filamentous fungi and their subsequent binding with the cations of zinc, copper and cadmium can lead to immobilization of these potentially toxic elements in the crystalline phase of biogenic minerals (Fomina et al., 2005). Therefore, increasing production of such metabolites leads to a significant reduction of the adverse effect of metal cations on the filamentous fungi (Sazanova et al., 2015). However, extracellular metal chelating agents may also be macromolecular organic substances, such as glomalin.

Glomalin is a glycoprotein produced by mycorrhizal fungi (Wright and Upadhyaya, 1996) and is highly effective biostabilizer of potentially toxic metals (González-Chávez et al., 2004).

4.1 BIOLEACHING

Unfortunately, microbial organic acid exudation has also significant impact on metal mobilization in fungal microenvironment; and thus, affects bioavailability of hazardous substances and their further transfer to other organisms (**Fig. 6**). Our published data (Urík et al., 2014a), depicted in **Fig. 7**, support this observation as fungus *A. niger* was capable to release (pre-adsorbed) arsenic from surface of amorphous ferric oxohydroxide phase. This biologically induced extraction of metals and metalloids from solid phases is generally termed *bioleaching*. Although, this process is well studied in bacteria and mostly focuses on metal extraction from low-grade ore and mineral phases concentrates by autotrophic bacterial strains (Bosecker, 1997), application of (heterotrophic) filamentous fungi has become lately a hot topic in relevance to biogeochemistry of metals (Boriová et al., 2016), as well as metal recovery from highly alkaline substrates which are not suitable for bacterial leaching (Ramanathan and Ting, 2013; Urík et al., 2015). To be more specific, efficiency of 2-hour bio-assisted extraction of metals from 1% fly ash suspension using *A. niger* supernatant (Jadhav and Hocheng, 2015) is comparable to that of sulphur-oxidizing and iron-oxidizing bacteria mixed cultures incubated with fly ash for 5 days (Ishigaki et al., 2005).

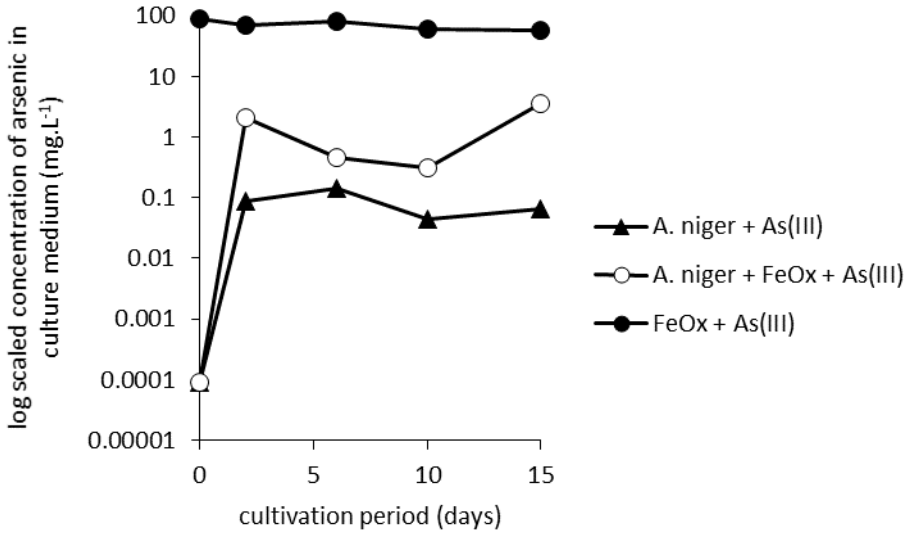

Fig. 7 Changes in concentration (log scale) of arsenic in culture medium during the 15-day cultivation of *Aspergillus niger* strain in presence of ferric oxohydroxides FeOx (○), in the absence of FeOx (●), and changes in arsenic concentration in the presence of FeOx without fungal strain (▲) (Urík et al., 2014a).

Fungal bioleaching is most likely mediated by two mechanisms: (i) decreasing the culture medium pH which induces the dissolution of substrate that binds the pollutant and (ii) exudation/production of metabolites which form readily dissolvable organo-metal complexes in the medium or compete with pollutant for sorption loci (Burgstaller and Schinner, 1992). Because of the intensity of these processes, after 15-day cultivation fungus *A. niger* extracted into culture medium, in aforementioned study of Urík et al. (2014a), almost 45% (1.8 mg) of arsenic pre-adsorbed onto ferric oxohydroxides.

This phenomenon may even occur in the environment where major environmental hazards were considered stable, and where general extraction techniques indicated only low concentrations of mobile metal fractions. This controversy also highlights the necessity to re-evaluate the significance of microbial exometabolites in mobilization of toxic metals and metalloids in the environment, as it is well known that their mobility and toxicity is affected by soil concentration of

low molecular weight organic compounds with chelating properties as well as by soil pH (Dijkstra and Fitzhugh, 2003; Van Hees et al., 2000). Thus, the pH stratification and microbial organic exometabolites' concentrations in the closest fungal environment should be considered when studying mobile metal and metalloid fractions in environmental samples. This was also the main objective of our previous study, more specifically, the implication of (biogenic) organic acids in determination of soil bioavailable aluminium fraction (Boriová et al., 2016). Our study clearly shows that the organic acid mixture, which mimics composition of *A. niger* strain's exudates, is more efficient in aluminium extraction compared to more concentrated or aggressive extractants applied in single extraction procedures (**Fig. 8**), such as 0.5 mol.L^{-1} HCl or 0.2 mol.L^{-1} $(NH_4)_2C_2O_4$ (Matúš et al., 2006).

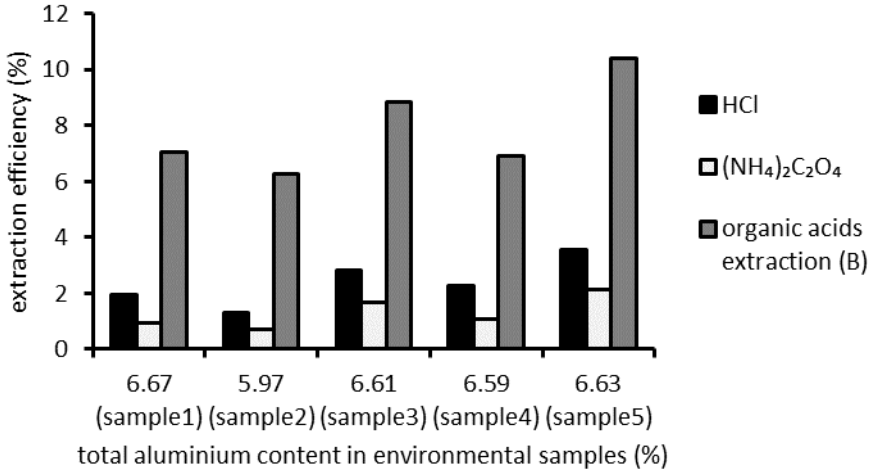

Fig. 8 Extraction efficiencies of aluminium from soil samples by single step extraction using 0.5 mol.L^{-1} HCl and 0.2 mol.L^{-1} $(NH_4)_2C_2O_4$ compared to extraction by mixture of organic acids prepared to according organic acid composition of culture media from the 12th cultivation day of *A. niger* (oxalic acid 52.2 mmol.L^{-1}; citric acid 2.0 mmol.L^{-1}; gluconic acid 11.6 mmol.L^{-1}; pH 1.45) (Boriová et al., 2016).

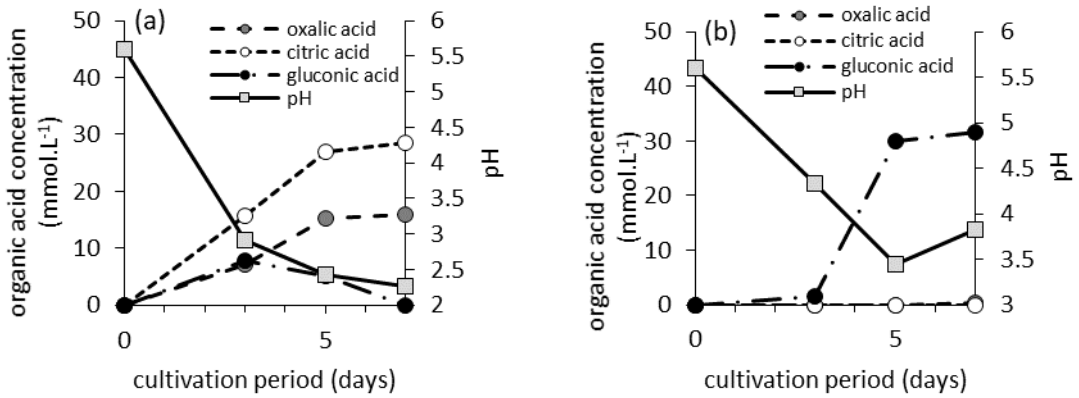

Fig. 9 *Aspergillus niger* G-10 (a) and *Penicillium crustosum* G-140 (b) extracellular organic acid concentrations and culture medium pH in 7-day incubation at red mud presence (Urík et al., 2015).

Some fungal strains, such as *A. niger*, are used in food and pharmaceutical industry for production of organic acids (Magnuson and Lasure, 2004) which accumulate in the extracellular environment and their concentrations easily reach up to hundreds of mmol.L^{-1} (Santhiya and Ting, 2006). As highlighted in **Fig. 9**, organic acid production is strain specific and time dependent. Because the fungal extraction efficiency most likely reflects the actual concentration of effective extracting agents in medium, it can significantly vary with strain applied in extraction procedure.

Therefore, the preliminary estimation of extraction efficiencies of desired element from specific substrates is always necessary (**Fig. 10**) in order to achieve research objective.

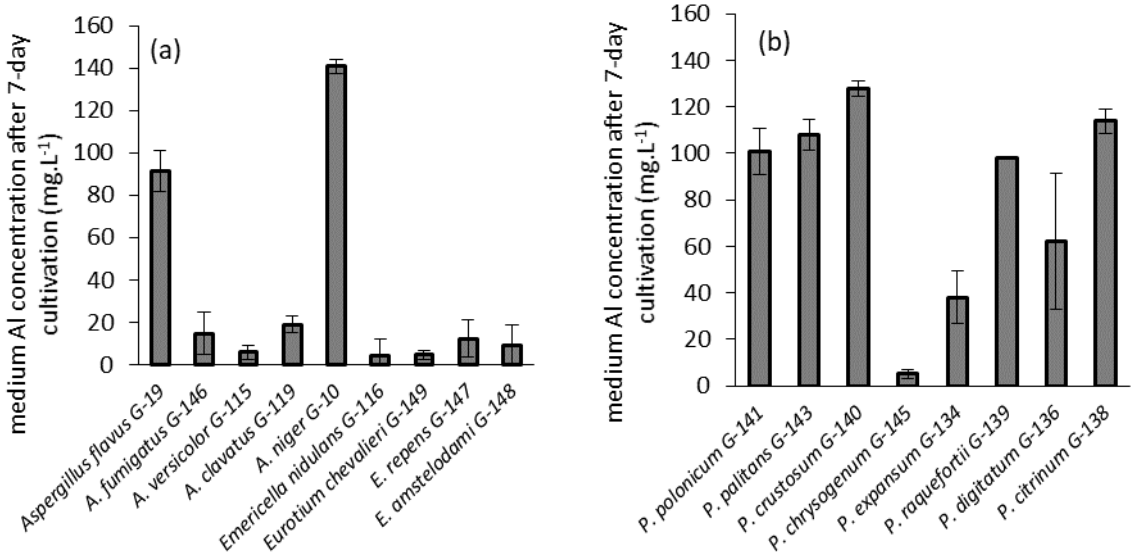

Fig. 10 Aluminium (bio)extracted from red-mud into the culture medium by fungal strains of genus *Aspergillus*, *Emericella* and *Eurotium* (a), and *Penicillium* (b) after 7-day cultivation (Urík et al., 2015).

Table 5 Extraction efficiency (%) of selected metals from electronic scrap by *Aspergillus niger*, 0.05 mol.L^{-1} oxalic and citric acids and distilled water (Kolenčík et al., 2013b).

Metal	*Aspergillus niger*	Oxalic acid	Citric acid	H_2O
Cu	68.3	13.3	67.4	0.10
Pb	27.9	7.4	91.4	0.01
Zn	4.1	1.8	92.0	0.45
Cd	21.9	38.9	70.8	0.10

Due to their capability to produce enormous amounts of organic acids and effectively acidify media, filamentous fungi have been applied for metal extraction from various waste materials in biohydrometallurgy (Amiri et al., 2012; Kolenčík et al., 2013a; Santhiya and Ting, 2006). This exceptional fungal quality has been also exploited in mineral phase and e-waste material processing by our research group (Kolenčík et al., 2013a; Kolenčík et al., 2013b; Kolenčík et al., 2011), and it was compared to extraction efficiencies by standard organic acids (**Table 5**).

5 BIOACCUMULATION

Extracellular immobilization of metal(loid)s is not ultimately effective; and a portion of these potentially harmful substances goes through cell wall barrier and cytoplasmic membrane directly to the cytosol. Here, the homeostasis must be kept to such an extent as not to disrupt cellular metabolism. Therefore, the regulation of free ions' intracellular concentration of potentially toxic substances must be addressed. This includes (i) their binding to various organic molecules within the cytosol, (ii) their sequestration in the specific membrane and non-membrane structures in the cell, (iii) their exudation from the cytosol across the outer membrane after (bio)chemical transformation, and (iv) regulation of their concentration by activity of membrane transporters responsible for removal of unwanted elements from cytosol (Eide, 2003; Ge et al., 2011; Su et al., 2011).

Intracellular sequestration of heavy metals in order to maintain homeostasis and detoxify the harmful metals is usually achieved by synthesis of metallothioneins (Averbeck et al., 2001). These low molecular weight polypeptides contain high percentage of cysteine (up to 33%) which is

involved in the complexation of the metal cations and their subsequent sequestration. Their synthesis is usually induced by oxidative stress which relates to metals' presence (Pagani et al., 2007; Waalkes and Goering, 1990).

Another efficient metal(loid) sequestering molecule is glutathione and its derivatives (Xu et al., 2014) which participate in the transport of undesirable element to the vacuole by specific molecular transporters (Lee et al., 2011). However, its function and thus its importance in the cell is much more diverse; and it is also involved in various cell stress responses. This includes non-enzymatic inactivation of reactive oxygen species (Pócsi et al., 2004).

In terms of long-term maintenance of the organism resistance, it is essential to continuously control the concentrations of the stress-inducing elements below a certain concentration level. This is allowed by intensification of the outflow of these elements from cytosol in the form of unchanged or (bio)transformed species through the cytoplasmic membrane. The transporters involved in this particular mechanism mostly belong to the group of CDF (*cation diffusion facilitators*). Their primary biological function is to discharge metals from the cytosol to various cell organelles or to the extracellular environment (González-Guerrero et al., 2005). Another significant group are ABC (ATP-*binding cassette*) transporters that use energy from ATP to transport a wide range of substances (Bauer et al., 1999). Their MRP subfamily (*multidrug resistance-associated proteins*) is involved in the detoxification of vacuolar glutathione conjugates (Li et al., 1996).

Synthesis of insoluble polyphosphate granules also appears to be associated with increased resistance to toxic metals and metalloids. Their exact role in the resistance of filamentous fungi is not entirely clear (de Lima et al., 2013) as various reports suggest that the presence of heavy metals decreases the polyphosphate content in the cell (de Lima Freitas et al., 2011).

Table 6 Bioaccumulation efficiency of fungal strains.

Fungus	Metal(loid)	Substrate type	Initial metal(loid) concentration ($mmol.L^{-1}$)	Removal efficiency (%)	Reference
Aspergillus flavus	Hg^{2+}	culture medium	0.005	97.5	Kurniati et al. (2014)
Aspergillus niger	Zn^{2+}	refinery effluent + peptone and glucose	not reported	72.4	Abdulmajeed et al. (2016)
Aspergillus niger	Cd^{2+}	culture medium	0.035	19.4	Pal et al. (2010)
Aspergillus niger	Cr(VI)	culture medium	1.9	41.0	Thippeswarny et al. (2012)
Aspergillus niger	Pb^{2+}	culture medium	0.12	91.1	George et al. (2012)
Fusarium oxysporum	As(III)	culture medium	0.53	5.3	Feng et al. (2015)
Microsporum nanum	Cd^{2+}	refinery effluent + peptone and glucose	not reported	87.8	Abdulmajeed et al. (2016)
Penicillium janthinellum	As(III)	culture medium	0.53	4.3	Feng et al. (2015)
Trichoderma harzianum	Ag^{+}	agar culture medium	3.06	46.4	Cecchi et al. (2017)

All previously mentioned processes which relate to active fungal resistance during toxic metal and metalloid exposure and, at the same time, result in intracellular and extracellular binding of metals and metalloids by living biomass are generally termed as *bioaccumulation* (**Table 6**). Chojnacka (2010) characterizes bioaccumulation as non-equilibrium process which occurs in two stages. While the first stage is a fast passive uptake which resembles biosorption, the second stage is relative slow and relates to (i) active or passive membrane transport (both efflux and influx), (ii) intracellular transformation and subsequent (iii) deposition of metals and metalloids in cellular structures, as discussed previously. This concept is very complex and involves deposition of ions within specific organelles, their enzymatic detoxification and influx/efflux processes (Srinath et al., 2002).

Actual distinction between bioaccumulation and biosorption can be very difficult, as indicate our published data (Urík et al., 2016) which show that apparent bioaccumulation capacity of *A.*

clavatus is very similar to maximum 2.6 $mg.g^{-1}$ sorption capacity calculated from Langmuir isotherm model using concentration data from 14-day cultivation (**Fig. 11**). Arguably this suggests passive sorption and formation of selenium monolayer on biomass surface, saturated over 60 $mg.L^{-1}$ Se(VI) concentration in medium, rather than its regulated storage in cell vacuoles. Therefore, we should expect cell's effective selenium efflux or its transformation into volatile form via biomethylation pathway (Eswayah et al., 2016).

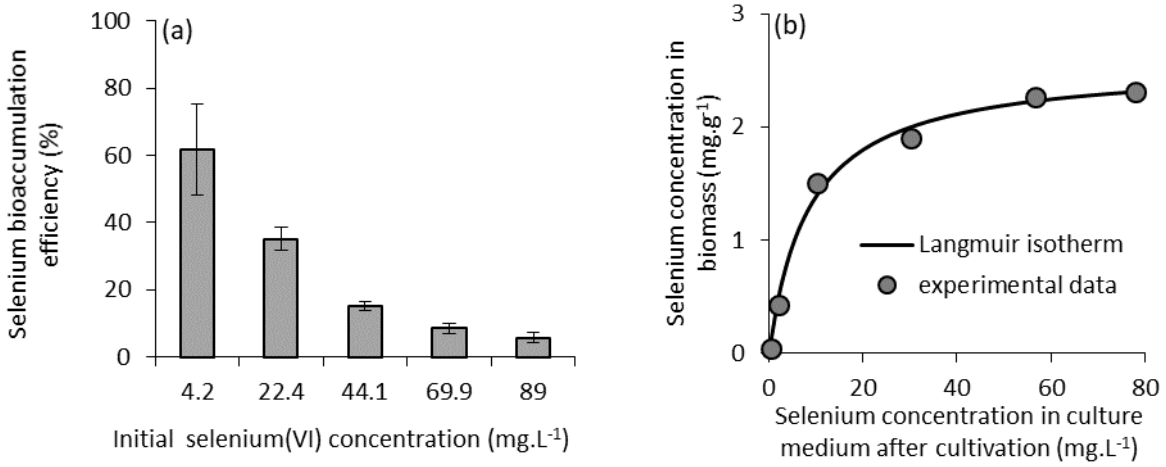

Fig. 11 (a) Bioaccumulation of Se(VI) after 14-day *A. clavatus* incubation and (b) experimental accumulation data evaluated by Langmuir isotherm with 2.56 $mg.g^{-1}$ calculated maximum sorption capacity (Urík et al., 2016)

6 BIOVOLATILIZATION OF METALS AND METALLOIDS

Most unique microbial mechanism for metal and metalloid removal from intracellular environment is biovolatilization via biomethylation or bioreduction pathway. Volatile derivatives of potentially toxic metals and metalloids were identified in samples of gases released from various natural and anthropogenic substrates (Meyer et al., 2007; Michalke et al., 2000). Some of these compounds are of anthropogenic origin or products of natural transalkylation (Mitra et al., 2005), while others originate from biologically induced formation of methylated derivatives or metal hydrides (Boriová et al., 2015b; Wang et al., 2015). Formation of volatile derivatives of metals and metalloids is an important part of biogeochemical cycles of various elements, especially since the resulting volatile forms are easily transported in the atmosphere (Jakob et al., 2010). Such volatile derivatives are of particular interest of environmental toxicology because their toxicity usually differs from their inorganic precursors (Kobayashi, 2010).

In case of microscopic filamentous fungi, the transformation of metals and metalloids into their respective volatile derivatives is often (and sometimes incorrectly) referred as *biomethylation*. A pioneer in this field of research was Gosio (1897) whose primary concern was the formation of volatile toxic derivatives of arsenic. The enzymatic transformation of inorganic arsenic into methylated compounds, however, was explained more than half a century later and is depicted on **Fig. 12**. This is the so-called oxidative methylation pathway which was suggested according to analytical study of Challenger (1951). Oxidative methylation involves the transfer of the methyl group from S-adenosylmethionine donor to a substrate containing trivalent arsenic while it is oxidized to its pentavalent form. Thus, the arsenic reduction is necessary before each methylation step. In this particular metabolic pathway, glutathione poses as a reducing agent (Thomas, 2007).

An alternative mechanism of arsenic's oxidative biomethylation was proposed currently, and since then has been generally accepted metabolic pathway. This biotransformation involves non-oxidative methylation via S-adenosylmethionine originated methyl group binding to a substrate (**Fig. 12**). To prevent oxidation, Hayakawa et al. (2005) assume the formation of complex with

glutathione. A similar mechanism is probably involved in methylation of antimony, bismuth, selenium and tellurium (Wuerfel et al., 2012)

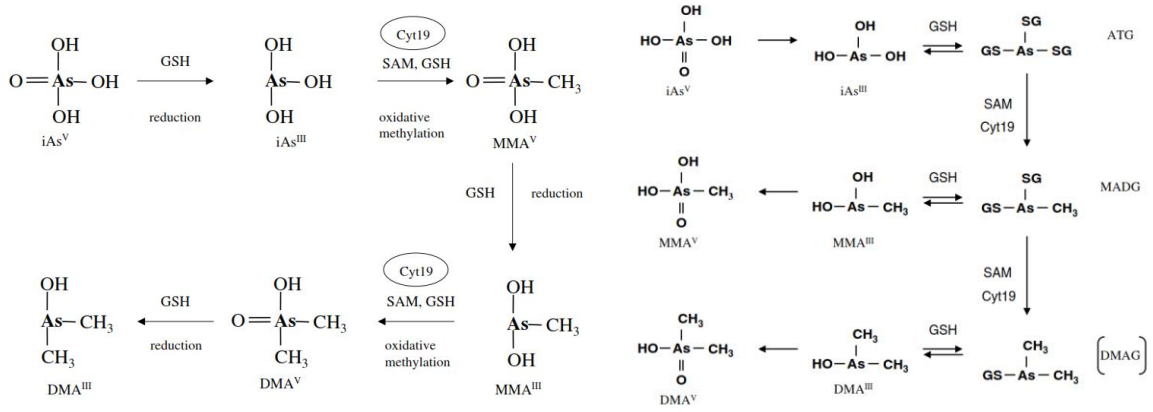

Fig. 12 Oxidative (left) and non-oxidative (right) methylation pathway of arsenic, adapted from Hayakawa et al. (2005). GSH, glutathione; SAM, S-adenosylmethionine; Cyt19, arsenic methyltransferase.

Filamentous fungi induce the formation of volatile forms of various metals and metalloids. This has been proven directly by analysis of the gases from fungal headspace, or indirectly by quantifying the loss of metal(loid) content from cultivation system (Boriová et al., 2014; Boriová et al., 2015a; Jenkins et al., 1998; Zeng et al., 2015). These authors also consistently consider biovolatilization an effective detoxifying mechanism which increases fungal resistance to available potential toxic metals and metalloids in the environment (Bentley and Chasteen, 2002; Urík et al., 2014c).

Our extensive research on arsenic biovolatilization by filamentous fungi, including isolates of *Penicillium glabrum*, *Neosartorya fischeri*, *A. niger*, *A. clavatus*, *Talaromyces wortmannii*, *T. flavus*, *Eupenicillium cinnamopurpureum*, shows that metabolic transformation of arsenic into volatile derivatives is relative conservative feature of this microbial group with relative biovolatilization efficiencies ranging from 6.7% (of initial 1.0 mg As(V) content) to 36.7% (of initial 0.2 mg As(V) content) (Čerňanský et al., 2009; Čerňanský et al., 2007; Urík et al., 2007). Therefore, it is most likely that application of microorganisms in remediation of arsenic contaminated substrates does not require cultivation of any particular fungal strain. Enhancing the activity of indigenous fungal strains may sufficiently serve the goal of lowering pollutant contentment in upper parts of soils. Fungal activity may be enhanced by optimizing environmental parameters which affect volatilization, such as content of soil nutrients, as well as moisture and aeration regulation (Frankenberger Jr and Arshad, 2001; Thompson-Eagle and Frankenberger, 1992). However, Edvantoro et al. (2004) successfully applied bioaugmentation of particular arsenic volatilizing fungal strains (*Penicillium* sp. and *Ulocladium* sp.) for remediation of cattle-dip site soils contaminated with arsenic.

Similarly, the importance of microbial processes in mercury volatilization has been questioned several times; highlighting the confrontation between direct biotic process and abiotic transformation induced by microbial products (Schlüter, 2000). Our published results (Urík et al., 2014c) confirm that soil filamentous fungi's contribution on mercury biovolatilization is significant (**Fig. 13**), although the precise mechanism of mercury volatilization remains unknown. It most likely involves both intracellular and extracellular reducing factors for formation of elemental mercury, and/or methylation agent (e.g. methylcobalamin) when considering mercury volatilization in dimethyl form (Jiménez-Moreno et al., 2013; Kelly et al., 2007; Yannai et al., 1991).

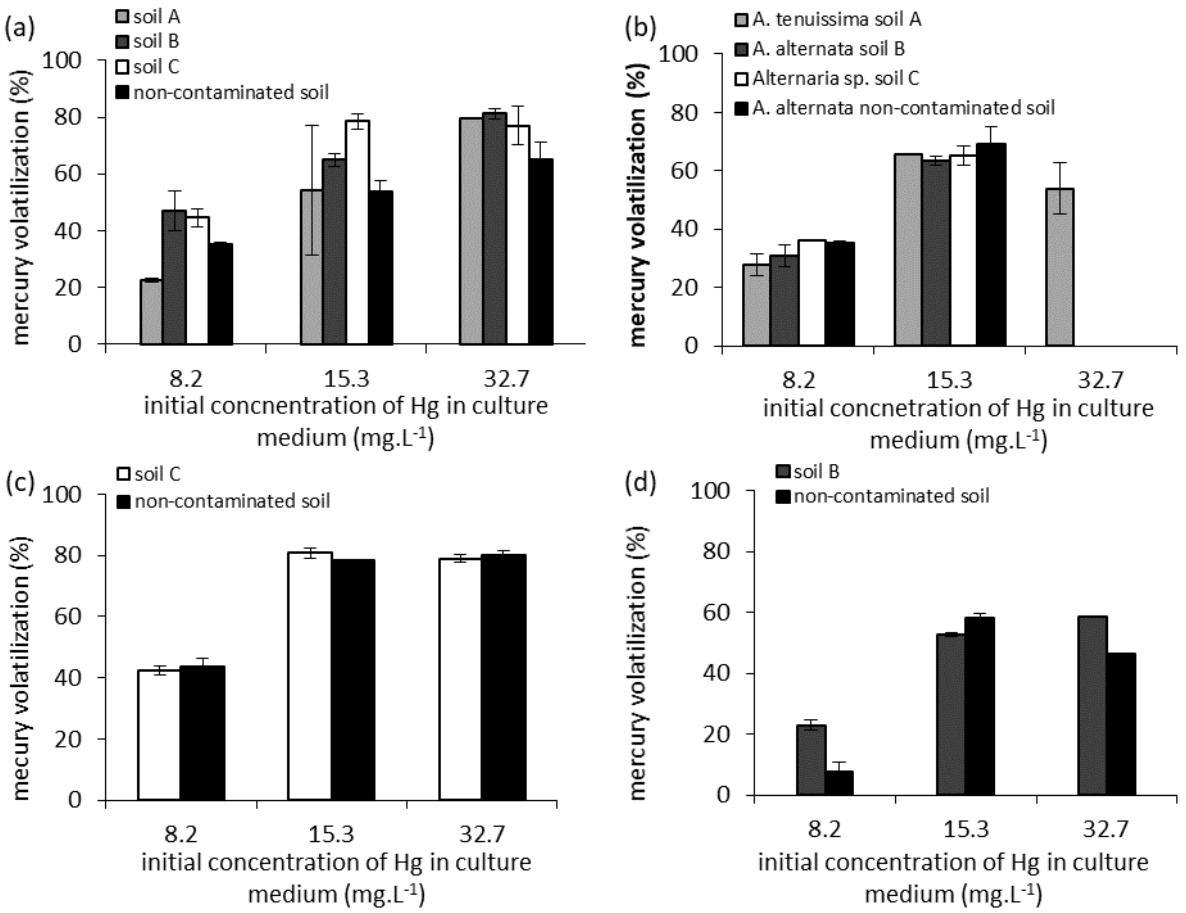

Fig. 13 Mercury biovolatilization efficiency during 7-day cultivation of fungal strains (a) *Cladosporium cladosporioides*, (b) *Alternaria* spp., (c) *Aspergillus niger* and (d) *Trichoderma atroviride*. Indicated strains were collected from mercury contaminated soils A, B and C with total mercury concentration of 20.2, 6.9 and 30.9 mg.kg^{-1}, respectively (Urík et al., 2014c).

While biovolatilization of arsenic and mercury via methylation pathway is controversial remediation technique, as all volatile methylated arsenic and mercury species are highly toxic (Dopp et al., 2011), selenium biomethylation is considered environmentally appropriate method for selenium removal from contaminated soils, as the methylated selenium species are hundreds time less toxic than inorganic Se(IV) and Se(VI) (Eswayah et al., 2016). Our results show (Urík et al., 2016) that biovolatilization of selenium by *A. clavatus* is triggered only over 4.2 mg.L^{-1} Se(VI) initial concentration in culture medium. Although there was up to 77% selenium removal efficiency by sorption/accumulation at low initial concentrations, no volatilization occurred. However, significant 2.8 mg.g^{-1} biovolatilization was achieved in the 14-day fungal incubation at initial 69.6 mg.L^{-1} Se(VI) concentration. In this case, selenium biovolatilization was proved to be advantageous remediation method delivering relatively stable and non-harmful volatile derivatives at higher initial concentrations of selenium, while standard biosorption treatment by inactive native or physico-chemically modified biomass leaves selenium concentrated residues (Nettem and Almusallam, 2013; Wasewar et al., 2009).

7 CONCLUDING REMARKS

This thesis highlights that filamentous fungi influence and transform their microenvironment, and thus, contribute to mobilization or immobilization of potentially toxic metals and metalloids. Microscopic filamentous fungi are capable of, to some extent, intensifying the degradation of solid phases, and to accumulating and volatilizing available metals and metalloids (Gadd, 2007). This

ability emerges from their effort (i) to obtain mineral nutrients with limited availability in the environment (e.g. phosphorus), (ii) to increase intake of organic substances bound to organo-mineral phases, and (iii) to maintain homeostasis of various elements in cytosol (Adeyemi and Gadd, 2005; Wengel et al., 2006).

These processes may be exploited in bioremediation (mycoremediation) of contaminated lands and waters as filamentous fungi effectively alter mobility and bioavailability of elements in soils and sediments; and may also serve as bio-filters for uptake of toxic metal(loid)s by other organisms (Schützendübel and Polle, 2002). Therefore, laboratory based experiments are the first step in understanding microbial activity which provide potential implications for biosorption, bioaccumulation, bioleaching and biovolatilization of hazardous metals and metalloids in remediation of areas burdened with natural or anthropogenic contamination.

Although the main emphasis of fungal application in remediation is on removal of potentially toxic elements from water bodies using chemically modified biomass (Littera et al., 2011; Urík et al., 2010), filamentous fungi have significant impact on mobilization and distribution of potentially toxic elements in soils and sediments with possible intracellular or extracellular transformation into volatile derivatives (Srivastava et al., 2011; Urík et al., 2014c). This can be successfully applied for remediation of contaminated areas, where toxic elements may undergo this unique microbial transformation (Lin and Terry, 2003). Also, inoculation of plants' substrates with mycorrhizal fungi affects the efficiency of metal(loid) uptake by plants, and their transport from roots to shoots (Audet and Charest, 2007; Göhre and Paszkowski, 2006) which can be exploited in phytostabilization and phytoremediation of hazardous metals and metalloids.

Inorganically contaminated soils are sometimes exposed to synthetically produced chelating agents to mobilize metals in decontamination processes (Wu et al., 2004). Alternatively, intensification of natural microbial activity in soils may provide more "environmentally friendly" extractions with higher efficiencies, even in comparison to ethylenediaminetetraacetic acid (Dimkpa et al., 2009). Microscopic filamentous fungi, an important component of microbial communities, are no exception to that, as their extracellular metabolites play significant role in desorption of metals and metalloids from mineral and amorphous phases (Adams et al., 2007; Urík et al., 2014a). Moreover, release of element from solid phase is only the first step of various transformations which pollutant can be subjected to due to diversity in metabolism of fungal strains, including formation of mycogenic mineral phases and biovolatilization (Pan et al., 2009).

The potential of filamentous fungi in remediation is indisputable, and thus, better understanding of environmental implications of their interaction with potentially toxic metals and metalloids, which is provided in our studies on biosorption, bioaccumulation, bioleaching and biovolatilization, advances our knowledge on their prospects in remediation of contaminated areas, and thus helps us to realize and exploit their abilities for our own ends..

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EDUCATION

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10/2010 — Ph.D. in *Environmental protection and utilization of land and nature*
Geological Institute, Faculty of Natural Sciences, Comenius University in Bratislava (Slovakia)
Thesis on "Biosorption and biovolatilization – alternative methods for arsenic removal" (advisor Dr. Jaroslav Ševc, PhD.)

06/2006 — M.Sc. in *Biology and Chemistry*
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AWARDS AND HONOURS

2017 — The Ministry of Education, Science, Research and Sport of the Slovak Republic's *Award for Science and Technology* in the category of Researchers under age of 35

2017 — Faculty of Natural Sciences Dean's *Medal for Excellent Young Researchers*

2016 — Award from the Slovak Spectroscopic Society for Young Researchers

2016 — *Danubius Young Scientist Award* from the Austrian Federal Ministry of Education, Science and Research (BMBWF) and the Institute for the Danube Region and Central Europe (IDM)

2016 — Comenius University's *Recognition of Excellent Young Researcher*

2014 — Scientific Grant Agency VEGA's *Recognition of Exceptional Achieved Results*

2008 — Comenius University Rector's *Recognition of Students on the Anniversary of Student Day*

2006 — Comenius University Rector's *Award for the Best Master's Thesis*

RESEARCH PROJECTS

01/2018-12/2021 — Effects of microbial extracellular metabolites and bio-transformation processes on mobility of Mn, Fe and Si, and other environmentally significant micro-nutrients (VEGA 1/0146/18) (principal investigator)

01/2014-12/2017 — Potential risk assessment of spread of inorganic contamination of geogenic or anthropogenic origin induced by biologically catalyzed release of toxic elements from humic matter (VEGA 1/0203/14) (principal investigator)

2013-2014 — Assessment of metals bioavailability in soils and sediments using diffusively gradients in thin-films technique, chemical extractions and biological methods (APVV SK-RO-0004-12) ((principal investigator)

01/2011-12/2013 — Microbially induced changes in redox properties of humic substances and their effect on microbial activity and higher plants in soils with organic or inorganic contaminants (VEGA 1/0778/11) (principal investigator))

01-12/2013 Nanosilver ecotoxicity and bioaccumulation evaluation using various cultural plants (UK/247/2013) (principal investigator)

01-12/2012 Influence of organic-mineral coatings on soil aggregate water repellency (UK/338/2012) (principal investigator)

01-12/2011 Humic acids – a redox barrier for non-melanized fungal strain? (UK/354/2010) (principal investigator))

01-12/2009 Biovolatilization and bioaccumulation of selenium by microscopic filamentous fungi (UK/216/2009) (principal investigator)

01-12/2008 Biosorption of selenium using fungal and plant biomass (UK/244/2008) (principal investigator)

01-12/2007 Biosorption of arsenic using fungal biomass from aqueous solutions (UK/197/2007) (principal investigator)

PUBLICATIONSs

2 monographs
4 monograph chapters
60 publications in peer-reviewed journals
378 citations (251 indexed in Scopus and WoS)
h-index: 9