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Assessment of Ni accumulation capability for a possible approach to remove metals from soils and waters.

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ABSTRACT

Abandoned industrial sites and mines may constitute possible hazards for surrounding environment due to the presence of toxic compounds that may contaminate soils and waters. The possibility to remove metal contaminants, specifically nickel (Ni), by means of fungi was presented exploiting a set of fungal strains isolated from a Ligurian dismissed mine. The achieved results demonstrate the high Ni(II) tolerance up to 500 mg Ni l^{-1}, and removal capability of *Trichoderma harzianum* Rifai. strain. This latter hyperaccumulates up to 11,000 mg Ni kg^{-1} suggesting its possible use in a bioremediation protocol to provide a sustainable reclamation of broad contaminated areas.

KEYWORDS: Mycoremediation, hyperaccumulation, metals, pollution, *Trichoderma harzianum.*

1. INTRODUCTION

Abandoned industrial sites and mines may constitute possible hazardous for surrounding environment due to the presence of toxic metal compounds that may contaminate soils and waters.

As a matter of fact, the European Environment Agency (EEA 2014) evaluates that contaminated sites will rise 50% up to 2025 and around 340,000 sites will possibly need urgent remediation. Conventional technologies for metal-contaminated soil remediation are often expensive and disruptive (SOER 2010). Thirteen trace metals and metalloids (specifically, Ag, As, Be, Cd, Cr, Cu, Hg, Ni, Pb, Sb, Se, Tl, and Zn) are considered priority pollutants (Gadd 2010; Brzostowski *et al.*, 2011). Among these, Ni occurs naturally in the environment, often combined with iron and sulphur, and it also has many industrial uses and anthropogenic sources (e.g. industrial waste materials, lime, fertilizer, and sewage sludge) that may contaminate the environment (Sharma and Agrawal 2005; Kabata-Pendias and Mukherjee 2007), mainly soils (Abedin 2014). Furthermore, this metal represents a threat to the human health. Soluble Ni salts and mixture of Ni sulphides and oxides from refinery dust are carcinogenic to the lung and nasal tissues (EA 2009). Conventional metal removal technologies include filtration, ion exchange, osmosis, chemical oxidation, reduction and precipitation, membrane technologies and evaporation recovery (Ahluwalia and

Goyal 2007). These processes are extremely expensive, and in most cases ineffective (e.g. removal of 1-100 mg l^{-1} of metals from wastewaters) (Moore *et al.* 2008). Table 1 lists the main remediation technologies, the related costs and application fields.

Recently, biological treatments, like metals biosorption or bioaccumulation, increased being cheap and highly efficient (Moore *et al.* 2008). Many microbial species, such as bacteria and fungi (including yeasts), are known to accumulate or adsorb metals (Johnson and du Plessis 2015). In extreme environments, fungi play a key role in biogeochemical cycles since they represent the major component of soil biota, especially owing to their polymorphism and reproduction by spores (SOER 2010). Some fungi can be defined as extremophyl microorganisms, which can survive and grow in strong metal-contaminated substrates. This capacity is due to some proteins called metallothioneins and phytochelatins, which bind and deactivate toxic metals (Anahid *et al.* 2011). These characteristics make fungi able to perform an efficient sustainable remediation of metals. In this context, Johnson (2014) defined biomining as a cheap and feasible technology to extract metals and remediate contaminated industrial sites using biological systems. Furthermore, the use of fungal accumulation allows both metal extraction (mycoextraction) from ores or waste materials and metal recovery (mycomining) from fungal biomass (Roccotiello *et al.* 2015). Mycomining could also allow the reuse of a raw material in the industry adopting metal reintegration. Ni has a relatively high price, estimated of US $ 10,000 per ton (www.lme.com). The Ni price fluctuation is similar to many other metals (http://www.infomine.com). The application of a on-site, full-scale, mycomining requires first providing scientific evidence via laboratory and large-scale experiment, most of which is still missing.

In this context our work was aimed at identifying and selecting native microfungal strains able to efficiently bioaccumulate Ni via mycoextraction, which can be exploited for sustainable environmental remediation in the future.

Table 1 Overview on the costs and fields of application of the traditional remediation and bioremediation techniques.

2. MATERIALS AND METHODS

2.1. Fungal isolation and screening test

A set of fungi, including *Aspergillus alliaceus* Thom & Church, *Trichoderma harzianum* Rifai., *Eurotium amstelodami* L. Mangin and *Clonostachys rosea* (Link) Schroers, was collected from the abandoned Libiola Cu-Fe sulphide mine (NW Italy), identified and stored in the culture collection of Mycological Laboratory of DISTAV (University of Genoa, Italy) (Zotti *et al.* 2014). Then, a screening test for Ni-tolerance was carried out to select the fungal strains able to survive at high toxic Ni concentrations. The microfungal strains were cultivated on malt extract agar (MEA) in test tubes. The strains were tested using a modified medium prepared adding different $NiSO_4 \cdot 6H_2O$ (SIGMA) concentrations (0; 100; 200; 400; 800 mg Ni l^{-1}) in the MEA solutions. These concentrations were chosen in the range of the European law limits for soil metals (CE 1986; Legislative Decree 2006), exceeding the highest limits in the screening test. All the media were sterilized by autoclaving (121°C, 20 min) and then inoculated with 0.5 ml fungal solutions, which were prepared by diluting conidia of each fungal strain in a semisolid suspension of 5 ml Tween 80 (polysorbitan 80). The conidia were counted using a Burker chamber to quantify the inocula (8×10^5 conidia ml^{-1}). After 12 days of incubation at 24°C in the dark, the most Ni-tolerant fungal strain with fastest growth was selected to the further Ni bioaccumulation tests.

2.2. Ni(II) mycoaccumulation test

The $NiSO_4 \cdot 6H_2O$ enriched solid media and the control medium were prepared (Ni 0; 100; 200; 400 mg Ni l^{-1}) to evaluate the Ni(II) uptake capability of the selected fungal strain. Sterilized microporous cellulose acetate membrane (BioRad) was placed on each Petri dish and inoculated with 0.5 ml fungal Tween 80 solution. These membranes assure the mycelia isolation from solid media. The experiments were conducted in triplicate.

After 14 days of incubation at 24°C in the dark, fungal biomass was removed by a sterile plastic scraper and dried at 60°C, 48 h. Fresh and dry fungal biomasses ware weighted for each Ni(II) concentration to evaluate the strain ability to grow under different Ni(II) concentrations.

Dry mycelia were analyzed by the ALS Analytical Lab (Sweden) in order to quantify Ni concentration in biomass.

2.3. Total Ni(II) concentration in fungi

The total Ni concentrations of the dry mycelia were analyzed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) at ALS Analytical Lab (Sweden). The ICP-MS detects metals and several non-metals at concentrations as low as one part in 10 (ppt) (Anahid *et al.* 2011). This is achieved by ionizing the sample to separate and quantify ions (Jenner *et al.* 1990). The Ni concentration was measured in triplicate for quality measurement assurance and the percentage coefficients of relative standard deviation were below 10%, reaching maximum values of about 25% only for those concentrations close to the detection limit of the element.

2.4. Data analysis

The fungal ability to cope with Ni was expressed with Tolerance Index (TI) (Fomina *et al.* 2005; Crane *et al.* 2010):

TI = (Dry Weight (DW) of treated mycelium/Dry Weight (DW) of control mycelium) × 100

The mobility of Ni(II) from culture medium to mycelium was evaluated by means of the Bioconcentration Factor (BCF), i.e., the ratio between the element concentration in the organism and the element concentration in the surrounding environment (Garcìa *et al.* 2009). Microfungi are able to bioconcentrate (BCF > 1) or to exclude (BCF < 1) specific metal ions from their cells.

The statistical analyses were performed using Statistica 8.0 (Statsoft Inc.) software.

The TI and BCF variability at different Ni(II) concentrations were evaluated by means of one-way ANOVA. A post-hoc Tuckey's Honest Significant Difference (HSD) test was performed to evaluate the data significance. The level of significance was considered at $P<0.05$.

3. RESULTS AND DISCUSSION

Table 2 shows the results of the growing test of the isolated fungal strains at different Ni concentrations. 3 Petri dishes were used to evaluate the accumulation capability for each strain and Ni concentration for a total of 48 dishes (samples). The test highlighted that *Trichoderma harzianum* is the most Ni-tolerant strain with the fastest growth (highest biomass after 2 week, data not shown). Hence, *T. harzianum* was employed in the Ni bioaccumulation test. However, the end point concentration (800 mg Ni l^{-1}) was not tested since it did not allow mycelia maturation in the test.

The ICP-MS results of *T. harzianum* dried biomass are summarized in Figure 1. Data, reported in a logarithmic scale, showed a significant Ni accumulation capability, which increases at higher Ni(II) concentrations (Figure 1) (P<0.05). Conversely, the biomass weight showed no significant difference between control and treatments (mean ± SD, Ni0: 1.6±0.41 g; Ni100: 1.7±0.33 g; Ni200: 1.6±0.35 g; Ni400: 1.6±0.30 g; P>0.05).

The Ni bioaccumulation test highlighted the highest Ni(II) uptake by *Trichoderma harzianum* with respect to the other native microfungi *Aspergillus alliaceus, Eurotium amstelodami,* and *Clonostachys rosea,* known to uptake other ecotoxic metals (Joseph *et al.* 2011; Ceci *et al.* 2012; Gazem and Nazareth 2013). *Trichoderma* species are highly resistant to a wide variety of toxins, xenobiotic compounds, and metals (Harman *et al.* 2004; Wang and Wang 2013). Specifically, some studies showed the high metal tolerance and uptake of *Trichoderma harzianum* for the lanthanides (i.e., Cd, Zn, Hg) (Krantz-Rülcker *et al.* 1993), and high Ni tolerance (Kredics *et al.* 2001). However, none is reported for Ni mycoextraction in *T. harzianum*. Some studies investigated and tested Ni(II) uptake by several fungal species (i.e. *Aspergillus niger* – Magyarosy *et al.* 2002; *Penicillium oxalicum* – Abedin 2014; *Candida* sp. - Dönmez and Aksu 2001). However, Dönmez and Aksu (2001) evaluated that *Candida* sp. growth was severely inhibited by 500 mg Ni l^{-1}.

Table 3 reports TI and BCF mean values. TI is strongly high and does not show any significant difference between control and treatments (P>0.05). The BCF values are always very higher than 1 for each Ni concentration and show no significant difference among treatments (Table 3) (P>0.05).

Our study proves the ability of *Trichoderma harzianum* to live and uptake Ni(II) at metal concentration usually considered toxic (400 mg Ni l^{-1}). The TI and BCF values supported this finding and showed a high fungal tolerance to Ni(II), which increased over time. ICP-MS results revealed also a high and exponential

Ni bioaccumulation capability (maximum 11,000 mg Ni kg^{-1}), and a high biomass growth. These results highlight the great role that this *T. harzianum* strain may have in Ni removal and recovery. Extremophile organisms like native fungi from contaminated or metalliferous sites can represent the turning point to a new and sustainable remediation concept (Zotti *et al.* 2014; Roccotiello *et al.* 2015). Ni mycoextraction by *T. harzianum* may be employable in a removal protocol useful for both soil and water restoration, and Ni recovery while respecting ecosystem biodiversity and stability. Furthermore, mycoextraction could allow Ni recycling and Ni reinsertion on the market via mycomining. While the majority of research in the last two decades was focused on phytomining (Brooks *et al.* 1998; Chaney *et al.* 2007; Van der Ent *et al.* 2015) little information is available about mycoextraction and its potential exploitation for mycomining. Metal removal by means of fungi from soils and waters is less energy intensive processes than conventional ones and can strongly reduce costs for the industry. Indeed, *T. harzianum* is able to actively accumulate up to 1.1% Ni kg^{-1} DW and can remove Ni from soils up to 1 m deep even with a slower but more prolonged growth with respect of the one of bacteria. We can estimate an operating cost comparable with biohydrometallurgy (i.e. 10-70 $/t) with a biomass yield of 11 kg Ni/t, with a possible net yield of 60 US $/t.

In addition some mining waste as ore deposit or some degraded lands may not be remediated with conventional approaches. Metal removal by fungi can provide a cost effective alternative to conventional remediation techniques.

However much research remains to be carried out, in order to estimate the actual value of this removal technology. Surely, the selection of the Ni mycoaccumulator *Trichoderma harzianum* strain represents the first step to an economic self-sufficient, sustainable remediation system.

Table 2 Screening of isolated fungal strains at different Ni concentrations (0, 100, 200, 400, 800 respectively). Experiments were conducted in triplicate to evaluate the accumulation capability for each strain and Ni concentration for a total amount of 48 dishes (samples). The sign plus ("+") indicates that the strain grew during the test.

Table 3 Summary of *Trichoderma harzianum* ability to tolerate (TIs = (DW of treated mycelium/DW of control mycelium) × 100) and bioconcentrate (BCFs= $[Ni_{fungi}]$ / $[Ni_{medium}]$) Ni(II) at different Ni treatments (0, 100, 200, 400 mg Ni l^{-1}, respectively). Means ± SD (*n*=12).

Figure 1 Ni(II) accumulation capability by *T. harzianum* (dried biomass) at different Ni treatment (0, 100, 200, 400, respectively). Means ± SD (*n*=12), data on Ni(II) accumulation are reported on a log scale.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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318 **Table 1** Overview on the costs and fields of application of the traditional remediation and bioremediation techniques.

Technique	**Localization**	**Pollutant type**	**Method**	**Intervention**	**Environmental matrix**	**Cost**	**Reference**
Separation pollutant is separated from polluted matrix and treated	*In situ*	Inorganic and organic compounds	Chemical-physical	dual phase extraction; sieving	Soil; Soil -Water; subsoil; groundwater	250-500 $/t	Bertelle and Beretta 2009; Bortone *et al.* 2013
				pump & treat	Groundwater	1-5 $/t	
				soil flushing	Topsoil	13-136 $/t	Khan *et al.* 2004
		Metals Cu, Zn, Cd, Pb, U, Fe, Ni, Ag, Th, Ra	Biological	biohydrometallurgy (bacteria and fungi)	water, leachate	10-70 $/t	Poulin and Lawrence 1996
				Biosorption by alive or dead fungal cells (e.g *Aspergillus niger, A. oryzae, Penicillium chrysogenum, P. spinulosum, Saccaromyces*	Wastewaters	1000-5000 $/t	Bishnoi and Garima 2005

	cerevisiae, Rhizopus nigricans, R. arrhizus, Mucor rouxii)			
Metals Ag, Cd, Co, Cr, Cu, Hg, Mn, Mo, Ni, Pb, Zn radionuclide (^{90}Sr, ^{137}Cs, 239Pu, ^{238,234}U)	Phytoextraction (e.g. *Brassica juncea, Thlapsi caerulescens, Helianthus annuus*, *Alyssum* sp., *Populus* sp.)	Soil, sediment, sludge	25-100 $/t	EPA 2000a; Khan *et al.* 2004
Inorganic and organic compounds	soil washing	Soil	170 $/t	Khan *et al.* 2004
Inorganic compound	Bioleaching (bacteria and fungi)	water and waste stream	40 $/t	Brown 1999; Harvey and Bath 2007
Fe, Cu, Zn, Au	Biomining by bacteria (*At. ferrooxidans* and *At. thiooxidans)*	waste stream	40 $/t	Brown 1999; Harvey and Bath 2007
Au, Pt, Ag, Pd	Biomining by fungi (*Aspergillus niger, Cladosporium cladosporoides, Mucor rouxii,*	aqueous solutions	-	Das 2010

				Rhizopus arrhizus)			
		Metals, radionuclides		Rhizofiltration	Water, groundwater	10-35 $/t	Schnoor 1998
	Ex situ	Inorganic and organic compounds	Chemical-physical	soil washing	Soil	130-140 $/t 42-115 $/t physical separation 240-1025 $/t Chemical extraction	FRTR 2007; Dermont 2008; Bertelle and Beretta 2009
				solvent extraction	Soil	360-440 $/t	Schnoor 1998
Transformation Pollutant is transformed into less toxic/harmless substances	*In situ*	Se, Hg, As, chlorinated solvents	Biological	Phytovolatilization (e.g. *Populus* sp., *Medicago sativa, Robinia pseudoacacia, Brassica juncea)*	Soil, sediment, sludge, water, groundwater	25-100 $/t	EPA 2000a
	Ex situ	Inorganic and organic compounds	Chemical-physical	Chemical treatment; inerting	Soil	360-600 $/t	EPA 2000b
Immobilization Pollutant is immobilized into a matrix or via	*In situ*	Mainly inorganic compounds	Chemical-physical	solidification; containment	Soil	240-340 $/t	Schnoor 1998
		Inorganic and	Thermal	Vitrification	Soil	110 $/t	Khan *et al.*

transformation into less		organic compounds					2004
mobile substances		As, Cd, Cr, Cu, Hg, Pb, Zn	Biological	Phytostabilization (e.g. *Brassica juncea*, *Populus* sp., etc.)	Soil, sediment, sludge	0.01-0.6 $/t	EPA 2000a
	Ex situ	Inorganic and organic compounds	Thermal	Vitrification	Soil	50-200 $/t	Khan *et al.* 2004
		Mainly inorganic compounds	Chemical-physical	Solidification	Soil	145-600 $/t	EPA 2000b

Table 2 Screening of isolated fungal strains at different Ni concentrations (0, 100, 200, 400, 800 respectively). Experiments were conducted in triplicate to evaluate the accumulation capability for each strain and Ni concentration for a total amount of 48 dishes (samples). The sign plus ("+") indicates that the strain grew during the test.

Strain	**Treatments (mg Ni l^{-1})**				
	0	**100**	**200**	**400**	**800**
Aspergillus alliaceus	+	+	+	-	-
Clonostachys rosea	+	+	+	-	-
Eurotium amstelodami	+	+	-	-	-
Trichoderma harzianum	+	+	+	+	+

Table 3 Summary of *Trichoderma harzianum* ability to tolerate (TIs = (DW of treated mycelium/DW of control mycelium) × 100) and bioconcentrate (BCFs= [Ni_{fungi}] / [Ni_{medium}]) Ni(II) at different Ni treatments (0, 100, 200, 400 mg Ni l^{-1}, respectively). Means ± SD (*n*=12).

Treatments (mg Ni l^{-1})	**TI (%)**	**BCF**
0	100	-
100	104.6 ± 9.7	25.4 ± 2.4
200	102.4 ± 11.1	26.0 ± 0.8
400	101.8 ± 7.1	27.3 ± 1.4

333 **Figure 1** Ni(II) accumulation capability by *T. harzianum* (dried biomass) at different Ni treatment (0, 100, 200, 400,

334 respectively). Means ± SD (*n*=12), data on Ni(II) accumulation are reported on a log scale.

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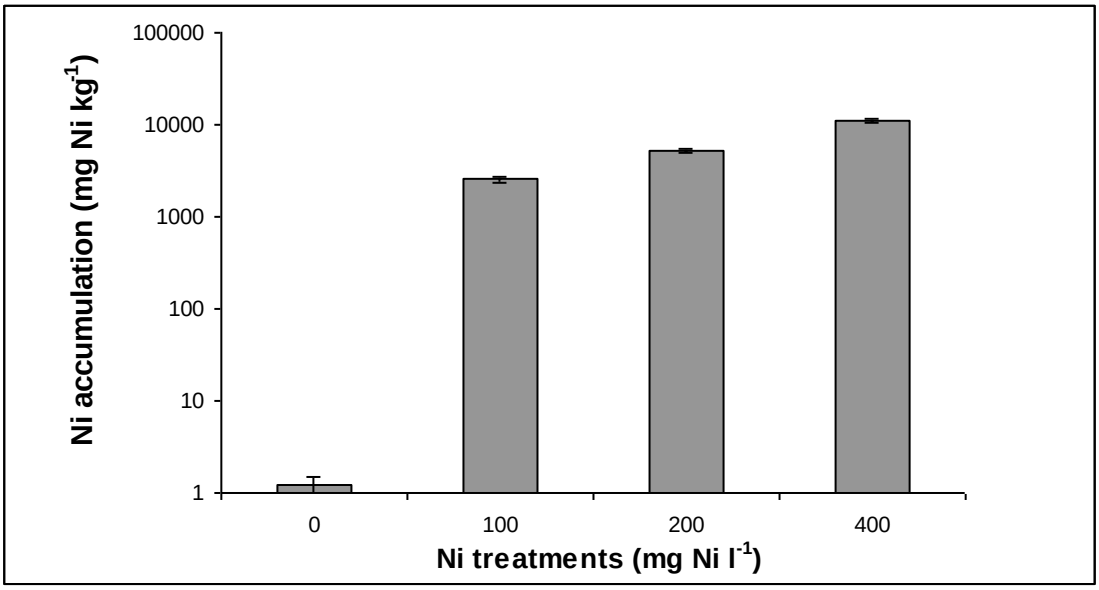

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