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Biotransformation of manganese oxides by fungi: solubilization and production of manganese oxalate biominerals

Zhan Wei,[1] Stephen Hillier[2] and Geoffrey M. Gadd[1]*
[1]*Division of Molecular Microbiology, College of Life Sciences, University of Dundee, Dundee DD1 5EH, UK*
[2]*The James Hutton Institute, Craigiebuckler, Aberdeen AB15 8QH, UK*

Summary

The ability of the soil fungi *Aspergillus niger* and *Serpula himantioides* to tolerate and solubilize manganese oxides, including a fungal-produced manganese oxide and birnessite, was investigated. *Aspergillus niger* and *S. himantioides* were capable of solubilizing all the insoluble oxides when incorporated into solid medium: MnO_2 and Mn_2O_3, mycogenic manganese oxide (MnO_x) and birnessite [$(Na_{0.3}Ca_{0.1}K_{0.1})(Mn^{4+},Mn^{3+})_2O_4{\cdot}1.5H_2O$]. Manganese oxides were of low toxicity and *A. niger* and *S. himantioides* were able to grow on 0.5% (w/v) of all the test compounds, with accompanying acidification of the media. Precipitation of insoluble manganese and calcium oxalate occurred under colonies growing on agar amended with all the test manganese oxides after growth of *A. niger* and *S. himantioides* at 25°C. The formation of manganese oxalate trihydrate was detected after growth of *S. himantioides* with birnessite which subsequently was transformed to manganese oxalate dihydrate. Our results represent a novel addition to our knowledge of the biogeochemical cycle of manganese, and the roles of fungi in effecting transformations of insoluble metal-containing compounds in the environment.

Introduction

Manganese is an important element in both terrestrial and aqueous environments. It is the fifth most abundant element in the Earth's crust and is an essential nutrient that can become toxic or deficient depending on environmental factors (Tebo *et al.*, 2005; Konhauser, 2007; Ehrlich and Newman, 2009). In nature, manganese can exist in the oxidation states +2, +3 and +4, and in soil it occurs as soluble and bioavailable Mn^{2+}, or as insoluble Mn^{3+} and Mn^{4+} oxides (Tebo *et al.*, 2005; Thompson *et al.*, 2005; Ehrlich and Newman, 2009). The role of manganese is significant in biological systems. It is an essential metal for most organisms, and is particularly important for plants and other photosynthetic microorganisms where it is required for photosynthetic oxygen evolution (Sigel, 2000; Tebo *et al.*, 2005; Dismukes and Willigen, 2006). Mn is also an activator of a number of important enzymes, e.g. isocitric dehydrogenase and malic enzyme (Ehrlich and Newman, 2009). In recent years, attention has focused on the biogeochemistry of manganese oxidation since Mn(III, IV) oxide minerals are ubiquitous in terrestrial and marine environments (Grote and Krumbein, 1992; Miyata *et al.*, 2004; 2006; 2007; Tebo *et al.*, 2004; Saratovsky *et al.*, 2009; Santelli *et al.*, 2011) and the formation of such oxides is a key process in the manganese cycle where microorganisms have a significant role.

Diverse microorganisms, including bacteria and fungi, can oxidize soluble Mn(II) and produce insoluble Mn(III)-Mn(IV) oxides (Grote and Krumbein, 1992; Tebo *et al.*, 2004; Miyata *et al.*, 2007; Ehrlich and Newman, 2009; Gadd *et al.*, 2012; Santelli *et al.*, 2011). Mn oxides play an important role in aquatic and terrestrial environments as they can affect nitrogen cycling (Thompson *et al.*, 2005), and also take part in a wide range of redox reactions with organic and inorganic chemical species and compounds (Tebo *et al.*, 2004). They are highly reactive and have high sorption capacities for numerous metal cations (e.g. Ni, Zn, Cu, Co, Mn, Pb and Cd), and also serve as strong oxidants for certain elements [e.g. As(III) to As(V); Cr(III) to Cr(IV)] and organic compounds such as humic substances (Tebo *et al.*, 2004; Thompson *et al.*, 2005; Miyata *et al.*, 2007; Jin *et al.*, 2009; Gadd *et al.*, 2012; Santelli *et al.*, 2011). Many bacterial species can oxidize manganese which is then deposited on cells, sheaths, or spores as oxides. Several fungi can also promote Mn(II) oxidation to $Mn(IV)O_2$ including *Acremonium* spp. (Miyata *et al.*, 2004; 2006; 2007; Saratovsky *et al.*, 2009).

A number of taxonomically unrelated bacteria can reduce manganese enzymatically or non-enzymatically (Lovley, 2000; Ehrlich and Newman, 2009; Gadd, 2010). Some bacteria and most of those fungi that reduce Mn(IV)

Received 20 February, 2012; revised 17 April, 2012; accepted 17 April, 2012. *For correspondence. E-mail g.m.gadd@dundee.ac.uk; Tel. (+44) 1382 384767; Fax (+44) 1382 388216.

Table 1. Growth on and solubilization of manganese oxides by *Aspergillus niger* and *Serpula himantioides*.

	A. niger		*S. himantioides*	
	$R_m : R_c$	$R_s : R_m$	$R_m : R_c$	$R_s : R_m$
MnO_2	0.80	–	0.98	–
Mn_2O_3	0.82	–	0.94	–
Biogenic MnO_x	0.90	0.89	0.95	0.87
Birnessite	0.94	0.80	0.80	0.96

Results are presented in the form of ratios of the rate of growth on manganese oxide-amended MEA medium in relation to growth on control medium ($R_m : R_c$) and the rate of extension of the solubilization zone in relation to growth on that given mineral ($R_s : R_m$). A ratio of 1.0 indicates that the colony extension rate in the presence of a metal compound (R_m) is the same as the control extension rate (R_c) and that the rate of extension of the clear zone of solubilization (R_s) on a given manganese compound is the same as the colony extension rate (R_m). The control growth rate for *A. niger* was 5.9 $\pm$ 0.12 mm day^{-1} over 15 days incubation at 25°C, that of *S. himantioides* was 4.3 $\pm$ 0.16 mm day^{-1} over 23 days incubation at 25°C (average of three replicates $\pm$ standard error of the mean).

oxides such as MnO_2 reduce them indirectly (non-enzymatically), with the likely mechanism being the production of metabolic products that can act as reductants for Mn(IV) oxides such as formic acid, pyruvate, H_2S, sulfite, Fe(II) (bacteria) and oxalate (fungi) (Ehrlich and Newman, 2009; Gadd, 2010). Reduction of manganese oxides releases mobile Mn(II) and this can result in dissolution of the oxide. However, little attention has been given to fungal reduction (solubilization) of manganese oxides including mycogenic manganese oxide.

The solubilization of insoluble metal compounds and minerals by fungi is of environmental and biotechnological significance (Sayer *et al*., 1995; Sayer and Gadd, 1997; Gharieb *et al*., 1998; Fomina *et al*., 2005a; Gadd, 2007) and particularly important in the soil environment because of the release of potentially toxic and/or nutrient metals and associated anionic components, e.g. phosphate (Gadd, 2011). Many researchers have highlighted fungal tolerance to high metal concentrations and the ability to leach metals in low or high pH environments (Burgstaller and Schinner, 1993; Sayer *et al*., 1995; Sayer and Gadd, 1997; 2001; Gharieb *et al*., 1998; Gharieb and Gadd, 1999; Fomina *et al*., 2005a). Solubilization can occur by protonation of the anion of the metal compound, and/or the formation of a soluble metal-ligand complex, and an important mechanism is the production of organic acids (Sayer *et al*., 1995; Sayer and Gadd, 1997; Gadd, 1999; 2007; Gadd *et al*., 2012).

The objective of this research was to investigate and demonstrate fungal solubilization of biogenic and synthetic manganese oxides, including birnessite which is one of the commonest manganese minerals found in soil. To our knowledge, this is the first study of the solubilization of these substances by fungi, and their subsequent transformation into manganese oxalates, manganese oxalate trihydrate preceding manganese oxalate dihydrate formation. Our results contribute to a more detailed understanding of the roles of fungi as geoactive agents and their possible contributions to the biogeochemical cycling of manganese.

Results

Growth on, and solubilization of manganese oxides by Aspergillus niger *and* Serpula himantioides

The results in Table 1 show that *A. niger* and *S. himantioides* were able to grow on all the manganese oxides tested, although *A. niger* was affected by the commercial manganese oxides relative to the control (Table 1), and tolerance index values were around 70% for both biogenic MnO_x and birnessite (Table 2). *Serpula himantioides* was highly tolerant to all the manganese oxides tested with tolerance index values exceeding 100% (Table 2). However, the growth rate of *S. himantioides* on birnessite-amended medium was slower than on other growth media (Table 1). There were no significant differences in growth rate for *S. himantioides* grown on media containing commercial manganese oxide (Table 1).

The capacity of an active strain to solubilize the insoluble Mn compounds was manifest by the production of a halo around the growing colony (Gharieb *et al*., 1998). In this study, the solubilization activity of both *A. niger* and *S. himantioides* was clearly observed (Table 1). Both *A. niger* and *S. himantioides* were able to solubilize all the insoluble manganese oxides provided (Table 1, Fig. 1). For colonies grown with commercial manganese oxide, the appearance of solubilization was different in that the medium became lighter around the growing colony instead of a clear halo forming (Fig. 1A and B). After 15 days and 25 days growth of *A. niger* and *S. himantioides* respectively, the oxides were all completely solubilized by the fungi. There were no significant differences for extension of the solubilization zone (R_s) on the given metal compound compared with colony

Table 2. Tolerance indices for test fungi grown on manganese oxides.

	MnO_2	Mn_2O_3	Biogenic MnO_x	Birnessite
Aspergillus niger	70.0	65.7	72.0	72.8
Serpula himantioides	144.0	136.4	164.6	196.4

Values shown are tolerance indices derived from the dry biomass weight of colonies grown on MEA media amended with different manganese oxide minerals. *Aspergillus niger* was grown for 15 days, *S. himantioides* was grown for 23 days at 25°C in the dark. All values shown are percentages derived by comparison with the mean control biomass yield (averages from three measurements are shown with typical relative standard deviations of about 5%).

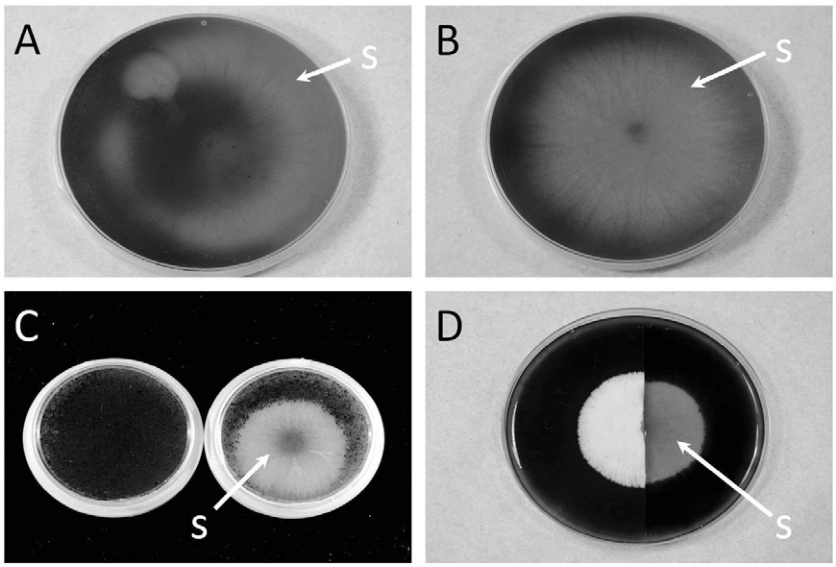

Fig. 1. Solubilization of manganese oxides in MEA medium by *Aspergillus niger* and *Serpula himantioides*.
A. MnO_2 solubilization by *A. niger*.
B. Mn_2O_3 solubilization by *A. niger*.
C. Biogenic MnO_x solubilization by *S. himantioides*, control (left), biogenic MnO_x containing medium (right).
D. Birnessite solubilization by *S. himantioides*: half of the overlying cellulose membrane with fungal mycelium was cut out to show the solubilization zone underneath the colony (S).

extension rate (R_m) after growth of *A. niger* and *S. himantioides* on biogenic MnO_x and birnessite (Table 1). *Serpula himantioides* showed a stronger capacity for solubilization of birnessite (Table 1). After inoculation and growth of *A. niger* and *S. himantioides*, the final media pH values were markedly decreased, especially for biogenic manganese oxide and birnessite-containing medium (Table 3).

Formation of manganese oxalate by Aspergillus niger *and* Serpula himantioides *during growth on manganese oxides*

Crystals formed under colonies of *A. niger* and *S. himantioides* growing on agar medium amended with MnO_2 (Fig. 2A) and Mn_2O_3 (Fig. 2B), and were visible after the agar medium cleared (Fig. 2C and D)., Crystals also formed in the agar amended with biogenic MnO_x and birnessite under and around the fungal colonies, appearing after 3–4 days growth at 25°C in the dark (Fig. 2E–L). There appeared to be four different patterns of crystals produced by *A. niger* grown on manganese oxide-containing medium (Fig. 2C, F, H and J). The sizes of these two groups of crystals were approximately 200 μm and 25 μm in diameter respectively. Energy dispersive X-ray analysis showed that the larger crystal type contained manganese while the smaller crystals contained calcium (Fig. 3). All the crystals that formed under colonies of *S. himantioides* (Fig. 2D, G, K and L) had approximately the same components as the biominerals produced by *A. niger* grown on manganese oxide-amended medium and contained manganese. In the medium with 0.5% (w/v) birnessite, large crystals formed after growth of *S. himantioides* for 5 days (Fig. 2K), but these changed into small crystals after this time (Fig. 2L).

X-ray powder diffraction (XRPD) analyses of manganese-containing biominerals produced by *A. niger* and *S. himantioides* confirmed the presence of well-crystallized compounds with a match to reference patterns for manganese oxalate trihydrate (Fig. 4A) and manganese oxalate dihydrate (Fig. 4B). Manganese oxalate trihydrate (Fig. 4A) was found after 5 days growth of *S. himantioides* on birnessite-amended agar at 25°C (shown in Fig. 2K), but subsequently was replaced by manganese oxalate dihydrate (shown in Fig. 2L). We can conclude therefore that the larger crystals are the trihydrate form.

Table 3. Surface pH values of agar underneath growing colonies on control and manganese oxide-containing medium.

	MEA	+MnO_2	+Mn_2O_3	+Biogenic MnO_x	+Birnessite
Initial pH	5.35	6.35	5.96	7.01	7.26
Aspergillus niger	3.47	2.90	2.78	2.96	3.08
Serpula himantioides	2.40	2.29	2.26	2.42	2.93

Values shown are averages from six measurements (with typical relative standard deviations of about 5%) across the agar plates. *Aspergillus niger* was grown for 15 days, *S. himantioides* was grown for 25 days at 25°C in the dark.

Solubilization of manganese oxides by organic acids

Aspergillus niger and *S. himantioides* are prolific producers of organic acids such as citric and oxalic acid (Gharieb *et al.*, 1998; Gadd, 1999; 2011; Karaffa and Kubicek, 2003; Jarosz-Wilkolazka and Gadd, 2003; Plassard and Fransson, 2009; Arwidsson *et al.*, 2010). Both oxalic acid and citric acid were able to solubilize biogenic MnO_x and birnessite, producing obvious solubilization haloes in amended media (Fig. 5). Significant solubilization haloes

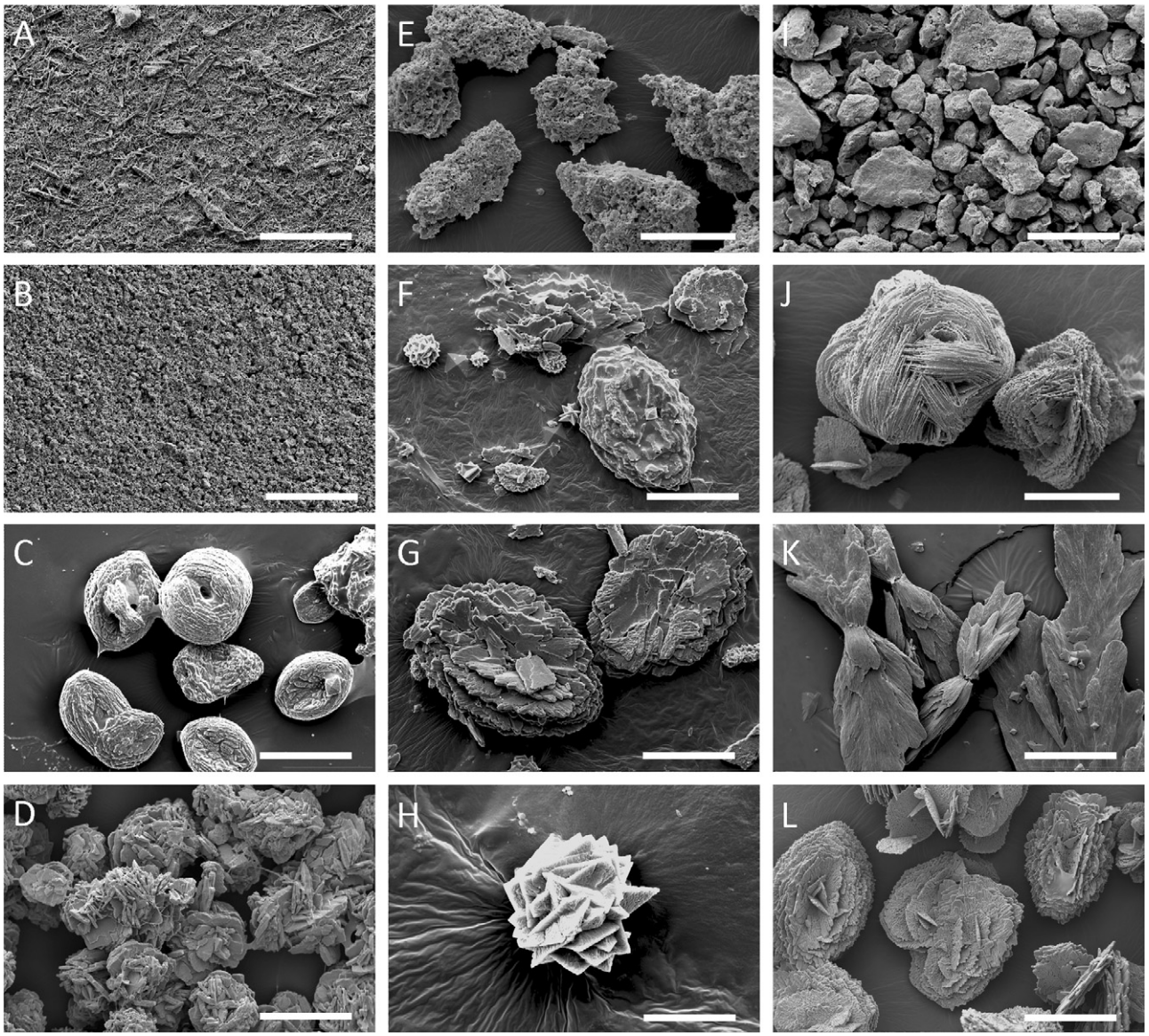

Fig. 2. Scanning electron microscopy of manganese oxides and the biominerals extracted from manganese oxide-amended MEA after fungal growth on (A–D) MnO_2 and Mn_2O_3 (E–H) biogenic MnO_x, and (I–L) birnessite.
A. MnO_2, scale bar = 500 μm.
B. Mn_2O_3, scale bar = 200 μm.
C. Biomineral produced by *Aspergillus niger* on MnO_2 amended agar after 15 days incubation at 25°C, scale bar = 200 μm.
D. Biomineral appearing in MnO_2 amended agar with *Serpula himantioides* after 23 days incubation at 25°C, scale bar = 200 μm.
E. Initial biogenic MnO_x, scale bar = 100 μm.
F. Biominerals extracted from biogenic MnO_x-amended medium after growth of *A. niger* for 15 days at 25°C, scale bar = 100 μm.
G. Biominerals produced by *S. himantioides* after growth on biogenic MnO_x-amended medium for 23 days at 25°C, scale bar = 100 μm.
H. Biominerals extracted from biogenic MnO_x-amended medium after growth of *A. niger* for 15 days at 25°C in the dark, scale bar = 25 μm.
I. Birnessite extracted from abiotic plates.
J. Biominerals produced by *A. niger* grown on birnessite mineral-amended medium for 15 days at 25°C.
K and L. Biominerals extracted from birnessite mineral-amended medium after growth of *S. himantioides* for (K) 5 days and (L) 23 days at 25°C. Scale bars, (I, J, L) = 200 μm, (K) = 500 μm.
All oxides were incorporated in the media to 0.5% (w/v) and all incubations were in the dark: typical images are shown from several examinations.

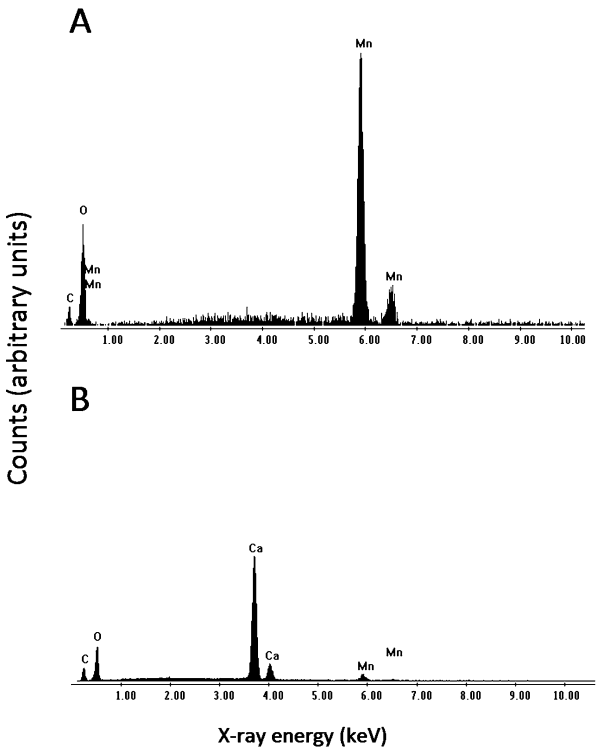

Fig. 3. X-ray microanalysis of crystals produced by *Aspergillus niger* and *Serpula himantioides* during growth with biogenic MnO_x. A. Manganese-containing biomineral produced by *S. himantioides*. B. Calcium-containing biomineral (shown in Fig. 2H) produced by *A. niger*. Typical spectra are shown from one of at least three determinations.

were however not formed in MnO_2- and Mn_2O_3-amended malt extract agar (MEA) medium.

Discussion

Many bacterial species can oxidize manganese, by enzymatic and non-enzymatic mechanisms, which is then deposited on cells, sheaths or spores as oxides (Tebo *et al*., 2005). Several fungi can also take part in manganese oxidation including *Acremonium* spp. (Miyata *et al*., 2004; 2006; 2007; Saratovsky *et al*., 2009; Hennebel *et al*., 2009; Santelli *et al*., 2011). For some basidiomycete white-rot fungi like *Phanerochaete chrysosporium*, redox transformations of Mn occur during lignin degradation but this oxidation is thought to be of minor significance regarding manganese biogeochemistry (Ehrlich and Newman, 2009). Bacteria and fungi also play an important role in manganese reduction and they may mobilize oxidized or fixed manganese enzymatically or non-enzymatically (Lovley, 2000; Tebo *et al*., 2004; Ehrlich and Newman, 2009; Das *et al*., 2011). In addition to direct enzymatic reduction by multicopper oxidases, many microorganisms excrete metabolites that can act as reductants for Mn oxides such as formic acid, pyruvate, and H_2S (bacteria) and oxalate (fungi), that lead indirectly to manganese reduction (Konhauser, 2007; Gadd, 2010). However, relatively few studies have examined the environmental importance of biological manganese oxide reduction although it is known that biological Mn oxide reduction is linked with a significant fraction of the total amount of organic matter degraded in coastal sediments (Canfield *et al*., 1993).

In this work, we have investigated the fungal reduction (solubilization) of a biogenic manganese oxide (produced by a fungus, *Acremonium strictum*), a manganese oxide mineral (birnessite) and commercial Mn oxides. We have clearly demonstrated that the test fungi *A. niger* and *S. himantioides*, possessed a high capacity to solubilize all these Mn oxides, and this is likely to be a property found in several other fungal species. Free-living and symbiotic fungi can be involved in mineral formation through precipitation of organic and inorganic secondary minerals and through nucleation and deposition of crystalline material on and within cell walls, such as oxalates and carbonates (Burford *et al*., 2003; 2006; Gadd, 2007; 2010; Gadd and Raven, 2010). Many fungal species can produce metal oxalates on interacting with a variety of different metals and metal-bearing minerals, e.g. Ca, Cd, Co, Cu, Mg, Mn, Sr, Zn, Ni and Pb (Sayer and Gadd, 1997; Gadd, 1999; 2007; 2010; Fomina *et al*., 2005b). It is often suggested that the formation of toxic metal oxalates may provide a mechanism that enables oxalate-producing fungi to tolerate high concentrations of toxic metals

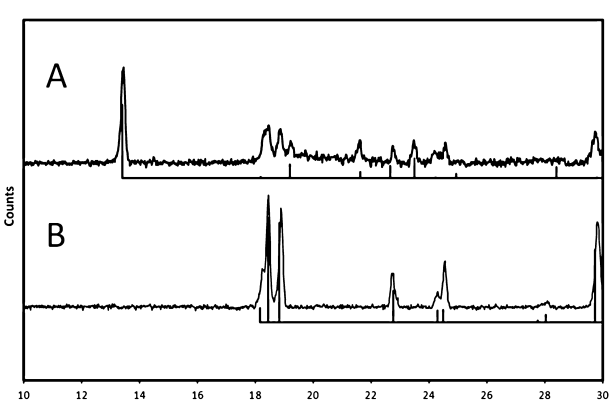

Fig. 4. X-ray powder diffraction patterns of biominerals extracted from birnessite-amended medium. Diffraction patterns were collected from crystals harvested from birnessite-amended MEA medium after growth of *Serpula himantioides* for (A) 5 days and (B) 23 days. (A) is the sample containing the large crystals (shown in Fig. 2K) and contains Mn-oxalate-trihydrate in addition to the dihydrate (lindbergite). (B) only contains Mn-oxalate dihydrate after longer incubation (crystals shown in Fig. 2L). The larger crystals are therefore identified as the trihydrate form. The powder diffraction file reference 'stick' patterns are (A) 012-0663 (Mn-oxalate trihydrate) and (B) 025-0544 (Mn-oxalate-dihydrate). Typical patterns are shown from one of at least three determinations.

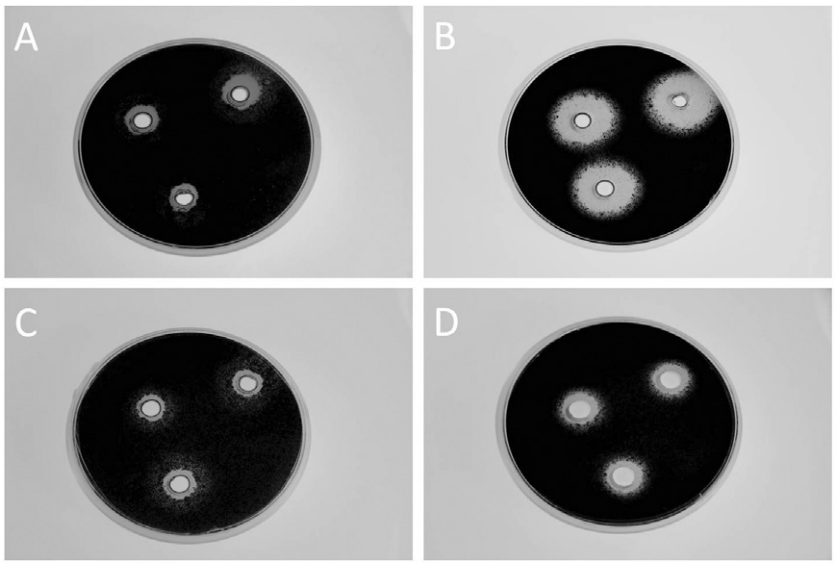

Fig. 5. The ability of organic acids to solubilize manganese oxide incorporated into MEA. Images show results of an abiotic test for solubilization of manganese oxides by addition of organic acids to 5 mm diameter wells cut in the agar medium. (A) and (B) are biogenic MnO_x and birnessite amended medium respectively with addition of 50 µl 0.5 M citric acid. (C) and (D) are biogenic MnO_x and birnessite amended medium respectively with addition of 50 µl 0.5 M oxalic acid. Typical results are shown from one of at least three different experiments.

(Fomina *et al*., 2005b; Gadd, 2007; 2010). Mycorrhizal fungi are involved in proton-promoted and ligand-promoted metal mobilization and immobilization via biosorption and accumulation within biomass as well as extracellular precipitation of mycogenic toxic metal oxalates (Fomina *et al*., 2005a). The pH can therefore be a vital factor in the processes involved in mineral transformations by fungi. Metal biosorption, precipitation, nucleation and deposition of crystalline material are influenced by pH (Burford *et al*., 2003; 2006; Parvathi *et al*., 2007; Gadd, 2010), while the nature and amount of organic acids excreted by fungi are also influenced by the pH and buffering capacity of the environment (Gadd, 1999; Sayer and Gadd, 2001; Fomina *et al*., 2007; 2008; Gadd and Fomina, 2011). The natural dihydrate form of calcium sulphate, ($CaSO_4.2H_2O$) (gypsum), found in gypsiferous soils and certain building construction materials, was effectively solubilized by *A. niger* and *S. himantioides* with the production of oxalic acid resulting in precipitation of calcium oxalate (Gharieb *et al*., 1998). Burford and colleagues (2006) found that a mixture of calcite ($CaCO_3$) and calcium oxalate monohydrate (whewellite; $CaC_2O_4.H_2O$) was precipitated on hyphae of *S. himantioides* when grown in simulated limestone microcosms. Our experiments have shown that oxalic acid producing *A. niger* and *S. himantioides* are capable of transforming insoluble manganese oxide minerals, including those produced biogenically, into manganese oxalates via a ligand-mediated solubilization process, in some cases manganese oxalate trihydrate resulting in the initial solubilization process, followed by conversion to manganese oxalate dihydrate (Fig. 6), which to our knowledge has not been observed before. It is possible that the monohydrate may also result, as for calcium oxalate biominerals (Gadd, 1999), although we did not detect this in any of our samples. Figure 6 summarizes Mn biotrans-

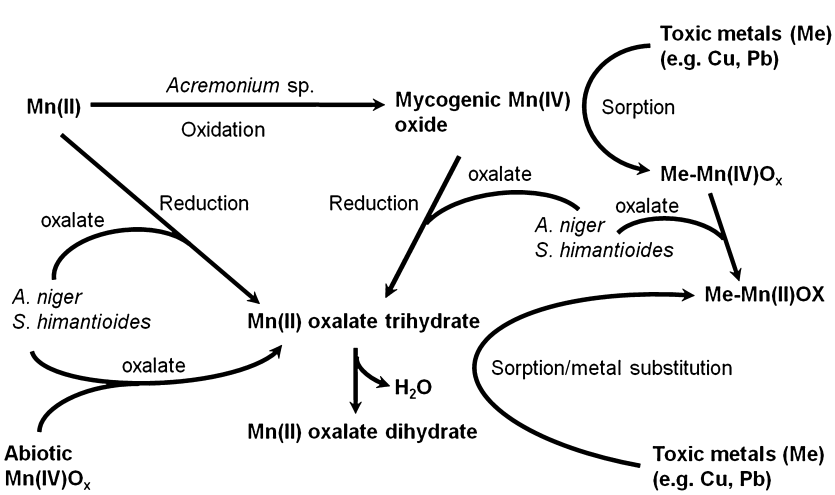

Fig. 6. A conceptual model for fungal transformations of manganese based on the work reported here. Fungal oxidation of Mn(II) to Mn(IV), and reduction of Mn(IV) to Mn(II) are shown as well as the organisms mediating such transformations. Microbial decomposition and utilization of manganese oxalate is not shown, but this will result in re-entry of Mn(II) into the biogeochemical cycles. The possible formation of manganese oxalate monohydrate is also omitted. Sorption of toxic metals to Mn(IV) oxides is also indicated (Miyata *et al*., 2007) and we speculate on such sorption/metal substitution reactions with the mycogenic oxalates, and conversion of metal-loaded manganese oxides to oxalates.

formations to oxalates but also highlights the further environmental relevance of these interactions, including those with Mn oxides. Microbially produced Mn oxides have significant sorption properties and several studies have examined the significant sorption capacity of biogenic Mn oxides for metal cations, e.g. Ni, Zn, Cu, Co, Mn, Pb and Cd (Nelson *et al*., 1999; Tebo *et al*., 2004; Toner *et al*., 2006; Miyata *et al*., 2007). Mn oxides are also able to oxidize numerous inorganic [e.g. As(III) to As(V); Cr(III) to Cr(IV)] and organic substrates (Tani *et al*., 2004; Murray *et al*., 2005; Murray and Tebo, 2007). Moreover, Mn oxides [and also Fe(III) (hydr)oxides] can act as terminal electron acceptors for bacterial respiration in anoxic environments (Tebo and Obraztsova, 1998), and this can determine the fate of a wide variety of organic compounds such as organic acids, fatty acids, and aromatics in the anaerobic subsurface (Lovley, 2000). Interactions of metal-loaded Mn oxides with oxalate may lead to mixed-metal oxalate formation. Metal oxalates themselves can interact with other metals and again we can speculate on the significance of sorption phenomena (Gadd, 2009) or metal-substitution reactions with these biominerals (Fig. 6). Clearly, little is known about the chemical characteristics of mycogenic manganese oxalates, but the thermal behaviour and magnetic properties of synthetic Mn(II) oxalate crystallohydrates (both trihydrate and dihydrate) have a wide application as precursors for the preparation of nano-materials, superconductors, sorbents and catalysts (Donkova and Mehandjiev, 2004). Further research on mycogenic oxalates may therefore be useful for the potential applications of such biominerals. Finally, microbial degradation of manganese oxalates will lead to recycling of the metal for interaction with the environment and biota, and possible formation of other secondary minerals. In conclusion, our observations represent a novel addition to our knowledge of biogenic components of the biogeochemical cycling of manganese, and again highlight the roles of fungi in effecting transformations of metals and minerals.

Experimental procedures

Organism, medium and growth conditions

The organisms used were *A. niger* van Tieghem (ATCC201373) and *S. himantioides* (Fries: Fries) P. Karst (originally provided by Dr N. White, University of Abertay, Dundee), which were maintained on MEA (LabM, Bury, UK) at 25°C. All experiments were carried out at 25°C in the dark using MEA with the addition of appropriate manganese compounds and minerals to the desired final concentration.

Metal compounds

Biogenic manganese oxide was produced by a strain of *A. strictum* (isolated and identified by Dr M. Fomina, University of Dundee) grown in Hepes-buffered AY medium (HAY), pH 7.0, containing 2 mM $MnSO_4$ (Miyata *et al*., 2004). HAY medium contains 3 mmol of sodium acetate, 150 mg of yeast extract, 50 mg of $MgSO_4.7H_2O$, 5 mg of K_2HPO_4, and 2 ml of a trace mineral salts solution per litre of 20 mM Hepes buffer (pH 7.0), and 2 mmol of $MnSO_4$. A stock K_2HPO_4 solution (30 mM) was separately autoclaved (121°C, 25 min) and added to the prescribed concentration. The trace mineral salts solution contained (per litre): 3.7 g of $CaCl_2.2H_2O$, 2.5 g of H_3BO_3, 0.87 g of $MnCl_2.4H_2O$, 1.0 g of $FeCl_3.6H_2O$, 0.44 g of $ZnSO_4.7H_2O$, 0.29 g of $Na_2MoO_4.2H_2O$, and 5 mg of $CuSO_4.5H_2O$. The production of biogenic manganese oxide was initiated when HAY medium containing 2 mM $MnSO_4$ was inoculated with *A. strictum* conidia (10^5 ml^{-1}) and incubated on a shaking incubator (105 r.p.m.). In order to prepare the conidial suspension, *A. strictum* was grown in 50 ml of HAY, pH 7.0, for 1 week at 25°C in a shaking incubator (105 r.p.m.) (Miyata *et al*., 2004). Fungal biomass was removed by filtration through sterilized muslin cloth (autoclaved twice at 121°C for 25 min), and the filtrates were stored at 4°C prior to use as conidial suspensions after appropriate dilution with sterile Milli-Q water. Biogenic manganese oxide was separated from the liquid medium by filtration through plastic mesh (100 μm mesh size) and washed with Milli-Q water several times. Samples were oven-dried at 105°C until constant weight, and ground to a powder using a pestle and mortar (Milton Brook, Dorset, UK). The synthetic manganese oxide mineral, birnessite (sodium form), was provided by Dr. S. Hillier (The James Hutton Institute, Aberdeen, AB15 8QH) and prepared from synthetic Na-buserite by freeze drying. Mn_2O_3 and MnO_2 were purchased from Sigma-Aldrich, UK.

Preparation of metal-amended plates and inoculation

Test fungi were grown on 20 cm^3 of MEA amended with 0.5% (w/v) metal compounds/minerals in 90 mm diameter Petri dishes. Prior to inoculation, 84 mm diameter discs of sterile cellophane membrane (Focus Packaging and Design Ltd, UK) were placed aseptically on the surface of the agar in each Petri dish. Inoculation was carried out by using 5 mm diameter discs of mycelium cut from the leading edge of colonies which had been maintained on MEA at 25°C for at least 7 days for *A. niger* and 14 days for *S. himantioides*. *Aspergillus niger* and *S. himantioides* were inoculated onto each metal compound (at least three replicates) and incubated at 25°C in the dark. Daily measurements were made of colony size and of any clear zones present. Measurements were discontinued when the colonies or clear zones had reached the edge of the Petri dish. Fungal colonies were removed from the agar plates by peeling the biomass from the cellophane membrane. The mycelia were dried in an oven to constant weight at 105°C for at least 2 days. Metal tolerance was evaluated using a tolerance index (TI) based on the dry weight of fungal biomass as follows: TI = (dry weight of treated mycelium/dry weight of control mycelium) × 100% (Sayer *et al*., 1995). SigmaPlot 12 (Systat Software) was used for statistical analysis, and at least three replicate determinations were used in experiments. Statistical tests including the Student's *t*-test and ANOVA were also conducted on data using SigmaPlot 12.

Purification of crystals produced by Aspergillus niger *and* Serpula himantioides *during growth on selected metal compounds*

Crystals were formed in the agar under fungal colonies and in the clear zones of solubilization when *A. niger* and *S. himantioides* were grown on commercial and biogenic manganese oxide, and birnessite. Crystals appeared after approximately 3 days growth of *A. niger* and 5 days growth of *S. himantioides* at 25°C. Crystals were extracted from the agar by gently homogenizing the agar with Milli-Q water at 80°C in a crystallizing dish. Crystals were dried at least overnight in a vacuum desiccator prior to examination by scanning electronic microscopy (SEM), energy dispersive X-ray analysis (EDXA) and XRPD.

Analysis of mineral transformation products

Scanning electronic microscopy and energy dispersive X-ray analysis. For SEM, biominerals were mounted on double-sided carbon adhesive tape on 10 mm diameter aluminum stubs and sputter coated for 5 min with gold and platinum (30 nm) using a Cressington 208HR sputter coater. Specimens were examined using an environmental SEM (ESEM) (Philips XL30 ESEM FEG) operating at an accelerating voltage of 15 kV. For EDXA, uncoated samples were used, and operation was at an accelerating voltage of 20 kV for at least 100 s.

X-ray powder diffraction. Mineralogical identification by XRPD was carried out using a Panalytical X-pert Pro diffractometer. Diffraction patterns were recorded from 3° to 60° 2 theta using Ni-filtered Cu K-alpha radiation, counting for 30 s per 0.167° step using an X-celerator position sensitive detector. Phases were identified by reference to patterns in the International Centre for Diffraction Data, Powder Diffraction File (PDF-4+2010).

Abiotic tests for solubilization of manganese oxides

Experimental agar plates with 20 cm^3 of MEA amended with 0.5% (w/v) metal compounds were utilized for this abiotic test. 50 μl of 0.5 M pure oxalic acid and citric acid (both Sigma-Aldrich LLC, UK) were added to a 5 mm diameter well bored in the agar using a sterilized cork-borer (autoclaved at 121°C for 25 min), and each test plate contained three wells. We also examined the effect of 50 μl of 0.5 M HCl acid (Sigma-Aldrich LLC, UK) on the manganese oxides in the same way: in this case no significant solubilization haloes resulted around the well.

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