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Inorganic Materials and Living Organisms: Surface Modifications and Fungal Responses to Various Asbestos Forms

Stefania Daghino,[b] Elena Martino,[b] Ivana Fenoglio,[a] Maura Tomatis,[a] Silvia Perotto,[b] and Bice Fubini*[a]

Abstract: In a previous study several strains of soil fungi were reported to remove iron in vitro from crocidolite asbestos, a process that was envisaged as a possible bioremediation route for asbestos-polluted soils. Here, we get some new insight into the chemical basis of the fiber/fungi interaction by comparing the action of the most active fungal strain *Fusarium oxysporum* on three kind of asbestos fibers—chrysotile, amosite, and crocidolite—and on a surface-modified crocidolite. None of the fibers examined significantly inhibited biomass production. Even the smallest fibrils were visibly removed from the supernatant following adhesion to fungal hyphae. *F. oxysporum*, through release of chelators, extracted iron from all fibers; the higher the amount of iron at the exposed surface, the larger the amount removed, that is, crocidolite > amosite ≫ chrysotile. When considering the fraction of total iron extracted, however, the ranking was chrysotile > crocidolite > amosite > heated crocidolite, because of the different accessibility of the chelators to the metal ions in the crystal structure. Chrysotile was the easiest to deplete of its metal content. Iron removal fully blunted HO· radical release from crocidolite and chrysotile but only partially from amosite. The removal, in a long-term experiment, of more iron than is expected to be at the surface suggests a diffusion of ions from the bulk solid towards the surface depleted of iron by fungal activity. Thus, if the fibers could be treated with a continuous source of chelators, iron extraction would proceed up to a full inactivation of free radical release. The fungal metabolic response of *F. oxysporum* grown in the presence of chrysotile, amosite and crocidolite revealed that new extracellular proteins are induced—including manganese-superoxide dismutase, the typical antioxidant defense—and others are repressed, upon direct contact with the fibers. The protein profile induced by heated crocidolite was different, a result suggesting a key role for the state of the fiber/hyphae interface in protein induction.

Keywords: asbestos · bioremediation · fungi · iron · radicals

Introduction

Asbestos fibers are mobilized from their natural deposits—the rocks to which they are associated—mainly following human activities. Excavation in mines or tunnel constructions through asbestos-containing rocks generates harmful airborne fibrils, which may be deposited in large soil areas. No current technology is available for the remediation of such sites. Serpentine rocks, naturally rich in chrysotile, or asbestos-contaminated soils are thus a serious environmental issue, recognized by environmental protection agencies (for example, the USA Environmental Protection Agency at the "Asbestos Health Effects Colloquium" (Oakland, CA, 24–27 May 2001) and the "Asbestos mechanism of toxicity workshop", (Chicago, IL, 12–13 June 2003)).

Asbestos is a commercial term encompassing two groups of magnesium silicates which often crystallize in fibrous form: amphiboles and serpentines. The crystal structure of the amphiboles can be described in terms of a basic structural unit formed by a double-tetrahedral chain (corner-linked SiO_4 tetrahedra) of composition $(Si_4O_{11})_n^{6n-}$. These silicate double chains share oxygen atoms with alternate layers of edge-sharing MO_6 octahedra, where M stands for a variety of cations: mostly Mg^{2+}, Ca^{2+}, Fe^{2+}, or Fe^{3+}. In most common amphiboles—amosite (fibrous grunerite), $(Fe^{2+},Mg)_7$-

[a] Dr. I. Fenoglio, Dr. M. Tomatis, Prof. B. Fubini
University of Torino, Dipartimento di Chimica IFM and
Centre of Excellence of Nanostructured Interfaces and Surface (NIS)
via Pietro Giuria 7, 10125 Torino (Italy)
Fax: (+39) 011-670-7855
E-mail: bice.fubini@unito.it

[b] S. Daghino, Dr. E. Martino, Prof. S. Perotto
University of Torino, Dipartimento Biologia Vegetale and
Center of Excellence for Plant and Microbial Biosensing (CEBIOVEM)
viale Mattioli 25, 10125 Torino (Italy)

$Si_8O_{22}(OH)_2$, and crocidolite (fibrous riebeckite), $Na_2(Fe^{3+})_2(Fe^{2+},Mg)_3Si_8O_{22}(OH)_2$—iron is a major stoichiometric component. The crystal structure of all the minerals in the serpentine group can be thought of as being formed by a double layer consisting of a tetrahedral (silicate) sheet of composition $(Si_4O_{11})_n^{6n-}$, in which three of the oxygen atoms in each SiO_4 tetrahedron are shared by adjacent tetrahedra, and an octahedral (brucite) sheet of composition $[Mg_3O_2(OH)_4]_n^{2+}$ formed by edge-sharing $MgO_2(OH)_4$ octahedra (Fe^{2+} can substitute for Mg^{2+} in this layer because of the similarity in size of the two divalent ions, Table 1). The two sheets are bonded together forming a double layer in

Table 1. Specific surface area and total iron content of asbestos fibers.

	Specific surface area [m^2g^{-1}]	Iron content [w/w%]
chrysotile, $Mg_3Si_2O_5(OH)_4$	21[a]	1.92[b]
amosite, $(Fe^{II},Mg)_7Si_8O_{22}(OH)_2$	5[a]	28.5[a]
crocidolite, $Na_2Fe_2^{III}(Fe^{II},Mg)_3Si_8O_{22}(OH)_2$	8[a]	27.3[a]
heated crocidolite, $Na_2Fe_2^{III}(Fe^{II},Mg)_3Si_8O_{22}(OH)_2$	5[a]	27.3[a]

[a] See references [9,27,53]. [b] See the Experimental Section.

which the apical oxygen atoms of the $(Si_2O_5)_n^{2n-}$ sheet are shared with the brucite layer.

Chrysotile, $Mg_6Si_4O_{10}(OH)_8$, the most common serpentine, can contain Fe^{2+} ions, replacing Mg^{2+} ions, to a large or small extent depending on the geological source.[1] Natural chrysotile may contain up to 5% iron. Such fibers are all very poorly soluble in water, but previous work[1–3] has shown that treatments in vitro with various chelators may extract iron from the fiber, modify surface properties,[4,5] and even promote, over a long period of time, the disruption of several subsurface layers.[6] Iron ions are directly involved in the accepted mechanism of fiber toxicity[7–10] because at the fiber/lung interface of the inhaled fiber they constitute active centers where release of free radicals and reactive oxygen species (ROS) takes place.[5,11,12] These reactions initiate or contribute to the overall pathogenicity.[13,14]

Incubation of the fibers with an aqueous solution of some chelators was reported to inhibit surface reactivity[15] and to decrease DNA damage and lipid peroxidation in vitro.[16–18] Synthetic chrysotile with no iron in the structure was recently reported not to be cytotoxic toward human epithelial cells in a culture.[19] Iron extraction from asbestos fibers may also destabilize the lattice if the extraction proceeds for several layers below the fiber surface. As both the fibrous habit and the chemical reactivity are involved in asbestos pathogenicity, iron removal may be considered as a possible strategy to reduce asbestos-associated toxicity and to inactivate the fibers.

Many microorganisms can modify the chemical status and solubility of metals in the environment,[20,21] and some of these microorganisms are widely employed in the bioremediation of heavy-metal-polluted sites.[22] Soil fungi are very suitable as bioremediation agents since they are ubiquitous organisms and efficient substrate colonizers, they have exceptional degrading capabilities, and they show a good tolerance to extreme environments and polluted conditions. Like all organisms, soil fungi require iron for their own metabolism and have developed mechanisms to scavenge this element from difficult sources through the release of chelating molecules (for example, siderophores and some organic acids).[23] A multidisciplinary approach was therefore set up to investigate whether soil fungi could mobilize iron ions from asbestos fibers.

Previous experiments have shown that a variety of fungal strains were able, at least in vitro, to grow in the presence of crocidolite asbestos and to extract iron from the fibers with variable effectiveness.[24,25] Crocidolite, however, is not the most common type of asbestos, being confined mainly to relatively small deposits in South Africa, China, and Australia. The greatest percentage of the asbestos mined and manufactured is chrysotile, the most common fibrous serpentine.[26] We have therefore chosen *Fusarium oxysporum*, the most effective iron-extracting fungus,[24] and compared its effect on various asbestos forms and on thermally modified crocidolite, in order to understand the role played by the physico-chemical characteristics of the fibers in their potential to be degraded by fungi. In order to recover the asbestos fibers after exposure to the fungal activity, the fibers were separated from the mycelium in some cases by a dialysis membrane. The potential of the recovered fibers to generate free radicals was then compared with that of the original samples. Finally in order to clarify the biological mechanism whereby fungi modify asbestos, the metabolic activities of the fungus in the presence of the various types of asbestos were also investigated, with particular interest in extracellular proteins and/or proteins involved in an oxidative-stress response.

Results

Growth and morphological analysis: In the presence of the fungal mycelium, all asbestos fibers and fibrils were visibly removed from the suspension and tightly bound to the fungal hyphae, so that the supernatant was progressively cleared, in a similar manner to that already reported for crocidolite. Previous ultrastructural studies have shown that the fibrils are, in this case, in intimate contact with the fungus.[24,25]

None of the fibers examined significantly inhibited biomass production of *F. oxysporum* grown in liquid culture (Figure 1), either in direct contact with the fungus or when kept in a separate chamber by a dialysis membrane. The presence of asbestos fibers, however, caused the release of a dark yellow soluble pigment in the culture medium.

Iron removal: In the absence of fungi, none of the fiber types released any detectable iron into the medium. Conversely, iron was always detected in the supernatant of

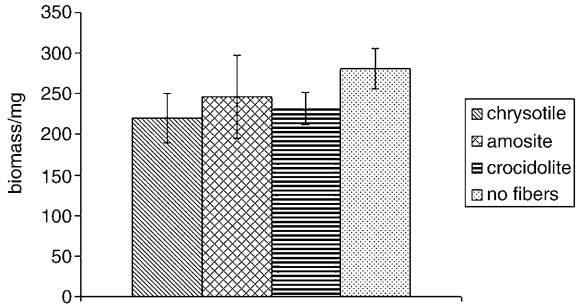

Figure 1. Fungal growth in direct contact with different kinds of fibers, expressed as biomass dry weight (mg) after an in vitro culture of 20 days. The control sample was grown in the absence of any fibers. Data are the mean of three independent experiments ± the standard deviation.

fungal cultures grown in the presence of the various kinds of fibers. The iron concentration in the medium was measured after 20 days of incubation and was found to be significantly increased in the presence of the mineral, either in direct contact with the mycelium or when kept in a separate chamber by a dialysis membrane. In an in vitro treatment with chelators[1] the amount of iron removed depends on both the surface area of the fibers and their chemical composition, namely the amount of surface iron. As expected, the amount of iron extracted was significantly greater for both amphiboles, crocidolite and amosite, than for chrysotile (Table 2). As previously reported, a thermal treatment of

Table 2. Iron extraction from the asbestos fibers, either kept in direct contact with *F. oxysporum* (A columns) or physically separated by a dialysis membrane (B columns).[a]

	Iron released [μM]		Iron released per unit surface [ions nm^{-2}]		Iron extracted from the fibers [%]	
	A	B	A	B	A	B
chrysotile	42.6	37.6	0.55	0.49	5.57	4.62
amosite	127.4	84.5	6.9	4.58	1.12	0.74
crocidolite	151.7	129	5.14	4.35	1.39	1.19
heated crocidolite	68.3	56.6	3.7	3.06	0.63	0.52

[a] The concentration of iron measured in the filtered culture medium was normalized by considering either the specific surface area or the total iron content of the fibers.

crocidolite at 800 °C in air leads to a 37 % reduction of the surface area of the fibers and modifies the coordination status of surface iron ions, which saturate their coordination valencies within the silica framework. The lower number of poorly coordinated iron ions at the surface of the heated sample is consistent with a reduced iron availability.[27] As expected, *F. oxysporum* is able to remove from heated crocidolite only about half the amount of iron removed from the original fibers.

To investigate whether iron extraction could proceed over a long period of time of contact between fungus and fibers and whether the accumulation of soluble iron in the medium could affect fungal growth, *F. oxysporum* was cultivated with crocidolite (the fiber richest in removable iron) for 56 days in a larger culture system (500 mL of culture medium instead of 80 mL). The fungal biomass, measured as dry weight at the end of the incubation, was similar in the presence of the fibers and in the control samples, thus indicating that there is no growth inhibition even after prolonged exposure. Aliquots of the culture medium were sampled weekly under sterile conditions to monitor the iron concentration. After 20 days the concentration of iron attained the value already measured in the smaller culture system, while after 56 days the iron released was twice as much (Figure 2). The final value attained, 11.45 ions nm^{-2}, is considerably more than what would be expected to be located at the surface of the fibers.[6]

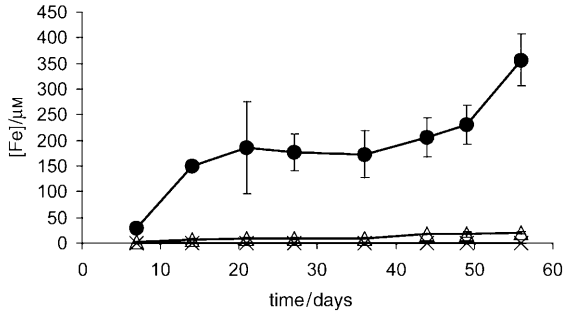

Figure 2. Variation of iron concentration in the supernatant of *F. oxysporum* grown in the presence (●) or absence (△) of crocidolite fibers over a period of 56 days. The concentration in the supernatant with the fibers alone (×) under the same conditions is also shown. Data are expressed as μM concentration of iron in filtered culture media and are the mean of three independent experiments ± the standard deviation.

The activity of *F. oxysporum* on crocidolite was also compared with that of the strong siderophore desferrioxamine in order to evaluate the amount of iron available for further extraction (that is, potentially mobilized in the human body by endogenous chelators). Crocidolite fibers, incubated either in the culture medium alone or with *F. oxysporum*, were thus further incubated with desferrioxamine (1 mM) for 37 days. Aliquots of the supernatant were taken at subsequent time periods to measure the iron concentration. Comparison of the kinetics of iron release (Figure 3) showed that the iron removed by desferrioxamine is substantially reduced following contact with the fungus but is not fully suppressed. The difference between the two curves is an indirect measure of the extent of iron extracted during fungal growth in the experimental conditions adopted.

HO· radical generation from the fibers: The EPR spectra of the 5,5′-dimethyl-1-pirroline-*N*-oxide (DMPO) hydroxyl radical (DMPO/OH·), obtained from aqueous suspensions of the fibers incubated in the culture medium alone and of fibers incubated for 20 days with *F. oxysporum*, are shown in Figure 4. All the fibers incubated in the culture medium alone generated substantial HO· radicals (Figure 4, A) a and B) a), as previously reported.[5,11] Conversely, treatment with

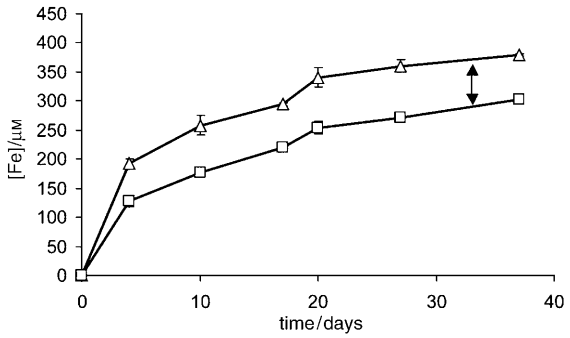

Figure 3. Iron extraction by desferrioxamine from untreated crocidolite fibers (△) and from fibers preincubated with *F. oxysporum* (□). The difference between the curves is due to the previous iron extraction by the fungus. Data are expressed as μM concentration of iron in supernatants and are the mean of three independent experiments ± the standard deviation.

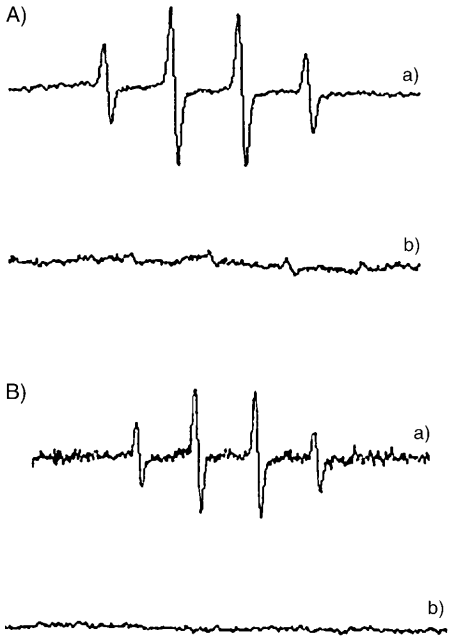

Figure 4. EPR spectra of the [DMPO-OH]• adduct of crocidolite (A) and of chrysotile (B). The spectra indicate free radical release from aqueous suspension of fibers incubated in the culture medium alone (A)a and B)a) or previously incubated with *F. oxysporum* (A)b and B)b).

Modification in fungal protein expression: The production of extracellular proteins, as quantified by using the Bradford assay, did not show any significant differences in asbestos-treated and control samples (data not shown). However, when separated by sodium dodecylsulfate (SDS) PAGE, the protein profiles revealed substantial differences for fungi grown in the presence and absence of asbestos fibers, with new extracellular proteins being induced and others being repressed following incubation with the fibers (Figure 5).

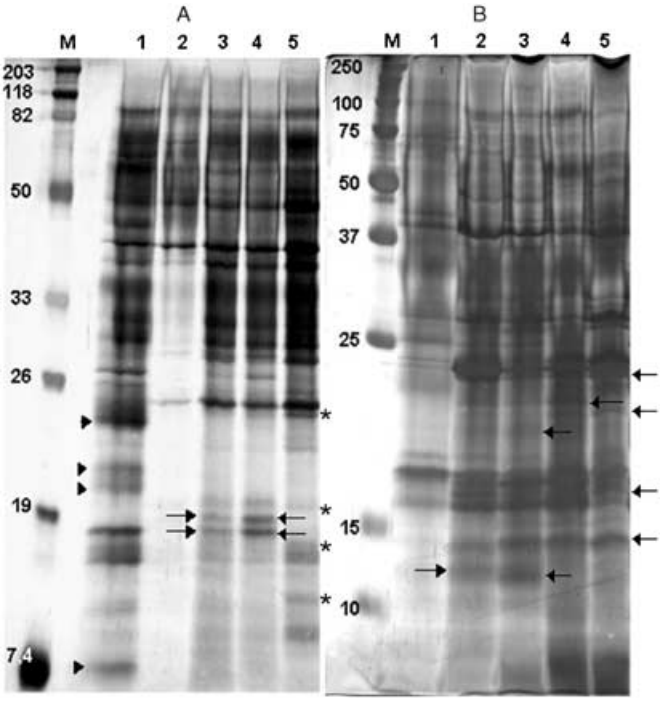

Figure 5. Extracellular protein profiles of *F. oxysporum* grown in Czapek glucose medium in the absence of fibers (lane 1) or in the presence of different kinds of asbestos (lane 2: chrysotile; lane 3: amosite; lane 4: crocidolite; lane 5: heated crocidolite). Mycelia were grown either in direct contact with the fibers (A) or separated from the fibers by a dialysis membrane (B). After SDS PAGE separation of the culture filtrates, proteins were visualized by silver staining. M = molecular weight marker. Arrowheads show proteins inhibited by the presence of the fibers, arrows show proteins induced in the presence of the fibers, and asterisks show proteins expressed after growth in direct contact with heated crocidolite.

F. oxysporum always inhibited, either partially or completely, radical release from crocidolite and chrysotile (Figure 4, A)b and B)b). The effect of the fungus on amosite and on heated crocidolite was less clear, as it markedly varied from one experiment to another. In one case radical generation from amosite was even enhanced following incubation (data not shown). Such variability often occurs in the presence of iron-removal/deposition equilibria because only a small fraction of isolated iron ions, in a well-defined coordinative and redox state, are catalytic centers for radical generation. During iron removal the number of such sites is bound to vary, occasionally increasing and then declining, on a given patch of fiber surface.

The protein patterns of samples grown in direct contact with crocidolite and amosite were very similar, while the sample grown with heated crocidolite showed peculiar protein bands (between 10–28 KDa). The sample grown in direct contact with chrysotile shows a lower intensity of protein bands than the other samples, probably due to minor protein precipitation, since the amount of proteins measured by the Bradford assay in the culture medium before precipitation is comparable to the other samples (data not shown). Conversely, the extracellular protein patterns observed after incubation with fibers kept in a dialysis membrane, and thus not in direct contact with the mycelium, did not show substantial differences among the different types of asbestos,

either natural or thermally modified. However they did reveal several induced bands that were not expressed in the control mycelia.

Isoelectric focusing followed by SDS PAGE and Western blotting with an antibody raised against manganese-superoxide dismutase (Mn-SOD) identified a band at just under 33 KDa in the basic fractions of the culture medium of *F. oxysporum* grown in the presence of crocidolite (Figure 6A). The SOD enzymes are involved in the cell's response to oxidative stress and catalyze the dismutation of the superoxide $O_2^{-\bullet}$ radical into water and dioxygen. The presence of SOD activity in the culture medium of *F. oxysporum* grown with crocidolite was also verified by native gel electrophoresis (Figure 6B), according to the method reported by Beauchamp and Fridovich.[28]

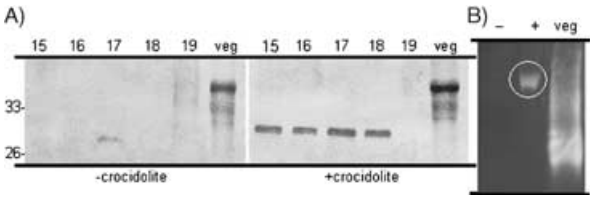

Figure 6. A) Western blot analysis of isoelectric focusing basic fractions (pH 6.5–9) of the culture media of *F. oxysporum* grown in the presence or absence of crocidolite. The Mn-SOD antibody recognized a band at 26–33 KDa only in the sample treated with crocidolite. B) Native gel electrophoresis showed SOD activity in a filtered culture medium of *F. oxysporum* grown in the presence (+), but not in the absence (−), of crocidolite. Veg: purified vegetal SOD (Sigma) as a positive control.

Discussion

Fungal growth: Several factors are involved in asbestos toxicity to humans, including free radical generation and damage to biomolecules. Such reactions do not appear to affect *F. oxysporum*. Its growth was not inhibited by any of the types of asbestos tested, so the fibers seem not to be harmful to fungal microorganisms. This may be due to the physical resistance of the hyphae cell wall or to radical scavenging systems, which are already known for some fungal strains.[29,30]

Fungi driven depletion in iron content of the various asbestos fibers: The different types of asbestos are all poorly soluble minerals, but organic chelators can extract iron ions from the surface of the fibers and take them into solution. Such a process is regulated by, besides the chemical nature of the chelator, several features of the mineral including fiber micromorphology, surface area, chemical composition, and coordination and oxidation state of iron.[1,3,6]

The extent of soil-fungi-mediated iron extraction also depends on the type of asbestos tested. Different asbestos minerals in fact contain a different amount of iron, have different crystalline structures, and consequently have different surface topology. Table 2 shows the relationship between these factors and the amount of iron removed by *F. oxysporum*. Amosite and crocidolite contain a similar amount of iron, much greater than chrysotile (Table 1). Crocidolite has a larger surface area than amosite, which accounts for the larger amount of iron extracted by the fungus under the same conditions. Heating crocidolite at 800 °C, which does not obviously modify the amount of iron within the fibers, lowers the extent to which iron ions are being removed from the fiber. Heating reduces the extension of the surface area (Table 1) following sintering and yields to a greater engagement of the iron ions with the silica framework. A reduction in the exposed surface as well as in the fraction of poorly coordinated surface ions, which are the first to be removed,[31] may account for the reduced mobilization of iron on the heated fibers. As the reduction in iron removal is larger than that expected as a consequence of the lower amount of exposed surface, we assume that the thermal treatment also reduces the accessibility of the chelators to the iron ions.[27]

Finally, although chrysotile contains iron only as a non-stoichiometric component, that is, to much lesser extent than the two amphiboles considered, the extraction from this fiber was much greater than expected, both because of the wide surface area exposed and the open serpentine structure, where the brucite layers $[Mg(OH)_2]$, in which iron replaces magnesium, are largely accessible.[14] If the amount of solubilized iron is expressed as a ratio between the iron extracted and the iron contained in the fiber (Table 2), the ranking is chrysotile > crocidolite > amosite > heated crocidolite.

The question arises of which factors, in an open system such as would occur in nature, would limit the process of iron extraction. The prolonged incubation of crocidolite with *F. oxysporum*, for as long as allowed by the culture-system volume (Figure 2), reveals that iron release may take place over a long period of time (56 days) and would have proceeded further if the fungal growth had not been limited. The shape of the curve shown in Figure 2 suggests a mechanism of iron removal involving three steps. In fact, iron removal proceeds at an approximately constant rate for the first 20 days, maybe involving the surface iron ions that are more readily available to the chelators. Iron concentration in the supernatant then levels off until the 45th day, when it starts to rise again. This suggests that progressive iron removal leads, after several weeks, to a collapse of the structure, observed as a sharp increase in iron concentration. The removal, in this long-term experiment, of more iron than is expected to be at the surface is consistent with a diffusion of ions from the bulk solid towards the surface depleted of iron by fungal activity, in a similar manner to that previously observed in the case of prolonged asbestos incubation with desferrioxamine.[6]

The incubation of fibers in a desferrioxamine solution showed a remarkable difference between fibers preincubated in the presence and absence of the fungus. Even when the fungus had already removed some surface iron, how-

ever, desferrioxamine could continue the removal process, a result confirming that if the fibers could be treated with a continuous source of chelators the iron extraction would proceed.

Soil fungi have developed several systems to take into solution and to absorb in the mycelium poorly soluble forms of iron, which is an essential element. Chelator release is one of these iron-interaction strategies, which is likely to mediate iron mobilization from asbestos. Indeed, a previous paper showed the release of specific (siderophores) and aspecific (organic acids) chelators into the culture medium by soil fungi growing with asbestos.[24] Taken together all these data suggest that fungal chelators secreted in soils may have an effect similar to desferrioxamine in vitro and may be continuously produced by mycelia growing close to asbestos fibers.

Modification of the surface reactivity of the fibers after contact with fungi in culture: The role played by iron in asbestos reactivity and toxicity is well established.[8,9,19] Iron chelators reduce free radical release from asbestos,[5,14] although no direct correlation may be established between the amount of iron removal and the reactivity at the fiber surface, which depends not only on the amount of surface iron but also on its coordination and oxidation state and on the fiber micromorphology.[15,27,32,33]

The incubation with *F. oxysporum*, similarly to the treatment with chelators, causes a decrement in $HO^{\bullet}$ release, with some differences among the different asbestos types examined. In the case of crocidolite and chrysotile, iron removal by the fungus fully blunted $HO^{\bullet}$ radical activity. With amosite, iron cycling at the surface gave somewhat inconsistent results; however, it was shown that contact with the fungus interfered with $HO^{\bullet}$ release. The differences in the $HO^{\bullet}$ radical activity for the various asbestos types may be ascribed to variations in the redox and coordination states of surface iron due to the different bulk composition (Fe^{III}/Fe^{II} in crocidolite, Fe^{II} in amosite). In particular, it was reported that amosite has about twice the amount of redox active iron that crocidolite has.[34] Crocidolite heated at 800 °C looses its potential to generate $HO^{\bullet}$.[27,35] However, following incubation with the fungus, similarly to the results with amosite, this potential was partially restored in some cases and not in others (data not shown). The variability observed with amosite and heated crocidolite, on the one hand, confirms the capability of the fungus to modify the surface status of iron in asbestos and, on the other hand, suggests that, alongside modification, the potential to generate $HO^{\bullet}$ may be depressed or enhanced by subtle surface reactions progressively taking place during iron removal.[31] We may however expect that in the long term, when almost all iron will be removed, no $HO^{\bullet}$ release will take place on what will remain of the original fibers. Radical scavenging has been related with reduction of in vitro DNA damage and lipid peroxidation and, thus, in an overall decrement in the toxic potential of the fibers.[2,16,17,36,37]

Biochemical response of fungi to asbestos fibers: Modification in protein expression: The modification in protein expression following interaction with asbestos may be a fungal response either to physical contact with the fibers or to a possible oxidative stress caused by the fiber or by mobilized iron. The SEM analysis of mycelia grown in direct contact with the fibers showed the physical integrity of hyphae;[25] thus, the extracellular protein pool changes in the presence of fibers are likely to be due to active synthesis. Protein induction or inhibition due to stress is reported in several studies on fungi and other microorganisms,[38–41] particularly for metal-induced stress, for example, extracellular protein induction in ericoid fungi grown in the presence of zinc.[42]

The samples grown in the presence of chrysotile, amosite, and crocidolite, either in direct contact with the mycelium or kept in a separated chamber, show a similar profile of protein expression which is different from the control samples. However, the direct contact of mycelia with heat-modified crocidolite leads to a peculiar protein profile, not seen when the same fiber is separated from the mycelium by a membrane. This indicates that, in the case of a physical interaction between hyphae and fibers, the fungal metabolic response, here observed as protein expression, depends on the surface characteristics of the fibers, more than on their mere bulk chemical composition. Phenomena of surface recognition by fungi have already been observed.[43–45]

The induction of superoxide dismutase activity, identified as coming from Mn-SOD by Western blotting with a specific antibody, was in agreement with that previously obtained with *Geomyces pannorum* var. *pannorum*.[25] Superoxide dismutases are oxidative-stress response enzymes: Mn-SOD expression is increased in rat lung epithelial cells after asbestos-fiber inhalation[46] and in vitro treatment of human lymphocytes with SOD inhibited cell death due to previous crocidolite incubation.[47] In this respect, mammals and fungal cells seem to have developed a homologous reaction in the presence of asbestos fibers.

Conclusion

Appropriate remediation routes for asbestos fibers dispersed in soil are still lacking. Soil fungi may provide an "environmental friendly" bioremediation approach, by continuously releasing chelators into the soil, close to the fibers, which is one of the strategies they naturally employ to extract metal ions from their substrate.

The development of environment-decontamination strategies requires a profound knowledge of the complexity of the interaction between the variety of asbestos fibers and mycelia. The present study shows, on the one hand, that the same fungus induces different modifications on various asbestos forms and, on the other hand, that the metabolic fungal response is strongly influenced, not only by the crystallo-chemical structure of the fibers but also by their surface state.

None of the fiber types inhibit fungal growth. The three fibers considered are largely depleted in their iron content, with a remarkable decrease in their potential to release free radicals. The amount of iron extracted by the fungus and the consequent variation in fiber reactivity, including the occasional variability of this result, however, depends on the physico-chemical characteristics of the fibers.

The present study shows that, in the very long term, with all forms of asbestos, iron depletion would involve modifications in the crystal and chemical nature of the mineral, with consequent inactivation of the fibers. The time required, however, would be different for the various forms, with chrysotile being more likely to be rapidly modified, due to the larger accessibility of the metal ions to the chelators.

The chemical and biochemical approach followed gives some insight into the peculiar interaction between a living remediation agent and an inorganic contaminant, but many points have still to be investigated before the application of this approach in the field. A crucial step will be to find out whether the fungi are able to modify asbestos fibers dispersed in a complex natural matrix, such as real soil. More knowledge is required on the chemical nature of the chelating molecules involved in iron removal from the fibers and on their strength and affinity for iron and for the other metal ions in the fibers (mainly magnesium). Finally, the fibers submitted to such treatment with soil fungi should be tested for their toxic potential (for example, cytotoxicity, genotoxicity) in cellular systems and validated in vivo, to verify whether, as is reasonably expected, toxicity correlates with the chemical modification induced in the fibers by the fungi.

Experimental Section

Asbestos fibers: Two types of amphibole asbestos fibers, crocidolite and amosite from Union International Contre le Cancer (UICC), and a serpentine asbestos, chrysotile UICC A (Rhodesian) were used. In all cases the asbestos fibers were suspended and dispersed in distilled water (1.84 g per 80 mL), prior to addition to the fungal cultures. The chemical formulae and the Brunauer–Emmett–Teller (BET) surface areas of these different asbestos types are reported in Table 1.

Thermal treatment of the fibers: Crocidolite fibers were heated in air at 800 °C for 3 h. The fibers were then cooled, suspended, and dispersed in distilled water (1.84 g per 80 mL) with the same procedure as that reported above.

Fungal isolate: The soil fungus *Fusarium oxysporum*[48] was chosen because of its good growth rate in the presence of crocidolite fibers and its effectiveness in iron extraction as reported in previous experiments.[24–25]

Culture conditions: Fungi were grown on liquid Czapek mineral medium supplemented with 2% (w/v) glucose (Carlo Erba, Milano, Italy). Czapek medium contained $NaNO_3$ (3 g L^{-1}), K_2HPO_4 (1.31 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}), KCl (0.5 g L^{-1}), and $FeSO_4 \cdot 7H_2O$ (0.01 g L^{-1}) and was adjusted to pH 5.5 with 2-[*N*-morpholino]ethanesulfonic acid (MES, 20 mM). Asbestos fibers were suspended in distilled water and autoclaved. After sterilization, an aliquot of the suspension (8.7 mL), shaken on a vortex to keep it homogeneous, was added to the culture medium (80 mL) and inoculated with the fungus. A larger batch culture system (500 mL of culture medium instead of 80 mL) was also used, but the ratio between the fibers and the volume of the liquid medium was maintained by adding 50 mL of suspension. In order to recover the asbestos fibers after exposure to the fungal activity, the suspension was sometimes separated from the mycelium by a dialysis membrane (cut off 12000–14000 Da) when added to the culture medium. A corresponding area of dialysis membrane was added to all samples, including the control samples that were run in parallel with the fungal mycelium growing in the same medium in the absence of the fibers. The fungal cultures were grown at 25 °C with shaking at 120 rpm for up to 20 days. Cultures were then filtered in a Buchner funnel and the mycelium was dried at room temperature and then weighed to obtain the biomass value. For fungal cultures growing in direct contact with the crocidolite fibers, the amount of fibers in the fungal biomass was estimated by running parallel samples containing only the culture medium and the added fibers. These samples (three replicates) were filtered along with the experimental cultures, and the weight of the fibers collected after filtration was subtracted from the biomass obtained for the fungal cultures.

Aliquots of the culture medium were used to measure iron concentration and for SDS PAGE analysis. At least three replicates were used for each set of experimental conditions.

Spectrophotometric determination of the iron concentration in the supernatant: The concentration of iron in the culture supernatant after incubation of asbestos fibers with the fungal mycelium was determined spectrophotometrically by measuring the formation of the violet-colored Fe^{2+}–3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*′-disulfonic acid (ferrozine, Sigma) complex at 562 nm by using a Uvikon 930 spectrophotometer, according to the method proposed by Lund and Aust.[1,24] The sensitivity of this detection method is around 1–2 μM.

Iron extraction by desferrioxamine: Untreated crocidolite fibers and a certain amount of crocidolite fibers preincubated with *F. oxysporum* were suspended (1 mg mL^{-1}, final volume 200 mL) in 0.15 M NaCl solution (pH 4.5) containing 1 mM desferrioxamine B mesylate at 37 °C, and the mixture was continuously shaken in the dark. The pH value was readjusted at regular time intervals throughout the incubation period to prevent alteration in the rates of iron mobilization with NaOH or HCl solutions. Aliquots of 2.50 mL were taken, at regular time intervals, and centrifuged at 5000 rpm to separate the supernatant from the fibers. The concentration was determined by measuring the absorbance of the Fe^{3+}–desferrioxamine complex at 428 nm by using a Uvikon 930 UV/Vis spectrophotometer.

Determination of the total iron in chrysotile: Since iron is present in chrysotile fibers as a magnesium substitute, various chrysotile samples contain very different amounts of iron. The amount of iron contained in the chrysotile used was determined by measuring the formation of the Fe^{2+}–ferrozine and the Fe^{3+}–desferrioxamine complexes at 562 and 428 nm, respectively, (with a Uvikon 930 spectrophotometer) after dissolving mineral (100 mg) in 27.5 M hydrofluoric acid (10 mL) and distilled water (50 mL).

Free radical detection by means of the spin-trapping technique: The HO• radical generation upon incubation of fibers (30 mg) with H_2O_2 (0.196 M) and phosphate buffer (0.5 M) solution was detected by using the spin-trapping technique with 0.15 M DMPO as the trapping agent, as described in previous papers.[5] The radical adducts formed, DMPO/OH•, were monitored by EPR spectroscopy with a PS100.X Adani EPR spectrometer. The number of radicals released is proportional to the intensity of the EPR signal measured by double integration. Kinetics of free radical generation were followed for up to 30 min.

Protein analysis: Aliquots of the culture media (200 μL or 16 mL depending on gel size) were analyzed by SDS PAGE to reveal the pattern of secreted proteins. Before loading, proteins were precipitated with 100% trichloroacetic acid (TCA)/0.4% deoxycholic acid (DOC) and acetone according to the protocol of Perotto et al.[49] After electrophoresis, performed according to the method of Laemmli[50] on 10% and 12.5% acrylamide separating gels, proteins were revealed by silver staining. The concentration of proteins in the culture media was detected by using the Bradford assay.[51]

Western blotting: After SDS PAGE, proteins were blotted on nitrocellulose paper (Highbond C-super, Amersham) under an electric field by using the MINI TRANS-BLOT Electrophoretic Transfer Cell (Bio Rad). The blot was carried on for 50 min at 100 V. The protein Mn-SOD was

detected by an anti-Mn-SOD antibody (raised in rabbit, EnVirtue) and an anti-rabbit secondary antibody conjugated with alkaline phosphatase (Sigma).

Liquid isoelectric focusing (Rotofor): Culture filtrates were concentrated about 10-fold by using a rotary evaporator system (Rotavapor) and were dialyzed for 24 h against water at 4 °C. An aliquot of 50 mL was subjected to liquid-phase preparative isoelectric focusing (IEF) in a BioRad Rotofor system, for 4 h at constant power (12 W), with BioLyte ampholines (BioRad, pH range 3–10 (4% v/v)). The pH value was measured for each Rotofor fraction before protein precipitation, separation by SDS PAGE, and Western blotting.

Superoxide dismutase activity: Proteins from filtered liquid culture were separated on 10% polyacrylamide gel in native conditions according to the method of Davis.[52] The loading buffer (tris(hydroxymethyl)aminomethane (Tris)/saccarose), was added to the samples (1:5 v/v) before electrophoresis. The running conditions were 15 mA in running buffer (Tris/glycine at pH 8.3). The superoxide dismutase (SOD) enzyme activity was revealed directly on the gel, as reported in Beauchamp and Fridovich.[28]

Statistical analysis: Statistical analysis of data was performed by using the program one-way ANOVA with the Tukey test as a post-hoc test.

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