Fungal weathering and implications in the solubilization of metals from soil and from asbestos fibres

S. Daghino1,2, E. Martino1,2 and S. Perotto1,2

1 Dipartimento di Biologia Vegetale, Università di Torino and Istituto per la Protezione delle Piante del CNR, v.le Mattioli 25-10125 Torino, Italy.
2 Interdepartmental Centre “G. Scansetti” for Studies on Asbestos and Other Toxic Particulates, Università di Torino, via P. Giuria 7-10125 Torino, Italy.

Geomycology investigates the impact of fungi on geological processes, including the alteration and weathering of rocks and minerals, mediated both by biomechanical and by biochemical activities. Several functional groups of fungi are involved in mineral bioweathering, including saprotrophic and mycorrhizal fungi. Serpentine sites provide interesting environmental cases for geomycology, because they are naturally enriched in heavy metals and can bear asbestos-rich rocks. An otherwise uncommon species, *Verticillium leptobactrum*, was found to be abundant in several serpentine sites in the Western Alps. *V. leptobactrum* and other fungal strains were shown to be able to extract different amounts of iron and magnesium from asbestos in vitro. The amount of iron mobilized from the fibres depended on the fungal activity but also on the physical characteristics of the mineral. Iron is of particular importance because one of the reactions determining asbestos toxicity is the iron-catalyzed generation of free radicals leading to biomolecules oxidation. Asbestos fibres modified in vitro by fungi were less chemically reactive and lost their oxidative activity against DNA.

Keywords: serpentine; fungi; asbestos; metals; bioweathering; iron; magnesium.

1. Definition of geomycology

The interactions between minerals and microorganisms may potentially affect any biological, geological and biogeochemical process occurring at or near the earth surface. The “weathering” of rocks and minerals is their deterioration or erosion, mediated by several different processes, acting synergistically to determine the composition of lithosphere, biosphere, hydrosphere and atmosphere [1]. Among these processes, there is an important biological component, due to the activities of pioneer organisms, such as lichens, but also bacteria and fungi. Filamentous fungi, plants and animals can enhance the disgregation of rock substrates by physical forces, such as the pressure of growing hyphae and roots or the burrowing [2]. However the biochemical activities of microorganisms are likely to play a more important role in rocks weathering than their biomechanical effects [3]. Bacteria, algae, fungi and plants contribute to chemical rocks weathering by releasing organic acids and other metabolites, as well as CO$_2$, that leads to carbonic acid formation, thus determining the soil acidification [3, 4, 5].

In the last years, geomicrobiology, defined as the study of the interactions between minerals and microorganisms, has largely focused on prokaryotes. The role of fungi in geological processes has been neglected, with the exception of lichens [3, 6]. In spite of this, the typical filamentous growth and the secretion of protons, organic acids and other metabolites, make fungi particularly suitable as biological weathering mediators of rocks, minerals and building materials [7, 8]. *Geomycology* has been defined by Burford et al. [9] as “the impact of fungi on geological processes, including the alteration and weathering of rocks and minerals, the accumulation of metals and their role in nutrient cycling and influence on proliferation of microbial communities in mineral substrates”.

2. Fungal mechanisms involved in the weathering process

The interaction of fungi with rocks and minerals is mediated either by biomechanical activities (biogeophysical weathering), or by biochemical activities (biogeochemical weathering) [10, 11]. The biomechanical deterioration of rocks is caused by the deep penetration of fungal hyphae into cracks, fissures and cavities already formed, but also by active burrowing into the intact mineral (e.g. into the mineral cleavage planes) [12, 3]. However the pressure force would not be enough to allow the hyphae to penetrate into the rocks, if not helped by biochemical solubilization of the substrate [10].

There are four mechanisms of metals and minerals solubilization by fungi: acidolysis and complexolysis are the most important, but also redoxolysis and mycelial metal accumulation. Low molecular weight organic acids are the most important chemical species for rocks biodeterioration [7, 13] and they have the double function to acidify the substrate, thus enhancing ions solubility, and to form complexes with solubilized ions, thus mobilizing them. Fungi are also able to synthesize and release siderophores that can contribute to bioweathering thanks to their chelating activity [11] (Fig. 1). Siderophores are low molecular weight molecules (500-1000 Da), with a high specificity for iron [14]. Regulation of the biosynthetic pathways allows the microorganism to reach high siderophores concentration close to the cell in...

Hoffland [10] indicated five functional groups of fungi involved in mineral bioweathering: lichen-forming fungi, mycorrhizal fungi, saprotrophic fungi, meristematic black yeasts, saprotrophic organisms that use the lignocellulose complex, such as white and brown rot fungi.

Mycorrhizae are mutualistic, symbiotic associations between fungi and plant roots, they concern the majority of the vascular plants and they are classified into different groups according to their morphological characteristics and to the plant species involved: arbuscular mycorrhiza, ericoid mycorrhiza, ectomycorrhiza, orchid mycorrhiza, monotropoid mycorrhiza, arbutoid mycorrhiza and ectendomycorrhiza [16]. A number of experimental studies have shown the ability of ectomycorrhizal (ECM) fungi to dissolve minerals (see also below) [17, 18, 19]. The ability of some ericoid mycorrhizal fungi (mycorrhizal endophytes of *Woollsia pungens* (Epacridaceae), *Hymenoscyphus ericae* and *Oidiodendron maius*) to dissolve hydroxyapatite, and Zn oxide and phosphate have also been reported [20, 21].

Fungi contribute to the deterioration of silicate-bearing rocks and iron- and magnesium-bearing minerals [12]. Silicates are easily degraded mainly by the production of organic and inorganic acids, alkalis, CO$_2$ and complexing agents [3]. Fungi were demonstrated to leach metals from different silicates faster than bacteria [22]. For instance *Aspergillus niger* weathers olivine, serpentine, feldspar and other minerals, *Penicillium simplicissimum* disgregates basalt, *Penicillium expansum* and *Scopulariopsis brevicaulis* extract aluminium from alumino-silicates [3]. *Piloderma* was able to extract potassium and/or magnesium from biotite, microcline, and chlorite to satisfy nutritional requirements [23]. Biodeterioration of rock surfaces can also be mediated by fungi by the redox attack of mineral constituents such as manganese and iron [24].

3. Fungal weathering and metal speciation

The biochemical activity of fungi and other microorganisms can also affect metals speciation and mobility in the soil, modifying their biogeochemical cycles as well as the C, N, P, and S cycling. These processes are relevant for bioweathering [25]. In the soil environment, metals and radionuclides can be dissolved in solution, held on inorganic soil constituents through various sorption or ion exchange reactions, complexed with soil organic materials, or precipitated as pure or mixed solids [26]. The potential of microbial processes for bioremediation may depend on the physical and chemical nature of the site, which influences the form in which metals occur. Furthermore, mineral components contain considerable quantities of metals which are biologically unavailable. Certain microbial processes dissolve minerals thereby increasing metal bioavailability and potential toxicity, whereas others immobilize them and reduce bioavailability [27].

The two main mechanisms of metal mineral dissolution by fungi are proton-promoted and ligand-promoted, respectively [13]. Organic acids provide both source of protons for solubilization and metal-chelating anion to complex the metal cation with complexation being dependent on factors such as relative concentrations of the anions and metals, pH, and the stability constants of the various complexes [28]. Conversely, immobilization can result from sorption to cell components or exopolymers, transport and intracellular sequestration or precipitation as insoluble compounds, e.g. oxalates [29, 13, 30].

A peculiar role in the metal solubilization process is played by mycorrhizal fungi. The physical, chemical and biological weathering of minerals by ECM fungi play a major role in forest ecosystems, as these processes release numerous nutrients required for tree growth (e.g., phosphorus, potassium, magnesium, calcium and iron) [31]. Several studies have shown an increased uptake of mineral-derived nutrients by trees when in symbiosis with ECM fungi [32, 33, 34, 35, 36, 37].

The ability to solubilize poorly soluble calcium phosphates, such as hydroxyapatite, has been described for a range of ECM fungi (*Pisolithus, Paxillus, Trichoderma* and *Suillus*) [38, 39, 35, 36]. The same fungi proved to be able to
weather silicates as well [39, 35, 36]. Concerning arbuscular mycorrhizal (AM) fungi, AM plants utilize more soluble phosphorus from soil mineral phosphate than non-inoculated plants [40].

The solubilization process by mycorrhizal fungi can potentially increase the availability and uptake of toxic metals by the plant. By contrast, ecto- and endomycorrhizal symbioses can play a crucial role in protecting plants from these toxic compounds [41, 42]. The ability of mycorrhizal associations to ameliorate metal toxicity to higher plants has been shown for ericoid [43], ecto- [44, 45, 46, 47, 48, 49, 50] and arbuscular mycorrhizas [51, 52]. The efficiency of protection, however, differs between distinct isolates of mycorrhizal fungi and different toxic metals and protective effects cannot be demonstrated for all associations in all circumstances [41]. What is clear is that plant roots and their associated free-living and symbiotic microbial populations significantly alter the physico-chemical characteristics of the rhizosphere by metabolic activities, resulting in a geochemical environment that can be very different from the bulk soil [33, 53]. This will have significant consequences for the biogeochemical mobility of metals and associated elements in such an environment.

4. Serpentine soils: ecology and characterization of the fungal community

Soils rich in metals, naturally or because of human activity, offer environmental cases for the study of the interactions between minerals, or metals, and fungi. Such soils are naturally found in ophiolitic sites that are recognized for their scientific and ecological relevance [54, 55]. The term ophiolite denotes a group of oceanic rocks, exposed on the continental crust because of the orogenic movements of earth surface, and including serpentine rocks. Serpentine loosely refers to a broad group of minerals associated with the weathering of ultramafic rocks. Serpentine is not the exclusive mafic mineral in these soils, however this term is accepted in the literature [54]. Whittaker [56] identified three traits shared among serpentine sites: poor plant productivity, high rates of endemism and vegetation types distinct from those of neighbouring areas, sometime characterized by morphological adaptation. Nutrient deficiency (especially in nitrogen and phosphorous) and magnesium abundance are typical features of serpentine soils, together with high levels of phytotoxic heavy metal concentrations, low pH and slow rock weathering [57, 54]. All together, these chemical and physical properties determine the so called “serpentine syndrome” [58, 59]. Serpentine sites, with their peculiar features, have naturally selected endemic plant species [60, 57] with physiological and ecological mechanisms of stress-tolerance. For example, roughly the 50% of the native flora of New Caledonia, is endemic to serpentine soils. Similar restrictions and remarkable floristic associations are also found in serpentine areas of the Mediterranean Sea, Africa, Australia–New Zealand, and Asia [61]. The importance of soil features to plants explains the strong serpentine edaphic factor’s influence on plant ecology and evolution. By contrast the ability of serpentine soils to affect the evolution and distribution of organisms other than plants is less well known.

Only few studies on the microbial diversity in serpentine soils are available. Oline and colleagues [62] demonstrated that bacterial communities from serpentine soils resulted to be more similar to each other than they were to communities from geographically close non-serpentine soils. DeGrood and colleagues suggested, on the basis of PFLA analysis, that the community composition of revegetated soils was similar to that of serpentine undisturbed soils with similar soil chemistry [63]. Some studies have focused on the diversity of root-associated fungi, either arbuscular mycorrhizal [64, 65] or ECM [66, 67] fungi, and on their role in plants adaptation to such edaphic factors. Fitzsimons and Miller [65] indicated that adaptation of plants to serpentine soil does not involve adaptation to a unique community of soil mutualists. Urban and colleagues [67] identified a broad taxonomic range of roots colonizing fungi, but they did not draw conclusions about a possible serpentine-driven speciation. On the other hand Gonçalves and colleagues [68] demonstrated that Ni exposure in serpentine soils induced Ni tolerance in some isolates of Cenococcum geophilum as an adaptive response. A similar result was obtained for the ericoid mycorrhizal fungus Oidiodendron maius [69]. The growth of O. maius isolates from a serpentine site and from other heavy metal-polluted and non polluted sites in the presence of different metal contaminants identified some tolerant strains, and suggested a site specific adaptation depending on the kind and degree of contamination of the soil of origin. Despite the high Cr and Ni soil concentrations, the same isolates showed a high level of genetic diversity by AFLP analyses.

4.1 Characterization of the fungal community in asbestos mines

Serpentine rocks can be associated with different fibrous minerals, including asbestos minerals such as chrysotile (serpentine asbestos) or tremolite (amphibole asbestos), which are known for their toxicity and pose serious environmental [70] and epidemiologic concern [71]. Such sites offer batches for the isolation of microorganisms naturally adapted to the soil features described above, possibly able to weather such kind of minerals.

Samples of serpentine rocks collected in two dismissed asbestos mines (Balangero, Torino and Emarese, Aosta) in the Western Alps area were collected to characterize the associated fungal population. The fungal community isolated from the soil and rock samples was quite abundant and featured an estimate of 60 and 38 different species in the two sites, respectively. Among the different species identified, three dominated in both sites: Verticillium leptobactrum, Paecilomyces lilacinus and Aspergillus fumigatus. Species belonging to the genera Penicillium, Mortierella, Myrothecium and Cladosporium were also found to be abundant in at least one site [72]. Most are ubiquitous fungal
species, already isolated from a variety of different substrates, including rocks and soils [2]. By contrast, the rare species *V. leptobactrum* has seldom been isolated before and only from rotten wood, insect exoskeletons, nematodes and other fungi. *V. leptobactrum* was subsequently isolated from serpentine rocks collected in other sites in the Western Alps [73]. The presence of a fungal species may be affected by many factors (e.g. nutrient and water availability, temperature, interactions with other microorganisms or plants), and the abundant occurrence of an otherwise rare species in sites that share the geographic location and a specific lithology raised the interest for this fungus.

*V. leptobactrum* was therefore investigated not only as a possible bioweathering agent on asbestos fibres, but also for its phylogenetic position. In fact, the taxonomy of the section Prostrata of the genus *Verticillium*, that includes *V. leptobactrum*, was revised by Gams and colleagues [74] which divided the section in five new genera: *Simplicillium*, *Lecanicillium*, *Pochonia*, *Haptocillium* and *Rotiferophtora*. ITS ribosomal sequences of serpentinicolous isolates of *V. leptobactrum* were aligned with GenBank sequences of reference strains of *V. leptobactrum* (collected from diverse substrates) and of the genera *Simplicillium* and *Lecanicillium*. The Neighbour Joining analysis showed that the serpentinicolous strains segregated in a clade close to but distinct from the *Simplicillium* clade, together with the *V. leptobactrum* reference strains [75]. The topology of the NJ tree was similar to the one obtained by Zare and Gams [76].

There was no correspondence between the clusters and the sampling sites, although the analysis of functional genes potentially involved in the adaptation of the fungus to the substrate, may allow us to clarify whether the site of sampling is affecting the intraspecific variability of *V. leptobactrum*.

Fig. 2 Culture system used for the study of the weathering effects of fungi on asbestos fibres. In the picture, *V. leptobactrum* was grown in a liquid medium in the presence of chrysotile fibres. A tight interaction was established between fungal hyphae and asbestos fibres (the inserted scanning electron microscopy picture shows *V. leptobactrum* hyphae in contact with crocidolite fibres). Soluble fungal metabolites are involved in the removal of Fe, Mg and Si ions from the fibres.

4.2 Fungal weathering of asbestos fibres

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 SiO4 tetrahedra) of composition (Si4O11)6n. These silicate double-chains share oxygen atoms with alternate layers of edge-sharing MO6 octahedra, where M stands for a variety of cations: mostly Mg2+, Ca2+, Fe2+ or Fe3+. In most common amphiboles - amosite (fibrous grunerite) (Fe2+,Mg)Si2O5(OH)4 and crocidolite (fibrous riebeckite) Na2(Fe3+)2(Fe2+,Mg)3Si2O5(OH)4 - iron is a major stoichiometric component. Chrysotile (Mg3Si2O5(OH)4) is a serpentine phyllosilicate composed of a layer of silica tetrahedra (Si2O3)n2n that share apical oxygen with Mg octahedra [Mg6O2(OH)4]n2n, the latter forming a outer faced
brucite-like layer [77]. In chrysotile, iron (generally as Fe\(^{2+}\)) substitutes Mg in the octahedral layer (from 1 to 6 oxide wt%) and its occurrence is suggested to modulate chrysotile toxicity [78, 79, 80]. Asbestos fibres are all very poorly soluble in water, but previous work [81, 82] have shown that treatments in vitro with various chelators may extract iron from the fibers, modify surface properties [83] and even promote, over a long period of time, the disruption of several subsurface layers [84]. Recent experiments show that the chelating activity of lichens growing in contact with serpentine rocks rich in chrysotile modify the morphology of the mineral, that loses its crystallinity, and its chemical reactivity [85]. Similarly, the in vitro incubation of chrysotile fibres with the aposymbiotic lichen-forming Xanthoparmelia tinctina fungus led to a significant decrease in the MgO %wt and increase of SiO\(_2\):MgO ratio of fibres, suggesting an incongruent dissolution of the surface layers, driven by a selective extraction of magnesium [86].

Although iron has a prominent role in determining the chemical reactivity and toxicity of asbestos fibres, the structure of the minerals has a Si backbone, and chrysotile in particular contains high amount of Mg. The ability of fungal strains of different origin to remove both Fe and Si and Mg ions from the mineral structure was investigated (Fig. 2).

Fusarium oxysporum, chosen for its known ability to release siderophores, was able to extract iron from crocidolite, chrysotile and amosite [87] and also showed a good ability to extract magnesium, while not silicon, from chrysotile [75]. A recent work performed by a proteomic approach by Chiapello and colleagues [88] showed no significant changes in the protein profiles of F. oxysporum when exposed to asbestos fibers. Nevertheless some up-regulated proteins suggest the induction of metabolic pathways involved in the protection from oxidative stress, possibly related to the increased concentration of iron leached from the fungus into the culture medium.

Among the fungal strains isolated from a serpentine soil, V. leptobactrum and P. lilacinus were able to extract iron from crocidolite [89] and V. leptobactrum also removed significant amounts of magnesium from chrysotile fibres bulk [75]. The amount of iron and magnesium extracted from the fibres not only depends on the activity of the fungus, but also on the physico-chemical characteristics of the different fibres, namely the amount of ion in the fibres and the surface area. Therefore the comparison of the normalized iron extraction data for crocidolite and chrysotile showed that fungi induce deeper modification in the latter [89] than in the former. These data confirm previous experiments done both with fungi and with purified organic chelators in vitro [87, 82]. In fact, previous studies had shown that chrysotile is more soluble than crocidolite, thus more easily altered by chelating treatment [78]. Besides, the different surface exposure of Mg and Si in pure chrysotile than in the mixed mineral chrysotile-balangeroite accounted for the different effects of oxalic acids on the two minerals [90].

Iron and magnesium extraction from fibres was also observed by direct analysis of the chemical composition of the fibres [89]. These data show that the effect of the fungal treatment involves mainly the fibres surface, while the bulk is only slightly modified. The decrease of iron and magnesium amount on the fibres parallels with the data of extraction. Iron and magnesium mobilization from asbestos fibres is likely to be mediated by low molecular weight chelators released into the medium by the fungus, because the extraction occurs even if fibres are separated from the mycelium by a dialysis membrane. Previous work showed that fungi able to extract iron from crocidolite could release two kind of chelators, siderophores and organic acids [91], but the exact identity of the molecules involved in these activity remains unclear as well as the molecules and the mechanisms undergoing magnesium extraction.

Reactivity and toxicity of asbestos fibres are related to the presence of iron at their surface [92]. For this reason, the reactivity of fibres depleted of iron by fungi was investigated by focusing on the ability of fibres to generate free radicals [93, 94] which depends on iron oxidation and coordination state [95] (Fig. 3a). The results obtained with fungi-weathered fibres suggest that the chelating activity of fungi involves reactive sites at the fibres surface, because there is a general decrease of reactivity for both chrysotile and crocidolite [96, 89]. The only exception observed was chrysotile, incubated with V. leptobactrum that exhibited enhanced reactivity. This could be caused by the strong chelating ability of the fungus that, by modifying the fibre surface, may lead to the exposure of poorly coordinated iron ions that may represent new reactive sites. A similar effect was observed after treatment of crocidolite with ascobic acid [97] and of chrysotile–balangeroite with oxalic acid [90]. V. leptobactrum does not modify the reactivity of crocidolite and chrysotile at the same extent, probably because of the different structure of the two minerals, as crocidolite is less soluble and has a smaller surface area than chrysotile [78].

Free radical release is one of the main causes of asbestos toxicity and carcinogenicity [98, 99]. In particular, genotoxic effects of fibres in vitro are mediated by free radicals [100] and correlate with the induction of mesothelioma in vivo [101]. The ability of fibres depleted of iron by fungi to induce an oxidative damage to naked DNA in the presence of hydrogen peroxide was investigated by measuring the formation of a marker of genotoxicity, the 8-oxo-7,8-dihydro-2'-deoxyguanosine (8oxodGuo) (Fig. 3b). Both untreated and control (i.e. fibre incubated in the fungal culture medium, without fungus) crocidolite and chrysotile induced DNA oxidation. Control fibres were even more active than the untreated, perhaps because of the higher amount of iron at their surface, as revealed by SEM-XPS analysis, and possibly due to iron deposition from the culture medium. Fibres treated with fungi induced significantly lower amount of 8oxodGuo than control fibres [89]. These results suggest that iron active in DNA damaging is mobilized from the fibres and that the oxidation occurs only in the presence of H\(_2\)O\(_2\), confirming the evidences provided by [102]. Thus, fungi decrease the amount of iron available for mobilization and active in DNA damage. The decrease in the oxidative ability of the fibres correlates with the blunting of radicals release, with the exception of V. leptobactrum-treated chrysotile (that generates more radicals, but do not oxidize DNA) and of control fibres (that oxidize DNA, but do not
show differences in radical release if compared with untreated fibres). These discrepancies are likely related to the differences between the two assays, the first depending on the reactivity of EDTA-chelated iron ions (Fig. 3b), the second depending on surface-coordinated iron ions reactivity (Fig. 3a). Altogether these results demonstrate that, with one exception, iron depletion from fibres mediated by fungi leads to an inhibition of the fibres surface reactivity. In parallel, the modified fibres have a lower oxidative activity on naked DNA.

Fig. 3 Asbestos fibres are able to generate hydroxyl radicals by an iron-catalyzed reaction involving ions at the fibres surface. The amount of iron available for oxidative reactions, both at the surface (a) and into solution (b), depends on the loss caused by the previous fungal treatment. (a) Hydroxyl radicals can be detected by electronic paramagnetic resonance (EPR) spectroscopy, using the spin-trapping technique that generates a signal (right section of the figure) in which the picks area depend on the amount of radicals detected (Martino E., Prandi L., Fenoglio I., Bonfante P., Perotto S., Fubini B. Soil fungal hyphae bind and attack asbestos fibers. Angewandte Chemie International Edition. 2003;42:219-222. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission). The loss of iron at the fibres surface leads to the blunting of the radicals release. (b) Iron ions taken into solution by chelators generate hydroxyl radicals that can oxidize DNA bases. The generation of 8-oxo-7,8-dihydro-2’-deoxyguanosine, a marker of DNA damage, is drastically reduced after the loss of iron caused by the weathering of the fibres by the fungi.

5. Conclusions and perspectives

In the last 10 years, research has focused on the role of fungi in the geological processes, related to their bioweathering activity and ability to modify metal ions [11]. In this frame, the experimental work reported here confirms the ability of different fungal strains to weather minerals. An in vitro approach was chosen, with the aim of analysing the effects of fungi on a single mineral with known composition and structure. This is far from the overall weathering processes happening in field, where atmospheric agents as well as other organisms can contribute to the alteration of the minerals. Studies of metal hyperaccumulating plants from serpentine soils have led to the discovery of novel physiological pathways but have also laid the foundation for the development of innovative technologies such as phytoremediation (i.e. the use of hyperaccumulators to extract heavy metals from contaminated soils) [61]. In the same way, the isolation and identification of the fungal population inhabiting ophiolite sites can contribute to the characterization of these
ecologically important sites, but also to the identification of metal tolerant strains, interesting for the development of bioremediation strategies of toxic minerals or metals.

For example, asbestos fibres in soil could be modified by chelators directly released by closely growing fungi. The interaction of fungi with iron in the soil is based on mechanisms of solubilization and adsorption, mediated by siderophores or by aspecific chelators like organic acids that could be biotechnologically exploited also for the bioremediation of a particular substrate such as asbestos minerals.

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