The potential of *Schizophyllum commune* for mycoremediation at the Chernobyl exclusion zone

Dissertation

To Fulfill the Requirements for the
Degree of
„doctor rerum naturalium“ (Dr. rer. nat.)

Submitted to the Council of the Faculty of Biological Sciences of the Friedrich Schiller University Jena

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Day of Public Defense (Tag der öffentlichen Verteidigung)

20.04.2022_____

“In der Wissenschaft gleichen wir alle nur den Kindern, die am Rande des Wissens hie und da einen Kiesel aufheben, während sich der weite Ozean des Unbekannten vor unseren Augen erstreckt.“

Sir Isaac Newton

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Abbreviations

Ca	Calcium
Cd	Cadmium
cDNA	Complementary desoxyribonucleic acid
CEZ	Chernobyl exclusion zone
Cs	Cesium
CYM	Complex yeast medium
DAG	Diacylglycerol
DNA	Desoxyribonucleic acid
et al.	et alii
evc	Empty vector control
Fig.	Figure
ICP-MS	Inductively coupled plasma - mass spectrometry
IMPase	Inositol monophosphatase
IP_1	Inositol monophosphate
IP_2	Inositol bisphosphate
IP_3	Inositol trisphosphate
JGI	Joint Genome Institute
MFS	Major facilitator superfamily
MM	Minimal Medium
MM + ura	Minimal medium supplemented with uracil
mRNA-Seq	Messenger ribonucleic acid sequencing
PCR	Polymerase chain reaction
PI	Phosphatidylinositol
PIP	Phosphatidylinositol phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C

PLC	Phospholipase C
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
RT	Room temperature
Sr	Strontium
Tab.	Table
Zn	Zinc

1. Summary

The pollution of the environment with metals is an omnipresent and growing problem. One possible approach to make such contaminated soils usable is the *in situ* remediation using fungi. This process is called mycoremediation. Ascomycetes and mycorrhizal fungi have been researched for this purpose, but first studies showed that wood-digesting fungi, to which the basidiomycete *Schizophyllum commune* belongs, appear to be potential candidates.

In order to investigate whether *S. commune* might be used in mycoremediation in the future, a test field was set up near the nuclear power plant in Chernobyl, which was damaged 35 years ago. *S. commune* and *Leucoagaricus naucinus*, another mushroom-forming basidiomycete, were cultivated in the field together with potato plants and winter rye. Using DNA isolation from soil samples and quantitative PCR with species-specific primers, it was possible to demonstrate that *S. commune* not only survived in the test field but also spread at a rate of around 8 mm/day. Survival and growth in Chernobyl soil for at least one year without the addition of any nutrients could be verified in a laboratory experiment. The DNA from soil samples from the test field was also used for community analysis. This showed that neither the inoculation with the fungi nor that of the two plants had a significant influence on the structure of the microbial community.

The examination of the metal tolerance showed that *S. commune*, even at suboptimal pH values and temperatures, can tolerate concentrations of Sr, Cs, Zn, and Cd that are well above the pollution of most contaminated soils.

To understand the mechanism behind this tolerance, an mRNA sequencing of *S. commune*, grown on two different intensely contaminated soils and minimal medium with and without the addition of metal, was carried out. This showed that growth on soil compared to medium has a greater influence on transcription than the different metal contents. In addition, it was found that genes that belong to transporters seem to be down-regulated under metal stress, whereas genes associated with secretion tend to be up-regulated.

Furthermore, the metal tolerance mechanism of *S. commune* was investigated using a Sr adapted strain. It was found here that both the wild-type and the adapted strain are able to

take up Cs and Sr into their hyphae and even transport it over a distance well above 100 cell lengths. Uptake and subsequent transport of metals in the adapted strain were significantly lower. This leads to the conclusion that the acquired metal tolerance is essentially an avoidance strategy. A qPCR experiment showed that a down-regulated MFS transporter, which is hypothetically involved in metal transport, is no longer down-regulated in the adapted strain under metal stress. Therefore, it was inferred that at least part of the avoidance mechanism could be depending on an increased efflux of metals.

The mRNA-Seq investigation also revealed a significant regulation of the genes encoding for myo-inositol oxygenase and inositol polyphosphate phosphatase, thus providing a connection to the inositol signaling cycle. The influence of metal stress on inositol signaling was investigated by combining qPCR of the two genes with different metal treatments and evaluating the data of a microarray using different metals and genetic backgrounds. All genes regulated under metal stress and associated with the inositol cycle were analyzed. A tendency to up-regulate kinases and down-regulate phosphatases resulting in a cycle shift towards phosphorylated inositol compounds could be seen. The influence of altered inositol signaling on metal tolerance was tested using an inositol monophosphatase overexpression mutant. Compared to the wild-type, the mutant is significantly more inhibited by Cs and Zn but tolerates Cd better. This indicated a connection of inositol signaling and metal tolerance with specific output for different metals.

Since Cd also was seen to induce a different response in metal transport and the qPCR investigation of a glutathione S-transferase compared to Zn, Cs, and Sr, the fungus probably reacts to this metal with a different mechanism.

Overall, an insight into the metal tolerance mechanisms of *S. commune* as a model wood-rotting basidiomycete could be obtained, and a connection to inositol signaling was established. It could also be confirmed that *S. commune* can be a potential candidate for mycoremediation, as it tolerates increased metal concentrations, radiation, and fluctuating environmental conditions over a long period, and it survives and even grows not only on wood, but also in natural and contaminated soil.

2. Zusammenfassung

Die Verschmutzung der Umwelt mit Metallen ist ein omnipräsentes und immer größer werdendes Problem. Ein möglicher Ansatz der Wiedernutzbarmachung solch kontaminierter Böden ist die *in situ* Sanierung mittels Pilzen. Dieser Prozess wird Mycoremediation genannt. Für diesen Zweck wurden bisher vor allem Ascomyzeten und Mykorrhiza-Pilze erforscht, jedoch zeigen erste Studien, dass auch Holz abbauende Pilze, zu denen der Basidiomyzet *Schizophyllum commune* gehört, potentielle Kandidaten zu sein scheinen.

Um zu erforschen, ob *S. commune* in Zukunft für Mycoremediation in Frage kommt, wurde ein Testfeld in der Nähe des vor 35 Jahren havarierten Atomkraftwerks in Tschernobyl errichtet. Auf dem Feld wurden *S. commune* und *Leucoagaricus naucinus*, ein weiterer fruchtkörperbildender Basidiomyzet, zusammen mit Kartoffelpflanzen und Winterroggen kultiviert. Mittels DNA Isolation aus Bodenproben und quantitativer PCR mit Spezies spezifischen Primern, konnte nachgewiesen werden, dass *S. commune* im Testfeld nicht nur überlebt, sondern sich auch mit einer Rate von circa 8 mm/Tag ausbreitet. Das Überleben und Wachstum für mindestens ein Jahr ohne die Zugabe zusätzlicher Nährstoffe konnte in einem Laborexperiment verifiziert werden. Die DNA aus Bodenproben des Testfelds wurde des Weiteren für eine Community Analyse genutzt. Diese ergab, dass weder die Inokulation der Pilze, noch die der beiden Pflanzen, signifikanten Einfluss auf die Struktur der mikrobiellen Gemeinschaft hatte.

Die Untersuchung der Metalltoleranz zeigte, dass *S. commune*, selbst bei nicht optimalen pH Werten und Temperaturen, Konzentrationen von Sr, Cs, Zn und Cd tolerieren kann, die weit über der Konzentration in den meisten kontaminierten Böden liegen.

Um den Mechanismus hinter dieser Toleranz zu verstehen wurde eine mRNA-Sequenzierung von *S. commune*, gewachsen auf zwei unterschiedlich intensiv kontaminierten Böden und Minimal-Medium mit und ohne Metallzugabe, durchgeführt. Diese ergab, dass das Wachstum auf Boden im Vergleich zu Medium einen größeren Einfluss auf die Transkription hat, als der unterschiedliche Metallgehalt. Zudem war zu erkennen, dass Gene, die Transporter kodieren,

tendenziell unter Metallstress herunterreguliert sind und Gene, die mit der Sekretion assoziiert sind, eher hochreguliert sind.

Der Metalltoleranzmechanismus von *S. commune* wurde zudem mittels eines Sr adaptierten Stammes untersucht. Hier konnte gezeigt werden, dass sowohl der Wildtyp also auch der adaptierte Stamm in der Lage sind Cs und Sr in ihre Hyphen aufzunehmen und sogar über eine Distanz von über 100 Zellen hinweg zu transportieren. Die Aufnahme und der anschließende Transport von Metallen ist in dem adaptieren Stamm deutlich verringert. Dies lässt den Schluss zu, dass es sich bei der erworbenen Metalltoleranz um eine Vermeidungsstrategie handelt. Ein qPCR Experiment zeigte, dass ein runterregulierter MFS Transporter, der hypothetisch am Metalltransport beteiligt ist, in dem adaptierten Stamm unter Metallstress nicht mehr herunterreguliert ist. Deshalb kann gefolgert werden, dass wenigstens ein Teil des Vermeidungsmechanismus auf erhöhtem Efflux von Metallen beruht.

Die mRNA-Sequenzierung ergab zudem eine signifikante Regulierung der Gene für eine Myo-Inositol Oxygenase und eine Inositol Polyphosphat Phosphatase und somit eine Verbindung zum Inositolzyklus. Der Einfluss von Metallstress auf das Inositol-Signaling wurde untersucht, indem eine qPCR der beiden regulierten Gene mit verschiedenen Metallbehandlungen mit der Auswertung der Daten eines Microarrays, das verschiedene Metallbehandlungen und genetische Hintergründe beinhaltet, kombiniert wurde. Hier wurden alle unter Metallstress regulierten Gene, die mit dem Inositolzyklus assoziiert sind, analysiert. Eine tendenzielle Hochregulierung von Kinasen und Herunterregulierung von Phosphatasen konnte beobachtet werden, was in einer Verschiebung des Zyklus in Richtung phosphorylierter Inositolverbindungen resultiert. Der Einfluss von verändertem Inositol-Signaling auf Metalltoleranz wurde mittels einer Inositol Monophosphatase Überexpressions-Mutante getestet. Die Mutante ist im Vergleich zum Wildtyp signifikant stärker durch Cs und Zn inhibiert, toleriert Cd jedoch besser. Dies deutete auf einen Zusammenhang von Inositol-Signaling und Metalltoleranz mit spezifischem Output für verschiedene Metalle hin.

Da bereits während des Transportexperiments und bei der qPCR Untersuchung einer Glutathion S-Transferase ein unterschiedliches Verhalten von *S. commune* in Bezug auf Cd

im Vergleich zu Zn, Cs und Sr zu beobachten wurde, reagiert der Pilz auf dieses Metall wahrscheinlich mit einem anderen Mechanismus.

Mit dieser Arbeit wurde ein Einblick in den Metalltoleranzmechanismus von *S. commune* als holzabbauenden Modellorganismus geschaffen und dessen Verbindung zum Inositol-Signaling wurde etabliert. Zudem konnte bestätigt werden, dass *S. commune* ein potentieller Kandidat für die Mycoremediation sein kann, da er über lange Zeit erhöhte Metallkonzentrationen, Strahlung und schwankende Umweltbedingungen toleriert und er überlebt und wächst sogar nicht nur auf Holz, sondern auch in natürlichen, kontaminierten Böden.

3. Introduction

3.1 The model fungus *Schizophyllum commune*

S. commune is a filamentously growing, mushroom-forming basidiomycete (Fig. 1) which is distributed over all continents except Antarctica. It has been used as a model in research for over a century. Thus, many aspects of its development, physiology, and genetics are explored (Raper and Miles 1958; Wessels 1965).

Fig. 1: *S. commune* fruiting bodies on dead wood (left, Bernhard Sprang, New Zealand 2017) and on artificial medium (right).

S. commune can be cultivated well under laboratory conditions and can complete its life cycle within 10-14 days on artificial medium (Stankis and Specht 2007). The fungus has a haploid phase in its life cycle and genetic modifications are possible (Munoz-Rivas et al. 1986). In addition, the genome of *S. commune* H-48 has been completely sequenced (Ohm et al. 2010). This makes *S. commune* an appropriate model organism.

S. commune is a white-rot fungus, which means that it grows saprotrophically on dead wood (Fig. 1). It produces extracellular ligninolytic enzymes that break down the complex organic polymer lignin (Schmidt and Liese 1980). This creates the whitish-yellowish color of the rotting wood that gave the white-rot its name. *S. commune* forms a variety of enzymes for lignin catabolism, e.g. laccases and cellobiose dehydrogenases (Ohm et al. 2010). It is also well equipped to break down cellulose, hemicellulose, and pectin (Ohm et al. 2010).

Other white-rot fungi such as *Phanerochaete chrysosporium, Trametes versicolor,* or *Pleurotus ostreatus* have been shown to survive saprotrophically in soil, if a suitable carbon source is

provided (Morgan et al. 1993; Canet et al. 2001). Hemicellulotic substrates such as wheat straw were best suited for this purpose (Hultgren et al. 2009). *S. commune* is a very frugal saprotrophic fungus and can grow in a variety of climates and substrates like grape residue, bread crumps but has also been observed as a human pathogen in immune-compromised patients (Rihs et al. 1996; Basso et al. 2020; Ivanova et al. 2014). It is, therefore, conceivable that it could survive even without the addition of nutrients in soil.

3.2 Metal tolerance in fungi

Heavy metals can be divided into essential (e.g. Zn, Cu, Ni, Fe, Mn, Mo), also called micronutrients, and non-essential (e.g. Cd, Pb, Hg, Ag, Sr, Cs). All of them are toxic above a certain threshold. The toxic effect occurs, among other things, by blocking functional groups of important molecules, e.g. enzymes, polynucleotides, transport systems for essential nutrients and ions, and disrupting the integrity of cell and organelle membranes (Ochiai 1987). For example, heavy metals can change the conformation of DNA and proteins and can also attack these molecules by forming radicals. Radical formation involves the interaction with reactive oxygen species, e.g. hydrogen peroxide, according to the type of the Fenton reaction (Lloyd and Phillips 1999).

It is assumed that environmental presence of heavy metals can lead to the acquisition of tolerance mechanisms or metal resistance in fungi. Some fungi are naturally less susceptible to heavy metal stress than others, with survival depending largely on the biochemical and structural capabilities of the microorganism. This includes genetic and physiological adaptations (Gadd and Griffiths 1978). Possible properties that increase metal tolerance include impermeable and/or pigmented cell walls, the binding of metals to the cell wall or extracellular polysaccharides, and the excretion of metabolites, that bind or precipitate metals e.g. oxalates (Fig. 2; Gadd 1992; Gadd et al. 2014). Among the intracellular metal tolerance are a decreased uptake or increased efflux of metals, intracellular precipitation or complexation, chelation by glutathiones or metallothioneins, and sequestration into cell compartments (Fig. 2; Landeweert et al. 2001; Zafar et al. 2007; Xu et al. 2014). Intracellular

oxidative stress caused by the Fenton reaction of metals can be counteracted with superoxide dismutase and catalase (Ott et al. 2002).

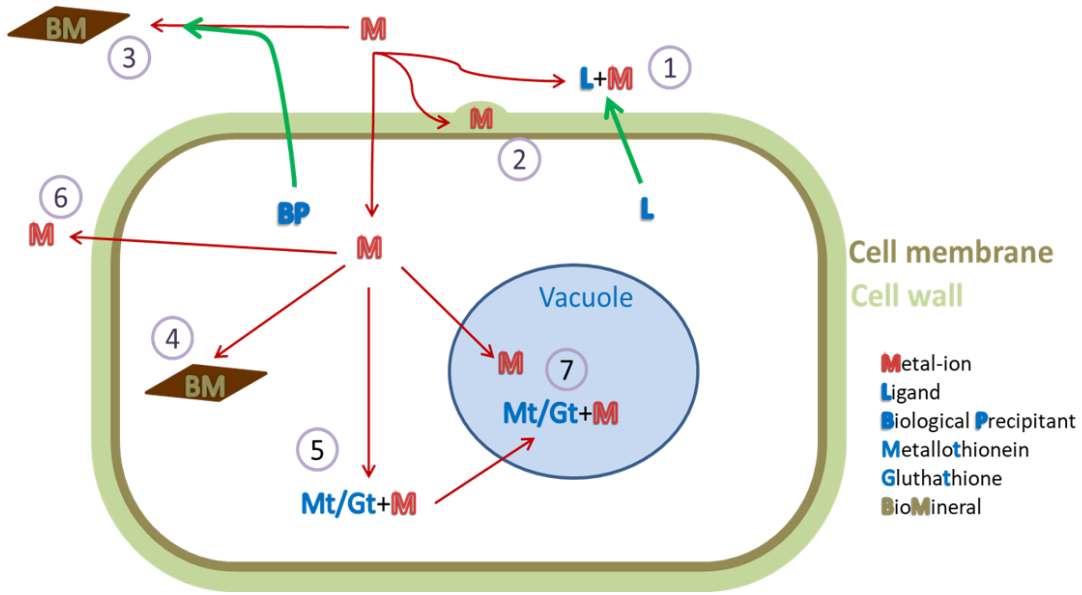

Fig. 2: Metal tolerance mechanisms in fungi. 1) Extracellular chelation by ligands, 2) Metal-binding to cell wall, 3) Extracellular biomineralization, 4) Intracellular biomineralization, 5) Intracellular chelation by glutathiones or metallothioneins, 6) enhanced efflux, and 7) Sequestration into cellular compartments. Modified from Mauz 2017; after Bellion et al. 2006.

S. commune can survive reasonable levels of cadmium, lead, and uranium (Gabriel et al. 1994; Günther et al. 2014). It is also able to accumulate metals through biosorption and even to dissolve them from rock material and sequester them into cellular compartments (Kirtzel et al. 2019; Javaid et al. 2010). However, it is not well established, which mechanism prevails in *S. commune* at increased heavy metal concentrations.

3.3 Inositol signaling

Inositol signaling is strongly connected in a complex network with other intracellular signaling pathways, and it is highly conserved in eukaryotes. It is involved in a wide variety of physiological processes including metabolic adaptation, apoptosis, fungal virulence, vesicle trafficking, and sexual development in ascomycete and yeast-forming fungi (Li et al. 2016; Lev et al. 2015; Xie et al. 2017). In *S. commune*, cross-talk between inositol and Ras signaling and involvement in sexual development has been shown (Knabe et al. 2013; Murry et al. 2019).

An extracellular stimulus that activates a membrane-associated phospholipase C (PLC) leads to cleavage of phosphatidylinositol 4-5-bisphosphate (PIP_2) into two second messengers: diacylglycerol (DAG), which stays at the membrane, and inositol triphosphate (IP_3), which is released to the cytoplasm (Fig. 3; Berridge and Irvine 1984). DAG activates a protein kinase C (PKC), which then phosphorylates specific target proteins. Amongst others, PKC can activate specific Ca^{2+} channels, leading to enhanced calcium influx into the cytoplasm (Nishizuka 1988).

A variety of functions was found associated with IP_3 signaling: 1) mobilizing intracellular calcium ions from the endoplasmic reticulum or vacuoles (Berridge 1993), 2) being phosphorylated by inositol multikinase (IMK) up to 5-bis-diphosphoinositol-tetrakisphosphate (IP8), which has a function in phosphate storage (Saiardi 2012), and 3) going on in the inositol signaling cycle by dephosphorylation to Inositol di- and monophosphate (IP_2 and IP_1). The last dephosphorylation from IP_1 to inositol is performed by the key enzyme inositol monophosphatase (IMPase) that is specifically inhibited by lithium, which leads to lower inositol and hence calcium levels within the cell (Teo et al. 2009). The inositol cycle is closed by phosphorylation of inositol by phosphatidylinositol synthase (PIS) to phosphatidylinositol (PI) and finally to phosphatidylinositol 4-phosphate (PIP; Fig. 3).

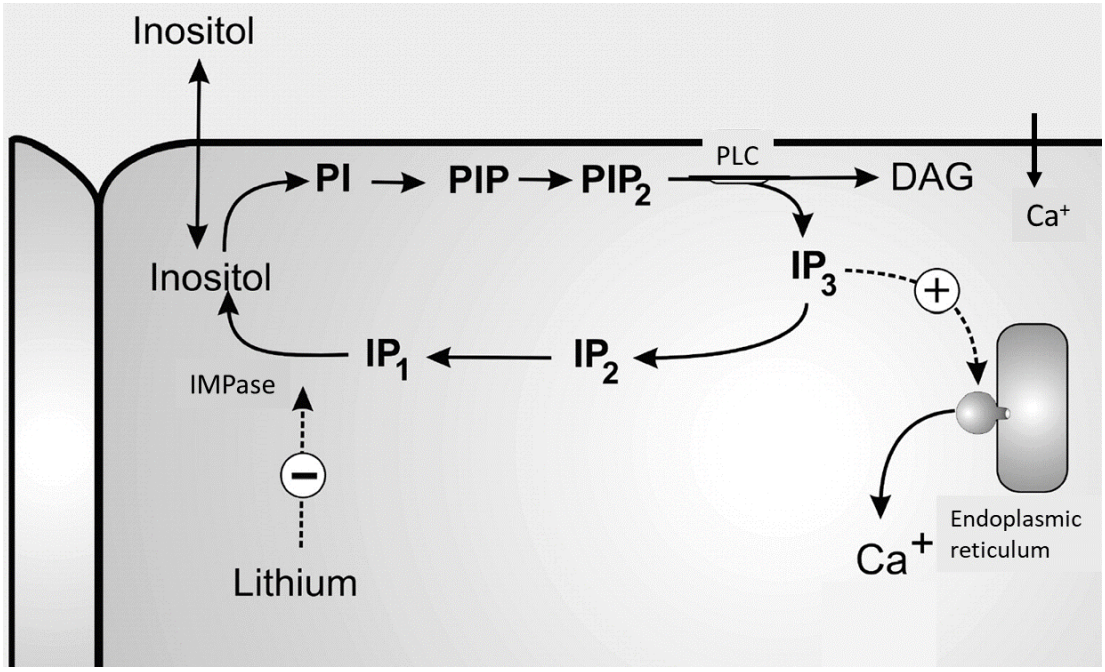

Fig. 3: Schematic representation of phosphoinositol signaling. Phosphatidylinositol-specific phospholipase C (PLC) cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) and produces two second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). Both can lead to calcium influx. IP_3 can be converted into higher phosphorylated IP_8 via IP_4 or is dephosphorylated to inositol *via* inositol di.- and monophosphate (IP_2 and IP_1) phosphatases. Dephosphorylation of IP1 to inositol is catalyzed by the key enzyme inositol monophosphatase (IMPase), which is inhibited by lithium. Inositol is phosphorylated again to membrane-bound PIP_2 via PI and PIP to close the cycle (modified after Berridge 2009. For more details see Figure 1 within manuscript 4).

While it has been reported that inositol signaling is impaired under metal stress (Ritter et al. 2014), the exact routes of regulation are unknown. It is therefore of interest to investigate this connection with the help of a genetically tractable model organism like *S. commune.*

3.4 Mycoremediation

Metals are omnipresent pollution in the environment that is mostly caused anthropogenically, for example through the burning of fossil fuels, mining, agriculture, and waste disposal (Nriagu and Pacyna 1988). Besides bacteria and plants, fungi are the most widespread organisms in nature and it is known that they have a major influence on the behavior of metals in the environment. They have the ability to immobilize and detoxify metallic pollutants (Gray 1998). This makes fungi suitable organisms for decontaminating polluted soil and water. This process is called mycoremediation.

As detailed above, fungi can change the bioavailability of metals in soil in many ways. Immobilization of metals can occur by 1) biosorption to biomass, 2) precipitation around the hyphae, and 3) accumulation and sequestration inside the hyphae. The ability of selective sorption of heavy metal ions from the environment plays a decisive role in metal remediation (Baldrian 2003). The biosorption to fungal biomass, including the cell wall, extracellular polysaccharides, or pigments, does not depend on the survival of the fungus (Volesky 1990). Extracellular polysaccharides such as schizophyllan, which is an extracellular polymer synthesized by *S. commune*, are potent biosorbents for heavy metals (Jiang et al. 2019). Secondary mineral formation through precipitation is often achieved through the secretion of oxalates (Gadd et al. 2014), but precipitation through the reduction of metals is also possible with fungi (Gadd 2004). However, lower bioavailability not always means lower toxicity. For example, an acidic pH increases the chemical bioavailability of metals but lowers the toxicity for fungi (Gadd and Griffiths 1980).

For heterogeneously contaminated landscapes, the wide hyphal network can also help to make land usable again due to the translocation of metals (Colpaert et al. 2011).

White-rot fungi are additionally useful for the remediation of xenobiotic substances. They can degrade a wide range of persistent compounds due to their extracellular lignin-modifying enzymes like manganese peroxidase or laccases. These enzymes are not very substrate-specific, thus are able to degrade other molecules which are broadly similar to lignin (Rhodes 2015). Some of these persistent substances are polyaromatic carbohydrates, phenols, pesticides, and additionally heavy metals (Singh 2006).

All these possible strategies, together with the ability to grow in very harsh environments, make fungi useful organisms to remediate contaminated areas and make them a target for improving crop yields in agriculture, to name just one example.

3.5 Significance of mycoremediation for the environment at the Chernobyl exclusion zone

On the 26th of April, 1986 the most severe radiological accident to date occurred at reactor block 4 of the nuclear power plant near Chernobyl, Ukraine. A test of the control system resulted in an unstable state of the reactor with strongly fluctuating core temperature and cooling water flow rates. This led to a steam explosion of the reactor core and wide distribution of radioactive debris. Radiation exposure was initially due to ^{131}I, a short-live radionuclide, and long-term ^{134}Cs and ^{137}Cs, as well as to a lesser extent ^{90}Sr. It is estimated that 4×10^{16} Bq ^{137}Cs have been released worldwide (United-Nations 2000). The most severely affected areas are, besides the region around Chernobyl, the east of Belarus, bordering areas of Russia, the south of Finland, and part of Austria (De Cort 1998). The distribution of strontium is more limited to the area around the reactor. In a radius of 30 km, which is the Chernobyl exclusion zone (CEZ), where no taxable stay is allowed, over 1.1×10^5 Bq m^{-2} ^{90}Sr were found (United-Nations 2000).

Radiation causes DNA damage and drastically increases cancer rate in the long term. As a result, the population of the severely affected regions moved away. The CEZ has been abandoned for over 35 years, creating a unique ecosystem in Europe that, since then, remains nearly untouched by humans (Beresford et al. 2016). Still, the CEZ is a harsh environment for animals, plants, and microbiota. A possibility for future land use is mycoremediation. It has already been shown various times that fungi can absorb large amounts of metals and radionuclides and survive under adverse conditions (e.g. Guillén and Baeza 2014; Árvay et al. 2014; Zhdanova et al. 2003). In addition, it has been shown that the uptake of radionuclides is much higher in fungi compared to plants (Borio et al. 1991 and citations therein).

The mechanisms of fungi to remediate radionuclides from contaminated soil are the same as for any other metal. For the tolerance against the radiation, a role of melanin pigmentation had been proposed (Zhdanova et al. 2000). Although mycorrhizal fungi seem to accumulate the greatest amount of radiocesium, saprotrophic basidiomycetes such as *S. commune* take up significant amounts (Smith et al. 1993; Wasser et al. 1991). Most of the accumulated cesium

is usually stored in the fruiting body (Malinowska et al. 2006). This leads to an immobilization of the radionuclides, and the soil can be used for e.g., agriculture again.

To further investigate the process of mycoremediation using basidiomycetes, a test field was set up with two different crop plants and the addition of *S. commune* within the CEZ. The test field is located 5 km south of the exploded reactor block near a former village (51°20′54″N; 30°07′41″O). Since *S. commune* is not usually reported to occur naturally in soil, contamination with environmental strains was not to be expected. It thus could be investigated whether this fungus survives and grows in the ground. The inoculated fungus was used in its haploid, unmated form which excludes the formation of fruiting bodies and therefore not leading to excess accumulation. The outcome of this field experiment could lead to future use in bioremediation based on *S. commune* metal accumulation inside or outside the hyphae, thereby changing their bioavailability. Additionally, the effects on the microbial community and phytoremediation were investigated.

3.6 Aim of the study

Environmental pollution with metals is diverse and widespread, and radionuclides are also a serious problem. One way to deal with this problem is by using fungi for remediation. For that, it is advantageous to know their metal tolerance and the mechanism behind it. So far, ascomycetes and mycorrhizal fungi have been used preferentially for the investigation of bioremediation, but studies have shown that basidiomycetes such as *S. commune* can also be useful for this purpose (Kulshreshtha et al. 2014; Rhodes 2015). Within this study we tried to clarify the following aspects:

1. Does *S. commune* survive at all in contaminated soil and how does its inoculation affect the existing microbial community?
2. What is the mechanism behind *S. commune*'s metal tolerance?
3. Is there a link between the inositol signaling pathway and the response to metal stress in *S. commune*?

To answer the first question, community analysis of soil from the test field in CEZ was carried out and the survival of *S. commune* was demonstrated using quantitative PCR with species-specific primers. To research the metal tolerance mechanism, an adapted strain was produced. Various influencing factors on the metal tolerance were tested, the transport of metals was measured with divided Petri dishes and ICP-MS, mRNA sequencing was carried out to detect changes in gene expression under metal stress and different substrates, and genes of interest were examined more closely using qPCR. The results of the mRNA sequencing showed significant regulation of some genes involved in the inositol cycle and thus led to the third question. This was answered by evaluating a microarray analysis and the growth behavior of an IMPase overexpression mutant in the presence of metals.

In summary, this study provides an overview of the response of *S. commune* to heavy metal stress and the mechanism to tolerate it. In addition, its ability to bioremediate contaminated soils was examined.

4. Manuscripts

4.1 Manuscript 1: Survival of the basidiomycete *Schizophyllum commune* in soil under hostile environmental conditions in the Chernobyl exclusion zone

Manuscript number: 1

Title of the manuscript: Survival of the basidiomycete *Schizophyllum commune* in soil under hostile environmental conditions in the Chernobyl exclusion zone

Authors: Lea Traxler (contribution 40 %), Anne Wollenberg, Georg Steinhauser, Ihor Chyzhevskyi, Sergiy Dubchak, Sina Großmann, Alix Günther, Dharmendra Kumar Gupta, Karl-Heinz Iwannek, Serhii Kirieiev, Falk Lehmann, Wolfgang Schulz, Clemens Walther, Johannes Raff, Erika Kothe

Bibliographic Reference: Traxler, L., Wollenberg, A., Steinhauser, G., Chyzhevskyi, I., Dubchak, S., Großmann, S., Günther, A., Gupta, DK., Iwannek, KH., Kirieiev, S., Lehmann, F., Schulz, W., Walther, C., Raff, J., Kothe E., (2021). Survival of the basidiomycete *Schizophyllum commune* in soil under hostile environmental conditions in the Chernobyl exclusion zone. Journal of Hazardous Materials, 403, 124002.

The candidate is:

x First author, □ Co-First author, □ Corresponding author, □ Co-author.

Status: Published in *Journal of Hazardous Materials*

Author	Concept	Data analysis	Experiments	Writing of manuscript	Provision of material
Lea Traxler	x 10%	x 30%	x 50%	x 60%	
Anne Wollenberg		x 20%	x 10%	x 10%	
Georg Steinhauser		x 10%		x 10%	
Ihor Chyzhevskyi			x		
Sergiy Dubchak			x		x 20%
Sina Großmann		x 15%			
Alix Günther			x		
Dharmendra Kumar Gupta		x 10%	x 10%		
Karl-Heinz Iwannek			x 10%		
Serhii Kirieiev			x		
Falk Lehmann			x		
Wolfgang Schulz		x 15%	x 10%	x 10%	
Clemens Walther	x 20%				x 25%
Johannes Raff	x 20%				x 25%
Erika Kothe	x 50%			x 10%	x 30%

4.1.1 Summary

The Chernobyl exclusion zone (CEZ) is still a highly contaminated area over 35 years after the accident. To find out whether the basidiomycete *S. commune* has the potential to help remediate contaminated soils, it was inoculated in a test field 5 km from the damaged reactor. By quantifying DNA from soil samples using species-specific primers, it was possible to prove that *S. commune* not only survives in the soil but also spreads at a rate of 8 mm/day. Additionally, laboratory experiments showed survival of *S. commune* for at least one year without added carbon source in soil from the CEZ. This shows that the fungus that otherwise lives on wood can tolerate both the soil as a substrate and the radiation as well as metal pollution and thus is a candidate for mycoremediation.

Journal of Hazardous Materials 403 (2021) 124002

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Survival of the basidiomycete *Schizophyllum commune* in soil under hostile environmental conditions in the Chernobyl Exclusion Zone

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ARTICLE INFO

Keywords:
Chernobyl exclusion zone
Fungi
Soil
Environment
Schizophyllum commune

ABSTRACT

Radioactive contamination resulting from major nuclear accidents presents harsh environmental conditions. Inside the Chernobyl exclusion zone, even more than 30 years after the accident, the resulting contamination levels still does not allow land-use or human dwellings. To study the potential of basidiomycete fungi to survive the conditions, a field trial was set up 5 km south-south-west of the destroyed reactor unit. A model basidiomycete, the lignicolous fungus *Schizophyllum commune*, was inoculated and survival in the soil could be verified. Indeed, one year after inoculation, the fungus was still observed using DNA-dependent techniques. Growth led to spread at a high rate, with approximately 8 mm per day. This shows that also white-rot basidiomycetes can survive the harsh conditions in soil inside the Chernobyl exclusion zone. The unadapted fungal strain showed the ability to grow and thrive in the contaminated soil where both stress from radiation and heavy metals were present.

1. Introduction

Microbes are omnipresent, and bacteria and fungi in soil are essential for element cycling and soil formation processes (Fierer, 2017 and citations therein). Hence, their impact on agriculture, human nutrition, health and renewable energy production cannot be overestimated and land-use changes will, in turn, impact the fungal and bacterial diversity (see, e.g., Tian et al., 2019). The decomposition of plant material depends on fungi that are able to degrade the lignin present in plant matter (Morgan et al., 1993; Patel et al., 2019). Hence, their contribution to mineralization processes in soil is essential for soil fertility.

Since the Chernobyl nuclear accident on April 26, 1986, the Chernobyl Exclusion Zone (CEZ) near the city of Pripyat has been largely abandoned and, over the course of more than three decades, has formed a unique ecosystem in Europe (Beresford et al., 2016). The area is characterized by minimal impact by humans (apart from the anthropogenically increased radiation levels; Beaugelin-Seiller et al., 2018) and has become a valued habitat for rare animal and plant species.

The impact of the harsh environment in the CEZ exerts stress that can impact plants and animals as well as microbiota. Thus, an effect on the soil fungal community can be expected. A model basidiomycete, *Schizophyllum commune* (Raper, 1966; Erdmann et al., 2012), was chosen because it is a mushroom that has the ability to degrade lignin as well as cellulose (Alma´si et al., 2019; Madhavan et al., 2014). It is an Agaricomycete, but phylogenetically distinct from most mushroom-forming basidiomycetes (Ebersberger et al., 2012). This allowed to design primers that will amplify the taxonomically important rRNA gene sequence ITS1/2 (Buzina et al., 2001). In addition, the tree-dwelling

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https://doi.org/10.1016/j.jhazmat.2020.124002
Received 20 February 2020; Received in revised form 30 August 2020; Accepted 14 September 2020
Available online 17 September 2020

fungus usually is not found in soil (Boyle et al., 1992; Naumann et al., 2005; Ohm et al., 2010). Therefore, a contamination of non-inoculated *Schizophyllum* was less likely as compared to other fungi that are found in soil regularly.

These lines of argument led to the inoculation of *S. commune* in a test field site set up within the CEZ. A spread of the fungus via spores could be ruled out, since only in timber, fruiting bodies are observed in nature (Esser et al., 1979; Ohm et al., 2014). In addition, the life cycle requires mating before a fertile mycelium is formed that would be able to produce spores (Raudaskoski and Kothe, 2010; Jung et al., 2018). Hence, a haploid, unmated mycelium was inoculated to protect the wild-life in the already stressed environment. This also meant that mycoremediation with extraction of metals and radionuclides into fruiting bodies could not be performed (compare Schindler et al., 2012). Rather, the effects of adding the fungus to the mycobiome present at the site was tested for phytoremediation as well as mycoremediation approaches. The effect that could be expected and used for future bioremediation approaches is that *S. commune* could accumulate metals inside or outside the cell, thereby changing their bioavailability. Consequently, the polluted area could be used again for crop planting. Therefore, the ability of *S. commune* to survive for prolonged time at the test field site was essential, as previous data on survival of a timber-growing fungus in soil had not been available.

We tested whether survival in soil is possible in laboratory conditions to verify that not the inoculation into soil alone would lead to cell death with this fungus. In the native soil, additional biotic stress is imposed on the inoculated fungus, as it has to deal with the microflora present, bacteria and other fungi alike. Earlier investigations have shown that *S. commune* is able to compete in many environments including timber, artificial media and metal contamination (Kirtzel et al., 2018).

The CEZ was chosen as a location for this study as it combines a multitude of stress factors relevant for the survival of the white-rot fungus in the soil. Apart from a significantly increased radiation background, a relatively dry climate, harsh winters, and sandy soil with a low content of organic material and nitrogen are presenting continuous stress factors. The huge areas impacted call for bio-geo methods of remediation. As mushrooms have been found to show high uptake of heavy metals and radionuclides (e.g., Arvay et al., 2014; Guill´en and Baeza, 2014), a mycoremediation approach may be feasible (see Chatterjee et al., 2017; Kapahi and Sachdeva, 2017 for reviews).

The uptake of radionuclides into fungi has been shown to exceed that by any plant, with many reports specifically after the Chernobyl accident (compare Borio et al., 1991, and citations therein). Our findings supports the use of mushrooms in mycoremediation approaches as the changed bioavailability of radionuclides will profit co-existing plants and microbes in the soil environment. As methods to establish future land-use in Chernobyl as well as Fukushima and other radiation-impacted sites have not yet been established, this may be of large importance in future application.

2. Materials and methods

1. Cultivation

Schizophyllum commune 12–43 (Jena Microbial Resource Collection, Jena, Germany) was cultivated in CYM-T (glucose 20 g/L, tryptone 2 g/L, yeast extract 2 g/L, K_2HPO_4 1 g/L, tryptophan 1 g/L, KH_2PO_4 0.5 g/L, $MgSO_4$ 0.5 g/L; for solid media: agar 18 g/L) at room temperature of approx. 23 °C.

In order to be able to assess the expansion of mycelium, experiments were carried out in columns (33 mm × 200 mm) filled with cube-shaped agar pieces (5 mm × 5 mm) of CYM-T. The inoculation with the corresponding fungus was performed in the middle of the agar column. The test tubes were closed with cotton and aluminium foil to ensure a sterile environment and incubated at room temperature for 30 days.

For cultivation on soil-medium, *S. commune* was inoculated on medium containing 18 g/L agar and 200 g/L sieved and dried soil from the test field site. Incubation was performed at 16 °C for 365 days to mimic more natural conditions. To test for survival, every 10 days a piece from the growing perimeter was inoculated into fresh complex yeast medium (Schwalb & Miles, 1967) and incubated for 5 days at 28 °C.

For a large-scale cultivation of *S. commune*, a bioreactor (Applikon, Bedford, MA, USA) with a working volume of 5 L was used. Incubation was carried out in CYM-T medium with antifoam agent (1 drop/L) at room temperature for 1 week. For the dissolved oxygen concentration in the medium, the reactor was gassed with 1.5 L air per minute. Two axially offset propeller stirrers with an initial speed of 300 rpm carried out the mixing. The stirring speed was increased when the dissolved oxygen concentration dropped. The pH value was adjusted to 6.5 and controlled using H_2SO_4 and NaOH. At the end of cultivation, the biomass was obtained by centrifugation (15,000 x g, 60 min and 4 °C) and used as inoculum for column and field experiments.

2.2. Soil columns

For column experiments, 2.5 kg of soil (pH 7.5 with composition of 90.4% sand, 6.3% silt, 3.3% clay) contaminated with Sr-90 (22 ± 3 Bq/g wet weight) and Cs-137 (0.0057 ± 0.0007 Bq/g wet weight) was mixed with 0.5 kg wet biomass of *S. commune* and filled into the column body (300 mm × 100 mm). The columns were gassed with air and watered with sterile tap water. Incubation took place at room temperature for a period of 6 months. No additional nutrients were supplied for the fungus. Excess water from the column could run off via a drain at the bottom of the column.

3. Experiments at the test field site in CEZ

1. Field site

Field experiments were conducted inside the CEZ at a test site near Kopachi village, approx. 5 km south of Chernobyl nuclear power plant (51° 20′ 54′′ N: 30° 07′ 41′′ O). In the aftermath of the accident, Kopachi village was evacuated and subsequently destroyed to prevent inhabitants to return to this severely contaminated area. The experiments were conducted on one of the agricultural areas belonging to Kopachi village that have also been abandoned since 1986 and has been lying fallow since then. The time-span of more than 30 years has allowed for the establishing of ecological equilibria of plant, animal and microbial communities in the area. However, since the area had been intensely used for agricultural purposes, the area is a unique analogue of a non-anthropogenic type of an agricultural area. The area was fenced with wooden planks to prevent wild boar and other large animals to disturb the pedological structure and stratigraphy during the experiments.

2. Inoculation

The soil was inoculated with *S. commune* in multiple locations within two stripes inside the fenced area. Four more such stripes to the right of the inoculated subplots were devised and planted as well to allow for control. In order to allow for the even spreading of *S. commune* in the soil, the inoculation was arranged within the two stripes with ten holes of 15–20 cm depth (Fig. 1). Vegetation on the stripes was removed, and the top soil was carefully tilled to aerate the top soil. The pre-grown fungal mycelium was suspended in water in a large plastic barrel by stirring, until the mycelium was evenly suspended. The control strips were prepared similarly, with the holes filled with water instead of the fungal suspension. The inoculation with *S. commune* in stripe one was 12 months and on stripe two 6 months before the sampling of the soil. As crop plants that allow for adding nutrients to the soil during their growth phase, potato and rye crops were planted in alternating stripes. As root exudates by plants can stimulate growth of fungi and bacteria in the rhizosphere, planting was chosen. Cultivation of crops was tested to allow for more a setting that might allow for future bioremediation

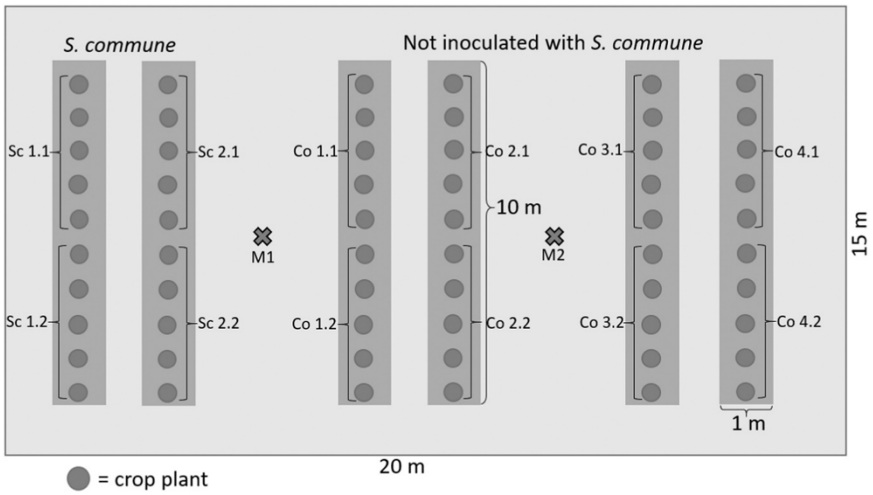

Fig. 1. Set-up and sampling at the test field site within the CEZ. In an area of 15 × 20 m, six rows were put into place, two of which were inoculated with *S. commune*. DNA was extracted from mixed soil samples at all test sites Sc, Co, and M to allow for re-isolation of the fungus 6 months and 1 year after inoculation.

applications. Root crops such as potato will respond specifically with uptake of radionuclides into the root biomass, which was expected to show a stronger effect of contamination present.

For sampling, soil was taken from 2 cm depth on May 23, 2018. Per hole approximately 0.5 g soil were sampled, and the soil of each 5 holes were mixed in a Falcon tube (Fisher Scientific, Schwerte, Germany). Thus, 2 mixed soil samples were taken per row, one each for the upper and lower part of the row. Additionally, two samples were taken in between the planted rows (see Fig. 1). Consequently, a total of 14 soil samples were used for the DNA extraction.

2.3.3. DNA-dependent identification of S. commune from CEZ soil

For DNA isolation, the DNeasy PowerSoil Isolation Kit from Qiagen (Hilden, Germany) was used as per manufacturer instructions. Due to the laboratory conditions on-site, the centrifugation steps were performed with 4700 x g for 1 min, and the incubation at 4 °C was prolonged to 7 min. The extraction was interrupted before the elution step, and the DNA was transported to Germany dry on the membrane. The elution occurred after approximately 55 h cooled transport, with 100 µl eluting buffer (10 mM Tris-HCl pH 8,5) incubated for 10 min on the membrane and centrifugation at 16,060 x g for 1 min.

To identify *S. commune* DNA in the isolated DNA samples, species specific primers (Eurofins Genomics, Ebersberg, Germany) were used (Buzina et al., 2001; forward scom1 5′GTTGACTACGTCTACCTCAC 3′; reverse scom2r 5′ GTTAGGCTCCAGCAGACCT 3′) which result in a product of 305 bp. For direct amplification, a reaction mixture of 2.5 µl of 10 pmol/µl of the primers, 1.25 µl of 10 mM dNTPs, 5 µl 10x DreamTaq Green Buffer, 0.63 µl 5 U/µl DreamTaq Polymerase (ThermoFischer Scientific, Waltham, USA), 33.12 µl nuclease-free water and 50 ng of DNA was used. As a negative control, water was added instead of DNA, and *S. commune* DNA was added as a positive control in place of the soil sample DNA. For PCR, 34 cycles of 30 s 95 °C, 30 s 50 °C and 50 s 72 °C with an initial denaturation of 5 min at 95 °C and a final elongation of 10 min at 72 °C was used.

To avoid unspecific PCR products for subsequent sequencing, a nested PCR with primers ITS1 and ITS4 (Innis et al., 1990) was performed (5 min initial denaturation at 95 °C, 35 cycles of 30 s 95 °C, 30 s 56 °C, 50 s 72 °C, followed by 72 °C for 10 min) followed by the use of primers scom1 and scom2r as before was performed and the resulting product was sent to sequencing (Eurofins Genomics, Ebersberg, Germany).

Quantification of the DNA samples (Ferre, 1992) was achieved using SYBR green fluorescence with the qTOWER^3 from AnalytikJena (Jena, Germany). Each qPCR reaction consisted of 6.25 µl Maxima SYBR Green/ROX qPCR Mastermix (2x; ThermoFischer Scientific, Waltham, USA), 1 µl each of 10 pmol/µl scom1 and scom2r primers, 2.25 µl water and 2 µl DNA. The qPCR was run with 10 min initial denaturation at 95 °C, 35 cycles of 20 s 95 °C, 20 s 50 °C and 20 s 72 °C, followed by 2 min final elongation. For absolute quantification of the *S. commune* DNA within the soil DNA, DNA samples and standards were run in simultaneous triplicates. An external standard consisting of a serial dilution from 0.1 to 10 ng of *S. commune* DNA was measured to obtain a standard curve from the logarithmic Ct values. The absolute DNA amount could be calculated using the incline of the standard curve. For an absolute quantification of *S. commune* DNA in the samples, a standard curve was used that produced an incline of

$$y = -3.8237x + 22.056. \quad (1)$$

2.3.4. Radiological assessment

Dose rates were measured at ground level immediately prior to inoculation with *S. commune* and over the period of more than 8 months (from September 26, 2017 to May 24, 2018) using a total of 8 thermoluminescence dosimeters (TLD) that were installed at 1 m above ground at the fence of the test field. One dosimeter was stolen during the experiment, hence only 7 dosimeters could be evaluated. The dosimeters were transported from Germany to Ukraine by airplane (without X-ray security scanning) and back from Ukraine to Germany by train to avoid cosmic radiation. They were set to 0 a few days before their installation and evaluated a few days after the removal from the test field. The additional dose during the (single) flight as well as the exposure to ambient radiation before and after the experiment can be regarded as negligible compared with the high dose rates during the experiments. Each dosimeter consisted of two TLD crystals, one of which was pre-irradiated to correct for fading effects over the long exposure time.

For gamma measurements of drillcores, two drillcores have been randomly sampled from the test field in October 2015 using a drill core auger and a polyethylene plastic tube lining of 30 cm length and 3.5 cm diameter. In the laboratory, the drillcores were deep-frozen to − 21 °C and cut into slices of 2.5 cm height. The soil slices were oven-dried at 105 °C until weight constancy was reached. The samples were sieved to 2 mm, packed into plastic dishes of 3.5 cm diameter and weighted for the subsequent gamma measurement.

Activity concentrations of the fission product ^{137}Cs (half-life 30.08 a) and of the activation product ^{241}Am (half-life 432.2 a) were quantified using the 662 keV and the 59 keV lines, respectively, on a 57 cm^3 Canberra planar n-type high purity germanium detector with a relative efficiency of 23% (at 1332 keV). The efficiency for each sample was

individually determined by using a sealed paper filter disc standard of the same diameter as the petri dishes carrying a multi-element standard with a known amount of ^{241}Am and ^{137}Cs. The total efficiency (ε_{total}) was then determined by performing two separate calibration measurements for each sample, one on top (ε_{top}) and one at the bottom (ε_{bot}) of the petri dish and the calculation of their logarithmic mean:

$$\varepsilon_{total} = \frac{\varepsilon_{bot} - \varepsilon_{top}}{\ln\left(\frac{\varepsilon_{bot}}{\varepsilon_{top}}\right)} \quad (2)$$

All measurements were decay corrected to the date of sampling (July 8, 2015).

3. Results

1. Evaluation of growth direction and dimension of S. commune

Experiments to evaluate the growth direction and dimension of the mycelium demonstrate that *S. commune* is able to grow through the test tube, both horizontally and vertically, within 2 weeks. Figure 2 compares the mycelium penetration of the test tube by different fungi after 4 weeks of incubation. The other selected and soil-living fungi, *Leucoagaricus naucinus* (DSMZ 8667, Braunschweig, Germany), *Xerocomus badius* (DSMZ 4436, Braunschweig, Germany) and *Suillus variegatus* (DSMZ 1752, Braunschweig, Germany), were not able to grow through the entire test tube. During the same incubation period, they either grew vertically around the inoculation site or showed one spreading within the column.

2. Evaluation of S. commune survival in soil

The cultivation of *S. commune* on water agar with added soil resulted in vital mycelium until the end of the experiment after 365 days. *S. commune* also showed in the column experiments that the contamination and the absence of nutrients in the used sandy soil is no impediment to the growth of the column.

After PCR with species-specific primers, the expected product of the samples of the field site could be shown (Fig. 3). While the samples from the inoculated strip in both rows (samples Sc 1.2, Sc 2.1, Sc 2.2) and the sample next to the inoculated strip (M1), as well as one control (Co 3.2) clearly showed higher amplification, while the remaining resulted in very weak (Sc 1.1, Co 1.1, Co 4.1 and Co 4.2) or no visible bands.

The specificity was verified by sequencing the specific band products, which could be clearly identified as the addressed part of the *S. commune* ITS region.

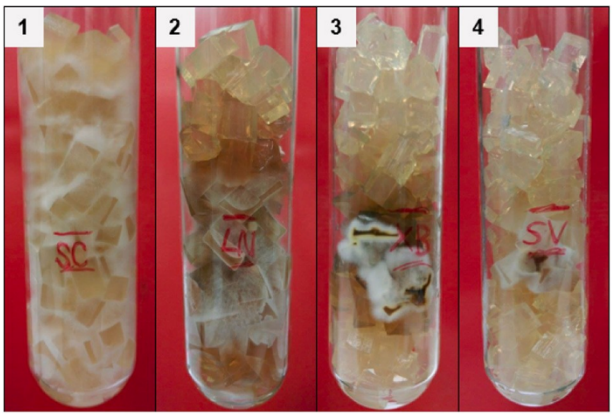

Fig. 2. Growth of (1) *S. commune*, (2) L. *naucinus*, (3) *X. badius* and (4) *S. variegatus* 4 weeks after inoculation in test tubes.

3. Quantification of S. commune DNA for estimation of spread

The resulting DNA concentrations showed more than 4 ng in the samples Sc 1.2, Sc 2.1, Sc 2.2 and M1 per g soil (Table 1), the not inoculated sampling site Co 4.1 showed an elevated *S. commune* DNA concentration of 1.16 ng/g, while all other samples resulted in lower concentrations than 1 ng/g soil. A mixed soil sample was taken to allow for some standardization. However, depending on sampling, e.g. wood parts in the specific sampling spot might well result in elevated DNA amounts of the wood rotting fungus. However, the spots not inoculated are expected to not profit even from wood present.

4. Surviving at a radiologically contaminated site

To evaluate the conditions that pertained to *S. commune* surviving under the condition at the CEZ, the radiological contamination had to be determined. Dose rates at ground level ranged between approx. 2 and 6 µGy/h with significant variation. The 7 dose rate meters installed 1 m above ground revealed a dose between 18 and 22 µGy per day (Table 2).

While the ambient dose rate 1 m above the ground is rather constant over the expanse of the test field, an in-depth gamma-analysis of the drillcores indeed revealed a very heterogeneous distribution of radionuclides not only in the vertical but also in the horizontal dimensions (Fig. 4).

The high heterogeneity of radionuclide distribution as well as the presence of significant amounts of alpha/gamma-emitting ^{241}Am revealed a considerable fraction of the contamination to be of particulate origin (so called 'hot particles') that were heterogeneously deposited on the test field in the immediate aftermath of the Chernobyl accident and have largely been dissolved in the soil since then (Steinhauser, 2018; Walther and Denecke, 2013). Being a high-energy alpha-emitter, ^{241}Am is highly dose relevant for living organisms in close vicinity to the radionuclide.

4. Discussion

In this work, we were able to show that *S. commune* is not only able to survive and even thrive in the soil, but, unlike the other tested soil-living fungi, it spreads and shows very extensive growth. Since extraction of RNA is impossible under the field laboratory conditions, we used DNA-based qPCR to determine the remaining load of *S. commune* DNA in the field soil. Supporting our notion that not remaining DNA from the original inoculum was extracted, one part of the inoculated field was lacking *S. commune*. In contrast, a non-inoculated spot showed abundances that were similar to those present within the inoculated plot.

Although a fungus was chosen that is not common in soil, *S. commune* is still able to survive saprotrophically and therefore exist in organic soil layers. The lignicolous life-style includes laccase formation, which may induce changes even in mineral composition (Kirtzel et al., 2017), and hence bioavailability of radionuclides and other heavy metals (Günther et al., 2014). The reduced bioavailability, in turn, could explain survival even at high levels of metals or radionuclides present in soil (Krauße et al., 2019). Indeed, the white-rot basidiomycetes *S. commune* was present one year after inoculation. It had been reported earlier that white-rot fungi, such as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus*, and many more need an added carbon source to survive in soil (Morgan et al., 1993; Canet et al., 2001). As best carbon source, hemicellulose-containing substrates, like chopped wheat straw, were reported (Hultgren et al., 2010). *S. commune* seemed to outperform these fungi, as it survived in this experiment for several months without additional carbon source. In addition, experiments under laboratory conditions could show survival without added nutrients.

During the time it had grown, if only the higher DNA amounts in soil are counted as successful growth, from the *S. commune* rows of inoculation to the non-inoculated point M1, but not as far as the control row 1.

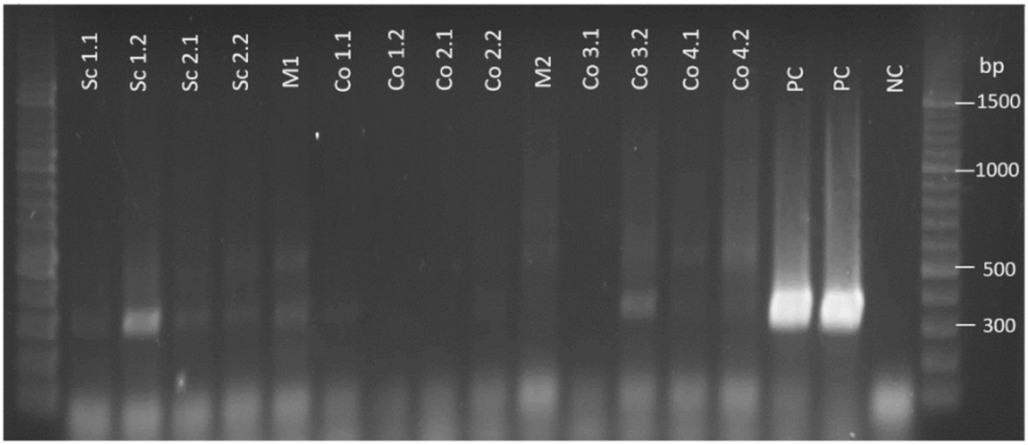

Fig. 3. Species specific PCR products from DNA extracted from soil samples. Sc1 was extracted 12 months and Sc2 6 months after inoculation. PC, positive control using *S. commune* 12-43 DNA; NC, negative control using *Aqua dest.*

Table 1
Quantitative PCR for absolute DNA quantification of *S. commune* at the test field site. From the measured Ct values, the DNA concentration was calculated and the results normalized per gram of soil to allow for a comparison of samples taken from the field site (compare Fig. 1).

Sample	Ct value	DNA concentration [ng/µl]	DNA concentration per g soil [ng/g]
Sc 1.1	27.19 ± 0.33	0.0000215	0.008594
Sc 1.2	24.67 ± 0.57	0.0432846	17.3138
Sc 2.1	25.14 ± 0.26	0.0104270	4.170806
Sc 2.2	25.15 ± 0.45	0.0100172	4.006883
M1	25.23 ± 0.19	0.0108536	4.341435
Co 1.1	26.25 ± 0.49	0.0003703	0.148102
Co 1.2	25.99 ± 0.35	0.0007932	0.317259
Co 2.1	25.78 ± 0.07	0.0015370	0.614799
Co 2.2	25.84 ± 0.19	0.0012833	0.513302
M2	26.37 ± 0.54	0.0002581	0.10324
Co 3.1	26.17 ± 0.07	0.0004709	0.188382
Co 3.2	25.97 ± 0.25	0.0008680	0.347211
Co 4.1	25.57 ± 0.42	0.0028902	1.156094
Co 4.2	26.11 ± 0.36	0.0005585	0.223381

Table 2
Dose rates 1 m above ground at the test field site within the CEZ.

Dosimeter no.	Mean dose after fading correction H* 10 [mGy]	Daily dose [µGy]	Rel. Uncertainty [%]
MP1	4.48	18.6	0.9
MP2	4.56	18.9	2.3
MP3	4.65	19.3	2.6
MP4	4.64	19.3	2.6
MP5	5.30	22.0	2.3
MP6	5.35	22.2	3.1
MP8	5.20	21.6	1.8

Time span of evaluation: September 24, 2017–June 4, 2018, no correction for transport dosage.

Therefore, it seems that the fungus could cross a distance of approximately 1 m within 6 months. Thus, a growth of approx. 8 mm per day can be calculated, which corresponds well with growth of the fungus on artificial media, which is 5–10 mm per day reported by Brunsch et al. (2015). It has been reported that the mycorrhizal fungus *Glomus mosseae* can cross a distance of 2.3 cm per week when grown in sandy soil (Camel et al., 1991). This would sum up to 60 cm in 6 months; thus, this strictly biotrophic fungus would not have reached the point M1 within the time passed between inoculation and sampling. A calculation for the largest organism on earth, the fungus *Armillaria*, conservatively judged 20 cm per year, which would translate into 0.5 mm per day. However, here the

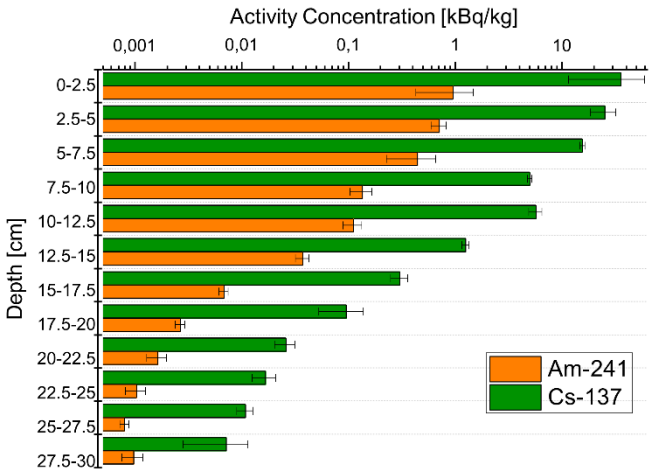

Fig. 4. Vertical distribution of gamma-emitting radionuclides. Average of two randomly sampled drillcores. Error bars indicate standard deviations within the two samples.

growth of rhizomorphs was tested, which on agar media also only developed with a speed of 0.2 mm per day (Smith et al., 1992). Since the formation of such intricate structures of the fungus seems unnecessary to obtain new nutrients by radial growth, and since in soil no dense mycelium but rather long, small runner hyphae are formed, our estimation seems feasible.

As can be seen from the DNA concentrations obtained, 4 ng *S. commune* genomic ITS region per g soil were found. In the sample Sc 1.2, a much higher value was detected, which might be due to variation in DNA extraction, but could also mean that inadvertently some parts of surviving inoculum had been sampled along with the intended soil outside the inoculation spots. The increased value of the sample Co 4.1 might, in contrast, also be due to rotting wood in the soil before preparation of the field site. In 30 years, bushes and trees had been growing which might attract *S. commune* in a natural way. This caveat cannot be completely ruled out for the other spots. However, the spacial distribution did clearly show that this sampling point Co 4.1 is an outlier.

Since the test site showed a high level of ionizing radiation with approximately 20 µGy daily dose, the fungus must have adapted to these environmental conditions as well. A laboratory strain, not adapted to high radiation, had been inoculated, which means that if adaptation strategies are employed by the fungus, these mechanisms must have prevented excessive killing right from the time of inoculation, because a longer time of adaptation would have meant that growth rates would be

even higher. Therefore, more physiological investigations are needed to find the molecular basis for the observed resilience against ionizing radiation.

Our study could show that even mycelium, and not like formerly mostly suggested mushrooms, can contribute to mycoremediation approaches. The uptake of heavy metals including radionuclides would lead to reduced bioavailability and hence reduced risk for feed and food production in impacted areas (e.g., Damodaran et al., 2014; Giovani et al., 2004). In addition to already developed agricultural counter-measures (Fesenko et al., 2007), future land-use can be possible in lost areas of increased radiation.

5. Conclusions

Here, we could show that even a saprotrophic fungus not usually found in soil can survive in soil at the CEZ test field site. In previous research, *S. commune* could be shown to take up heavy metals and radionuclides (Günther et al., 2014) and therefore was chosen for this experiment. Not only could the basidiomycete withstand the harsh conditions, it even was able to compete with other soil fungi under these conditions. The use of a wood-rotting fungus enabled us to exclude natural occurrence before inoculation that was prone to compromise results with any other, typical soil basidiomycete if we had used one of those. Indeed, *S. commune* was not found in spaces not inoculated with one exception, where either wood must have been sampled or in inoculation, a mistakenly lost part of mycelium might have been placed unintendedly. Using this set-up, we were able to show spread of the fungus in the native soil. Part of the area were planted to allow to address, whether plants might be positively or negatively affected. This was not the case. Thus, we could open routes for bioremediation approaches.

As this model experiment could show, basidiomycete fungi - and specifically typical soil fungi other than a wood-rotter - may be used in multiple ways for bioremediation. First, mycorrhizal fungi might allow for better plant performance under stress conditions. Second, the intracellular or extracellular uptake/adsorption of radionuclides well known for fungi will allow for radiation protection from radionuclide transport with wind or water, including distribution to lower soil horizons. And thirdly, using mushroom forming saprotrophs like the button and related agarics on contaminated land provides a means of reducing the levels of contaminants by consequent mushroom harvesting, as transport into above-ground biomass with fungi does not require the root-shoot transfer that limits radionuclide transport into above-ground tissue for plants.

CRediT authorship contribution statement

Lea Traxler: Conceptualization, investigation (DNA based experiments), validation (DNA based experiments), Writing - original draft. **Anne Wollenberg:** Investigation (large scale cultivation, agar and soil columns), Validation (agar and soil columns), Conceptualization, Writing - original draft. **Georg Steinhauser:** Supervision, conceptualization, Writing - original draft. **Ihor Chyzhevskyi:** Performing the field experiment and provide comprehensive assistance of collection the samples in the Chernobyl Exclusion Zone. **Sergiy Dubchak:** Resources, investigation (participation in field experiments at the trial site), project administration at CEZ. **Sina Großmann:** Formal analysis and investigation (column experiments – performance and analysis of inorganic parameters). **Alix Günther:** Supervision, investigation (soil columns). **Dharmendra Kumar Gupta:** Investigation (field experiments). **Karl-Heinz Iwannek:** Investigation (field experiments). **Serhii Kirieiev:** Provision of laboratory rooms and equipments as well as all permissions for joint research in the Chernobyl Exclusion Zone. **Falk Lehmann:** Investigation (large scale cultivation). **Wolfgang Schulz:** Investigation (radioanalytics), validation (radioanalytics), Writing - original draft. **Clemens Walther:** Conceptualization, supervision, acquisition of the financial support for the project leading to this publication. **Johannes Raff:** Project administration and conceptualization, supervision, acquisition of the financial support for the project leading to this publication. **Erika Kothe:** Writing - original draft, editing; supervision, subproject administration, acquisition of the financial support for the project leading to this publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Financial support of this work by the German Federal Ministry of Education and Research (contract number 02S9276D) is gratefully acknowledged. EK and LD were supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - SFB 1127/2 ChemBioSys - 239748522 and the Graduate School Jena School for Microbial Communication.

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4.2 Manuscript 2: Microbial community analysis in the radionuclide contaminated Chernobyl exclusion zone

Manuscript number: 2

Title of the manuscript: Microbial community analysis in the radionuclide contaminated Chernobyl exclusion zone

Authors: Lea Traxler (contribution 60%), Sebastian Pietschmann, and Erika Kothe

Bibliographic Reference: -

The candidate is:

x First author, □ Co-First author, □ Corresponding author, □ Co-author.

Status: In preparation

Author	Concept	Data analysis	Experiments	Writing of manuscript	Provision of material
Lea Traxler	x 40%	x 20%	x 100%	x 80%	
Sebastian Pietschmann		x 80%		x 20%	
Erika Kothe	x 60%				x 100%

4.2.1 Summary

Soil samples were taken from a test field in the vicinity of the exploded nuclear power plant in Chernobyl. To investigate bioremediation, the test field was inoculated with the two crops potatoes and winter rye, and the two basidiomycetes *Schizophyllum commune* and *Leucoagaricus naucinus*. DNA was isolated from the soil samples and community analysis for bacteria and fungi was carried out. This showed that the various inoculations of the test field

have no significant influence on the structure of the microbial community at phylum level. The bacterial community showed greater fluctuations in richness and diversity depending on the sampling location compared to the fungal community. Since the test field is very heterogeneously contaminated, this may be due to the lower metal and radionuclide tolerance of bacteria.

Microbial community analysis in the radionuclide contaminated Chernobyl exclusion zone

Running Title: Community analysis in the Chernobyl exclusion zone

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Abstract

The Chernobyl Exclusion Zone (CEZ) is a unique habitat which is highly contaminated and undisturbed for over 30 years. The contamination with radionuclides is very heterogeneous which makes future land use particularly difficult. As part of an investigation into bioremediation using crops and basidiomycetes, a test field was established in the immediate vicinity of the destroyed nuclear power plant. An analysis of the microbial community showed that the treatments in the test field had no significant influence on the structure of the communities. The richness and diversity of the bacterial community fluctuate more than that of the fungi. This suggests that fungal communities are less affected by the vastly different intensity of contamination at different sampling points. Consequently, remediation by fungi could be a suitable attempt.

Keywords

Pollution, heavy metal, radionuclide, Chernobyl, remediation, community analysis

Introduction

Soil contamination by heavy metals and radionuclides is a global problem. The majority of this pollution is of human origin from sources like agriculture, mining, and waste disposal (Nriagu and Pacyna 1988). A serious example of this is the nuclear reactor accident in Chernobyl, Ukraine that occurred in April 1986. Approximately 4 x 10^{16} Bq ^{137}Cs were released worldwide and more precisely distributed around the damaged reactor over 1.1 10^{5} Bq m^{-2} ^{90}Sr (United-Nations 2000). In addition, highly radioactive particles (so-called hot particles) were released, which still ensure a very inhomogeneous distribution of radiation. This made the area unusable for humans and a restricted zone with a radius of 30 km was created, the so-called Chernobyl exclusion zone (CEZ).

One approach to make contaminated land usable again for agriculture is remediation through fungi (mycoremediation). To research this approach more closely, a test field was set up 5 km southeast of the damaged nuclear reactor (see Figure 1). The two crops, winter rye (*Secale cereale*) and potato (*Solanum tuberosum*), were selected, which were also grown in this area before the accident. The two basidiomycetes *Schizophyllum commune* and *Leucoagaricus naucinus* were selected as fungal inocula. Both were chosen because they were heavy metal and radionuclide tolerant in previous experiments (Günther et al. 2014; Wollenberg et al. 2017). The natural habitat of *L. naucinus* is the soil of grasslands, which applies to the CEZ. *S. commune* is a white-rot fungus and therefore grows naturally on rotting wood and not in the ground. Nevertheless, it has been proven that it can survive and grow in the soil of the CEZ for at least several months (Traxler et al. 2021).

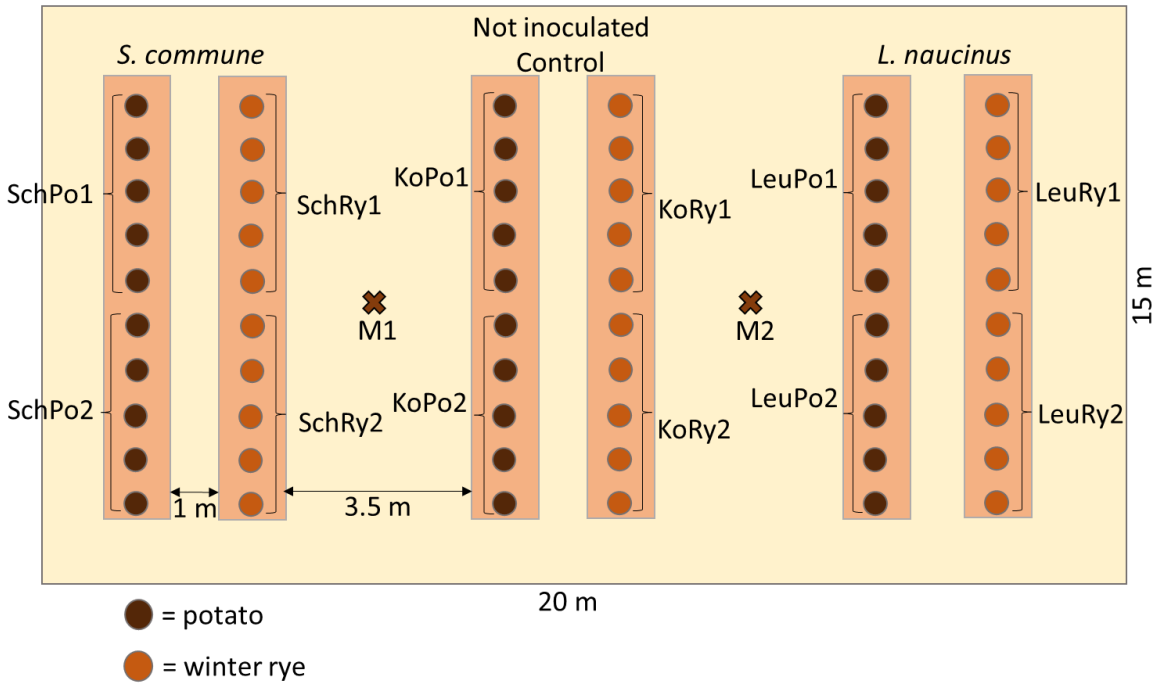


Figure 1: Inoculation scheme of the test field in CEZ. Sample designation: Sch= *Schizophyllum commune*; Ko= not inoculated with fungi, control; Leu= *Leucoagaricus naucinus*; Po= Potato; Ry= Winter rye; M= Middle, neither with fungi nor with plants inoculated.

Fungi can change the bioavailability of metals and thus change their mobility. This happens, for example, through the secretion of oxalic acid or a change in the redox potential (Gadd 2007; Gadd et al. 2014). It has also been shown that fungi can change the activity of antioxidant enzymes in plants (Vuković et al. 2020). Another advantage of mycoremediation is that fungi can contribute to the translocation of heavy metals and hot particles through their extensive hyphal network (Colpaert et al. 2011). This makes soil contamination more homogeneous and thus protects plants from excessive contamination. This aspect is particularly important in areas as inhomogeneously contaminated by reactor particles as the CEZ (Schulz 2020). It was recently demonstrated that *S. commune* is able to transport Cs and Sr (Manuscript 3).

The location of the CEZ is particularly interesting as it was abandoned by humans over 30 years ago and thus offers a unique, undisturbed soil habitat (Beresford et al. 2016). Studies of microbial communities in radionuclide polluted soils in connection with remediation of multi-metal contaminated sites in the real world have rarely been conducted. We, therefore,

investigated the microbial community of fungi and bacteria at the test field. In this way, it can be discovered whether and how the community has changed through the inoculation with fungi and plants, or whether the adverse circumstances in the CEZ, including long-time contamination, are the limiting factor for the soil community and keep it stable. Thus, conclusions can be drawn about the microbial connections of bioremediation.

Material and Methods

Test field and soil sampling

The test field was set up in 2017 5 km southeast of the destroyed reactor block 4 and is located near a former village (51°20'54'' N; 30°07'41'' O). Due to the proximity to the reactor, it can be assumed that there are reactor particles in the field, which leads to very heterogeneous radionuclide contamination. The soil of the test field has not been moved or flooded since 1986. A detailed soil characterization, soil profile, and a sequential extraction (Zeien and Brümmer 1989) were done by Schulz 2020. Gamma measurements showed an average contamination with A_{Cs137} = 1216 [700] Bq / m^2 (33 Ci / km^2) and A_{Am241} = 36 [16] Bq / m^2 (1.0 Ci / km^2) (Vuković et al. 2020). The soil is a sandy Podzol with a pH of 6, which was probably used for agriculture until 1986.

The test field is 15 x 20 m in size and protected from animals by a wooden fence. It consists of two double rows that were planted with potato (*Solanum tuberosum*) and winter rye (*Secale cereale*) (see Figure 1). The plants were surrounded by a weed fleece that is supposed to hold back natural vegetation. The first two rows were additionally inoculated with a liquid culture of *Schizophyllum commune* 12-43 and the last two rows with *Leucoagaricus naucinus*. Both cultures were diluted with tap water (approx. 15 g mycelium/ liter) and poured directly onto each plant. Rye was always sawn in autumn and potatoes in spring. The inoculation of the fungi took place a few cm below the surface and at the same time as the planting of the respective plant.

The inoculation of the fungi together with potatoes was 12 months and together with rye 6 months before the soil was sampled. For sampling, the soil was taken from a depth of 2 cm on

May 23, 2018. Approximately 0.5 g of soil was taken from each hole and the soil of five holes was mixed in a Falcon tube (Fisher Scientific, Schwerte, Germany). Thus, two mixed soil samples were taken per row, one each for the upper and lower part of the row. In addition, two samples were taken between the planted rows (see Figure 1). As a result, a total of 14 soil samples were used for DNA extraction and named as described in Figure 1.

DNA extraction

For DNA isolation, the DNeasy PowerSoil Isolation Kit from Qiagen (Hilden, Germany) was used as per manufacturer instructions. Due to the laboratory conditions in Chernobyl Eco-center, the centrifugation steps were performed with 4700 x g for 1 min, and the incubation at 4°C was prolonged to 7 min. The extraction was interrupted before the elution step, and the DNA was transported to Germany dry on the membrane. The elution occurred after approximately 55 hours of cooled transport, with 100 µl eluting buffer (10 mM Tris-HCl pH 8.5) incubated for 10 minutes on the membrane and centrifugation at 16 060 x g for 1 min.

ITS and 16S rRNA gene library sequencing

From these 14 DNA samples, amplicons were generated using 515F-806bR (Apprill et al. 2015) for the construction of the 16S rRNA gene libraries and ITS1F-ITS2 (White et al. 1990) for the construction of the ITS gene libraries. Before the normalization and pooling, amplicons were quality checked with the Qiaxcel capillary electrophoresis system. Those libraries were sequenced on the Illumina MiSeq Platform using MiSeq reagent V3 with 300 bp paired-end reads. The library preparation, as well as the sequencing, were carried out by the StarSeq GmbH (Mainz, Germany).

Sequence analyses

Untrimmed but demultiplexed sequences were delivered from the StarSeq GmbH. Denoising, trimming, and generation of amplicon sequence variant tables (ASV) were carried out using DADA2 (Callahan et al. 2016) in QIIME2 (Bolyen et al. 2019). For taxonomic classification of

the 16S rRNA gene libraries, the QIIME2 feature-classifier (Bokulich et al. 2018) was used with a 515F-806bR trained, 99% similarity clustered version of the SILVA release 132 databases (Quast et al. 2012; Yilmaz et al. 2014). For the taxonomic classification of the ITS libraries, a QIIME2 compatible version of the UNITE v8 database was used. Alpha diversity indices such as Chao1, Shannon, Simpson were calculated using the microbiome package (Lahti et al. 2018) in R (Team 2021). For beta diversity analyses, the 16S sequence data were rarefied to 27500 sequences and the ITS sequences to a number of 65180. Principle component analyses were performed with the QIIME2 diversity plugin and visualized using the QIIME2 emperor plugin (Vázquez-Baeza et al. 2017; Vázquez-Baeza et al. 2013).

Results

Structure of fungal and bacterial communities

The alpha rarefaction shows that the sequencing was done to a sufficient depth (Figure S1). Since the curves for both the sequencing of the fungal DNA (ITS) and those of the bacterial DNA (16S) were asymptotic, it can be assumed that only a few species have not been considered for the community analysis.

We measured the richness of the microbial communities. Here three indices were used to describe the richness. Observed species, which is the count of OTUs per sample, Chao1, which additionally estimates the abundance of species by correcting the variance and abundance-based coverage estimator (ACE), which uses thresholds for abundance and rare taxa and thus also estimates diversity. Two separate groups can be seen for the bacterial communities - one with a high degree of richness (KoRy1, KoRy2, LeuRy2, M2, SchPo2, and SchRy1) and one with a low level (Figure 2A). However, the two groups seem to be distributed independently of both the inoculation with plants (potato and rye) and that with fungi (*Schizophyllum commune* and *Leucoagaricus naucinus*). It is also noticeable that the two replicas of a respective treatment are often not very similar. No separate groups can be recognized in the fungal community (Figure 2B). Sample M2 is noticeable for its particularly low richness, with SchPo1 and SchRy2 notable for their particularly high richness. There does

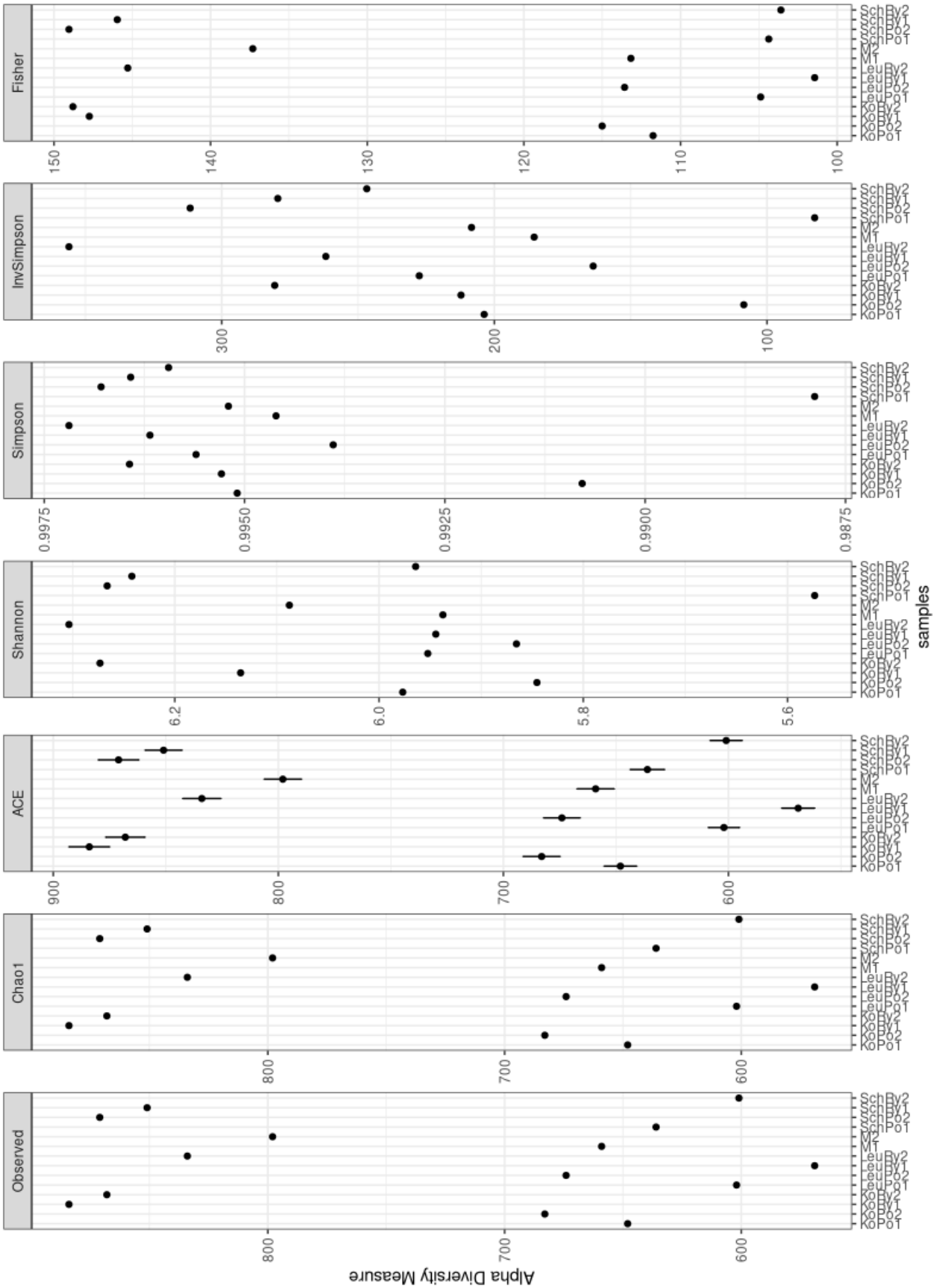
not appear to be a connection between the richness of the fungal and bacterial communities at the different sampling sites.

The diversity of the community within each sample is shown by the Shannon index. It includes the richness as well the equitability. The sample SchPo1 stands out with especially low diversity in the bacterial community (Figure 2A). In addition, this sample, together with KoPo2, has a particularly low value in the Simpson index. This index describes the dominance of an individual species within the community. High diversity and complexity of the bacterial community could be demonstrated especially in sample M1, which was neither inoculated with fungi nor with plants.

Within the fungal community, the two samples KoPo1 and M2 stand out because of their particularly low diversity and, in addition, M1 because of their low dominance (Figure 2B). M2 also shows a low value in the fisher diversity. This describes the relationship between the species abundance and the number of individuals per species.

Overall, all samples show higher values for the Fishers, Simpson, and Shannon index and thus diversity in their bacterial community compared to that of the fungi. Additionally, the Simpson index of the bacterial community shows a greater probability for all samples and thus a higher dominance of a few highly abundant taxa compared to the fungal community.

A


Fisher
InvSimpson
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Chao1
Observed
Alpha Diversity Measure
samples
SchBv2
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SchPo2
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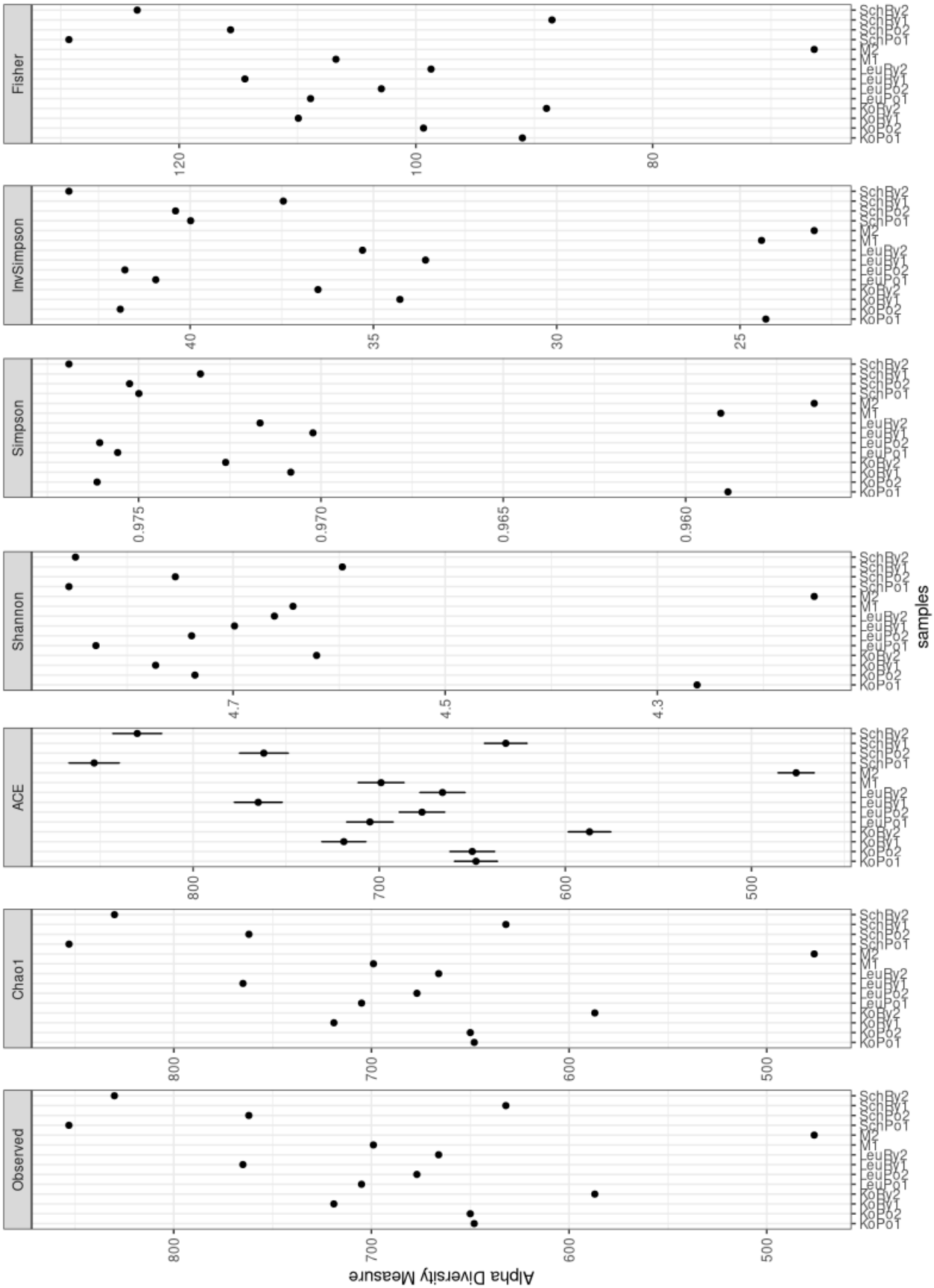
LeuBv1
LeuPo2
LeuPo1
KoBv2
KoBv1
KoPo2
KoPo1

B


Observed
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Fisher
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samples
SchBv2
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M2
M1
LeuBv2
LeuBv1
LeuPo2
LeuPo1
KoBv2
KoBv1
KoPo2
KoPo1

Figure 2: Alpha diversity indices for bacterial (A) and fungal (B) sequences. Sample designation: Sch= *Schizophyllum commune*; Ko= not inoculated with fungi, control; Leu= *Leucoagaricus naucinus*; Po= Potato; Ry= Winter rye; M= Middle, niether with fungi nor with plants inoculated.

The weighted UniFrac method was used to calculate beta diversity, which considers both the abundance of species and their phylogenetic distance. Here, too, there is no clear influence of the planting or inoculation with fungi on the composition of the community for both fungi and bacteria (Figure 3). It is noticeable for both bacteria and fungi that the community of sample M2 differs significantly from that of the other samples. One of the SchRy samples also differs clearly in its fungal community compared to all other samples (Figure 3B).

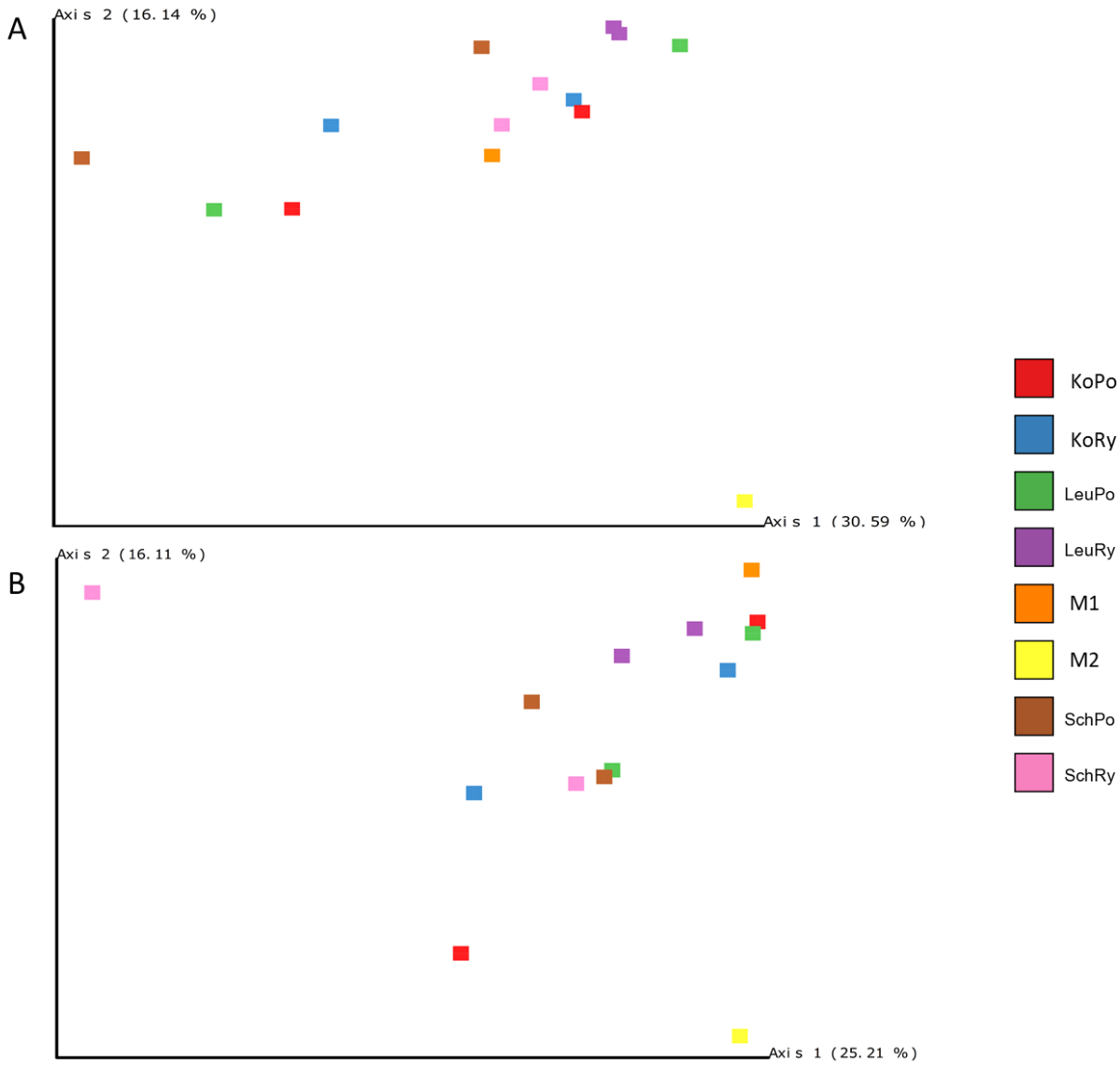

Figure 3: Beta-diversity analysis using principal component analysis based on weighted UniFrac distances. (A) Bacterial and (B) fungal sequences. Sample designation: Sch= *Schizophyllum commune*; Ko= not inoculated with fungi control; Leu= *Leucoagaricus naucinus*; Po= Potato; Ry= Winter rye; M= Middle, neither with fungi nor with plants inoculated.

Taxonomic analysis

The Taxa bar plot for the bacterial community shows all phyla which are represented more frequently than 1 % in the soil samples. The two most common phyla are the Acidobacteria with 15-28 % and the Proteobacteria with 17-28 % frequency (Figure 4). A slight tendency towards a higher prevalence of Firmicutes can be seen in the samples taken from near potato plants. However, overall, no clear correlation can be established between a changed composition of the bacterial community with the plants or the fungi added.

The two most common phyla in the fungal community are Ascomycota with 33-47 % and Mortierellomycota with 18-37 % prevalence (Figure 5). Basidiomycota, which also includes the two inoculated fungi, are in a range of 6- 20 % represented. Here, too, no shift can be identified in the community composition depending on the treatments. Sample M2, which showed a clear difference in beta diversity to all other samples, does not show any abnormalities in the taxa bar plots.

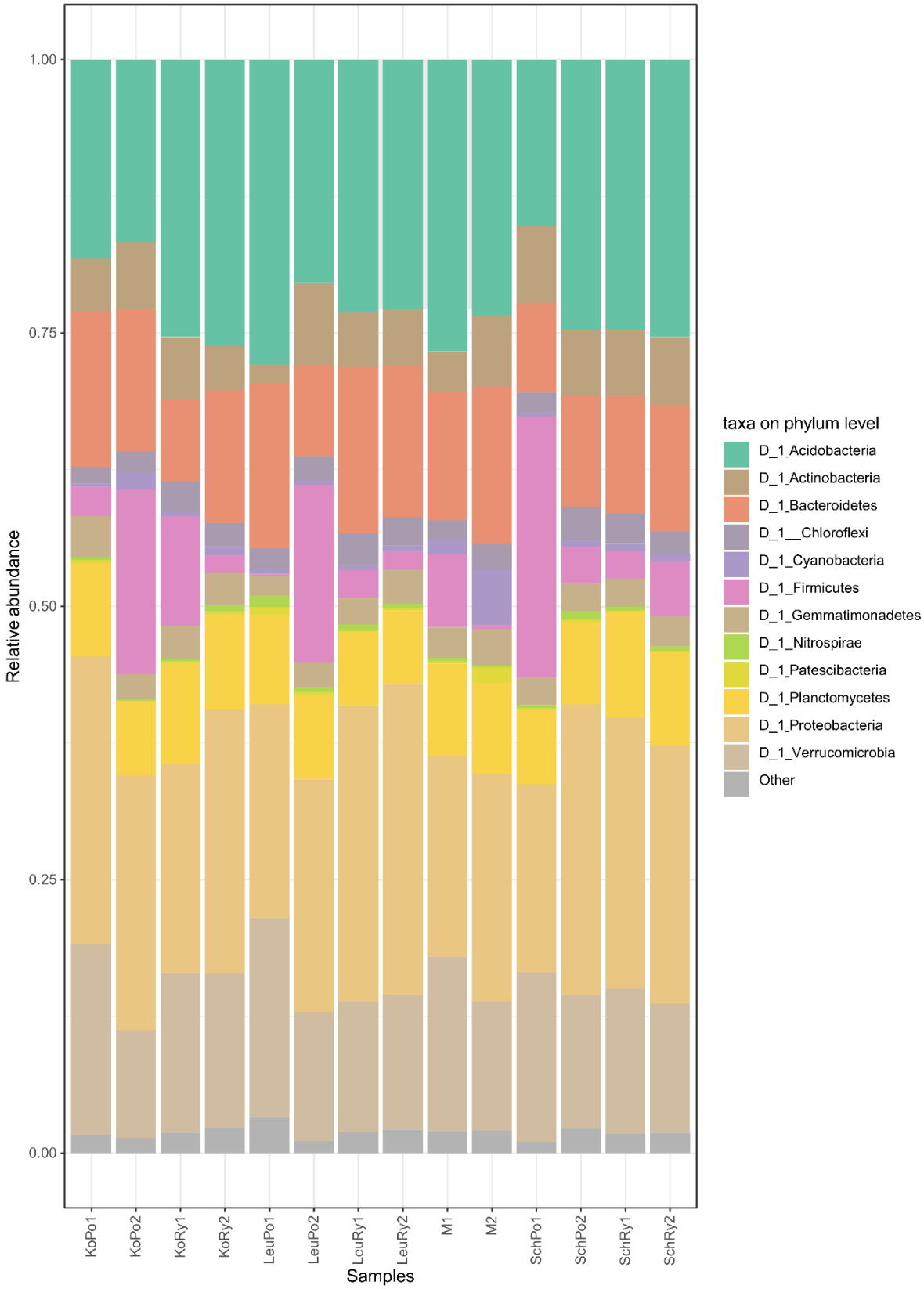

Figure 4: Taxa bar plot of the bacterial community at the level of phyla. Shown are just taxa with an abundance over 1 %. Sample designation: Sch= *Schizophyllum commune*; Ko= not

inoculated with fungi control; Leu= *Leucoagaricus naucinus*; Po= Potato; Ry= Winter rye; M= Middle, neither with fungi nor with plants inoculated.

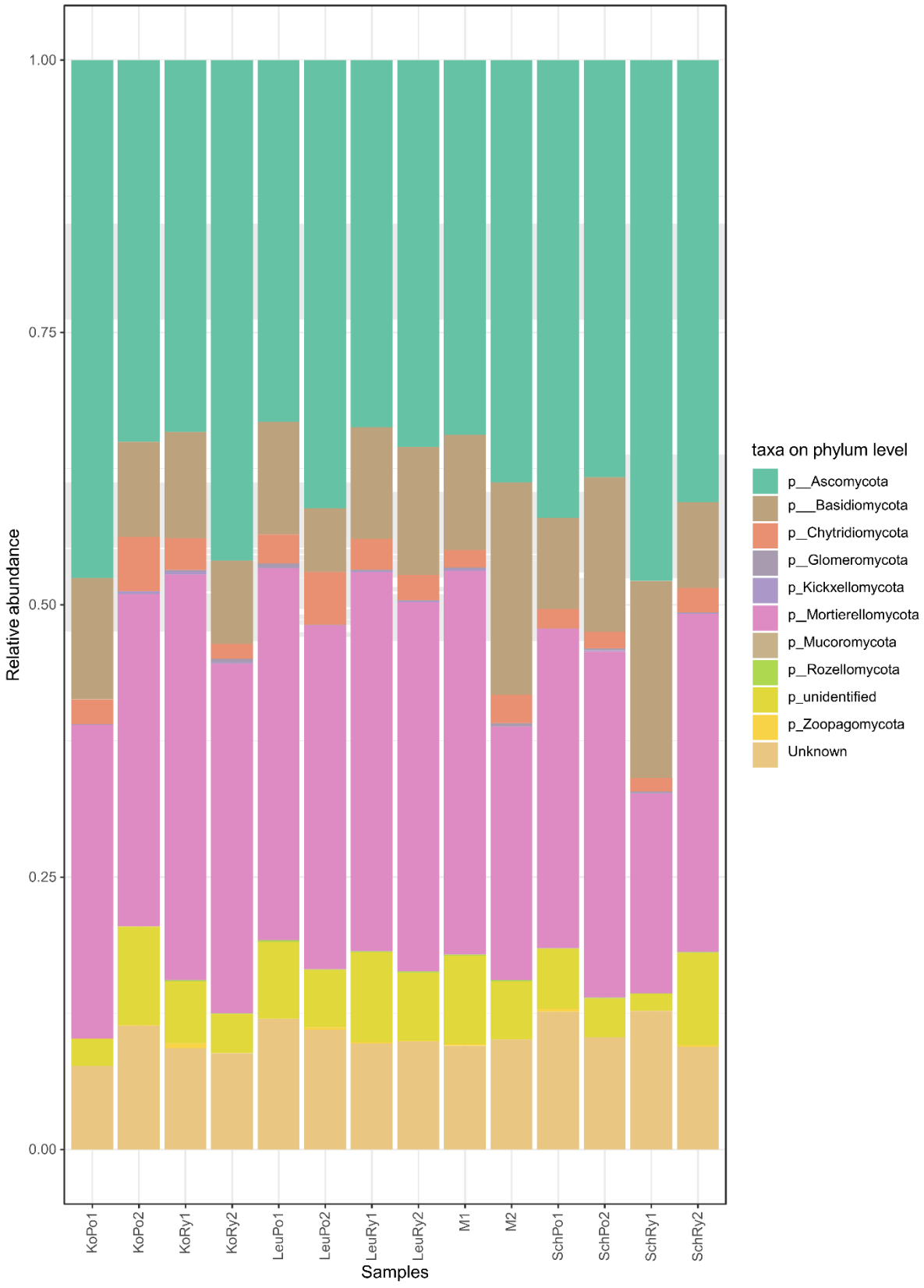

Figure 5: Taxa bar plot of the fungal community at the level of phyla. Sample designation: Sch= *Schizophyllum commune*; Ko= not inoculated with fungi control; Leu= *Leucoagaricus naucinus*; Po= Potato; Ry= Winter rye; M= Middle, neither with fungi nor with plants inoculated.

Discussion

Our investigation of the microbial community on a test field inoculated with fungi and crop plants in the CEZ revealed no change in the composition of the microbial community at phylum level depending on the treatments.

The composition of the bacterial and fungal community matches former reported microbial communities in contaminated soil (Shah et al. 2014; Qin et al. 2020; Shi et al. 2020). It has been reported that Firmicutes in particular are tolerant against metals (Desai et al. 2009; Zhao et al. 2019). The slightly increased proportion of Firmicutes in the samples nearby potato plants thus rather indicates metal mobilization by the plant. Since a clear increase in the proportion of Firmicutes could only be observed in three samples, this may also be due to a coincidentally higher level of contamination at these points.

The bacterial communities showed higher variability in their diversity depending on the location of the sampling compared to the fungi. One possible explanation for this is that bacteria are more sensitive to metal contamination than fungi (Singh et al. 2019). Since the region around the damaged reactor is very heterogeneously polluted by so-called hot particles, there are also high fluctuations in contamination within the sampling points (Figure S2; Schulz 2020). This also explains why the replicas of the various treatments are often not very similar in terms of their community composition (Figure 3). Overall, it is assumed that with the increasing pollution, the richness and diversity of microbial communities decreases (Agnello et al. 2018; Ying et al. 2008). This could be an explanation for the particularly low diversity and the large difference in the community structure of sample M2. Unfortunately, no radioactivity measurements are available for the areas between the inoculated rows, so it cannot be said with certainty that there is a higher level of contamination at this point. The separation into a group with high and one with lower richness within the bacterial community can also be

explained by an inhomogeneous distribution of contamination. The collection points that are low in richness are likely to be in higher contaminated areas. A clear separation cannot be seen for fungi, as this community seems to be more stable under metal and radionuclide stress. In addition, within the fungal community, lower alpha diversity was only found in samples that were not inoculated with fungi (Figure 2B). This is probably the case because fungi usually have a higher tolerance to metals than bacteria (Hiroki 1992). In addition, fungi can tolerate a wide range of different contaminants, including heavy metals, radionuclides, and organic contaminants (Gadd 2007; Günther et al. 2014; Hultgren et al. 2009; Ellouze and Sayadi 2016). It was also reported that inoculation with the basidiomycete *Tricholoma vaccinum* in soil led to an increase in OTUs under saprotroph conditions (Wagner et al. 2019). This effect also explains a higher diversity in samples that were inoculated with fungi.

In summary, it can be said that the inoculation of the two basidiomycetes *S. commune* and *L. naucinus* together with the two crops potato and winter rye did not change the microbial community on the phylum level in the time of observation. From this, it can be concluded that another factor is limiting the community in a way that a slight disruption does not lead to a significant change in it. Possible factors are besides the contamination, the sandy soil, which tends to dry out quickly, and the strong temperature fluctuations from -30 to +30° C in the CEZ (Schulz et al. 2019). The high stability of the fungal community under adverse conditions together with the knowledge that *S. commune* can survive in the soil at the test field for at least one year (Traxler et al. 2021) confirm the assumption that fungi, in particular basidiomycetes, are potentially suitable for the remediation of contaminated soils.

Acknowledgments

The financing of the project by the BMBF through the cooperative projects BioVeSTra and USER is gratefully acknowledged. We would also like to thank Alexander Mauz, Petra Mitscherlich, and Katrin Krause for the preparatory work on the growth of *S. commune* in soil and the employees of the Eco-Center of the CEZ for the maintenance of the test field.

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Supplemental Material

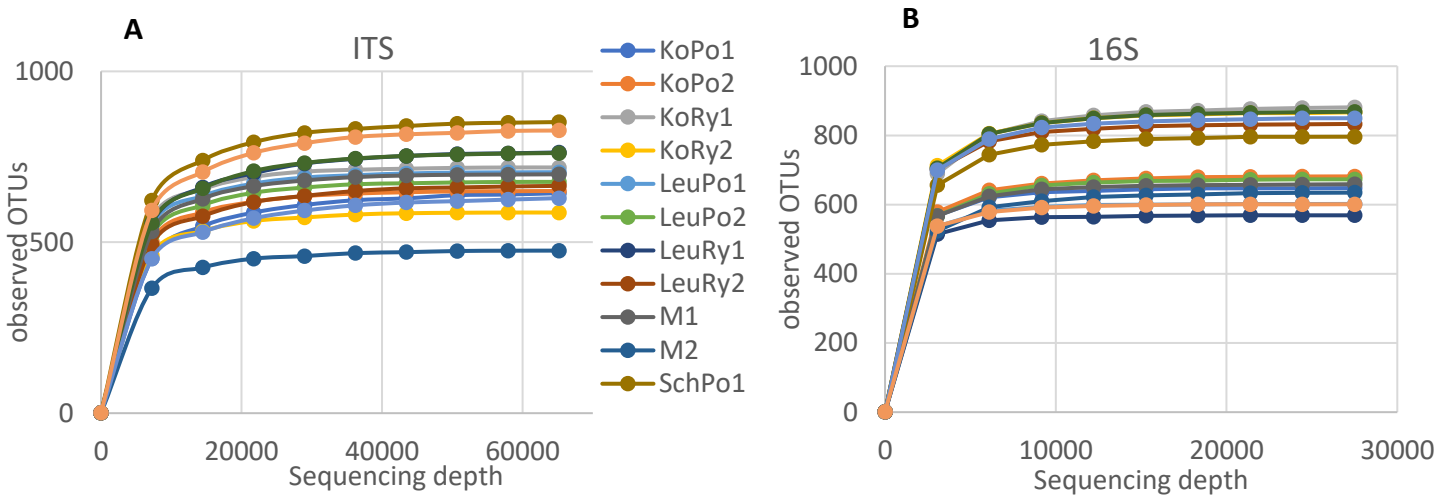
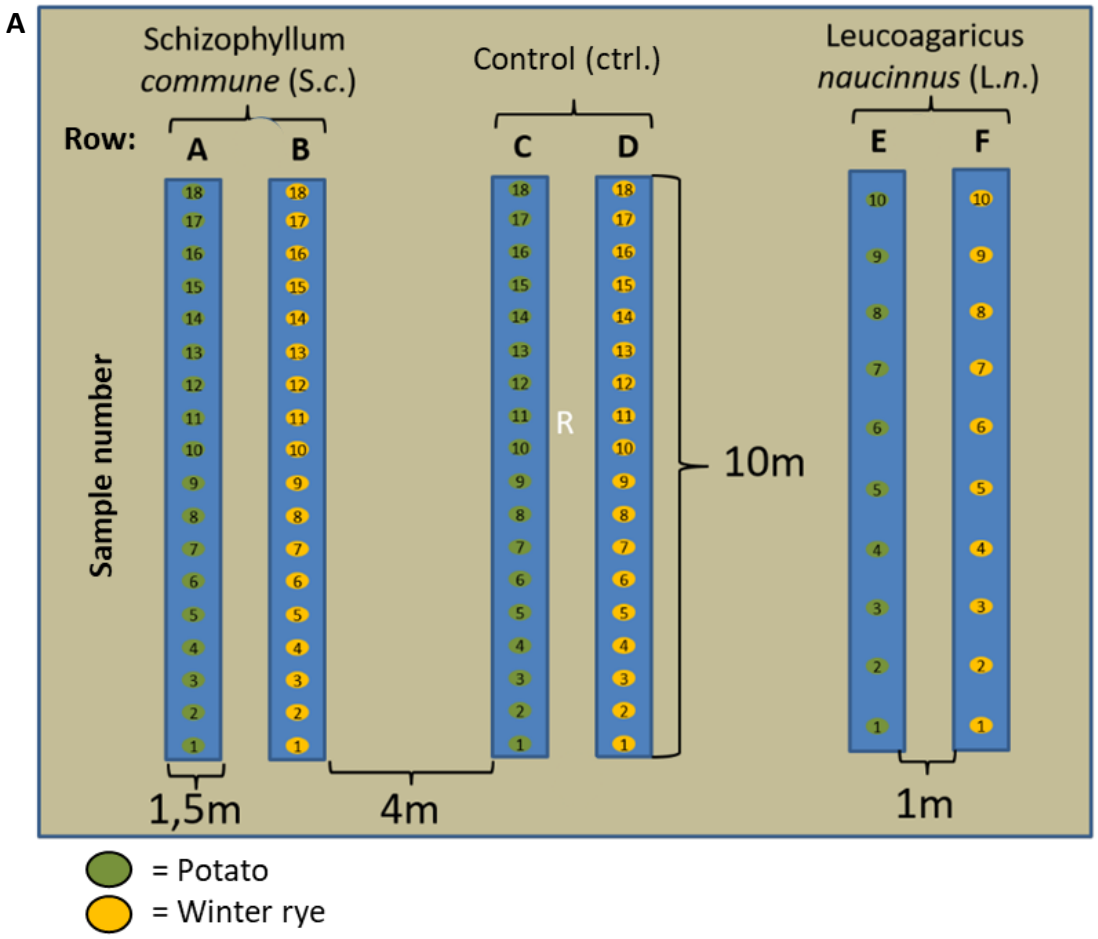


Figure S1: Alpha rarefaction of fungal (A) and bacterial (B) sequences. Sample designation: Sch= *Schizophyllum commune*; Ko= not inoculated with fungi control; Leu= *Leucoagaricus naucinus*; Po= Potato; Ry= Winter rye; M= Middle, neither with fungi nor with plants inoculated.



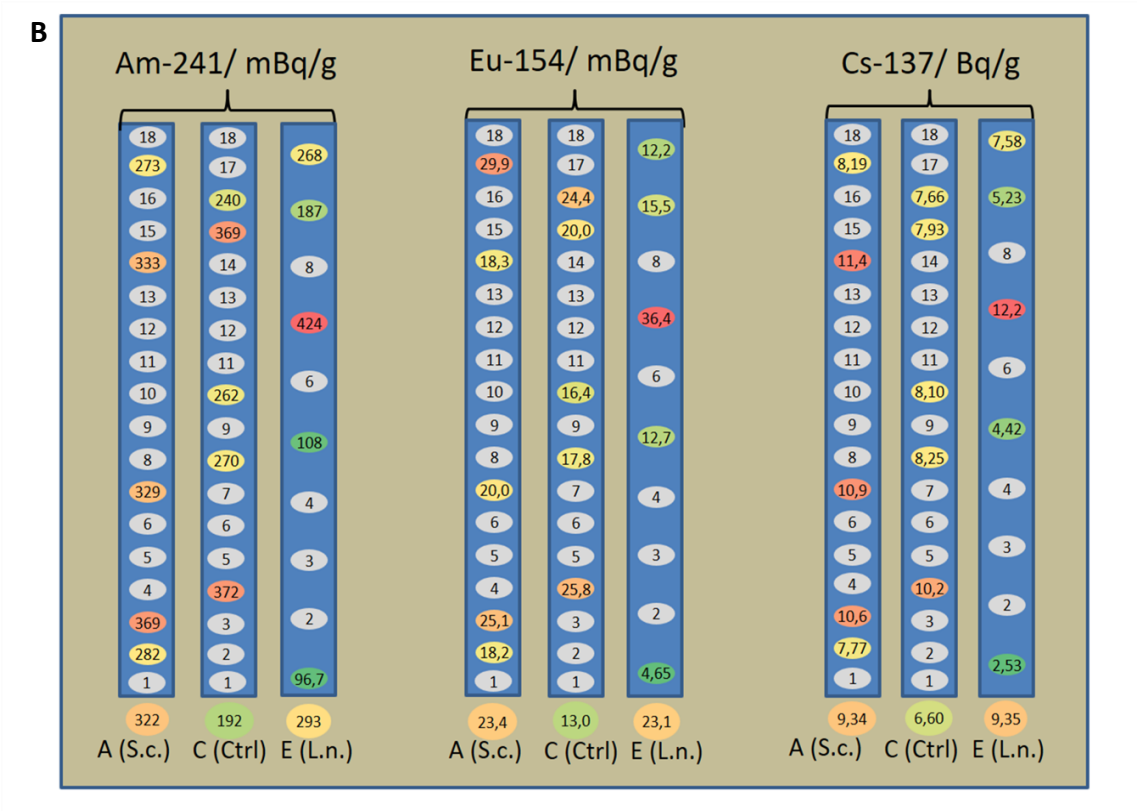


Figure S2: Distribution of radionuclides at the test field 2017. A= Sampling scheme. B= Measured radioactivity (Schulz, 2020).

4.3 Manuscript 3: Metal transport in hyphae of the basidiomycete *Schizophyllum commune*

Manuscript number: 3

Title of the manuscript: Metal transport in the hyphae of the basidiomycete *Schizophyllum commune*

Authors: Lea Traxler (contribution 60 %), Jenny Shrestha, Martin Richter, Katrin Krause, Thorsten Schäfer, Erika Kothe

Bibliographic Reference: -

The candidate is:

x First author, □ Co-First author, □ Corresponding author, □ Co-author.

Status: In revision in *Environmental Microbiology*

Author	Concept	Data analysis	Experiments	Writing of manuscript	Provision of material
Lea Traxler	x 30%	x 80%	x 50%	x 60%	
Jenny Shrestha		x 20%	x 20%		
Martin Richter			x 30%		
Katrin Krause	x 20%			x 10%	
Thorsten Schäfer					x 20%
Erika Kothe	x 50%			x 30%	x 80%

4.3.1 Summary

Within this work, a strontium-adapted strain from the wild-type *S. commune* 12-43 could be produced (called *S. commune* 12-43 Sr), which also showed increased resistance to Cs and Zn. Using the adapted strain, it could be shown that the improved metal tolerance mechanism must be an avoidance strategy since clearly less Sr and Cs were transported along the hyphae. Overall it could be shown that *S. commune* is able to transport metals over hundreds of cells. No increased tolerance could be established for Cd in the adapted strain and its localization in vacuoles was the same for both strains. Transcriptome analysis showed that *S. commune* 12-43 Sr has lower cellular stress in the presence of metals. Since an MFS transporter that is otherwise down-regulated under metal stress is not down-regulated in the adapted strain, the likely avoidance strategy seems to be increased efflux.

Metal transport in hyphae of the basidiomycete *Schizophyllum commune*

Running Title: Transport in fungal hyphae

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Originality statement:

We could show that the metals cesium, strontium, cadmium, and zinc, are tolerated at millimolar concentrations by the basidiomycete fungus *Schizophyllum commune* 12-43 and that transport occurs through hyphae. To address genes related to the tolerance, adapted strains were raised. Transcriptome analysis identified transporters and intracellular sequestration mechanisms. Specifically, a glutathione S-transferase is involved in the unexpectedly high natural tolerance, and metal sequestration in vacuoles could be shown using a fluorescence dye specific for cadmium. With this intracellular fractionation, transport through hyphal compartments is possible and it could be shown that the metals indeed are reaching cells some tens to hundreds of cellular compartments away from a metal source. Thus, this model fungus can be used to study metal tolerance and metal transport in basidiomycete fungi.

Summary

The basidiomycete *Schizophyllum commune* that is able to grow in soil was investigated for its metal tolerance mechanisms. Specifically, its ability to transport metals along its hyphae and effects of temperature and pH on tolerance of Cs, Sr, Cd, and Zn were tested. At concentrations allowing for half-maximal growth, adapted strains were raised. The adaptation did not yield changes in cell or colony morphology. The strontium-adapted strain, *S. commune* 12-43 Sr, showed transport of Sr through aerial hyphae over tens to hundreds of cellular compartments. Intracellular metal localization in vacuoles was shown for cadmium. Gene expression profiles under metal stress growing on soil versus artificial medium showed a higher impact of the soil structured surface than with different metal concentrations. A comparison of wild-type and adapted strains could confirm lower cellular stress levels leading to lack of glutathione S-transferase up-regulation in the adapted strain. Thus, we could show metal transport as well as specific mechanisms in metal avoidance using *S. commune* as a model.

Keywords: Metal tolerance, basidiomycete, *Schizophyllum commune*, metal transport, intracellular sequestration.

Introduction

Fungi can spread in soil for very large distances – the largest known organism is a wood decay basidiomycete (compare Sipos et al., 2018). At the same time, long hyphae comprised of hundreds of cellular compartments – in basidiomycetes mostly with a length of 100 to 500 µm (Jung et al., 2018; Greening and Moore, 2018) - can transport nutrients, water and ions over large distances (Krause et al. 2020). This is best studied for basidiomycetes living in ectomycorrhizal mutual symbiosis with host trees (Victor et al., 2017). Thus, they provide important ecosystem functions that influence biogeochemical cycles (Schlunk et al., 2015; Tome et al., 2015), and support and control microbial communities in soil (Wagner et al., 2019). The transport of heavy metals within hyphae could specifically be useful for

bioremediation approaches. Indeed, metal uptake into above-surface fruiting bodies has been well documented, e.g. with radiocesium after the Chernobyl reactor accident in 1986 that has released an estimated amount of 4 x 10^{16} Bq ^{137}Cs (United-Nations, 2000; Borio et al., 1991). The distribution of radiostrontium was more limited in distribution, but more than 1.1 10^{5} Bq m^{-2} ^{90}Sr were detected within the 30 km radius around the reactor block (United-Nations, 2000). In addition, and depending on the specific soil, metals including zinc, or even the more toxic cadmium are present (suppl. Tab. S1). Former studies revealed that fungi are present and show specific protection mechanisms (see Fuller et al., 2020; Vasileiou et al., 2020).

Like many other wood decay basidiomycetes, *Schizophyllum commune* is saprotrophic and can live not only in timber, but has previously been found to survive and grow over distances of meters in the soil of the Chernobyl Exclusion Zone (Traxler et al., 2021). It is easily cultivated in haploid as well as mated, fruiting body-forming dikaryotic stages, and can tolerate sufficient metal concentrations to provide a good model system (Kirtzel et al., 2019). The amount of data on cellular and molecular biology, bioweathering activities, gene expression analyses, and proteome studies makes this fungus an excellent target to study metal uptake and transport along the hyphal compartments (Erdmann et al., 2012; Knabe et al., 2013; Freihorst et al., 2018; Brunsch et al., 2015; Ohm et al., 2010). Here, we investigated the tolerance of *S. commune* 12-43 against Cs, Sr, Cd, and Zn, and their transport along the hyphal network. To identify the underlying mechanism(s), we created different metal adapted *S. commune* strains and compared metal localization, transport, and expression of selected genes with the wild-type.

Metal adaptation might be achieved by mutation (genetic mechanism of adaptation) or by changed expression pattern leading to altered gene expression profiles. This shift in metabolic flow and enzymatic activities (physiological adaptation) would be visible in transcriptome analyses. Hence, in a comparison of a metal adapted strain to its progenitor, differential gene regulation was expected. We further hypothesized that metal transport as

well as intracellular metal sequestration might explain the higher metal tolerance in an adapted strain.

Results & Discussion

Metal inhibition and cellular effects

In nature, abiotic factors are constantly changing. At the same time, metal toxicity is different for different ions, at different pH and temperature conditions, and with different effects on cell morphology and function. Therefore, we tested three different pH values and three different temperatures for each of five different concentrations of CsCl and $SrCl_2$. To compare the metal toxicity to other environmental ions, $CdCl_2$ and $ZnCl_2$ were studied as well, with Cd being toxic already at low concentrations, while Zn is essential and only overly high concentrations exert a detrimental effect. Both ions co-occur with the radionuclides at the site where the growth of *S. commune* could be shown in a field experiment at the Chernobyl Exclusion Zone (Traxler et al., 2021). While inhibition increased with higher metal concentrations, growth was still visible even at the highest concentrations used (suppl. Fig. S1). We defined half-maximum growth (effective concentration, EC_{50}) with 50 mM CsCl, 100 mM $SrCl_2$, 0.2 mM $CdCl_2$ or 10 mM $ZnCl_2$. Compared to the metal concentrations in the bioavailable fractions at Chernobyl that yielded approximately 0.2 µM Cs, 1 mM Sr, below 0.1 µM Cd and 0.13 mM Zn available (suppl. Tab. S1), the tolerated concentrations of the non-adapted fungus thus are between 100-fold and 2000-fold higher than required in the Chernobyl exclusion zone.

Environmental influences were tested for temperature and pH. For the Chernobyl Exclusion Zone, summer temperature with medians between 15 and 25°C is given, and the soil samples revealed a pH of 5.2. At the optimum growth temperature of 28°C under laboratory conditions, CsCl, $CdCl_2$, and $ZnCl_2$ were less toxic than at non-optimal conditions (Fig. 1). In contrast, $SrCl_2$ was more toxic at higher temperatures when combined with a low pH. This feature was unexpected and initiated a more detailed investigation of the effects of $SrCl_2$. The pH value did not show a significant impact on growth inhibition by $CdCl_2$, whereas it

affected the toxicity of the other metals tested. While $ZnCl_2$ inhibited the growth to a lesser extent with rising pH values, CsCl and $SrCl_2$ showed higher growth inhibition with rising pH values (see Fig. 1).

The effects of Sr were analyzed in more detail. An unexpected finding for the non-essential metal Sr was that $SrCl_2$ even had a growth-promoting effect at cold temperatures (suppl. Fig. S2). In addition to increased growth diameter, *S. commune* grew with more aerial mycelium at concentrations of 10 mM and 25 mM $SrCl_2$ indicating an even larger effect of growth promotion, if biomass and not the diameter of the mycelium was considered. While no essential function of Sr is known, this alkaline earth metal can substitute for Ca in signaling, even in humans with therapeutic applications (Saidak et al., 2012; Zhang et al., 2013). For *S. commune*, Ca signaling has been linked to hyphal growth as well as intracellular phosphate storage, which in turn is necessary for fast growth (Murry et al., 2018; Murry et al., 2021). Thus, extended hyphal growth can be easily explained by Sr replacing Ca for induction of fast cellular elongation and growth.

Metal adaption and cross-tolerance

To test for metal tolerance mechanisms, *S. commune* 12-43 was adapted to metals by ten serial inoculations on metal-containing plates. To investigate metal tolerance mechanisms, adapted strains were compared to the non-adapted parent, *S. commune* 12-43 in further experiments, to determine changes in their behavior towards heavy metal stress. For these experiments, plates with metal concentrations inhibiting the growth to half-maximum growth diameter of the substrate mycelium were used. While Cs, Cd, and Sr adaptation showed improved growth on the respective metal after ten transfers (Fig. 2), neither Zn nor the water control resulted in increasing growth after 10 weeks, and the macroscopic morphology of the mycelium did not change over the adaption process.

The cross-tolerance test revealed that the Cs-adapted strain (*S. commune* 12-43 Cs) grew worse than the wild-type on the water control and all other metals, and it did not grow at all on Sr (suppl Fig. S3). The cadmium adapted strain (*S. commune* 12-43 Cd) was not affected

for growth on any other tested metal, while *S. commune* 12-43 Sr grew faster than the wild-type on Cs and Cd as well as on the adaptation metal Sr. Thus, *S. commune* 12-43 Sr showed real cross-tolerance, and taken together with the unexpected growth promotion at low Sr concentrations, made this strain the optimal selection for further experiments.

Hyphal transport of metals

To test for uptake and transport of metals, divided plates were used. On one side of the split plates, metal-containing medium was supplied and the inoculum was placed there. The Petri dish divider allowed to fill the other half with metal-free medium. The hyphae crossing the barrier through their aerial mycelial growth and could be harvested from the metal-free side. For control of uptake, the metal-containing side of the plate was tested. Even there, only the aerial mycelium was harvested, meaning that the metal must have been taken from the agar below and accumulated in the aerial biomass. However, capillary transport on the cell wall, and cell wall adsorption, cannot be distinguished with this set-up from real intracellular uptake and transport. However, we could detect all four metals in the aerial biomass of *S. commune* 12-43 Sr. Interestingly, the adapted strain showed less uptake for Cs, Sr, and Cd and transport for Cs, Sr, and Zn, while the Cd transport with *S. commune* 12-43 Sr was even higher than in the non-adapted wild-type (Fig. 3, positive control).

For long-distance transport, the mycelium was harvested on the side of the plate that contained minimal medium without additional heavy metal. Thus, the metal detected could only have been brought there *via* the aerial mycelium from the source. Here, it was evident that the adapted *S. commune* 12-43 Sr transported less metal along the hyphal filaments to the other side of the split plate (Fig 3, transport). It seems noteworthy that, although all four metals were found in aerial mycelium on the side with metal, Cd and Zn were not transported for a larger distance, while this was the case for Sr and Cs. This indicates that the reason for the increased tolerance against Sr and Cs is rather export of the metals. This is in accordance with efflux transporters being up-regulated when these are localized within the vacuolar membrane or at the ER/Golgi vesicles (Blaudez et al., 2011).

168

169 *Metal compartmentalization*

170 To visualize the uptake of cadmium, laser scanning microscopy revealed intracellular
171 deposition of the metal in the hyphae of both adapted and wild-type strains. The co-staining
172 with Leadmium Green and Synapto Red suggested an intracellular localization within
173 vacuoles and vesicles, with the highest amount of deposition in the apical cells (suppl. Fig.
174 S4). Swelling of the hyphal tip was also seen. The localization of cadmium was the same in
175 both strains, going along with the finding that Cd was not tolerated significantly better by *S.*
176 *commune* 12-43 Sr in comparison to the wild-type. These findings are in accordance with
177 former studies (Salt and Wagner, 1993; Böhm, 2014; Janicka-Russak et al., 2012;
178 Wierzbicka et al., 2007; Spiridonova et a., 2019). Thus, transport along hyphae can be
179 explained with rates that are much higher than possible by simple diffusion mechanisms. In
180 addition, we were able to demonstrate that metals, indeed, can be transported from one part
181 of the mycelium to another. With this investigation, the white-rot fungus can be used for
182 further, more detailed studies, for which a model fungus can be proposed that can be
183 genetically modified and shows the completion of its life cycle on artificial media (Ohm et al.,
184 2010).

185

186 *Gene expression under metal stress*

187 The mRNA sequencing resulted in 264 significant gene regulations, of which 89 were up-
188 regulated, 160 were down-regulated and 13 were regulated in both directions depending on
189 the comparison (Tab. 1). Comparisons between *S. commune* grown on soil *versus* grown on
190 medium resulted in more significant changes than comparisons with the same growth
191 medium and different heavy metal contents. The sorting according to enzyme classes (Fig.
192 4) showed that most of the results belonged to the groups “hypothetical protein” and “poorly
193 characterized”. Genes belonging to the class of transporters were likely to be down-
194 regulated, and genes associated with the secretory pathway to be up-regulated under metal
195 stress, which could be part of a metal tolerance-system (Eide 2003).

196 Three genes were selected for further investigation: a down-regulated general transporter
197 gene, an up-regulated MFS general substrate transporter gene, and an up-regulated
198 glutathione S-transferase gene (suppl. Tab. S2). The verification of regulation by qPCR
199 confirmed induction and repression of the chosen genes as suggested from mRNA-Seq
200 (suppl. Fig. S5). For the metal-adapted *S. commune* 12-43 Sr, a changed regulation pattern
201 was seen (Fig. 5).
202 While the glutathione S-transferase gene was always up-regulated in the wild-type, this was
203 not the case for *S. commune* 12-43 Sr. Increased formation of glutathione has been
204 identified earlier as a result of intracellular metal stress (Ruytinx et al., 2013). Similarly, and
205 in accordance with the reduced cellular stress upon metal treatment of the adapted strain, a
206 less dominant regulatory pattern was visible. This confirmed our hypothesis that a changed
207 expression pattern resulted in metal adaptation.
208 The MFS general transporter gene is annotated as a conserved and poorly characterized
209 protein with unknown function by KOG, GO, and Interpro. Using BLAST comparisons, it
210 shows similarity to an MFS general substrate transporter, with an identity of 65% and a score
211 of 1575 bits in, e.g., *Stereum hirsutum* (Floudas et al., 2012). Transporters of the major
212 facilitator superfamily have been reported to be unspecific concerning their substrates. The
213 translocation of small molecules is mediated through chemiosmotic ionic gradients (Paulsen,
214 2003), and MFS transporters can secrete a variety of toxic compounds including organic and
215 inorganic ions. Thus, they can enhance tolerance against heavy metals (Stergiopoulos et al.,
216 2002). Since the gene for this transporter was higher expressed in the adapted strain,
217 increased excretion of toxic metals could be a reason for the improved growth under metal
218 stress. The hypothetical general substrate transporter is, according to Interpro and GO
219 descriptions, a sugar transporter and thus part of metabolic processes. This gene was up-
220 regulated in metal treatments in the mRNA-Seq and the microarray assay. This agrees with
221 previous research showing that metal stress often leads to an up-regulation of carbohydrate
222 metabolism (Chiapello et al., 2015).
223

Conclusion

Our findings support the role of basidiomycete fungi, and here specifically saprotrophic basidiomycetes like *S. commune*, in environmental interactions. They even can adapt to enhanced metal concentrations, with increased tolerance in *S. commune* 12-43 Sr being associated not with accumulation, but avoidance of intracellular metal accumulation. This puts the use for bioremediation into a different perspective. The fungus can take up and transport metals along the hyphae, leading to translocation of mobile cations. This would be specifically helpful in a contaminated area that most often shows high spatial heterogeneity of contamination. A re-distribution of metals, and the intracellular storage during transport, would lead to less strong effects of locally high metal contamination on plants growing in the vicinity (Colpaert et al., 2011).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The project was financed by the BMBF through the cooperative project BioVeSTra. The authors acknowledge support through the graduate school JSMC financed by the Carl-Zeiss-Foundation and the collaborative project USER financed by the BMBF. We thank Dirk Merten for the ICP-MS measurements.

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Tables

Table 1: Significant expression changes in mRNA sequencing sorted by comparisons.

Comparison	Significantly expressed gene changes
Paradies soil vs. Chernobyl soil	71
Paradies soil vs. heavy metal medium	115
Heavy metal medium vs. minimal medium	59
Chernobyl soil vs. minimal medium	119

Figures

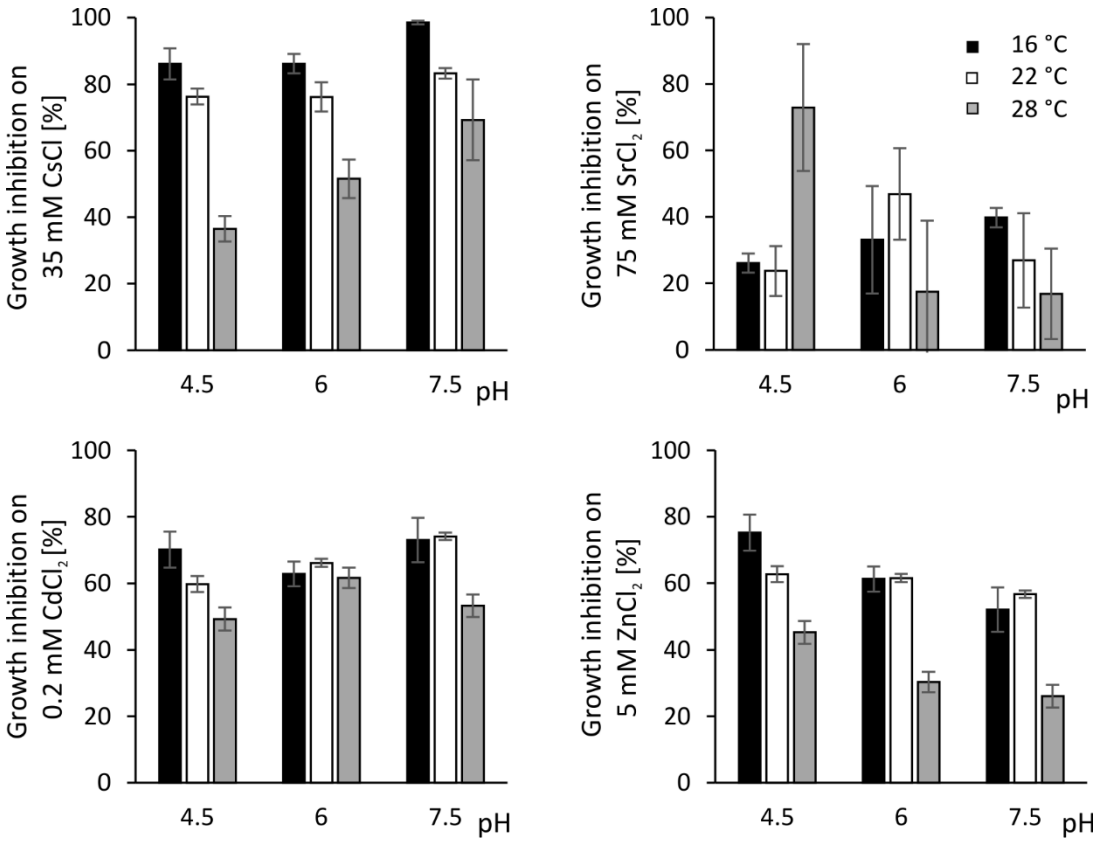


Fig. 1: Factors influencing growth inhibition by heavy metals. *S. commune* 12-43 (ATCC 38230) was cultivated in minimal medium (MM+ura containing 11.2 mg/l uracil and 16 g/l agar; Raper and Hoffman, 1974) for 14 days at different pH and temperatures (black, 16°C; white 22 °C; grey 28°C) with metal addition to check for percentage of growth inhibition as compared to the control grown at pH 5.3 and 28°C.

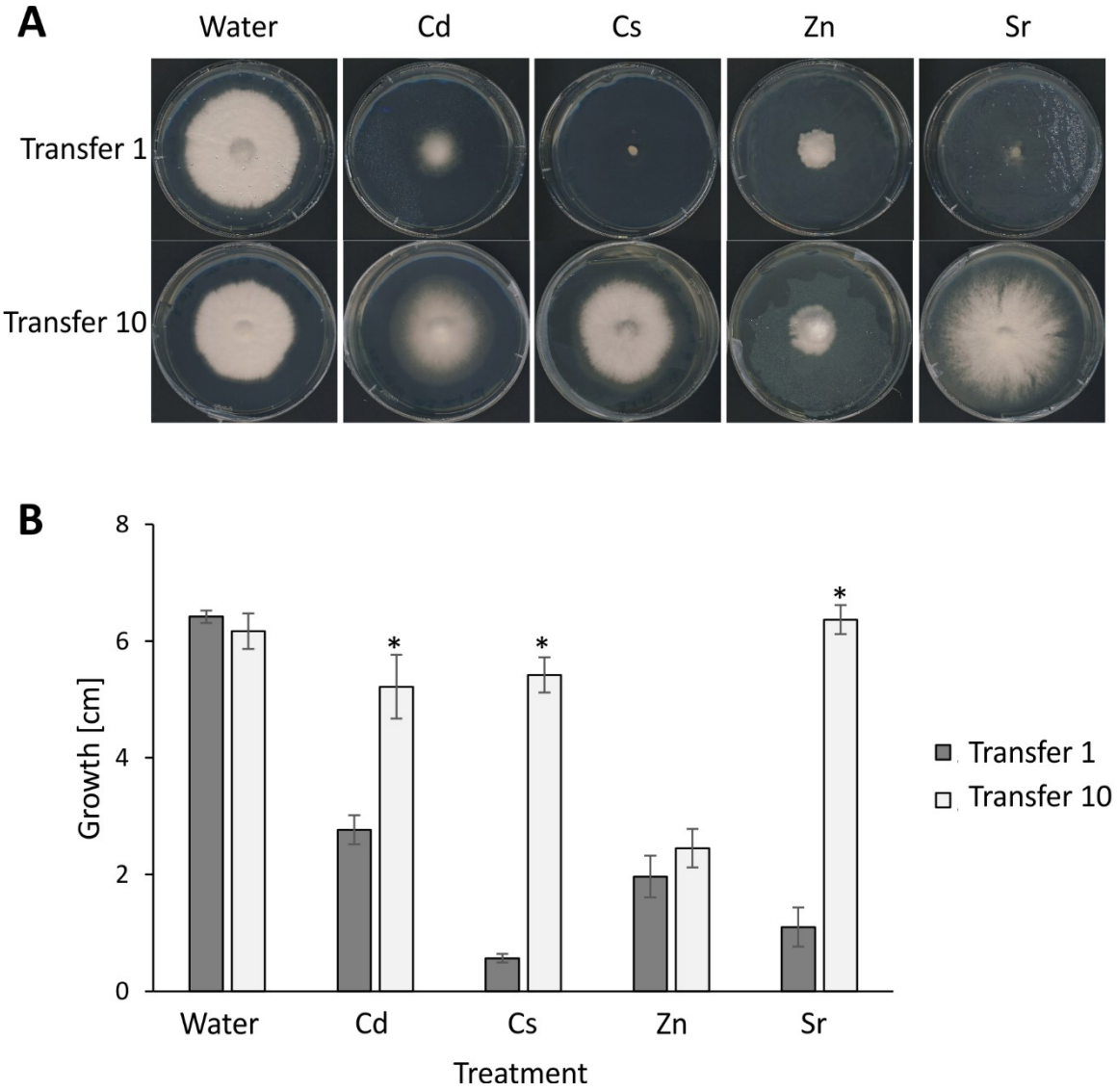


Fig. 2: Metal adaptation towards Cd, Cs, Zn, and Sr over 10 weeks after ten serial transfers. The colony morphology (A) and the mean growth after the first (grey) and weekly 10th transfer (white; B) of serial weekly transfers are shown on MM+ura with 50 mM CsCl, 100 mM $SrCl_2$, 0.2 mM $CdCl_2$, or 10 mM $ZnCl_2$ at 28°C. Water instead of metal solution was added in a mock adaptation experiment to exclude effects like selection of the fastest growing hyphae (n=3; significance $p \leq 0.05$ was calculated with a two-tailed t-test with unequal variance).

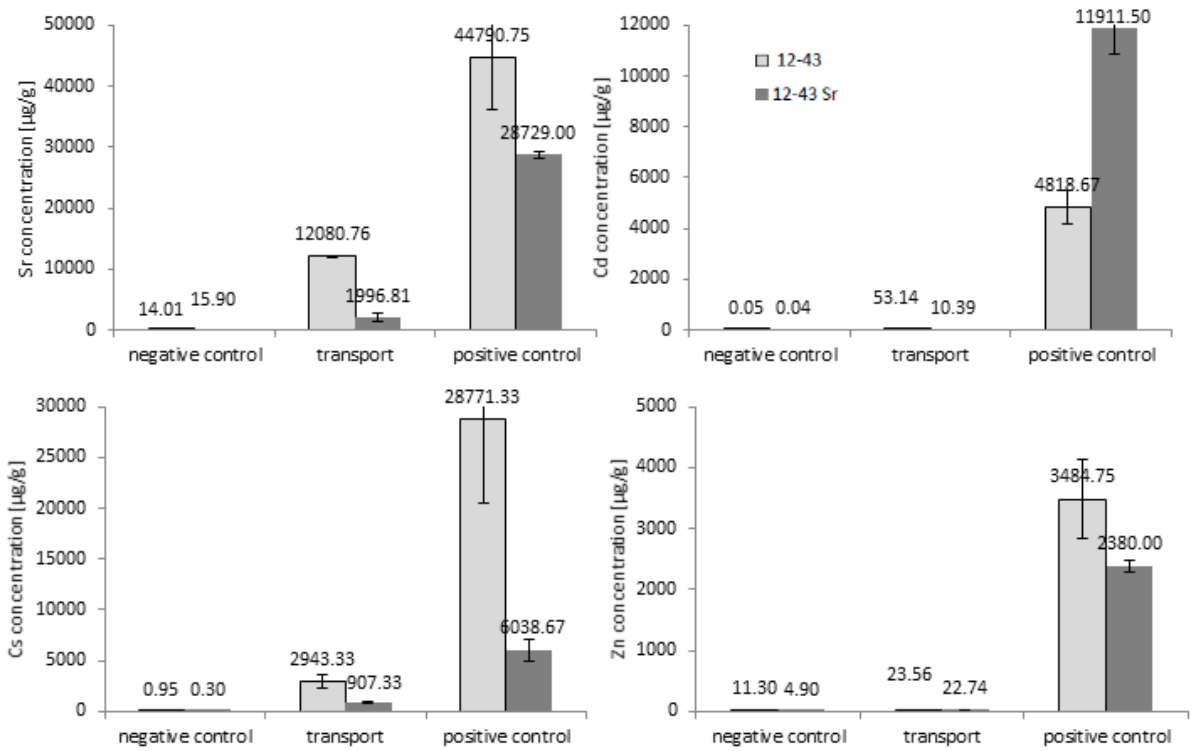


Fig. 3: Metal transport measured for hyphae obtained from split plates. The adapted *S. commune* 12-43 Sr (white) and the wild-type *S. commune* 12-43 (dark grey) were inoculated on a nylon-membrane to aid harvesting of aerial mycelium only (45 µm pores). Inoculation was performed at one side (MM+ura for negative control; MM+ura with 40 mM CsCl, 75 mM $SrCl_2$, 5 mM $ZnCl_2$ or 0.2 mM $CdCl_2$), and sampled after 14 days at 28°C from the same (positive control) side, or from the other half-plate that contained metal-free MM+ura (transfer). The mycelium was harvested, dried at 60°C and homogenized, and then analyzed by ICP-MS.

Diffusion of metals was impossible, because of the plastic division in the split plates that separated the two media, but allow for the fungus to grow over this barrier. The experiments were performed in five technical and three biological replicates.

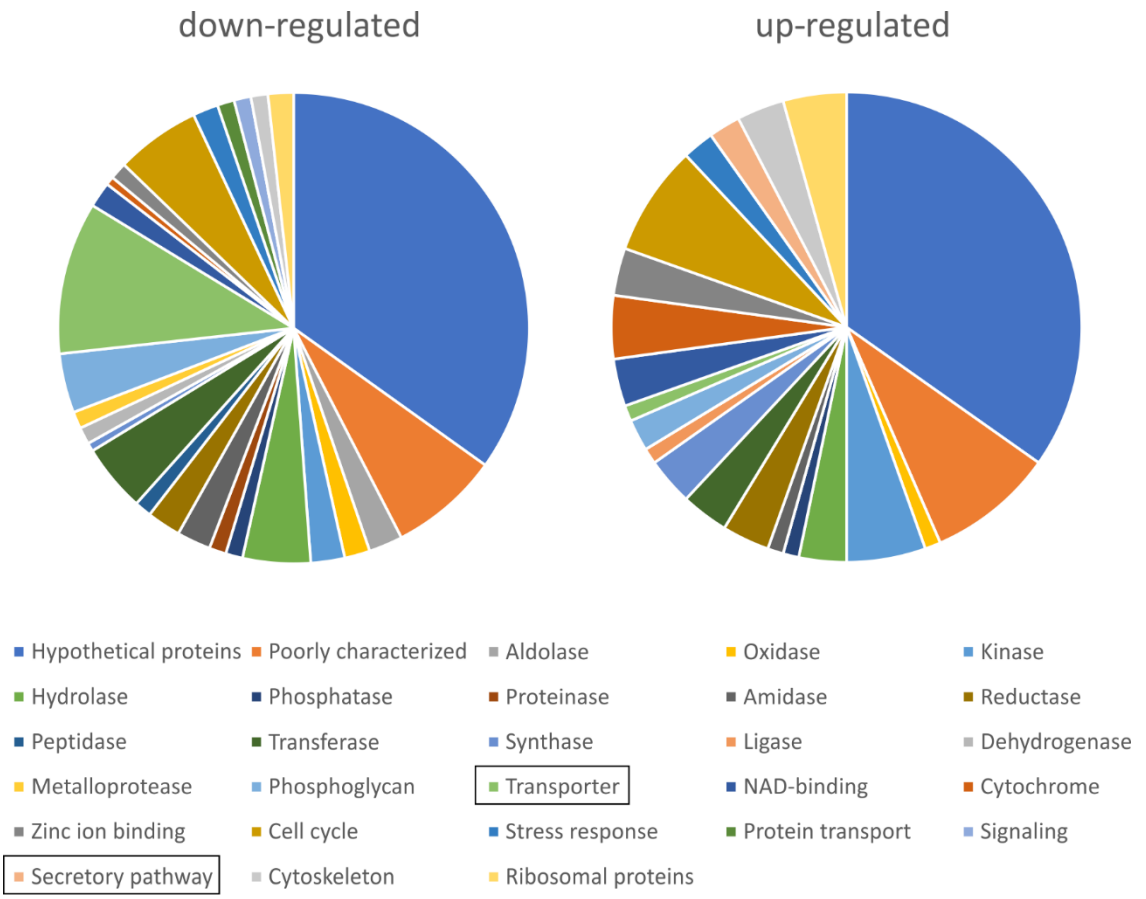


Fig. 4: Transcriptional regulation upon metal exposure of *S. commune* 12-43. The wild-type was grown on two soils (100 g each of autoclaved Chernobyl or Paradies park soil with 50 ml ½ MM+ura added to the soil to promote faster growth for 4 weeks) and in MM+ura without or with metal concentrations equivalent to those present in Paradies soil (see suppl. Tab. S1) for 2 weeks at 125 rpm and 16°C to mimick natural conditions. Harvested mycelium was homogenized and RNA extracted for library preparation, mRNA-Seq, and TopHat alignment to the *S. commune* genome (https://mycocosm.jgi.doe.gov/Schco3/Schco3.home.html). Bioinformatic pairwise comparisons with Cuffdiff 2 workflow (StarSeq; Trapnell et al., 2012) was used and significance assumed when $p \leq 0.005$ and $q \leq 0.05$. The sequenced hits were sorted by enzyme classes according to BLAST hits.

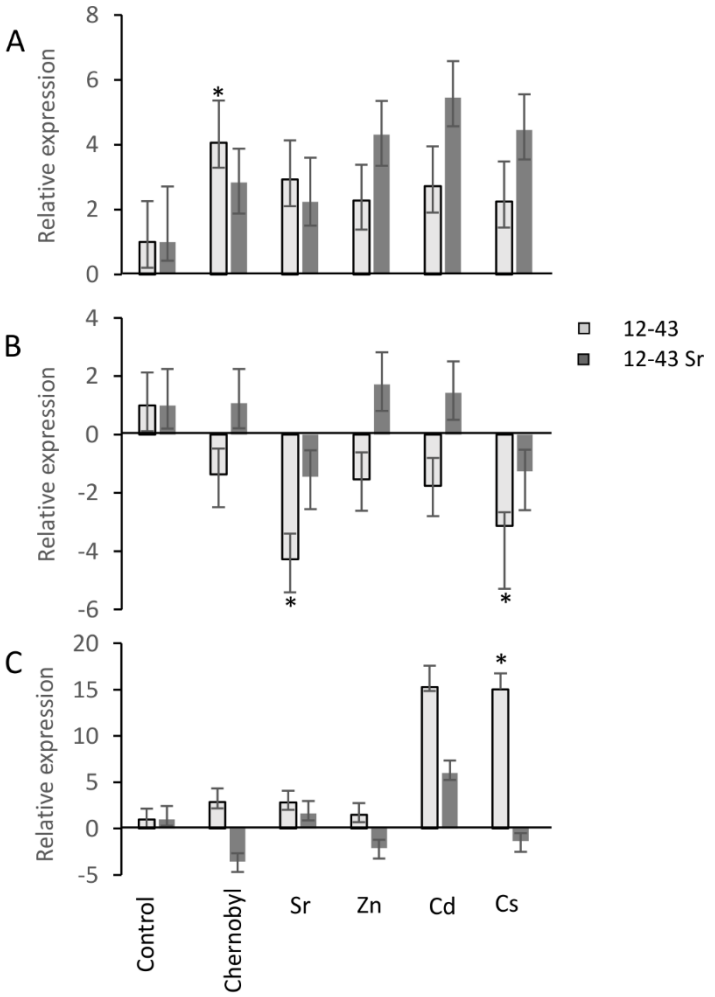
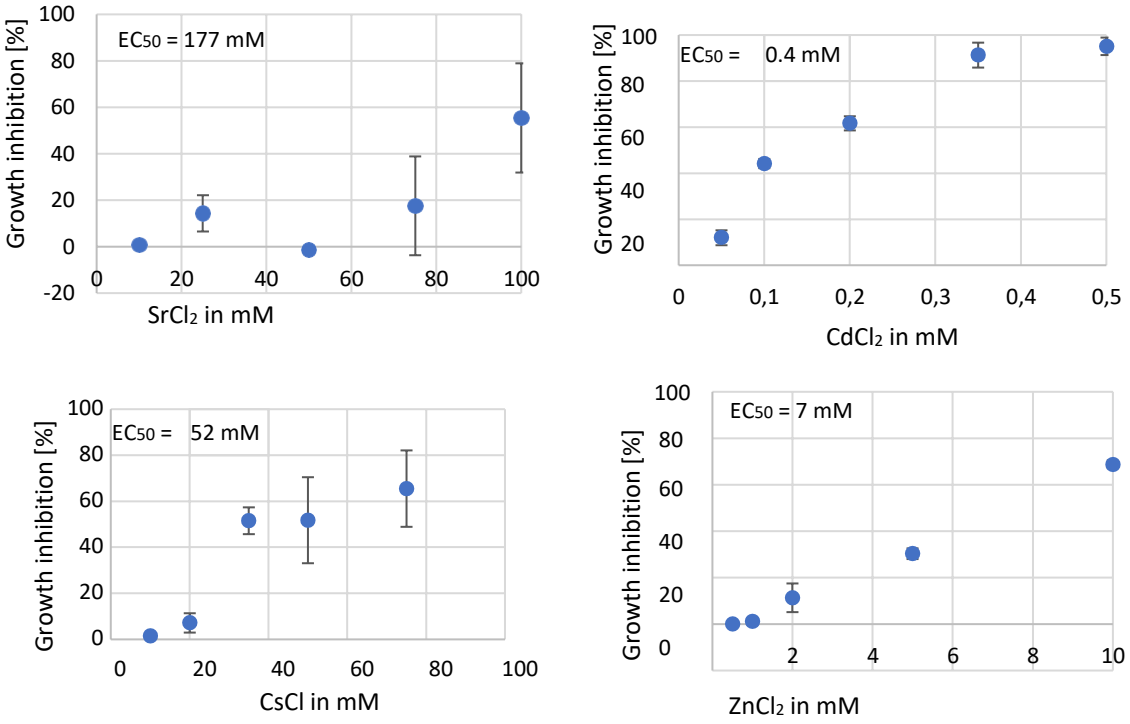


Fig. 5: Verification of metal-dependent expression changes by qPCR. Selected genes (A, general substrate transporter XM_003031963.1; B, MFS general substrate transporter XM_003027220.1; C, glutathione S-transferase XM_003027462.2) were analyzed in the wild-type and adapted strains grown at 28°C for 5 days on cellophane membrane-topped MM+ura plates with 75 mM $SrCl_2$, 50 mM CsCl, 5 mM $ZnCl_2$, 0.2 mM $CdCl_2$, or the concentration of the five most abundant bioavailable metals in Chernobyl soil (suppl. Tab S3). The cDNA synthesis was done from 500 ng total RNA and afterward qRT-PCR was (for details, see suppl. Material) performed using the respective primers (suppl. Tab. S4). A melting curve was measured after every run from 60 to 94°C with Δ1°C per 30 sec. All measurements were performed in three biological and three technical replicates with negative control and a “no reverse transcriptase” control each. Relative expression was calculated (Pfaffl, 2001) using the housekeeping genes actin1 (XM_003026104.1), ubiquitin

453 (XM_003036409.1) and translation elongation factor 1 (XM_003037215.1). Significance was

454 tested with a two-tailored t-test with unequal variances for significance at p-value ≤ 0.05.

Supplemental Material

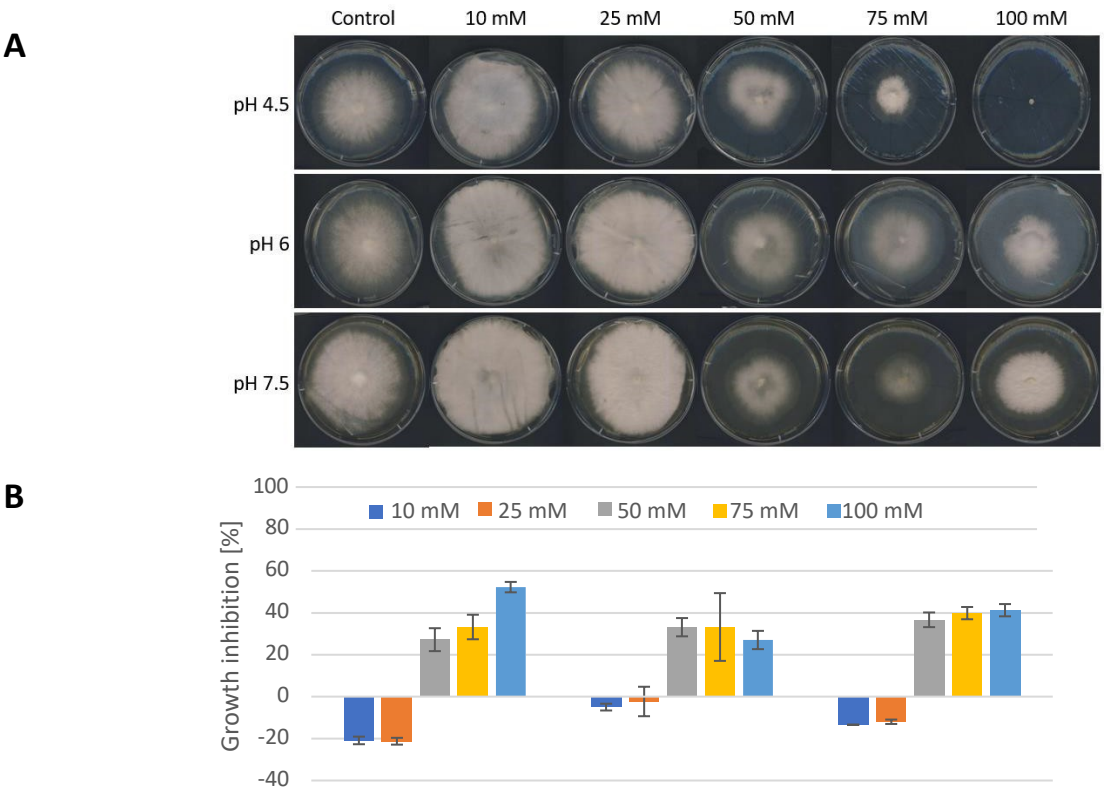


Suppl. Fig. S1: Growth inhibition by metals at pH 6 and 28°C. *S. commune* was cultivated for 14 days on MM+ura with added metals (n = 5). Half maximal growth inhibition (EC50) was calculated from a regression line [y=ax+b; y= 50].

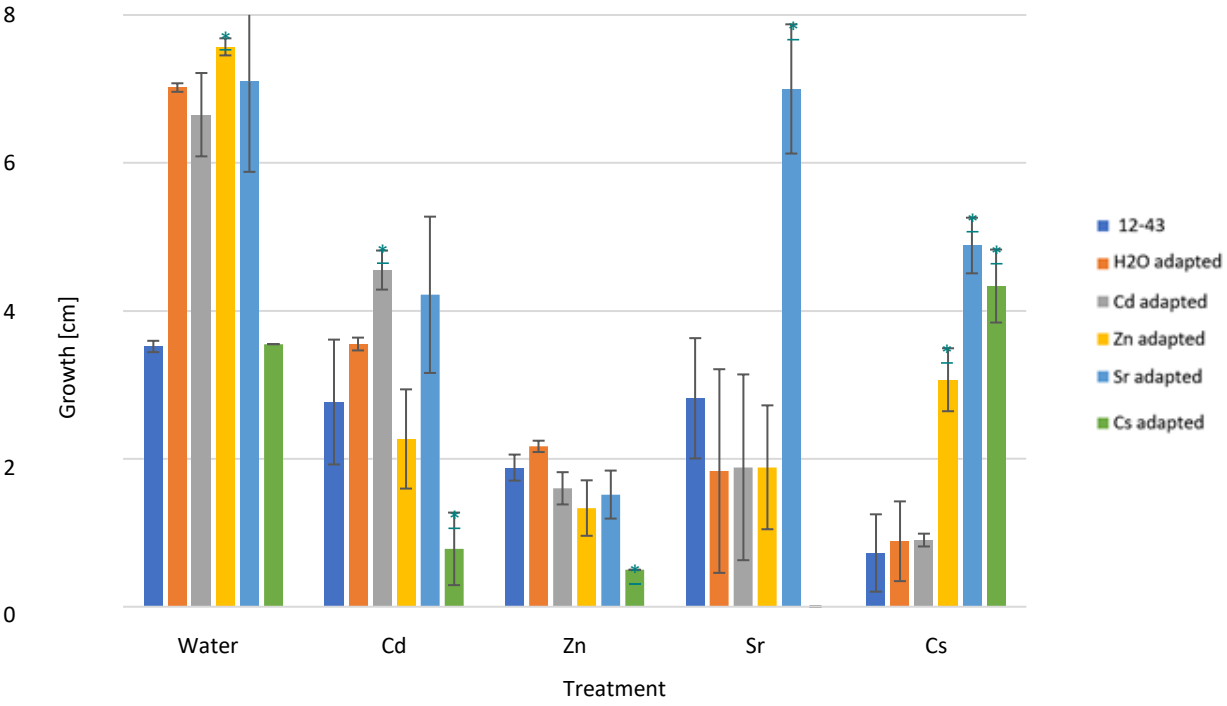
Suppl. Tab. S1: Metal content in soil samples from the Chernobyl Exclusion Zone (bold) and the Paradies soil (*italic*).

µg/g soil	Cs	Sr	Cd	Zn
fraction 1	**0.075** *0.0165*	**3.99** *51.03*	**0.03** *0.016*	**1.5** *1.2*
fraction 2	**0.015** *0.0026*	**0.29** *19.18*	**0.045** *0.216*	**1.6** *7.1*
sum 1 + 2	**0.09** *0.019*	**4.29** *70.22*	**0.075** *0.232*	**3.1** *8.3*

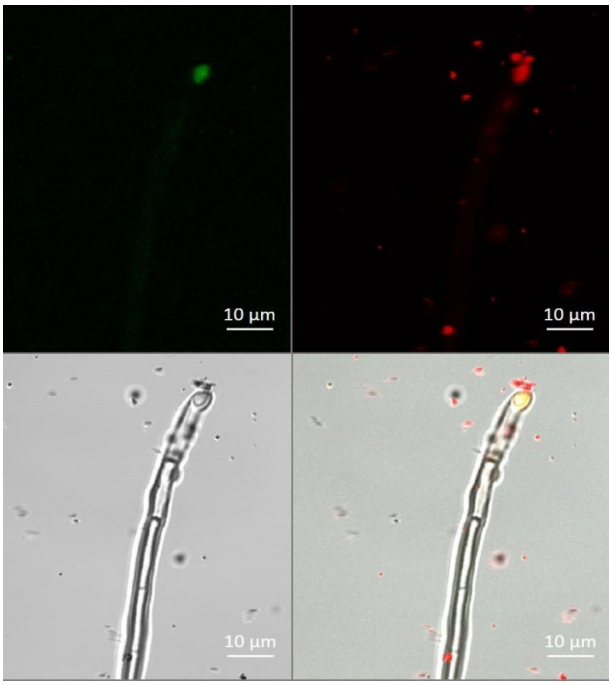
For comparison to the in vitro experiments, metal contents in the bioavailable fractions F1 and F2 determined by ICP-MS are given (compare Traxler et al., 2021; Zeien and Brümmer, 1987). Chernobyl soil was taken 10-20 cm below the surface for testing (51◦20′54″ N: 30◦07′41″ O) and the pH was 5.2. Paradies soil was taken 5 cm below surface (50°55'22.2"N 11°35'10.0"E) and the pH was 6.4.



Suppl. Fig. S2: Influence of pH on the growth inhibition by $SrCl_2$ at 16°C. The growth morphology of the mycelia (A) and the percentage of growth inhibition (B) are shown.



Suppl. Fig. S3: Cross tolerance of adapted strains to other metals. Supplementation (50 mM CsCl, 100 mM $SrCl_2$, 0.2 mM $CdCl_2$ and 10 mM $ZnCl_2$) was used and significant changes (*) calculated for the metal adapted strains compared to the water control with $p \leq 0.05$.



Suppl. Fig. S4: Cadmium localization within hyphae using co-staining with Leadmium Green and Synapto Red visualized by laser scanning microscopy. *S. commune* was grown at minimal medium with 0.2 mM $CdCl_2$. 30 µM Synapto Red (Fm4-64, Biotum, Apprieu,France) and 50 µg Leadmium green (ThermoFisher Scientific, Waltham, USA) dissolved in 50µl DMSO and diluted with 0.9% NaCl were added to overgrown coverslips. After 2 h incubationtime, it was washed three times with ½ MM+ura. Confocal laser scanning microscopy (LSM 780, Carl Zeiss, Jena, Germany) using 40x/1.30 NA EC Plan-Neofluar objective at a laser intensity of 0.2 % and a wavelength of 514 nm (Synapto red) and 488 nm (Leadmium green) was used with detection in a range of 474-518 nm. Images were calculated and finalized withthe program Zen2 (blue edition).

Suppl. Tab. S2: Significantly regulated genes determined by mRNA sequencing in *S. commune* 12-43 wild-type.

JGI transcript ID	2-fold change				Description KOG	BLAST hits at NCBI database
	Ch MM	Pa HM	Pa Ch	HM MM		
11173	*NA*	- 5.53	- 2.23	*NA*	*NA*	Proteophosphoglycan 5 in *Leishmania major* strain Friedlin; Score= 150; id= 16%
14899	*NA*	*NA*	2.25	*NA*	*NA*	Alternative oxidase in *Coniophora puteana*; Score 1120; id= 67%
62301	*NA*	*NA*	*NA*	2.00	1-Acyl dihydroxyacetone phosphate reductase and related dehydrogenases	NAD-P binding protein in *Stereum hirsutum*; Score= 655; id= 42%
85169	*NA*	*NA*	- 2.13	*NA*	*NA*	hypothetical proteins

JGI transcript ID	2-fold change				Description KOG	BLAST hits at NCBI database
	Ch MM	Pa HM	Pa Ch	HM MM		
106400	inf	*NA*	*NA*	*NA*	Serine/threonine-protein kinase	Vegetative cell wall protein gp1 in *Chlamydomonas reinhardtii*; Score= 123; id= 41%
231154	*NA*	*NA*	1.96	*NA*	*NA*	Carbohydrate-binding module family 13 protein/ putative endo-1,3-beta glucanase in *Agaricus bisporus*; Score= 664; id= 42%
250848	*NA*	- 4.29	- 2.75	*NA*	*NA*	
1078592	*NA*	*NA*	*NA*	- 1.86	*NA*	Related to acid phosphatase in *Sporisorium reilianum*; Score= 1036; id= 52%
1080019	*NA*	- 3.37	- 3.06	*NA*	*NA*	no match
1104156	6.05	*NA*	*NA*	4.88	Splicing coactivator SRm 160/300, subunit SRm300	no match
1126088	*NA*	*NA*	*NA*	1.87	*NA*	FMN-dependent NADH-azoreductase in *Desulfobaccter acetoxidans*; Score 115; id= 37%
1141276	*NA*	*NA*	*NA*	2.12	*NA*	hypothetical proteins, e.g. *Armillaria solidipes*; Score= 127; id= 32%
1143104	4.62	*NA*	*NA*	*NA*	*NA*	hypothetical proteins; 5 transmembrane helices
1145136	*NA*	*NA*	*NA*	- 1.76	*NA*	hypothetical proteins; ifi-6-16 superfamily
1150509	*NA*	*NA*	*NA*	1.81	Flavonol reductase/cinnamoyl-CoA reductase	NAD-binding protein in *Fomitiporia mediterranea*; Score= 928; id= 53%
1153664	*NA*	- 3.89	*NA*	*NA*	Transaldolase	Aldolase in *Trametes versicolor*; Score= 189; id= 24%
1166241	- 3.15	*NA*	*NA*	*NA*	Transaldolase	Aldolase in *Trametes versicolor*; Score= 189; id= 24%
1168136	*NA*	*NA*	*NA*	1.76	*NA*	hypothetical proteins; ifi-6-16 superfamily
1171639	- 3.39	*NA*	*NA*	*NA*	*NA*	Heat shock protein in *Rhizoctonia solani*; Score= 102; Id= 77%
1177211	- 3.83	- 3.22	*NA*	*NA*	*NA*	CREB-binding protein in *Homo sapiens*; Score= 99; id= 18%
1187442	4.54	*NA*	*NA*	2.20	*NA*	Dimeric alpha-beta barrel, partial in *Metarhizium majus*; Score 43; Id= 26%
1193121	- 2.59	*NA*	*NA*	*NA*	*NA*	hypothetical proteins
1194301	*NA*	- 3.57	*NA*	*NA*	*NA*	hypothetical proteins
1195633	*NA*	- 5.81	- 5.14	*NA*	*NA*	hypothetical proteins
1213227	- 5.63	- 8.09	*NA*	*NA*	*NA*	Leucine-rich repeat-containing protein in *Canis familiaris*; Score= 90; id= 36%
1216147	*NA*	- 4.03	*NA*	*NA*	*NA*	Ceacam20_predicted, CEA-related cell adhesion molecule 20 in *Rattus norvegicus*; Score= 95; id= 38%
2005284	-inf	*NA*	*NA*	*NA*	Casein kinase (serine/threonine/tyrosine protein kinase	Kinase-like protein in *Stereum hirsutum*; Score= 780; id= 41%
2086920	3.64	*NA*	*NA*	3.02	Predicted glutathione S-transferase	Glutathione S-transferase in *Stereum hirsutum*; Score= 1106; id= 62%
2086990	*NA*	*NA*	2.15	1.95	*NA*	Histidine kinase in *Candidatus vecturithrix granuli*; Score= 39; id= 33%
2088105	- 3.72	*NA*	*NA*	*NA*	1,2alpha-mannosidase	Glycoside hydrolase family 47 protein in *Serpula lacrymans*; Score 1632; id= 59%
2107770	*NA*	*NA*	*NA*	2.45	Uncharacterized conserved protein	MFS general substrate transporter in *Stereum hirsutum*; Score= 1575; id= 65%

JGI transcript ID	2-fold change				Description KOG	BLAST hits at NCBI database
	Ch MM	Pa HM	Pa Ch	HM MM		
2147438	-4.65	-5.41	*NA*	*NA*	*NA*	no match
2176489	*NA*	- 3.99	- 2.94	*NA*	*NA*	no match
2176878	*NA*	*NA*	- 2.99	*NA*	*NA*	Amino acid adenylation domain protein in *Hungateiclostridium thermocellum*; Score 35; id= 35%
2211287	*NA*	- 3.75	- 1.90	*NA*	*NA*	Similar to KIAA0447 protein in *Pan troglodytes*; Score= 95; id= 20%
2213880	3.89	*NA*	*NA*	4.08	Kynurenine 3-monooxygenase and related flavoprotein monooxygenases	FAD/NAD(P)-binding domain-containing protein in *Dichomitus squalens*; Score 667; id= 29%
2240204	*NA*	inf	*NA*	*NA*	*NA*	hypothetical proteins
2244565	*NA*	- 3.86	*NA*	*NA*	*NA*	Similar to solute carrier family 34 (sodium phosphate) member2; type 2 sodium-dependent phosphate transporter in *Pan troglodytes*; Score= 101; id= 36%
2245539	- 3.84	- 3.72	*NA*	*NA*	*NA*	N-acetylmuramoyl-L-alanine amidase Ain *Bacterium* HR16; Score= 91; id= 30%
2298796	- 3.89	*NA*	*NA*	*NA*	Serine/threonine-protein kinase	Vegetative cell wall protein gp1 in *Chlamydomonas reinhardtii*; Score 178; id= 27%
2307907	- 6.61	- 3.65	2.76	*NA*	Aspartyl protease	Aspartic proteinase precursor in *Trametes versicolor*; Score 1161; id= 51%
2319004	*NA*	- 3.78	*NA*	*NA*	*NA*	Sulfate transporter in *Burkholderia* sp.; Score= 83; id= 47%
2345748	*NA*	*NA*	*NA*	2.28	*NA*	Predicted: Protein purity of essence in *Drosophila eugracillis*; Score= 38; id= 30%
2357859	- 3.44	*NA*	*NA*	*NA*	*NA*	Predicted: chromosome-associated kinesin KIF4A in *Austrofundulus limnaeus*; Score= 87; id= 34%
2437752	*NA*	3.43	*NA*	*NA*	*NA*	just hypothetical proteins
2469817	*NA*	*NA*	*NA*	-inf	*NA*	Proteolysis and peptidolysis-related protein in *Cryptococcus neoformans*; Score= 1070; id= 71%
2482226	2.85	*NA*	- 2.03	*NA*	*NA*	Inositol polyphosphate phosphatase, partial in *Trametes versicolor*; Score= 1257; id= 56%
2482406	*NA*	*NA*	*NA*	3.10	*NA*	YpgE/AlgH family protein in *Liberibacter crescens*; Score= 94; id= 31%
2482494	*NA*	*NA*	*NA*	- 1.82	*NA*	Histone acetyltransferase ELP3 in *Punctularia strigosozonata*; Score= 2747; id= 94%
2482729	3.67	*NA*	*NA*	3.10	*NA*	Citrate synthase in *Coprinopsis cinerea okayama*; Score= 1784; id= 80%
2482860	- 3.65	*NA*	*NA*	*NA*	*NA*	Retrotransposon nucleocapsid protein in *Cryptococcus neoformans*; Score= 329; id= 17%
2483927	- 3.87	*NA*	*NA*	*NA*	*NA*	Predicted: Serine/Threonine-protein phosphatase 6 regulatory ankyrin repeat subunit A-like, partial in *Strongylocentrotus purpuratus*; Score= 341; id= 48%
2486595	*NA*	- 4.04	*NA*	*NA*	*NA*	tRNA-dihydrouridine synthase 2 in *Coprinopsis cinerea okayama*; Score= 1323; id= 55%
2486953	- 5.20	*NA*	*NA*	*NA*	*NA*	BTB/POZ domain protein in *Rhizoctonia solani*; Score=83; id= 31%

JGI transcript ID	2-fold change				Description KOG	BLAST hits at NCBI database
	Ch MM	Pa HM	Pa Ch	HM MM		
2489112	*NA*	4.11	2.48	*NA*	*NA*	Putative secretory pathway protein ysy6-like protein in *Eutypa lata*; Score= 119; id= 44%
2495032	*NA*	*NA*	1.97	*NA*	*NA*	Other 1 protein kinase in *Moniliophthora roreri*; Score= 120; id= 26%
2496335	- 3.07	- 4.68	*NA*	*NA*	*NA*	tat pathway signal sequence in *Beauveria bassiana*; Score= 386; id= 31%
2497507	*NA*	*NA*	1.93	*NA*	*NA*	Nitric oxide synthase-interacting in *Hypsizygus marmoreus*; Score= 1152; id= 71%
2498271	- 3.41	*NA*	*NA*	*NA*	*NA*	hypothetical proteins
2499969	*NA*	*NA*	*NA*	- 2.03	*NA*	Proteophosphoglycan ppg4 in *Leishmania major*; score= 126; id= 24%
2501258	*NA*	*NA*	*NA*	1.76	*NA*	WD40 repeat-like protein in *Neolentinus lepideus*; Score= 505; id= 48%
2501677	*NA*	*NA*	*NA*	2.42	*NA*	no match
2502055	3.67	*NA*	*NA*	*NA*	*NA*	Ankyrin, partial in *Stereum hirsutum*; Score= 472; id= 67%
2502252	- 3.22	- 5.48	- 2.64	*NA*	*NA*	General substrate transporter in *Punctularia strigosozonata*; Score= 1761; id= 59%
2502688	- 3.50	*NA*	*NA*	*NA*	*NA*	Pro-Pol partial in *Lentinula edodes*; Score= 757; id= 41%
2504225	3.96	*NA*	*NA*	*NA*	*NA*	Ribosome biogenesis protein Sqt1 in *Dichomitus squalens*; Score= 1342; id= 68%
2506764	- 8.54	- 3.95	*NA*	- 3.39	*NA*	hypothetical proteins
2507097	*NA*	-inf	*NA*	*NA*	*NA*	DUF323 domain-containing protein in *Coprinopsis cinerea okayama*; Score= 1736; id= 61%
2509722	*NA*	- 6.79	- 5.00	*NA*	*NA*	Marvel domain protein in *Rhizoctonia solani*; Score= 296; id= 41%
2511002	4.65	*NA*	*NA*	*NA*	*NA*	Transcriptional activator of proteases prtT in *Hypsizygus marmoreus*; Score= 2945; id= 78%
2512878	- 2.76	- 3.78	*NA*	*NA*	*NA*	DUF647 containing protein in *Coniophora puteana*; Score= 760; id= 36%
2513725	*NA*	*NA*	*NA*	2.03	*NA*	hypothetical proteins
2515739	- 6.02	- 5.48	*NA*	*NA*	*NA*	PrsW family intramembrane metalloprotease in *Streptomyces scabiei*; Score= 90; id= 31%
2516065	*NA*	4.66	*NA*	*NA*	*NA*	S-adenosyl-L-methionine-dependent methyltransferase, partial in *Coniophora putaneana*; Score= 446; id= 35%
2516444	- 4.67	- 4.81	*NA*	*NA*	*NA*	Transporter in *Glanoderma sinense*; Score= 138; id= 31%; has Atrophin 1 superfamily and DNA_po13_gamma3 superfamily
2516612	- 3.59	- 4.11	*NA*	*NA*	*NA*	Aldo/keto reductase in *Auricularia subglabra*; Score= 1152; id= 66%
2517867	*NA*	*NA*	*NA*	1.91	*NA*	Retro virus-related Pol polyprotein from transposon opus in *Hypsizygus marmoreus*; Score= 346; id= 36%
2518120	5.47	4.44	*NA*	*NA*	*NA*	hypothetical proteins
2519051	*NA*	- 2.78	*NA*	*NA*	*NA*	no match
2519067	- 4.18	- 4.25	*NA*	*NA*	*NA*	Poly ADP-ribose polymerase 3 in *Grifola frondosa*; Score= 176; id= 28%
2525305	*NA*	3.85	2.03	*NA*	*NA*	Proteophosphoglycan ppg4 in *Leishmania major*; Score= 628; id= 21%

JGI transcript ID	2-fold change				Description KOG	BLAST hits at NCBI database
	Ch MM	Pa HM	Pa Ch	HM MM		
2526826	*NA*	*NA*	*NA*	1.96	*NA*	no match
2528013	3.74	*NA*	*NA*	*NA*	*NA*	no match
2543225	4.51	*NA*	- 2.10	*NA*	*NA*	SNF-family ATP dependent chromatin remodeling factor snd21 in *Coprinopsis cinerea okayama*; Score= 2328; id= 48%
2545517	4.06	*NA*	*NA*	1.93	*NA*	hypothetical proteins
2550635	*NA*	*NA*	- 1.83	*NA*	*NA*	Zinc metalloprotease in *Moniliophthora roreri*; Score= 194; id= 42%
2550927	- 2.98	- 3.63	*NA*	*NA*	*NA*	hypothetical proteins
2556274	- 4.02	- 5.12	*NA*	*NA*	*NA*	Ankyrin, partial in *Lepidopterella palustris*; Score= 460; id= 30%
2559216	*NA*	*NA*	*NA*	1.94	*NA*	HET domain-containing protein in *Stagonospora* sp.; Score= 293; id= 26%; Sequence has GOODBYE, RlpA superfamily and P-loop_NTPase superfamily domain
2562512	4.69	3.80	*NA*	*NA*	*NA*	Phosphoglucomutase first 3 domain-containing protein in *Trametes versicolor*; Score = 1854; id= 54%
2568939	- 3.57	*NA*	*NA*	*NA*	*NA*	Alcohol oxidase in *Fomitiporia mediterranea*; Score= 1601; id= 51%
2571870	*NA*	- 3.98	*NA*	*NA*	*NA*	hypothetical proteins
2574146	*NA*	- 4.98	- 2.14	*NA*	*NA*	Integral membrane protein in *Metarhizium anisopliae*; Score= 506; id= 47%
2579958	- 5.50	- 4.57	*NA*	*NA*	*NA*	hypothetical proteins
2580814	5.90	*NA*	*NA*	3.34	*NA*	Cytochrome P450 in *Coniophora puteana*; Score= 927; id= 32%
2590387	*NA*	- 2.72	*NA*	*NA*	*NA*	Peptidase S41 family protein ustP in *Hypsizygus marmoreus*; Score= 2066; id= 60%
2591720	- 3.50	*NA*	*NA*	*NA*	*NA*	Redoxin domain-containing protein in *Deinococcus marmoris*; Score= 95%; id= 37%
2600011	*NA*	*NA*	*NA*	- 1.76	*NA*	Cell surface glycoprotein 1 in *Rhizoctonia solani*; Score= 99; id= 55%; sequence has DUF390 superfamily
2603640	5.02	3.60	*NA*	*NA*	*NA*	hypothetical proteins
2603998	*NA*	*NA*	2.11	*NA*	*NA*	hypothetical proteins but with Zn binding site and putative histone H3 binding site, has ING superfamily
2604035	*NA*	*NA*	*NA*	- 1.87	*NA*	Alpha/beta-hydrolase in *Stereum hirsutum*; Score= 437; id= 43%
2604043	5.03	*NA*	*NA*	2.18	*NA*	Long-chain-fatty-acid-CoA ligase in *Coprinopsis cinerea okayama*; Score= 1216; id= 38%
2604070	- 4.17	*NA*	1.90	*NA*	*NA*	Synembryn-A in *Hypsizygus marmoreus*; Score= 1445; id= 55%
2605066	4.03	*NA*	*NA*	2.51	*NA*	hypothetical proteins
2605229	*NA*	*NA*	*NA*	- 2.24	*NA*	NAD(P)-binding protein in *Coniophora puteana*; Score= 518; id= 57%
2605383	6.81	4.78	*NA*	*NA*	*NA*	no match
2605614	*NA*	*NA*	2.01	*NA*	*NA*	Multidrug resistance protein, putative in *Ixodes scapularis*; Score= 178; id= 43%
2605703	*NA*	*NA*	- 1.96	*NA*	*NA*	hypothetical proteins
2606453	*NA*	*NA*	- 2.54	*NA*	*NA*	Proteophosphoglycan ppg4 in *Leishmania major* strain; Score= 131; id= 31%
2606982	*NA*	*NA*	*NA*	2.09	*NA*	Protein HID1 in *Hypsizygus marmoreus*; Score= 2845; id= 60%

JGI transcript ID	2-fold change				Description KOG	BLAST hits at NCBI database
	Ch MM	Pa HM	Pa Ch	HM MM		
2607034	- 3.64	*NA*	*NA*	*NA*	*NA*	SNF family protein in *Stereum hirsutum*; Score= 741; id= 51%
2607237	*NA*	*NA*	2.07	*NA*	*NA*	no match
2608475	*NA*	- 3.88	- 2.61	*NA*	*NA*	hypothetical proteins with HERPES superfamily
2608547	*NA*	*NA*	- 2.33	*NA*	*NA*	HMG-box transcription factor in *Flammulina velutipes*; Score= 327; id= 47%
2608711	*NA*	*NA*	*NA*	1.78	*NA*	Dethiobiotin synthase in *Flavobacterium chilense*; Score= 81; id= 29%
2609461	*NA*	*NA*	1.83	*NA*	*NA*	no match
2609957	- 4.23	- 5.75	*NA*	*NA*	*NA*	hypothetical proteins
2610509	*NA*	*NA*	- 1.94	*NA*	*NA*	High osmolarity signaling protein Sho1 in *Neosartorya fischeri*; Score= 260; id= 24%
2610599	- 5.18	- 6.21	*NA*	*NA*	*NA*	NAD(P)-binding protein in *Punctualaria strigozonata*; Score= 920; id= 58%
2610732	- 4.09	*NA*	*NA*	*NA*	*NA*	Glycerol-3-phosphate dehydrogenase in *Laccaria bicolor*; Score= 1412; id= 71%
2610764	- 3.78	*NA*	*NA*	*NA*	*NA*	26S proteasome non-ATPase regulatory subunit 12 in *Hypsizygus marmoreus*; Score= 291; id= 65%
2610865	*NA*	*NA*	2.33	*NA*	*NA*	DNA repair and recombination protein pif1 in *Coprinopsis cinerea okayama*; Score= 261; id= 21%
2611974	- 4.35	- 4.80	*NA*	*NA*	*NA*	MFS general substrate transporter in *Trametes versicolor*; Score= 1275; id= 31%
2611979	- 3.58	- 4.38	*NA*	*NA*	*NA*	ATP-dependent RNA helicase DRS1 in *Tramitomyces* sp.; Score= 618; id= 46%
2612001	*NA*	- 4.09	*NA*	*NA*	*NA*	Putative transmembrane protein in *Rhizoctonia solani*; Score= 460; id= 74%
2612018	*NA*	- 3.78	*NA*	*NA*	*NA*	Cytochrome P450 family domain in *Rhizoctonia solani*; Score= 252; id= 34%
2612041	*NA*	*NA*	*NA*	2.06	*NA*	Putative 54S ribosomal protein L32 mitochondrial in *Hypsizygus marmoreus*; Score= 318; id= 56%
2612073	*NA*	*NA*	*NA*	2.01	*NA*	Translation initiation factor IF-2 in *Kineococcus radiotolerans*; Score= 104; id= 45%
2612719	- 4.48	- 5.57	- 1.85	*NA*	*NA*	MFS general substrate transporter in *Dichomitus squalens*; Score =1461; id= 56%
2612775	*NA*	*NA*	*NA*	- 1.89	*NA*	hypothetical proteins
2612880	*NA*	*NA*	- 1.87	*NA*	*NA*	no match
2613297	*NA*	*NA*	- 2.06	*NA*	*NA*	no match
2613412	- 3.32	*NA*	*NA*	*NA*	*NA*	Rsm22-domain-containing protein in *Dichomitus squalens*; Score= 1297; id= 43%
2613450	*NA*	- 3.59	*NA*	*NA*	*NA*	MYC1 in *Coprinopsis cinerea okayama*; Score= 1849; id= 75%
2615136	*NA*	- 3.96	*NA*	*NA*	*NA*	hypothetical proteins
2615345	*NA*	- 4.16	*NA*	*NA*	*NA*	hypothetical proteins
2615354	- 2.86	- 3.44	*NA*	*NA*	*NA*	Putative kinase-PK-like protein in *Rhizoctonia solani*; Score= 3368; id= 32%

JGI transcript ID	2-fold change				Description KOG	BLAST hits at NCBI database
	Ch MM	Pa HM	Pa Ch	HM MM		
2615369	- 3.03	*NA*	*NA*	*NA*	*NA*	DUF300 domain containing protein in *Fomitiporia mediterranea*; Score= 1332; id= 51%
2617924	*NA*	- 5.05	*NA*	*NA*	*NA*	Gamma-glutamyl phosphate reductase in *Dichomitus squalens*; Score= 1513; id= 68%
2618104	*NA*	*NA*	3.01	*NA*	*NA*	NAD(P)/FAD-dependent oxidoreductase in *Mycobacterium bohemicu*m; Score= 80; id= 38%
2618220	- 5.93	*NA*	2.62	*NA*	*NA*	Diguanylate cyclase in *Micromonospora* sp.; Score= 77; id= 49%
2620233	*NA*	*NA*	2.19	*NA*	*NA*	Putative Thiamin diphosphate-binding protein in *Moniliophthora roreri*; Score= 594; id= 53%
2620633	*NA*	*NA*	- 2.15	*NA*	*NA*	Dipeptidyl aminopeptidase in *Punctularia strigosozonata*; Score= 2631; id= 51%
2620649	*NA*	- 4.51	*NA*	*NA*	*NA*	P-loop containing nucleoside triphosphate hydrolase protein in *Dichomitus squalens*; Score= 820; id= 69%
2620672	- 4.49	- 3.80	*NA*	*NA*	*NA*	Cytidine deaminase in T*rametes versicolor*; Score= 521; id= 61%
2620687	- 5.11	*NA*	*NA*	*NA*	*NA*	no match
2620801	*NA*	3.48	*NA*	*NA*	*NA*	Predicted: flt3-interacting zinc finger protein 1- like in *Sus scrofa*; Score= 188; id= 19%
2620969	*NA*	*NA*	*NA*	1.92	*NA*	Cytochrome P450 in *Coniophora putaneana*; Score= 1779; id= 66%
2622299	*NA*	3.54	*NA*	*NA*	*NA*	Fasciclin-like protein in *Lenitinula edodes*; Score= 357; id= 42%
2622668	*NA*	*NA*	1.75	*NA*	*NA*	no match
2622778	*NA*	3.03	*NA*	*NA*	*NA*	Glycoside hydrolase in *Trametes versicolor*; Score= 826; id= 60%
2622931	- 4.07	*NA*	1.79	*NA*	*NA*	40 S ribosomal protein S1 in *Lenitula edodes*; Score= 1263; id= 96%
2623466	*NA*	*NA*	1.84	*NA*	*NA*	Spermidine synthase in *Laccaria bicolor*; Score= 1261; id= 36%
2623995	*NA*	- 3.08	*NA*	*NA*	*NA*	Growth arrest specific 2-like in *Mycena chlorophos*; Score= 790; id= 54%
2625127	- 1.02	- 5.66	*NA*	- 4.50	*NA*	Major facilitator superfamily domain-containing protein in *Coniella lustricola*; Score= 84; id= 57%
2625162	inf	*NA*	*NA*	*NA*	*NA*	Glycoside hydrolase family 13 protein in S*chizophyllum commune* H4-8; Score= 2468; id= 100%
2625300	*NA*	*NA*	*NA*	2.26	*NA*	HAMP domain-containing histidine kinase in *Micromonospora costi*; Score=87; id= 30%
2625317	*NA*	*NA*	*NA*	1.96	*NA*	Antibiotic biosynthesis monooxygenase in *Frateuria aurantia*; Score= 84; id= 26%
2625787	- 3.00	- 6.67	- 4.71	*NA*	*NA*	Splicing factor SF1 in *Coprinopsis cinerea okayama*; Score= 1759; id= 61%
2625792	*NA*	- 5.28	- 4.13	*NA*	*NA*	hypothetical proteins
2625917	*NA*	*NA*	- 2.03	*NA*	*NA*	no match
2626469	*NA*	*NA*	- 2.18	*NA*	*NA*	Predicted transporter (major facilitator superfamily) in *Aspergillus oryzae*; Score= 1759; id= 55%
2626537	*NA*	*NA*	- 1.70	*NA*	*NA*	no match
2626545	*NA*	3.78	*NA*	*NA*	*NA*	Serine/Threonine protein kinase in *Hahella chejuensis*; Score= 79; id= 38%

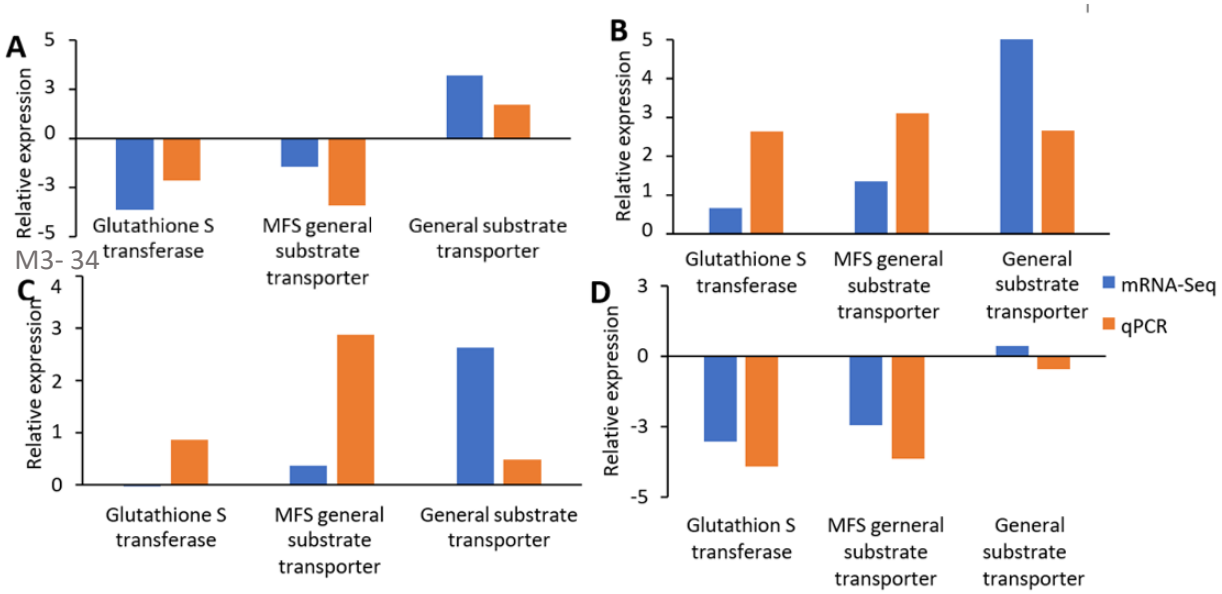
JGI transcript ID	2-fold change				Description KOG	BLAST hits at NCBI database
	Ch MM	Pa HM	Pa Ch	HM MM		
2626581	- 3.81	- 5.46	*NA*	2.86	*NA*	no match
2626803	- 3.12	- 3.76	*NA*	*NA*	*NA*	Bifunctional ornithine acetyltransferase/ N-acetylglutamate synthase in *Paenibacillus* sp.; Score= 92; id= 32%
2626893	*NA*	- 4.10	*NA*	*NA*	*NA*	hypothetical proteins
2626905	- 3.62	- 3.86	*NA*	*NA*	*NA*	Predicted: endothelial transcription factor GATA-2-like in *Acyrthosiphon pisum*; Score= 292; id= 26%
2627391	- 5.02	*NA*	*NA*	*NA*	*NA*	hypothetical proteins
2627477	- 3.76	*NA*	1.71	*NA*	Multidrug resistance-associated protein/mitoxantrone resistance protein, ABC superfamily	ABC transporter in *Laccaria bicollor*; Score= 4543; id= 61%
2627543	- 4.50	- 4.56	*NA*	*NA*	*NA*	Delta-sterol C-methyltransferase in *Punctularia strigosozonata*; Score= 1456; id= 77%
2627703	*NA*	*NA*	- 2.58	*NA*	*NA*	Adenine nucleotide transporter in *Coprinopsis cinerea okayama*; Score= 1169; id= 62%
2628645	- 4.19	- 5.30	*NA*	*NA*	*NA*	S-adenosyl-L-methionine-dependent methyltransferase, partial in *Coniophora putaneana*; Score= 531; id= 34%
2628670	*NA*	*NA*	*NA*	- 2.03	*NA*	Other/VPS15 protein kinase in *Coprinopsis cinerea okayama*; Score= 4988; id= 48%
2629026	*NA*	- 2.91	*NA*	*NA*	*NA*	Peptide methionine sulfoxide reductase in *Coprinopsis cinerea okayama*; Score= 840; id= 74%
2629146	*NA*	- 4.73	*NA*	*NA*	*NA*	Multidrug transporter in *Corallococcus* sp.; Score= 93; id= 34%
2629217	*NA*	*NA*	- 2.27	*NA*	*NA*	VPS9 domain protein in Rhizoctonia solani; Score= 658; id= 29%
2629242	*NA*	2.94	*NA*	*NA*	*NA*	Predicted: Parafibromin in *Drosophila eugracilis*; Score= 91; id= 33%
2629932	*NA*	- 5.20	- 2.63	*NA*	*NA*	C-4 methyl sterol oxidase in *Punctularia strigosozonata*; Score= 1200; id= 72%
2630516	- 4.29	- 4.31	*NA*	*NA*	*NA*	no match
2631634	- 4.39	*NA*	*NA*	*NA*	*NA*	High affinity nicotinic acid transporter in *Coprinopsis cinerea okayama*; Score= 1219; id= 51%
2631635	- 4.39	- 4.35	*NA*	*NA*	Permease of the major facilitator superfamily	High affinity nicotinic acid transporter in *Coprinopsis cinerea okayama*; Score= 1219; id= 51%
2632485	*NA*	- 3.96	*NA*	2.57	*NA*	Actin cytoskeleton-regulatory complex protein PAN1 *Ajellomyces capsulatus*; Score= 109; id= 10%
2633266	*NA*	- 3.85	*NA*	*NA*	*NA*	UV excision repair protein rhp23 in *Cryptococcus neofomrans*; Score= 503; id= 49%
2633361	- 6.72	- 7.81	*NA*	*NA*	*NA*	MFS general substrate transporter in *Coniophora putaneana*; Score= 251; id= 38%
2633382	*NA*	- 3.74	*NA*	*NA*	*NA*	Putative dehydrogenase E1 component subunit alpha, mitochndrial in *Apis mellifera*; Score= 83; id= 29%
2633499	- 4.89	- 3.84	*NA*	*NA*	Synaptic vesicle transporter SV2 (major facilitator superfamily)	MFS general substrate transporter in *Coniophora putaneana*; Score= 1332; id=52%

JGI transcript ID	2-fold change				Description KOG	BLAST hits at NCBI database
	Ch MM	Pa HM	Pa Ch	HM MM		
2633611	3.29	*NA*	*NA*	*NA*	*NA*	Sfk1 in *Hypsizygus marmoreus*; Score= 479; id= 62%
2633632	*NA*	- 3.90	*NA*	*NA*	*NA*	no match
2633699	- 6.53	- 6.64	- 2.09	*NA*	*NA*	EMILIN-3 (elastin microfibril interacter 3), partial in *Chlamydotis macqueenii*; Score= 80; id= 40%
2633705	*NA*	*NA*	- 3.48	*NA*	*NA*	hypothetical proteins
2633851	- 4.16	- 4.54	*NA*	*NA*	*NA*	hypothetical proteins
2633862	*NA*	- 3.95	*NA*	*NA*	*NA*	General substrate transporter in *Dichomitus squalens*; Score= 966; id= 70%
2634178	*NA*	*NA*	- 2.95	*NA*	*NA*	hypothetical proteins
2634274	*NA*	*NA*	- 2.47	*NA*	*NA*	Acetamidase Formamidase in *Coniophora puteana*; Score= 805; id= 82%
2634450	3.72	*NA*	*NA*	*NA*	*NA*	ECSIT domain containing protein in *Rhizoctonia solani*; Score= 340; id= 33%
2634654	*NA*	*NA*	2.13	- 2.70	*NA*	Non-structural maintenance of chromosome element 4 in *Hypsizygus marmoreus*; Score= 981; id= 56%
2636155	*NA*	*NA*	2.21	*NA*	*NA*	Cytochrome p450 in *Moniliophthora roreri*; Score= 1082; id= 47%
2636215	*NA*	*NA*	*NA*	2.35	*NA*	60 S ribosomal protein L18A in *Postia placenta*; Score= 807; id= 89%
2637065	- 3.48	- 4.09	*NA*	*NA*	*NA*	hypothetical proteins
2637255	*NA*	5.30	*NA*	*NA*	*NA*	RNA ligase in *Laccaria bicolor*; Score= 531; id= 69%
2637838	3.72	*NA*	*NA*	*NA*	*NA*	Ketosteroid isomerase-like protein in *Pseudomonas fluorescens*; Score= 125; id= 31%
2637900	- 5.00	- 5.63	*NA*	*NA*	*NA*	hypothetical proteins
2638021	*NA*	*NA*	- 1.84	*NA*	*NA*	hypothetical proteins
2638783	- 4.80	- 5.14	*NA*	*NA*	Synaptic vesicle transporter SV2 (major facilitator superfamily)	MFS general substrate transporter in *Coniophora puteana*; Score= 1630; id= 60%
2639986	*NA*	- 4.16	*NA*	*NA*	*NA*	hypothetical proteins
2640462	*NA*	- 4.72	*NA*	2.13	*NA*	no match
2640484	- 3.30	*NA*	*NA*	*NA*	*NA*	HNH endonuclease in *Rhizoctonia solani*; Score= 174; id= 31%
2640495	*NA*	- 4.36	- 2.04	*NA*	*NA*	Predicted: sodium channel *protein type 10 subunit alpha in Dipodomys ordii*; Score= 95; id= 28%
2640585	5.81	*NA*	*NA*	*NA*	*NA*	no match
2640596	4.73	*NA*	*NA*	*NA*	*NA*	hypothetical proteins
2640876	- 5.87	- 7.49	- 2.15	*NA*	*NA*	Tryptophan 2,3-dioxygenase in *Cobrinopsis mediterranea*; Score=848; id= 45%
2640893	*NA*	4.43	*NA*	*NA*	*NA*	hypothetical proteins
2640924	*NA*	*NA*	*NA*	1.87	*NA*	hypothetical proteins with DUF 4604 domain
2640999	- 4.68	- 6.31	*NA*	*NA*	*NA*	hypothetical protein
2641520	- 5.81	- 5.12	*NA*	*NA*	*NA*	Probable AMD2-amidase *Sporisorium reilianum*; Score= 232; id= 50%

JGI transcript ID	2-fold change				Description KOG	BLAST hits at NCBI database
	Ch MM	Pa HM	Pa Ch	HM MM		
2641735	*NA*	*NA*	*NA*	3.59	*NA*	Galactosyltransferase domain containing protein in *Rhizoctonia solani*; Score= 189; id= 37%
2642070	*NA*	- 3.60	*NA*	*NA*	*NA*	P-loop containing nucleoside triphosphate hydrolase protein in *Dichomitus squalens*; Score= 1551; id= 43%
2642686	- 4.15	- 4.64	*NA*	*NA*	*NA*	Heme peroxidase in *Punctularia strigosozonata*; Score= 2405; id= 41%
2642928	*NA*	inf	*NA*	*NA*	*NA*	Related to Actin-2 in *Armillaria ostoyae*; Score= 249; id= 33%
2644956	*NA*	*NA*	- 2.14	*NA*	*NA*	Glycoside hydrolase in *Auricularia delicata*; Score= 676; id= 50%
2645079	- 3.27	*NA*	*NA*	*NA*	*NA*	no match
2662082	- 3.71	*NA*	*NA*	*NA*	*NA*	N-myristoyl transferase in *Stereum hirsutum*; Score= 1770; id= 67%
2662683	- 3.53	- 5.29	*NA*	*NA*	*NA*	UBC-like protein in *Auricularia delicata*; Score= 515; id= 61%
2664319	2.90	*NA*	*NA*	*NA*	*NA*	Zinc ion binding in *Ascochyta rabiei*; Score= 129; id= 34%
2666269	*NA*	- 3.37	*NA*	*NA*	*NA*	Brt1 in *Schizophyllum commune*; Score= 669; id= 99%
2666280	- 3.73	- 4.55	*NA*	*NA*	*NA*	Ras-interacting protein 1 in *Bos taurus*; Score= 122; id= 24%
2666810	- 3.88	*NA*	*NA*	*NA*	*NA*	DUF1212 domain membrane protein in *Coprinopsis cinerea okayama*; Score= 1715; id= 56%
2667308	- 3.68	*NA*	*NA*	*NA*	*NA*	Proteophosphoglycan pp4 in *Leishmania mayor*; Score= 198; id= 22%
2668091	*NA*	- 3.43	*NA*	*NA*	*NA*	Alpha/beta-hydrolase in *Stereum hirsutum*; Score= 615; id= 48%
2668098	- 4.15	- 4.09	*NA*	*NA*	*NA*	hypothetical proteins
2668122	- 3.41	- 3.65	*NA*	*NA*	*NA*	Putative methyltransferase in *Punctularia strigoszonata*; Score= 972; id= 74%
2668417	- 3.40	*NA*	*NA*	*NA*	*NA*	Cysteine proteinase in *Punctularia strigosozonata*; Score= 1503; id= 52%
2668705	- 6.50	- 4.58	*NA*	*NA*	*NA*	Aldolase in *Trametes versicolor*; Score= 198; id= 19%
2669985	*NA*	*NA*	1.91	*NA*	*NA*	mtDNA inheritance protein Dml1 in *Laccaria bicolor*; Score= 891; id= 28%
2670889	- 3.12	*NA*	*NA*	*NA*	*NA*	Mannoprotein in *Coprinopsis cinerea okayama*; Score= 1092; id= 70%
2672425	*NA*	- 3.21	*NA*	*NA*	*NA*	HNH endonuclease in *Rhizoctonia solani*; Score= 174; id= 31%
2672502	*NA*	*NA*	*NA*	2.29	*NA*	Myo-inositol oxygenase in *Schizophyllum commune* ; Score= 1213; id= 100%
2672592	*NA*	- 3.40	*NA*	*NA*	*NA*	Glycosyltransferase family 32 protein in *Laccaria bicolor*; Score= 1425; id= 59%
2673438	*NA*	*NA*	1.83	*NA*	*NA*	GMC oxidoreductase in *Formitiporia mediterranea*; Score= 416; id= 49%
2677561	*NA*	*NA*	*NA*	- 4.30	*NA*	Proteophosphoglycan ppg3, putative in *Leishmania major*; Score= 204; id= 23%
2683542	2.95	*NA*	*NA*	2.16	*NA*	Enyl-CoA hydratase/isomerase family protein in *Erythrobacter* sp.; Score= 91; id= 61%
2685035	*NA*	*NA*	- 2.26	*NA*	*NA*	Amidohydrolase in *Coprinopsis cinerea okayama*; Score= 1482; id= 45%
2686334	- 3.77	*NA*	*NA*	*NA*	*NA*	Proteophosphoglycan 5 in *Leishmania major* strain Friedlin; Score= 431; id= 19%
2689334	*NA*	*NA*	2.24	*NA*	*NA*	Methionyl-tRNA synthase in *Trametes versicolor*; Score= 2466; id= 64%

JGI transcript ID	2-fold change				Description KOG	BLAST hits at NCBI database
	Ch MM	Pa HM	Pa Ch	HM MM		
2690020	-4.01	-5.68	*NA*	*NA*	*NA*	Alpha/beta-hydrolase in *Punctularia strigosozonata*; Score= 816; id= 38%
2694864	6.89	4.74	*NA*	*NA*	*NA*	Kinase-like protein in *Auricularia delicata*; Score= 710; id= 34%
2696261	3.74	*NA*	*NA*	*NA*	*NA*	hypothetical proteins
2696376	*NA*	*NA*	- 1.89	*NA*	*NA*	Duf domain containing protein in *Chromobacterium violaceum*; Score= 90; id= 23%
2697838	*NA*	*NA*	1.91	*NA*	*NA*	hypothetical proteins
2698689	*NA*	*NA*	*NA*	1.88	*NA*	hypothetical proteins
2700393	4.61	*NA*	- 2.24	*NA*	*NA*	UPF0183-domain containing protein in *Trametes versicolor*; Score= 1329; id= 66%
2701559	*NA*	*NA*	- 1.78	*NA*	*NA*	SH3-domain containing protein in *Dichomitus squalens*; Score= 387; id= 23%
2703529	- 5.88	- 3.23	2.48	*NA*	*NA*	FAD/NAD(P)-binding domain-containing protein in *Coniophora puteana*; Score= 1090; id= 22%
2703875	- 6.09	- 3.68	*NA*	- 1.95	*NA*	Proteophosphoglycan ppg4 in *Leishmania major;* Score= 256; id= 22%
2704336	- 3.43	*NA*	*NA*	*NA*	*NA*	hypothetical proteins
2705421	- 3.31	*NA*	*NA*	- 1.68	*NA*	Gag-pol polyprotein in *Moniliophthora rorei*; Score= 170; id= 28%
2706622	*NA*	*NA*	*NA*	1.71	*NA*	Nbs1 in *Moniliophthora roreri*; Score= 265; id= 35%
2706631	4.20	*NA*	*NA*	*NA*	*NA*	hypothetical proteins
2715898	*NA*	*NA*	- 1.74	*NA*	*NA*	HAD-IIIA family hydrolase in *Ruminococcus bromii*; Score= 91; id= 27%
2733368	*NA*	inf	*NA*	*NA*	*NA*	Possible amidase enhance in *Synechococcus* sp.; Score= 127; id= 8%
2746250	- 3.49	*NA*	*NA*	*NA*	*NA*	Transaldolase in *Punctularia strigosozonata*; Score= 1458; id= 87%

Shown are the four comparisons grown on Chernobyl soil compared to minimal medium (Ch MM), Paradies soil *versus* heavy metal-containing medium (Pa HM), Paradies soil *versus* Chernobyl soil (Pa Ch), heavy metal-containing *versus* minimal medium (HM MM). Data not available (NA), infinite (inf)



M3- 34

M3- 34

Suppl. Fig. S5: Verification of mRNA-Seq by qPCR. A: minimal medium *vs*. Chernobyl soil; B: heavy metal medium *vs*. Paradies soil; C: Paradies soil *vs*. Chernobyl soil; D: minimalmedium *vs*. heavy metal medium.

Suppl. Tab. S3: Comparisons for mRNA sequencing. Expression changes were compared between different cultivations and metal concentrations using mRNA sequencing (italics) andqPCR (bold) in the wild-type *S. commune* 12-43.

JGI Transcript ID, Description	2-fold change			
	MM vs. Ch	HM vs. Pa	Ch vs. Pa	MM vs. HM
2502252, General substrate transporter	*3.2* **1.7**	*5.5* **2.7**	*2.6* ***0.5***	*0.4** **-0.5**
2107770, MFS general substrate transporter	*-1.5** **-3.4**	*1.4** **3.1**	0.4* **2.9**	*-2.5* **-3.6**
2086920, Glutathione S-transferase	*-3.6* **-2.1**	*0.7** **2.6**	*-0.1** **-0.9**	*-3* **-3.9**

Values marked with * were not significant in the mRNA sequencing results. Ch= Chernobyl soil; MM=Minimal medium; Pa= Paradies soil; HM= Heavy metal medium.

Suppl. Tab. S4: Primers used in this study.

Primer name	NCBI ID	Gene	Sequence (5'-3')	Product length in bp	Primer efficiency (%)
mfs4	XM_003027220.1	Hypothetical Major Facilitator Superfamily general substrate transporter	For: GCAGCTAGATGCAAGAACGAGC Rev: GCCAGATAGGCCCCAATGTATAG	162	93
gtrans3	XM_003031963.1	Hypothetical general substrate transporter	For: GTCGAGAGATTCGGACGCAAG Rev: AACGACCCAAGGAACTGGTCC	198	95
gsto4	XM_003027462.2	Hypothetical gluthatione S-transferase	For: GCTTTTCGACGGCTGGGACAA Rev: CTTTAGCCACCTGTTGATCGCTG	193	98
act	XM_003026104.1	Actin-1	For: CTGCTCTTGTTATTGACAATGGTTCC Rev: AGGATACCACGCTTGGACTGAGC	178	96
tef	XM_003037215.1	Translation elongation factor 1a	For: AGCTCGGCAAGGGTTCCTTCA Rev: AACTTCCAGAGGGCGATATCA	97	97
ubi	XM_003036409.1	Ubiquitin-conjugating-protein	For: GAAGGAGTACGATGCGAAGG Rev: TCCTCCTCTGCCTTCTTGC	93	90

Experimental Procedures for working with RNAs

Metal determination by ICP-MS

Microwave digestion of 0.2 g dried and ground mycelium were mixed with 5 ml 65% nitric acid, 25 ml distilled water, and incubated for 10 min at RT. The samples were heated to 180°C for 15 minutes in an air-tight and pressure-tight vial in a microwave (MARS 5 Xpress, CEM, Matthews, USA), the temperature was maintained for a further 15 minutes and cooled for 30 minutes. The digested samples were centrifuged at 3000 rpm for 15 min and the supernatant was adjusted to a pH of 2 with nitric acid. Both total digestion and the bioavailable fractions 1 and 2 of a sequential extraction of Chernobyl soil (Zeien and Brümmer, 1989) were analyzed by ICP-MS (XSeries II, ThermoFischer Scientific, Waltham, USA).

Staining and microscopy

Both fungal strains were grown for 5 days on MM+ura with and without 0.2 mM $CdCl_2$ topped with 4 coverslips per petri dish. Single hyphae grew over the coverslips. Those were stained with Leadmium Green AM (ThermoFisher Scientific, Waltham, USA) that stains Cd and Pb, and Synapto Red (FM4-64, Biotium, Apprieu, France) to visualize vacuoles and vesicles. Forstaining, 30 µM Synapto Red was added to the coverslips and incubated for 20 min, before adding the Leadmium Green dye (50 µg Leadmium green dissolved in 50 µl DMSO and 1:10diluted with 0.9 % NaCl). After incubation for 2 h at RT, the coverslips were washed three times with ½ MM+ura.

For fluorescence microscopy, Axioplan 2 microscope (Carl Zeiss, Jena, Germany) was used and images were recorded with a SPOT Insight FireWire camera (Diagnostic Instruments, Munich, Germany). Confocal laser scanning microscopy (LSM 780, Carl Zeiss, Jena, Germany) using 40x/1.30 NA EC Plan-Neofluar objective at a laser intensity of 0.2 % and a wavelength of 514 nm (Synapto red)

and 488 nm (Leadmium green) was used with detectionin a range of 474-518 nm. Images were calculated and finalized with the program Zen2 (blueedition).

mRNA sequencing and microarray analysis

S. commune 12-43 was grown for 4 weeks on 100 g of sterilized soil from the Chernobyl Exclusion Zone (51°20’54’’ N 30°07’41’’E) and contaminated soil from a park (50°55'22.2"N 11°35'10.0"E) with 50 ml ½ MM+ura added to the soil to promote faster growth at 16°C to mimicking natural conditions. Autoclaving was used since previous experiments had confirmed sterility as well as low impact on changes in metal availability (Kraußе et al., 2019). The metal concentration in the soils was determined by ICP-MS (compare suppl. Tab. S1).

S. commune was inoculated and incubated to liquid MM+ura with and without the added heavy metal composition found in the soil analyses of the park soil with growth for 2 weeks at 125 rpm and 16°C, to exclude effects of growth on structured surfaces in soil. The harvested mycelium was homogenized by grinding with liquid nitrogen. From approx. 100 mg mycelium, RNA was extracted (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany). The mRNA isolation, library preparation, TopHat alignment to *S. commune* genome (https://mycocosm.jgi.doe.gov/Schco3/Schco3.home.html) and bioinformatic pairwise comparisons with Cuffdiff 2 workflow were done by StarSeq (Mainz, Germany; Trapnell et al.,2012). Significance was assumed when $p \leq 0.005$ and $q \leq 0.05$.

A microarray analysis previously published (Freihorst et al., 2018) was used to verify results. From that analysis, we used the comparisons between *S. commune* 12-43 grown on MM+ura and grown with contaminated seepage water (HSW), as well as the co-isogenic *S. commune* W22 grown with cadmium.

qRT-PCR

For verification of RNA sequencing results, the wild-type and adapted strains were grown at 28°C for 5 days on cellophane-topped MM+ura plates with 75 mM $SrCl_2$, 50 mM CsCl, 5 mM $ZnCl_2$, 0.2 mM $CdCl_2$, or the concentration of the 5 most abundant bioavailable metals in Chernobyl soil (suppl. Tab S3). 100 mg mycelium was homogenized with liquid nitrogen and total RNA was isolated (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany). cDNA was synthesized from 500 ng RNA (QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany).

qRT-PCR was performed with the qTOWER^3 from AnalytikJena (Jena, Germany). Each reaction consisted of 3.125 µl Maxima SYBR Green/ROX qPCR Mastermix (2x; ThermoFischer Scientific, Waltham, USA), 0.5 µl each of 10 pmol/µl primers (suppl. Tab. S4), 1.125 µl water, and 1 µl cDNA 1:10 diluted. The qPCR was run with 10 min initial denaturation at 94°C, 40 cycles of 20 sec 94°C, 20 sec 59°C, and 20 sec 72°C, followed by 2 min final elongation at 72°C. A melting curve was measured after every run from 60 to 94°C with Δ1°C per 30 sec. All measurements were performed in 3 biological and 3 technical replicates with a negative control and a “no reverse transcriptase” control each. Relative expression was calculated (Pfaffl, 2001) using the housekeeping genes actin1 (XM_003026104.1), ubiquitin (XM_003036409.1) and translation elongation factor 1α (XM_003037215.1). Significance was tested with a two-tailored t-test with unequal variancesand a p-value of ≤ 0.05.

4.4 Manuscript 4: Inositol signaling in the Basidiomycete fungus *Schizophyllum commune*

Manuscript number: 4

Title of the manuscript: Inositol signaling in the Basidiomycete fungus *Schizophyllum commune*

Authors: Reyna Murry, Lea Traxler (contribution 35 %), Jessica Pötschner, Thomas Krüger, Olaf Kniemeyer, Katrin Krause, Erika Kothe

Bibliographic Reference: Murry, R., Traxler, L., Pötschner, J., Krüger, T., Kniemeyer, O., Krause, K., Kothe, E. (2021) Inositol Signaling in the Basidiomycete Fungus *Schizophyllum commune*. *J. Fungi* 7, 470.

The candidate is:

□ **First author, x Co-First author,** □ **Corresponding author,** □ **Co-author.**

Status: Published in *Journal of Fungi*

Author	Concept	Data analysis	Experiments	Writing of manuscript	Provision of material
Reyna Murry	x 35%	x 40%	x 35%	x 35%	
Lea Traxler	x 15%	x 40%	x 50%	x 35%	
Jessica Pötschner			x 15%		
Thomas Krüger		x 20%			
Olaf Kniemeyer					x 20%
Katrin Krause	x 10%			x 10%	
Erika Kothe	x 40%			x 20%	x 80%

4.4.1 Summary

This study investigates the role of inositol signaling in basidiomycetes, since it is already known that inositol signaling is involved in a large number of physiological processes in other fungi. Therefore, the key enzyme inositol monophosphatase was overexpressed in *S. commune* T33 and a mutant was produced, which were used for the following experiments. We could show both that the inositol cycle is involved in metal tolerance, and also an influence of metal stress on this signaling pathway. An important role in cell wall integrity and vesicle trafficking could also be proven. The role of inositol signaling for cellular trafficking could be confirmed by means of proteome analysis. In addition, we were able to develop a model that links the Ras pathway, which has already been researched, with the inositol pathway in *S. commune*.

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Inositol Signaling in the Basidiomycete Fungus *Schizophyllum commune*

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Abstract: Intracellular signaling is conserved in eukaryotes to allow for response to extracellular signals and to regulate development and cellular functions. In fungi, inositol phosphate signaling has been shown to be involved in growth, sexual reproduction, and metabolic adaptation. However, reports on mushroom-forming fungi are lacking so far. In *Schizophyllum commune*, an inositol monophosphatase has been found up-regulated during sexual development. The enzyme is crucial for inositol cycling, where it catalyzes the last step of inositol phosphate metabolism, restoring the inositol pool from the monophosphorylated inositol monophosphate. We overexpressed the gene in this model basidiomycete and verified its involvement in cell wall integrity and intracellular trafficking. Strong phenotypes in mushroom formation and cell metabolism were evidenced by proteome analyses. In addition, altered inositol signaling was shown to be involved in tolerance towards cesium and zinc, and increased metal tolerance towards cadmium, associated with induced expression of kinases and repression of phosphatases within the inositol cycle. The presence of the heavy metals Sr, Cs, Cd, and Zn lowered intracellular calcium levels. We could develop a model integrating inositol signaling in the known signal transduction pathways governed by Ras, G-protein coupled receptors, and cAMP, and elucidate their different roles in development.

Keywords: inositol phosphate signaling; inositol monophosphatase; cell wall integrity; sexual development; intracellular trafficking; heavy metals

Citation: Murry, R.; Traxler, L.; Pötschner, J.; Krüger, T.; Kniemeyer, O.; Krause, K.; Kothe, E. Inositol Signaling in the Basidiomycete Fungus *Schizophyllum commune*. *J. Fungi* **2021**, *7*, 470. https://doi.org/10.3390/jof7060470

Academic Editor: Ulrich Kück

Received: 20 May 2021
Accepted: 7 June 2021
Published: 10 June 2021

1. Introduction

Schizophyllum commune is a saprotrophic white-rot fungus that can complete its life cycle within two weeks on artificial media. It has a long haploid growth phase, and the full genome of *S. commune* has been published [1]. The basidiomycete is accessible to transformation and genetic modification [2–4]. With regard to mating, *S. commune* has been used extensively to study signaling pathways [5,6] involving inositol signaling [7–10].

Inositol phosphate signaling has been intensively studied since the 1980s [11,12]. In ascomycete and yeast-forming fungi, inositol phosphate signaling plays a major role in a wide range of biological processes, including metabolic adaptation, apoptosis, fungal virulence, vesicle trafficking, and sexual development [13–16]. The filamentous basidiomycete fungus *S. commune* could be shown to include phosphate signaling in its life cycle. The first evidence for inositol and Ras signaling cross-talk was suggested to be in phosphate storage [8,10].

The tetrapolar mating system of basidiomycetes is controlled by two non-linked loci. The *A* locus encoding several pairs of homeodomain transcription factors with

multiple mating specificities in each allele, and the *B* loci with a pheromone receptor system allow for the recognition of multiple non-self-specificity pheromones with each of the two pheromone receptors. This elaborate mating system controls the formation of the dikaryon that is capable of forming fruiting bodies in response to extracellular signals under natural growth conditions. Since mushroom development is of importance for food production and ecosystem functions, the investigation of signal perception and transduction with its integration into sexual development is of broad interest (see [1]).

An extracellular stimulus that activates a membrane-associated phospholipase C (PLC) leads to cleavage of phosphatidylinositol-4,5-bisphosphate (PIP2) into diacylglycerol (DAG), which stays at the membrane, and soluble inositol 1,4,5-triphosphate IP_3 (Figure 1) [17,18]. Through DAG, protein kinase activation leads to Ca^{2+} channel opening and subsequent calcium influx [19]. IP_3 is involved in calcium release from the endoplasmic reticulum [20], phosphate storage via polyphosphorylated inositol phosphates (up to IP_7 and IP_8) [10,21,22] and connected to other signaling cascades, including Ras signaling [8,10,23]. The cycle is restored through IP_2 and IP_1, which are dephosphorylated to inositol by the key enzyme, inositol monophosphatase (IMPase). This enzyme is specifically inhibited by lithium, which leads to lower inositol and calcium levels in the cell [24]. The sugar inositol can enter catabolic pathways via inositol oxygenase activity forming glucuronic acid [25]. Inositol monophosphate (IP_1) and inositol monophosphatase (IMPase), thus, are key players in a highly complex network of signaling pathways.

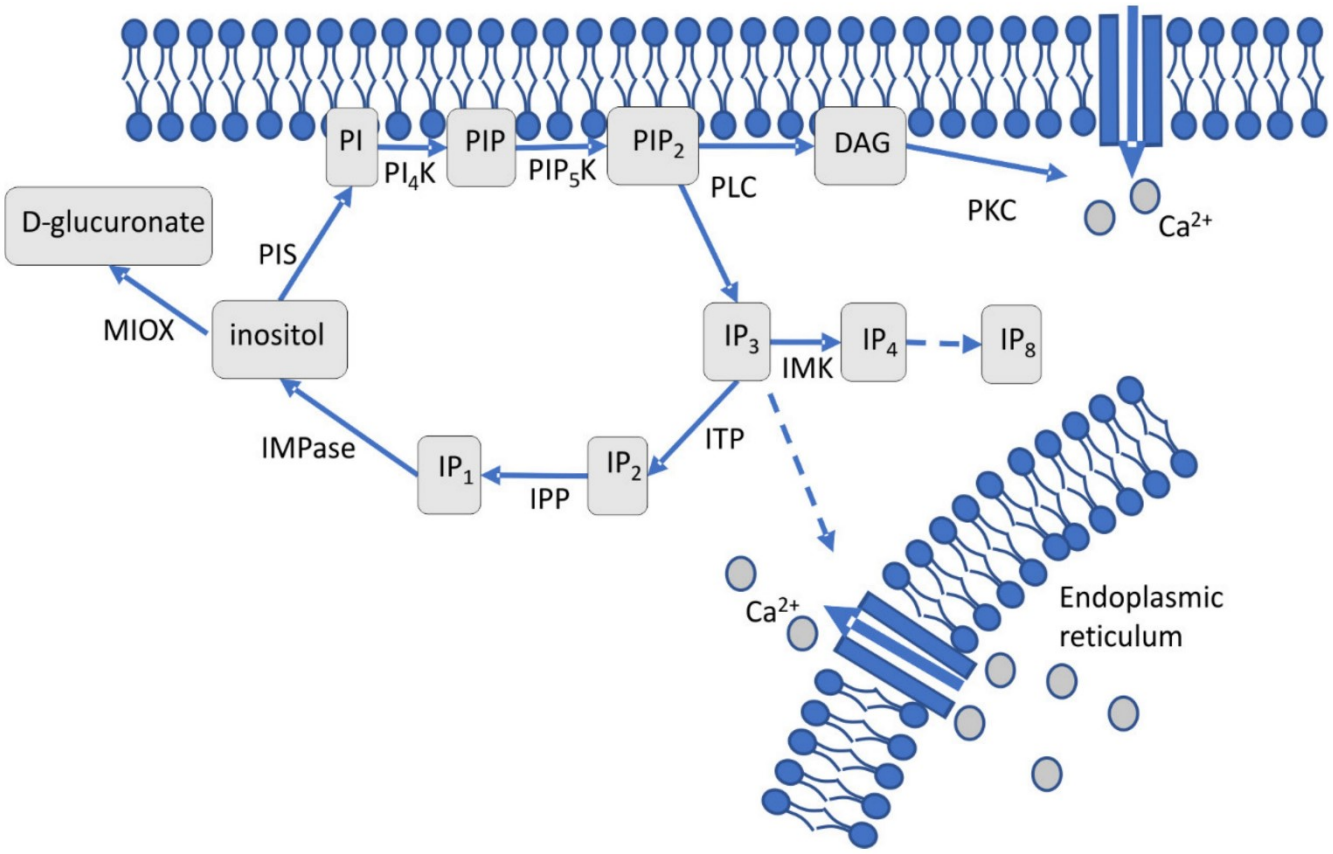

Figure 1. Schematic representation of cellular inositol signaling. Phosphatidylinositol specific phospholipase C (PLC) cleaves phosphatidylinositol (PI)-4,5-bisphosphate (PIP_2) generating two second messengers, diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3). IP_3 can be diverted into higher phosphorylated IP_4 through IP_8 or dephosphorylated to inositol through IP_2 and IP_1. Inositol, regenerated by IMPase from IP_1, is the precursor of membrane-bound PIP_2.

The enzyme IMPase is quite well studied and known to require magnesium and to be non-competitively inhibited by lithium [26]. Therefore, in humans, the enzyme is

addressed in the therapy of manic-depressive disorder with lithium-based drugs [27]. In euryhaline eel, IMPase plays a role in the adaptation to hypertonic conditions [28], while in plants, it contributes to drought and salt resistance, as well as response to pathogens and symbionts [29]. In ascomycetes, yeasts and the filamentous fungus *Podospora anserina* have been studied [16]. IMPase is highly conserved among basidiomycetes, including the yeast-forming *Cryptococcus neoformans* [13]. To understand the role in *S. commune*, we used overexpression of IMPase and discovered potential roles in cell wall integrity and cellular trafficking. Since it has been reported that inositol signaling is also impaired under metal stress [30], we were interested in inositol signaling in basidiomycete fungi, using the model organism *S. commune*. For this purpose, IMPase overexpression mutants were compared with wildtypes for their metal tolerance against Sr, Cs, Cd, and Zn. Regulation of the genes belonging to inositol signaling was assessed with respect to metal stress using microarray and proteome analyses [31].

2. Materials and Methods

1. *Phylogenetic Analysis and Chromosomal Organization of imp1*

To construct a phylogenetic tree, the genome sequence [1] was used for sequence searches at https://mycocosm.jgi.doe.gov and https://www.ncbi.nlm.nih.gov, visualized using Launch Jalview Desktop (http://www.jalview.org/; all accessed on 24 October 2018). Tree calculation with MrBayes was carried out via the online tool CIPRES Science Gateway and visualized using FigTree v1.4.3. If necessary, tree captions and information were edited using Adobe Illustrator.

The chromosomal organization based on predicted genes, size, and functional data from the genome sequence was displayed with ChromoMapper.

2. *Fungal Growth and Microscopy*

S. commune (strains listed in Supplementary Table S1) was used to construct *imp1* overexpressing strains OEIMP4 and OEIMP6 (primers, Supplementary Table S2; for construction, Supplementary Figure S1) and an empty vector control EVC. The gene *imp1* under control of the constitutive, strong *tef1* promotor was cloned using yeast recombination of amplified sequences (Q5 High-Fidelity polymerase, New England Biolabs, Ipswich, USA) using primers with 3′ and 5′ overlap (Supplementary Table S2). Shuttle plasmid pRS415 was linearized with *Sac*I and *Sac*II, the *Hind*III/*Pci*I fragment (2.9 kb) ligated into pSKtrp resulting in pOEIMP. Circular plasmid pOEIMP was introduced in *S. commune* T33 according to [32]. Transformants were screened by PCR using primers 5F and 5R (see Supplementary Table S2).

Strains were cultivated on CYM [33] complemented with 4 g/L tryptophan if needed. Calcofluor white (0, 125, 250, 500 µg/mL), Congo red (0, 125, 250, 500 µg/mL) and SDS (0, 20, 60, 100 µg/mL) were added for respective analyses for 10 days. Growth was checked measuring the mycelium diameter every day and significant differences between treated and control cultures were confirmed by Student's *t*-test (p-value ≤ 0.05).

To visualize cellular trafficking, SynaptoRed FM 4-64 (3.2 µM for 40 min, washed twice with PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) was used after 3 days of cultivation in 24 well plates (Greiner Bio One, Frickenhausen, Germany) at room temperature with 150 µL agar and 150 µL liquid medium to keep the culture moist. The sample was directly observed (CLSM Zeiss LSM 780, Jena, Germany; 40×/1.30 NA EC Plan-Neofluar objective and transmission light or fluorescence Ar/ML 488/514 nm laser with 0.2% intensity). The signal from 622 nm to 759 nm was detected with GaAsP detector. Pinhole size and laser dwell time were minimized. Finalization and intensity measurements of images were carried out with the program Zen2012 (Zeiss, Jena, Germany).

For growth of *S. commune* with metal stress, monokaryotic *S. commune* 12-43 (*ura*$^-$) and S. commune T33 (*trp*$^-$, *ura*$^-$), the empty vector control and the inositol monophosphatase overexpressing *S. commune* OEIMP4 were cultivated on minimal medium (MM + ura +

trp: 20 g/L glucose, 2 g/L aspartic acid, 1 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.5 g/L MgSO4, 60 µg/L thiamine hydrochloride, 11.2 mg uracil, 1 g tryptophan, 16 g/L agar; pH 6) [34] at 28 °C for 14 days. Metal salt solutions of $SrCl_2$, CsCl, $ZnCl_2$ or $CdCl_2$ were added to the medium after autoclaving. Growth rates were calculated from the mycelial diameter in two dimensions, with all experiments in triplicates and significant differences confirmed by *t*-test (*p*-value ≤ 0.05).

2.3. Overexpression of imp1

RNA isolation was performed based on manufacturer's protocol (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany). A total of 500 ng RNA were transcribed to cDNA (Quanti-Tect Reverse Transcription, Qiagen, Hilden, Germany). All experimental cDNA samples were assayed in triplicates. Expression of *imp1* was normalized using *tef1* as a reference after qRT-PCR (smart Cycler II and 25 µL Cepheid reaction tubes, Cepheid, Sunnyval, USA). The qPCR mix consisted of 12.5 µL Maxima SYBR Green/ROX qPCR Master mix (Thermo Fisher Scientific, Hamburg, Germany), 0.5 µL of 10 pmol/µL of each forward and reverse primers (see Table S2), 9.5 µL DNAse, RNAse, pyrogenase free water (Carl Roth, Karlsruhe, Germany), and 2 µL of 25 ng/µL cDNA. The cycling program contained a gradient: 95 °C for 120 s, 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 15 s; 40 cycles of 60–90 °C for 0.2 °C/s.

2.4. Calcium Levels under Metal Stress

S. commune 12-43 was grown on MM + ura with either 100 mM $SrCl_2$, 75 mM CsCl, 10 mM $ZnCl_2$, or 0.5 mM $CdCl_2$ topped with a cellophane foil. After 14 days at 28 °C, the mycelium was harvested with a plastic spatula and dried for 18 h at 70 °C until constant weight and the mycelium homogenized in an agate mortar. The experiment was performed in five replicates. Ca as well as metal contents were measured in a mixed sample of five biological replicates after microwave extraction using ICP-MS (Inductively Coupled Plasma - Mass Spectrometry, XSeries II, Thermo Fisher Scientific, Bremen, Germany; for details compare [35]).

2.5. Proteome Study

Empty vector control (EVC1) and *imp1* overexpressing *S. commune* OEIMP4 were incubated as before and proteins, enriched for membrane associated proteins, were extracted (ReadyPrep Protein Extraction Kit Signal, Bio-Rad, Munich, Germany) based on manufacturer's protocol after grinding mycelia with liquid nitrogen. A sonication step (VCX 130 PB, Sonics and Material, Newtown, CT, USA) was performed, and concentrations of isolated proteins were determined (Sigma-Aldrich, Darmstadt, Germany) [36]. To purify the protein, 50 µM protein were loaded onto 10% polyacrylamide gels. After electrophoresis, gels were washed two times with ultrapure water, protein bands were excised and cut into cubes of approx. 1 mm^3 and transferred to 1.5 mL tubes. In-gel digestion was performed [37] using 50 ng/µL trypsin-LysC (mass spec grade, Promega, Mannheim, Germany) in 50 mM NH_4HCO_3. Peptides were extracted with trifluoroacetic acid (TFA) and increasing concentrations of acetonitrile (50, 70, and 90%), purified with 10 kDa MWCO filters (VWR, Langenfeld, Germany), and dried peptides solubilized in MS buffer (0.05% TFA in 2% acetonitrile–98% H_2O) before being subjected to LC-MS/MS analysis (Ultimate 3000 nano RSLC, QExactive HF, Thermo Fisher Scientific, Waltham, MA, USA). Initial peptide trapping for 5 min on an Acclaim Pep Map 100 column (2 cm × 75 µm, 3 µm) at 5 µL/min was followed by separation on an analytical Acclaim Pep Map RSLC nano column (50 cm × 75 µm, 2 µm). Mobile phase gradient elution of eluent A (0.1% *v/v* formic acid in water) mixed with eluent B (0.1% *v/v* formic acid in 90/10 acetonitrile/water) was performed (buffer B for 0 min 4%, 5 min 5%, 15 min 6%, 100 min 8%, 150 min 12%, 250 min 23%, 300 min 34%, 320 min 41%, 340 min 52%, 350 min 60%, 360 min 75%, 365–375 min 96%, 375.1–400 min 4%). Positively charged ions were generated at a spray voltage of 2.2 kV using a stainless-steel emitter attached to the Nanospray Flex Ion Source (Thermo Fisher

Scientific, Waltham, USA). The quadrupole/orbitrap instrument was operated in Full MS/data-dependent MS2 (top10) mode. Precursor ions were monitored at m/z 300–1500 at a resolution of 120k FWHM using a maximum injection time of 100 msec and an automatic gain control target of 1e6. HCD fragmentation at 30% normalized collision energy generated MS2 ions, which were scanned at 15 k FWHM (ITmax = 100 ms, AGC = $2{\cdot}10^5$). Dynamic exclusion of precursor ions was set to 35 s. The LC-MS/MS instrument was controlled by Chromeleon 7.2, QExactive HF Tune 2.8, and Xcalibur 4.0 software (Thermo Fisher Scientific, Waltham, MA, USA).

The obtained tandem mass spectra were searched against the JGI database of *S. commune* H4-8 v3.0 (https://mycocosm.jgi.doe.gov/Schco3/Schco3.home.html; accessed on 2 February 2017) using Proteome Discoverer PD 2.1 and the algorithms of Mascot 2.4, Sequest HT, and MS Amanda. Two missed cleavages were allowed for trypsin digestion. The precursor mass tolerance was set to 10 ppm and the fragment mass tolerance was set to 0.02 Da. Modifications were defined as dynamic Met oxidation and static Cys carbamidomethylation. At least two peptides per protein and a strict false discovery rate < 1% (reverse decoy) were required. Label-free quantification of the Top 3 unique peptides per protein was based on the precursor ion area detector approach implemented in PD 2.1. For quantification, data were normalized using the total peptide amount approach. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [38] partner repository with the dataset identifier PXD026465.

2.6. Transcriptome Analyses

An mRNA sequencing approach was analyzed and enzymes of inositol signaling were found to be significantly regulated under metal stress [39]. The two significantly regulated genes, myo-inositol oxygenase (XM_003027475.1) and a hypothetical inositol polyphosphate phosphatase (XM_003036036.1) were selected to confirm regulation under metal stress using qPCR.

RNA was extracted from mycelium of *S. commune* 12-43 grown with 75 mM $SrCl_2$, 50 mM CsCl, 5 mM $ZnCl_2$, or 0.2 mM $CdCl_2$ (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany). From 500 ng RNA, cDNA was synthesized (QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany). The qRT-PCR was performed with the qTOWER3 from AnalytikJena (Jena, Germany). Each reaction consisted of 3.125 µL Maxima SYBR Green/ROX qPCR Mastermix (2x; ThermoFischer Scientific, Waltham, USA), 0.5 µL each of 10 pmol/µL primers, see Supplementary Table S2), 1.125 µL water and 1 µL cDNA 1:10 diluted. The qPCR was run with 10 min initial denaturation at 94 °C, 40 cycles of 20 s 94 °C, 20 s 59 °C, and 20 s 72 °C followed by 2 min final elongation. A melting curve was measured after every run from 60 to 94 °C with Δ1 °C per 30 s. All measurements were done in three biological and three technical replicates, with each a negative control and a "no reverse transcriptase" control. Relative expression was calculated using the genes coding for actin 1 (XM_003026104.1), ubiquitin (XM_003036409.1) and translation elongation factor1α (XM_003037215.1) [40]. Significance was tested with a two-tailored *t*-test with unequal variances and a *p*-value of ≤0.05.

Microarray data (GEO omnibus acc. no. GSE172373) were evaluated for regulation of enzymes involved in inositol signaling. In the microarray, the gene regulation of *S. commune* 12-43 grown on complex yeast medium [33] was compared with growth in presence of multi-heavy metal containing seepage water and to co-isogenic *S. commune* W22 grown with 0.01 mM cadmium nitrate. All enzymes of inositol signaling that were regulated under metal stress were selected from this microarray data.

3. Results

1. *A Basidiomycete Clade of IMPases*

The gene coding for IMPase, *imp1,* is located in scaffold 2 of the genome of *S. commune* H4-8 and has a coding region of 1247 bp interrupted by five introns. IMPase is highly conserved among basidiomycetes, and the single gene *imp1* in *S. commune* encodes

340 aa containing all three motifs for substrate and metal binding, and nucleophilic activation. Alignments and phylogenetic analysis including human, yeast, and basidiomycete sequences revealed the existence of different evolutionary clades in IMPases (Figure 2). The highest aa identity was shared between the *S. commune*, *Laccaria amethystina* (67%), *Laccaria bicolor* (67%), *Termitomyces* (66.5%), and *Fistulina hepatica* (64%) sequences, all of basidiomycete origin.

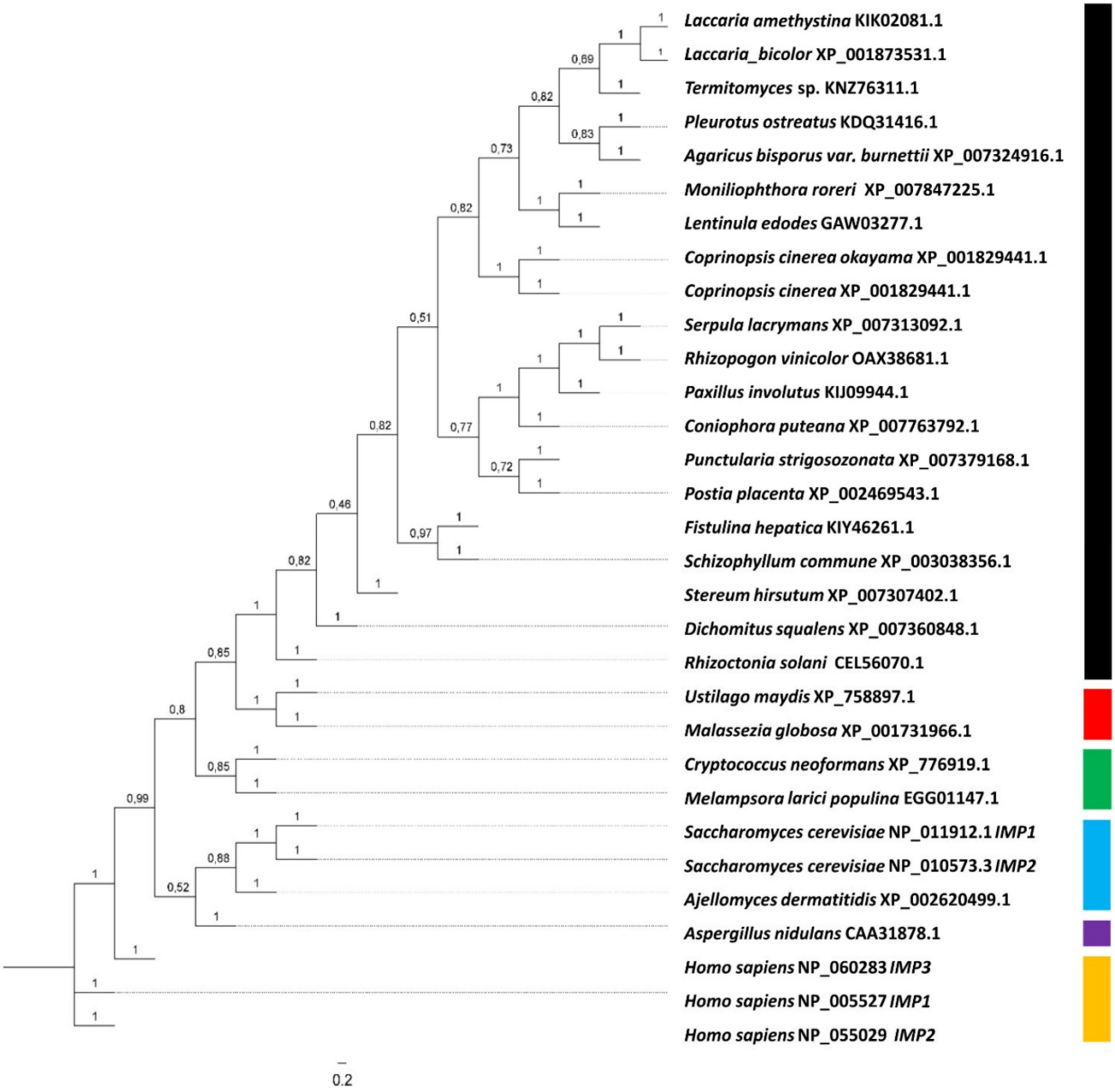

Figure 2. Phylogenetic tree for IMPases. Accession numbers (GeneBank) are given for all sequences. IMPase clades are indicated with filamentous Basidiomycota (black), yeast-like Basidiomycota I (red), yeast-like Basidiomycota II (green), yeast-like Ascomycota (blue), filamentous Ascomycota (purple) und human IMPases (yellow).

Surrounding genes included a conserved zinc finger transcription factor and a chaperone of the DnaJ superfamily. This direct neighborhood is conserved with a zinc finger protein, *zfand1,* and also with the human gene coding for inositol monophosphatase 1.

3.2. Imp1 Overexpression Leads to Improved Cell Wall Integrity

Overexpression was achieved with the gene *imp1* under the control of the strong promotor of *tef1*, originally leading to constitutive expression of translation elongation factor EF1α. The overexpression was verified by RT-qPCR (Supplementary Figure S2). A remarkable increase of 42- and 67-fold up-regulation was confirmed for the two independent transformants, OEIMP4 and OEIMP6, respectively. Growth was only slightly retarded upon long term incubation for *imp1* overexpressing strains as compared to a wildtype and the empty vector control without obvious changes in morphology of mycelia (Figure 3).

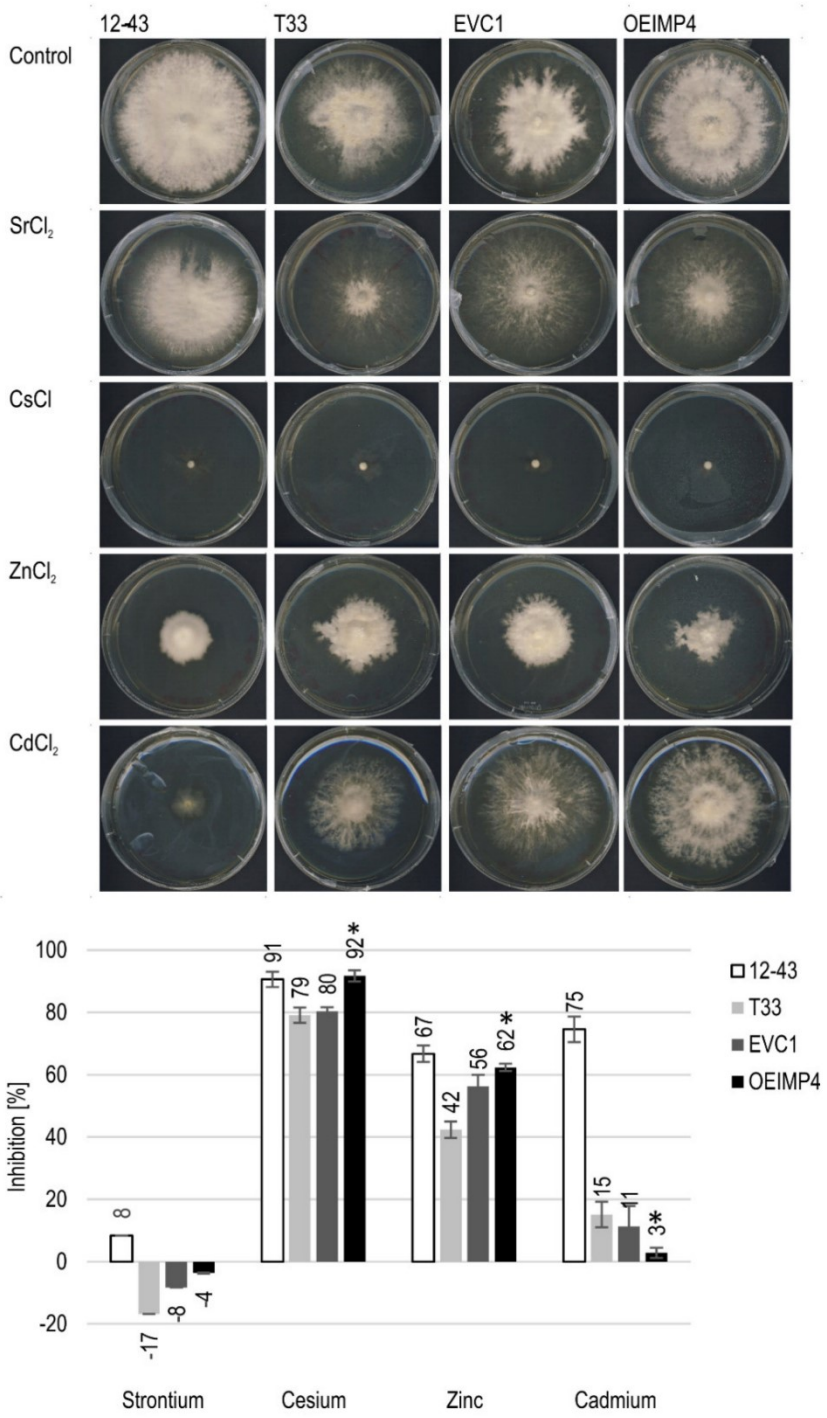

Figure 3. Growth inhibition of metals. Growth morphology (**top**) and inhibition calculated from mycelia diameter (**bottom**) of *S. commune* 12-43, T33, EVC1 and OEIMP4 in three biological replicates.

A more detailed analysis of cellular effects of overexpression revealed a role in cell wall integrity, as decreased susceptibility against calcofluor white, binding to chitin, Congo red for specific polysaccharide staining, and SDS as membrane-active detergent were observed (Supplementary Figure S4). The results indicate that Imp1 and inositol signaling might play a role in biosynthesis and organization of the cell wall of *S. commune*. Since stability against cell wall stress was observed, growth with heavy metals exerting such stress was screened.

3.3. Influence of Altered Inositol Signaling on Metal Tolerance

The influence of altered inositol signals on metal tolerance was investigated by exposing IMPase-overexpressing *S. commune* OEIMP4 to heavy metal stress. For all four tested metals, the strain *S. commune* 12-43 performed inferior as compared to *S. commune* T33 and its derivatives, *S. commune* EVC1 and *S. commune* OEIMP4 (Figure 3). In addition to higher inhibition through metals, *S. commune* 12-43 formed more aerial mycelium. The remaining three strains all showed, unexpectedly, improved growth with Sr. However, this is due to the fact that aerial mycelium was reduced in favor of more (and thus faster) substrate hyphae formation. The inositol monophosphatase overexpression led to a significantly higher inhibition by Cs and Zn, which was not seen with Cd.

The intracellular calcium levels were distinctly reduced in *S. commune* 12-43 grown in the presence of heavy metals. The difference was most evident in the presence of Sr, but clearly visible also for Cs and Cd, where calcium levels were reduced (Table 1).

Table 1. Calcium concentrations in *S. commune* 12-43 grown under metal stress.

	Control	100 mM $SrCl_2$	75 mM CsCl	10 mM $ZnCl_2$	0.5 mM $CdCl_2$
Ca [µg/g]	1626 ± 25	219.83 ± 0.02	282 ± 2	234 ± 6	393 ± 2

3.4. Inositol Signaling Related Gene Regulation in Metal Stress

The general direction of the gene regulation of the two genes of interest could be confirmed using qPCR. Exceptions were the comparisons of minimal medium versus Chernobyl soil for the hypothetical inositol polyphosphate phosphatase gene and heavy metal medium versus Paradies park soil for the *myo*-inositol oxygenase gene (Figure 4). The two microarray comparisons (Supplementary Table S3) showed that increased metal stress lead to a higher expression of both genes. This also tended to be the case for the first three comparisons of the mRNA sequencing, but not for the comparison minimal medium versus heavy metal medium. The qPCR investigation confirmed that the hypothetical polyphosphate phosphatase gene was highly up-regulated under the influence of all metals tested. The Myo-inositol oxygenase gene just tended to be up-regulated under metal stress. However, in the presence of zinc and cadmium, no regulation could be recognized and clear down-regulation in the case of strontium.

The two comparisons of the microarray data mostly reacted under metal stress with gene regulation in the same direction for the same genes. A difference was most likely to be seen in the regulation of protein kinase C, which occurs outside of the immediate inositol cycle (see Figure 1). It could also be seen that under metal stress, kinases tended to be up and phosphatase tended to be down-regulated (Table S3). Down-regulation was clearly observed for inositol triphosphate phosphatase, which catalyzes the phosphorylation of inositol triphosphate to inositol diphosphate.

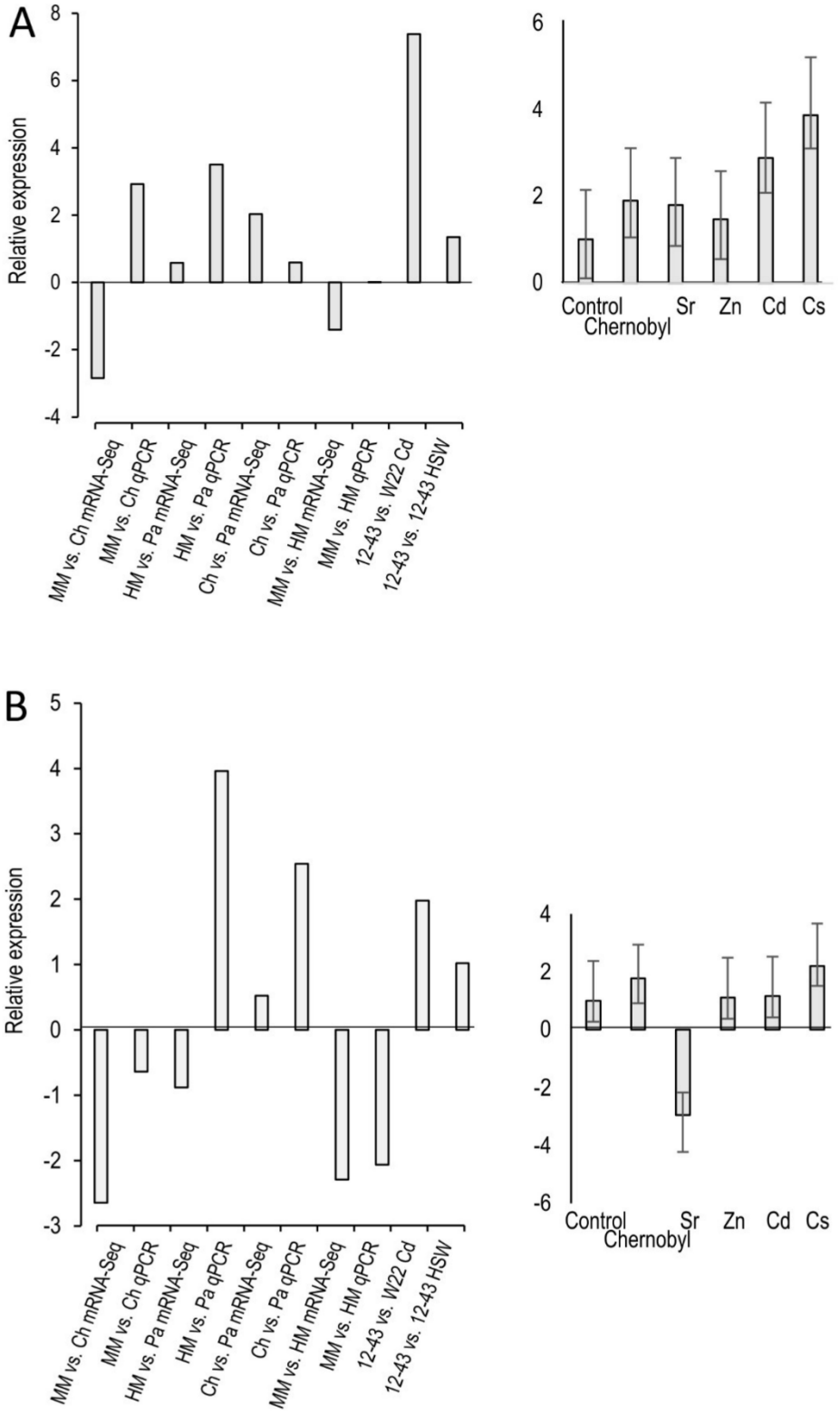

Figure 4. Validation of mRNA sequencing by microarray and qPCR (left) and further investigation by qPCR (right) for inositol polyphosphate phosphatase (**A**) and myo-inositol monooxygenase (**B**). *S. commune* 12-43 or *S. commune* W22 were grown on minimal medium (MM), minimal medium containing the heavy metals present in Paradies park soil (HM), directly in Chernobyl soil (Ch), Paradies park soil (PA) or seepage water (HSW).

5. Imp1 Overexpression and Changed Intracellular Trafficking

Fungal endocytosis and exocytosis are key processes in growth and cell wall synthesis at the growing tip. SynaptoRed FM 4-64 staining of vesicles and vacuoles in the hyphal apex showed altered vesicle size (Figure 5). In addition, overexpression of *imp1* in conjunction with Brefeldin A treatment, which is a potent inhibitor of exocytosis, increased size and variance in organelles, supporting the notion of involvement of Imp1 in vesicle trafficking. Wavy hyphae in the treated wildtype and *imp1* overexpressing strains show that directional growth might be co-afflicted. Since a strong phenotype thus could be assigned to *imp1* overexpression, changes in proteome were investigated to identify proteins with changed expression profiles in the transformant OEIMP4 as compared to EVC1.

6. Proteome Analysis Verifies a Function of imp1 in Cellular Trafficking

KOG classification of the 287 proteins with higher abundance upon *imp1* overexpression revealed 43 proteins involved in cellular processes and signaling, 41 in information storage and processing, and 58 in cell metabolism (31 were poorly and 114 not well characterized). Of the 127 proteins with lower abundance, 12 were classified into cellular processes and signaling, 7 in the information storage and processing, and 35 in cell metabolism, with 21 poorly and 52 not well characterized (Supplementary Table S4).

The highest regulation in the membrane-associated proteome was recognized with respect to cellular trafficking (Figure 6). Among those were membrane AAA^+-type ATPase containing the peptidase M41 domain, isopentenyl-diphosphate delta-isomerase involved in the mevalonate pathway, and the antioxidant thioredoxin. Another 14 proteins (signal recognition particle SRP19, ABC transporter, C5 cytosine-specific DNA methylase, acyltransferase, signal recognition particle SRP72, mitochondrial import inner membrane translocase, Tim8 containing zinc finger domain, armadillo type-fold Sec7-like, VPS5/SNX1 containing a PhoX domain, cysteine protease required for autophagy Apg4p/Aut2p, nuclear transport factor 2, t-SNARE, dynamin/VPS1, and two SNF7 proteins) decreased in abundance.

Higher levels were observed for cytoskeleton-associated proteins, three actin binding protein-related proteins and a kinesin-like protein. This supports a function in vesicle trafficking, since vesicles are cargo to the cytoskeleton and moved by motor proteins on the cytoskeletal tracks for directed intracellular movement.

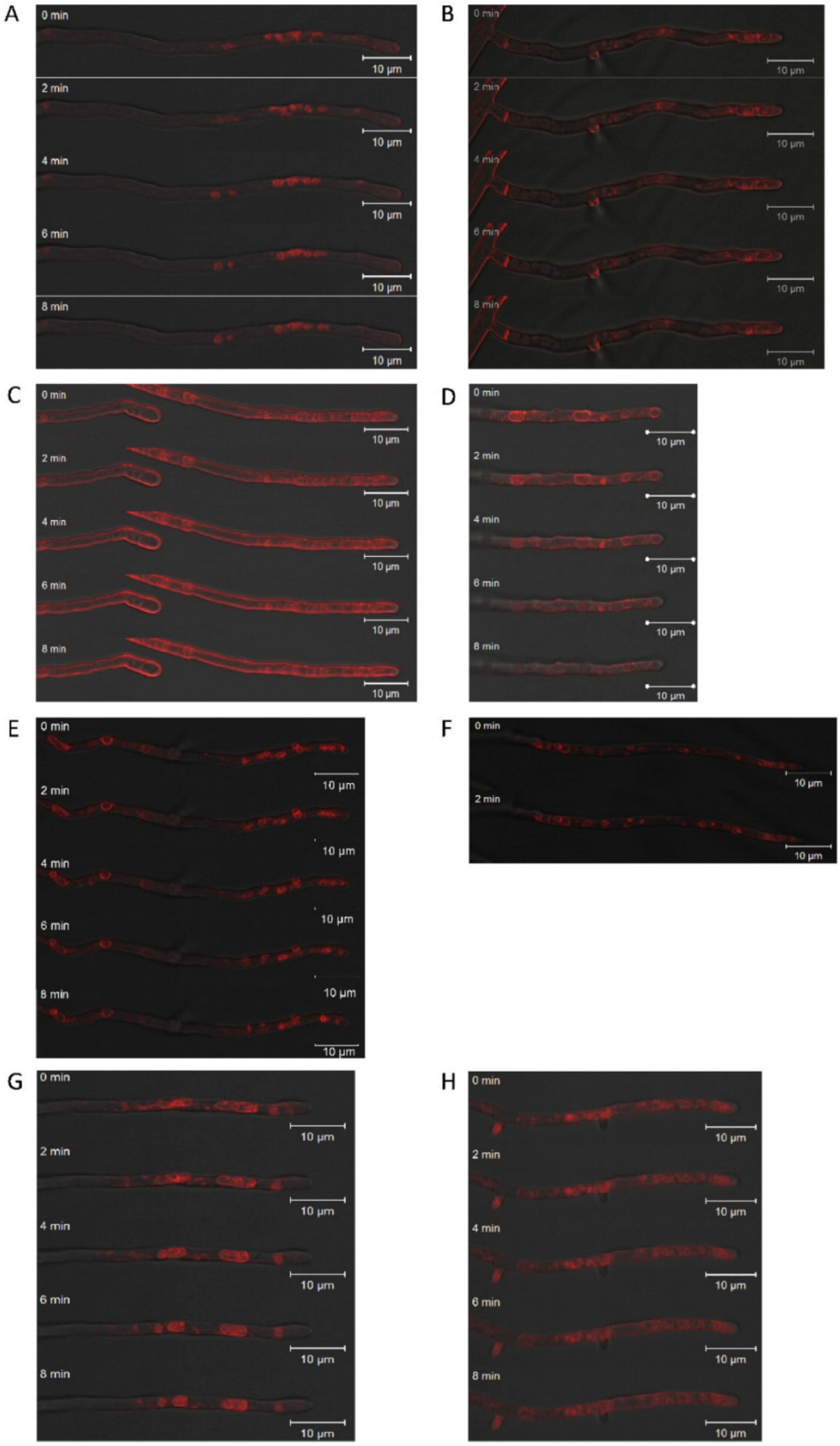

Figure 5. Effect of brefeldin A on intracellular and membrane trafficking. *S. commune* T33 (**A**,**B**), the empty vector control EVC1 (**C**,**D**), and overexpression transformants OEIMP4 and OEIMP6 (**E**–**H**) without (**A**,**C**,**E**,**G**) and with 5 µm brefeldin (**B**,**D**,**F**,**H**) were compared. Membranes were stained using FM4-64 and monitored with time-lapse mode for 0–8 min.

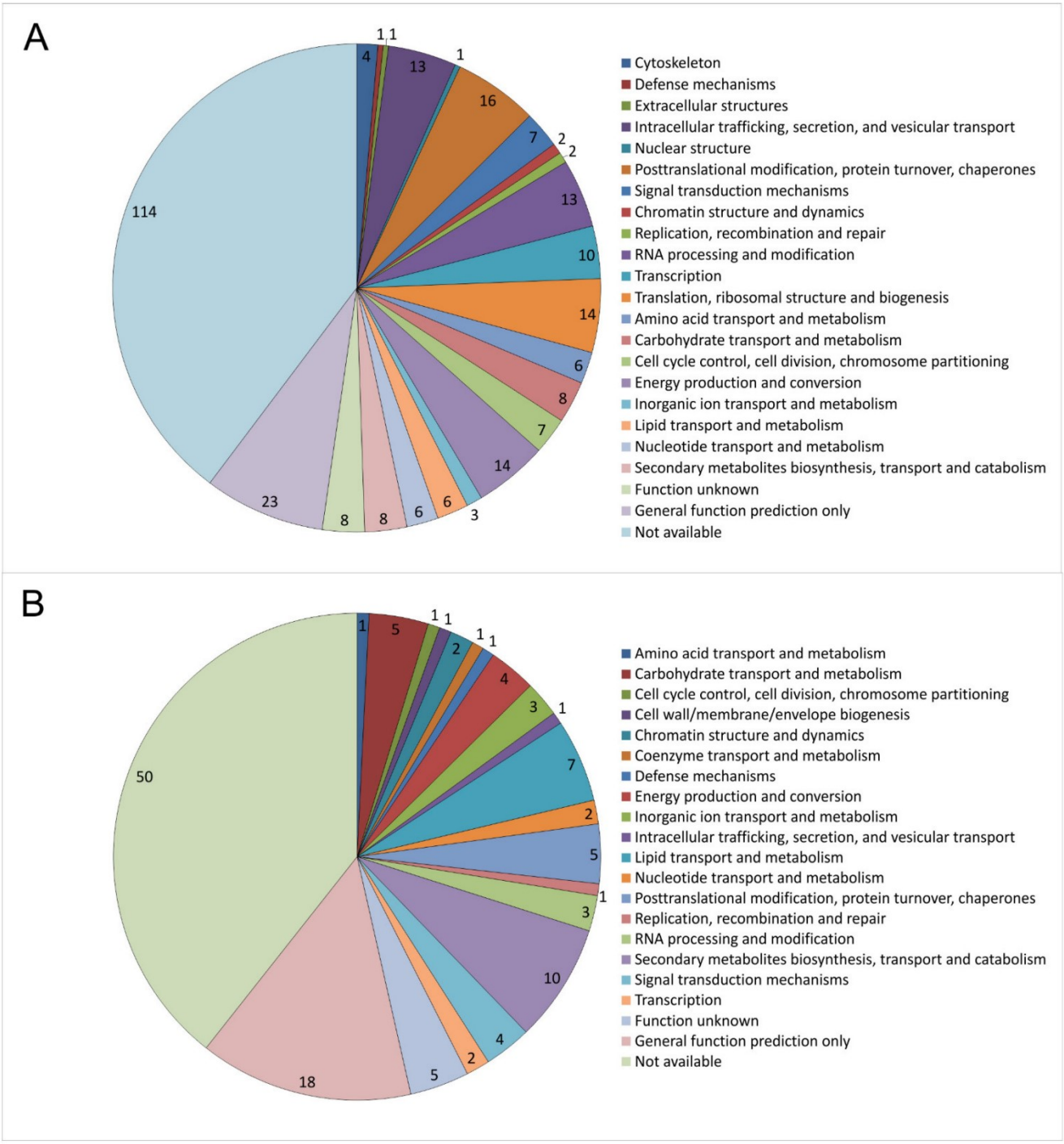

Figure 6. Membrane-associated proteins regulated with respect to *imp1* overexpression in *S. commune* OEIMP4 *versus* empty vector control *S. commune* EVC1. KOG classification of 287 induced (**A**) and 127 repressed proteins (**B**) are given.

4. Discussion

The inositol cycle depends on IMPase activity maintaining a moderate level of the cellular inositol pool [41]. The *imp1* gene encoding IMPase had been found to be up-regulated upon Ras1 activation, suggesting cross-talk between Ras and inositol signaling [8,10]. In addition, lithium as IMPase inhibitor induced a shift to inositol polyphosphates. The phylogenetic analysis obtained now shows conservation between basidiomycete IMPases, including the presence of only one gene for IMPase and linkage to zinc finger (see [42]) and

chaperone genes [43]. The conservation is also true for the three strains of *S. commune* with genome sequences available (Supplementary Figure S5).

Overexpression could not only be verified by qPCR, but also independently with the proteome study. A functional role in cell wall structure could be assigned in cell wall integrity and membrane stability, which confirms earlier studies showing particularly amino sugars contents being affected by changes in phosphoinositide in *Neurospora* [44]. Thus, cell wall biosynthesis through formation and membrane fusion of excretory vesicles involved in cell wall biosynthesis during tip growth of fungi is supported with this study.

Downstream of inositol signaling, cellular processes, and signaling related proteins were predominantly affected through *imp1* overexpression, which over-represented intracellular trafficking machinery and cytoskeleton associated proteins. This finding supports the notion that inositol signaling is involved in vesicle trafficking.

The up-regulated signal recognition particle subunits, SRP19 and SRP72, are involved in targeting secretory vesicles in eukaryotes [45–47]. Receptor-mediated activation of SRP results in signal peptide cleavage and release of proteins meant for secretion at the endoplasmic reticulum [48,49]. Another protein with elevated abundance, Snf7, is a component of the ESCRT (endosomal sorting complex required for transport)-III complex that is needed for the sorting of proteins into invaginating vesicles of the multivesicular body. The ESCRT complexes are released from the endosomal membrane with the help of an AAA-motif containing ATPase for further rounds of membrane invaginating [50]. Such an ATPase containing an M41 peptidase domain, Snf7, co-regulated with an ABC transporter responsible for export/import of a wide range of substrates including ions and macromolecules, was identified.

Heavy metals are omnipresent in the environment and exert toxic effects at higher concentrations involving oxidative stress [51]. In plants, inositol signaling has been shown to be involved in the reaction to oxidative stress. Here, *S. commune* kinases in inositol signaling were shown to be elevated under metal stress, while phosphatases mainly decreased in abundance. This would shift the cycle to more phosphoinositols like PIP_2 and less myo-inositol. This is in accordance with the finding that H_2O_2-induced cell death is associated with decreased myo-inositol amounts in *Arabidopsis thaliana* and brown algae [30,52]. The up-regulated inositol polyphosphate phosphatase has been shown to be involved in response to ROS and abiotic stress in both plants and animals [53,54]. We could show altered inositol signaling to influence metal tolerance in *S. commune*.

Our investigations showed a down-regulation of inositol multikinases under the influence of metals, which probably leads to lower amounts of highly phosphorylated inositol phosphates and pyrophosphates (IP_7 and IP_8; compare [23,55].

The calcium levels of *S. commune* were decreased 4- to 7-fold in the presence of different heavy metals. This could be on one hand due to the presence of extra amounts of metals which were taken up by the hyphae instead of calcium by calcium channels [56] or, on the other hand, could be in a relationship with altered inositol signaling and thus calcium as a second messenger [57]. Calcium has a protective role against ROS and supplemental added calcium leads to increased Cd tolerance [58].

As for vesicle transport, t-SNARE and Sec7-like proteins were induced upon *imp1* overexpression. SEC-7 is involved in vesicular budding and intracellular trafficking between compartments of the Golgi apparatus, and target membrane bound SNARE (t-SNARE) plays an important role in facilitating vesicle fusion in cargo trafficking [59]. For cargo transport, the cytoskeleton is needed that was up-regulated at the proteome level as well.

Long-distance transport along microtubules is suggested by the up-regulation of kinesin, while a plus-end directed microtubule-dependent motor protein [60], dynamin, connects the long-distance and short-distance transport, e.g., at Golgi compartments. Here, again the activity of small GTPases is involved [61,62]. Filamentous actin is organized by the activity of actin-bundling proteins, two of which were detected among the proteins with increased abundances under *imp1* overexpression. Finally, actin-binding proteins and cofilin/tropomyosin permit transport of cargo along actin filaments. This is connected to

exocytosis, where vesicles are brought to the vesicle supply center at the growing tip before fusing with the cell membrane to release cell wall components needed for tip growth. The role of actin transport in endocytosis in older hyphal compartments has been discussed controversially for fungi, but seems likely.

To further investigate the role of inositol signaling in intracellular trafficking, we further examined the effect of the strong protein secretion inhibitor brefeldin A in *imp1* overexpressing transformants [63]. Indeed, larger vacuoles were more abundant in the overexpressing strains, supporting the view that vesicle fusion is increased through IMPase activation, and hence the result of inositol signaling. With this study, the view on signal transduction could be expanded (Figure 7) to include inositol phosphate signaling in mushroom forming basidiomycetes.

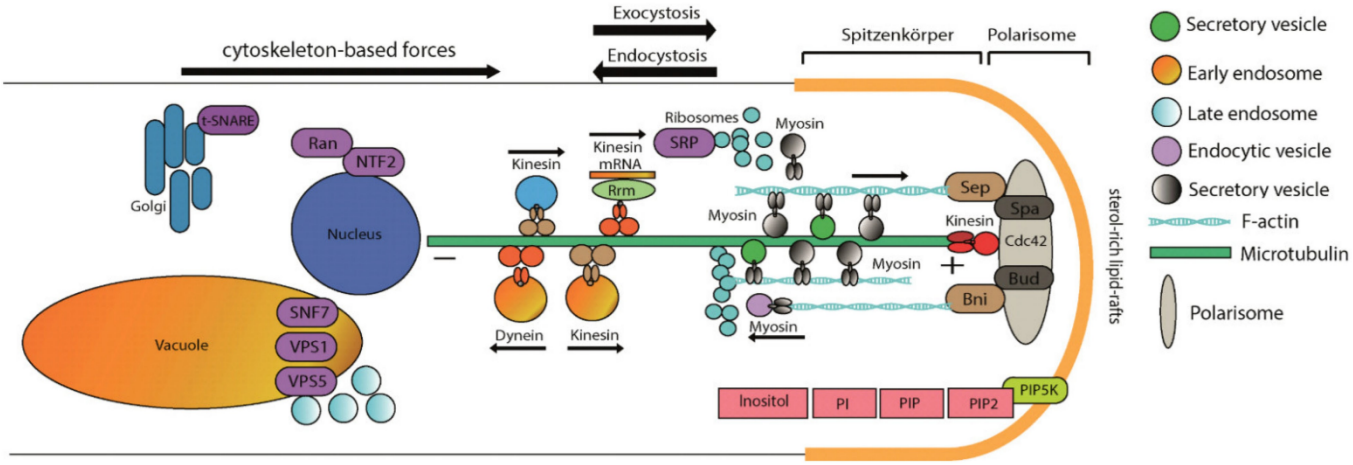

Figure 7. Model integrating inositol signaling in intracellular and membrane trafficking.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7060470/s1, Supplementary Table S1: *S. commune* strains used in this study, Supplementary Table S2: Primers used in this study, Supplementary Figure S1: Plasmid construction for generation of *imp1* overexpression strains, Supplementary Figure S2: Verification of *imp1* overexpression by RT-qPCR, Supplementary Figure S3: Effect of *imp1* overexpression on colony morphology (top) and growth (below) of *S. commune*, Supplementary Figure S4: Susceptibility towards calcofluor white (A), SDS (B), and Congo red (C), Supplementary Table S3: Regulation of genes involved in the inositol signaling cycle, Supplementary Table S4: Intracellular trafficking associated proteins regulated in *imp1* overexpressing transformant pOEIMP4 vs. EVC, Supplementary Figure S5: Chromosomal map of *imp1* surrounding genes in three different strains *S. commune*, H4-8, LoeD, and TatD.

Author Contributions: Conceptualization, E.K. and K.K.; methodology, L.T. and T.K.; validation, K.K.; investigation, L.T., J.P. and R.M.; writing—original draft preparation, R.M. and L.T.; writing—review and editing, L.T., K.K., T.K., O.K. and E.K.; visualization, R.M., L.T. and K.K.; supervision, E.K.; project administration, E.K.; funding acquisition, O.K. and E.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by International Leibniz Research School (ILRS) within the frame of DFG-GSC124 (JSMC) and DFG-CRC 1127 ChemBioSys (project number 239748522), and DFG-CRC-TR 124 FungiNet (project number 210879364, project Z2).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are publicly available.

Acknowledgments: We would like to acknowledge Simon Kügler for his work on imp overexpression plasmid construction and Dirk Merten for ICP-MS measurements.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplemental Material

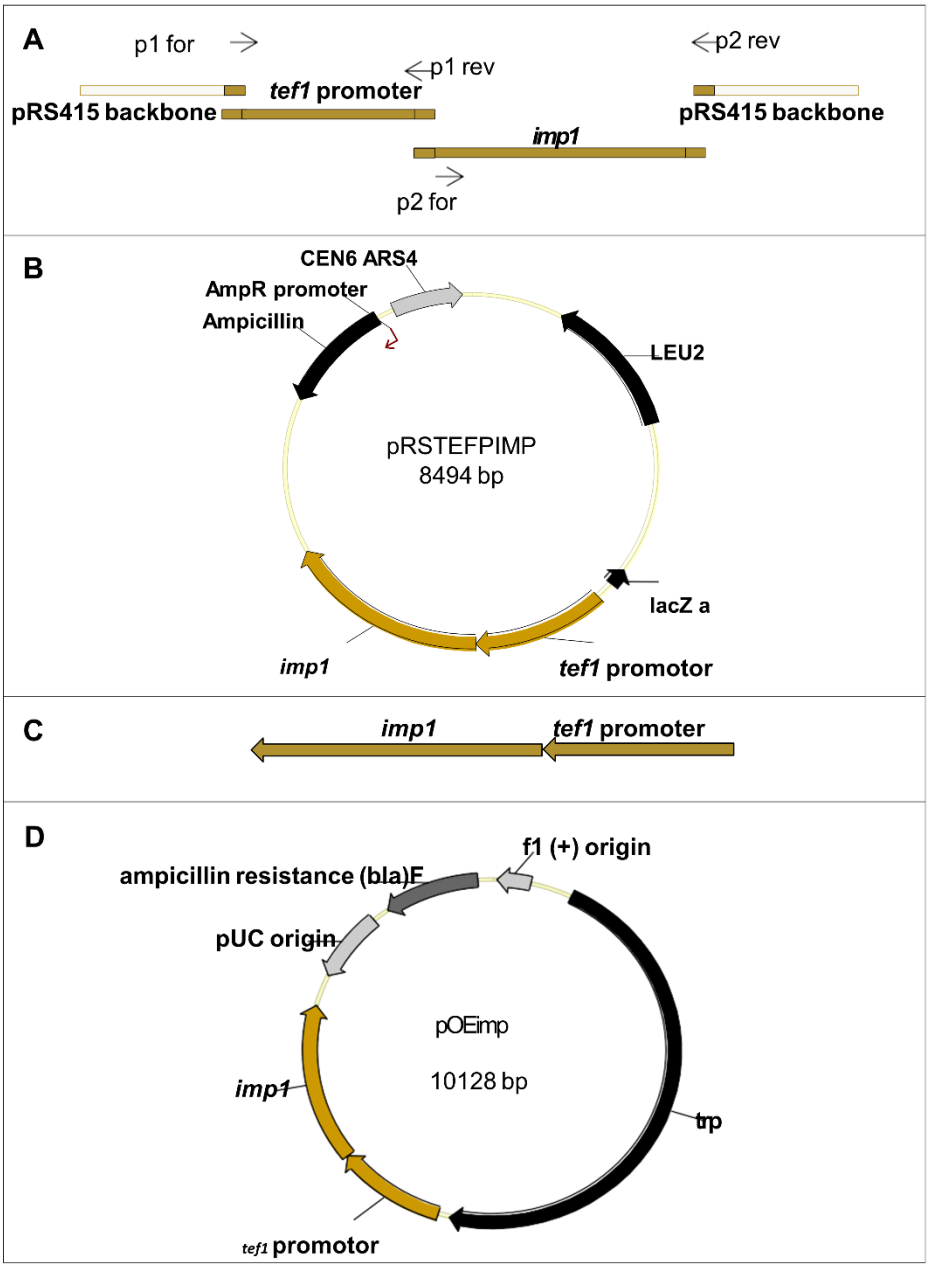
Suppl. Tab. S1: *S. commune* strains used in this study.

Strain	Genetic background	Source
12-43	*ura-*	Jena Microbial Resource Collection (JMRC), Germany
T33	*matA3,1; matB3,2; trp-; ura-*	JMRC
EVC1	*matA3,1; matB3,2*	JMRC
OEIMP4	*matA3,1; matB3,2; tef1p::imp1*	JMRC
OEIMP6	*matA3,1; matB3,2; tefp::imp1*	JMRC

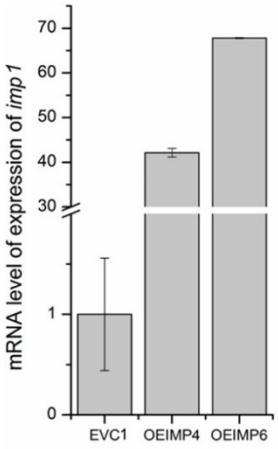
Suppl. Tab. S2: Primers used in this study.

Name	Gene	Sequence (5'-3')	Length (bp)	Efficiency
p1for	pRS415::*ptef1*	*CTAGTTCTAGAGCGGCCGCCACCGCCGGAAAAGAACAAGACGTGT**		
p1rev	*tef1::imp*	*TAGTCGGCGATGGTAAGGTCGGTGGGC*ATTTTGAGTGTTTTCTAAGTGAG*		
p2for	*tef1::imp*	*TCACTTAGAAAACACTCAAA* ATGCCCACCGACCTTACCAT*		
p2rev	*Imp*::pRS415	*CTAAAGGGAACAAAAGCTGGGACACACGAGGATGACGGTT**		
mio3	Myo-inositol oxygenase	For: GGATCTACAAGCCGCACTGT Rev: CACGGTGCCAGGGATAGAAG	149	92.80%
imp	Inositol monophos-phatase	For: GAAGCCCGTGCTCGGTG Rev: TGGTTGTTGATGCCCTCCGT		
ipp1	Hypothetical inositol polyphosphate phosphatase	For: GACCGCATAGCGGACTACAAC Rev: TCCATGAGGGAATAGCCCGA	188	109.95%
act	Actin-1	For: CTGCTCTTGTTATTGACAATGGTTCC Rev: AGGATACCACGCTTGGACTGAGC	178	96.31%
tef	Translation elongation factor 1a	For: AGCTCGGCAAGGGTTCCTTCA Rev: AACTTCCAGAGGGCGATATCA	97	97%
ubi	Ubiquitin-conjugating-protein	For: GAAGGAGTACGATGCGAAGG Rev: TCCTCCTCTGCCTTCTTGC	93	89.5%

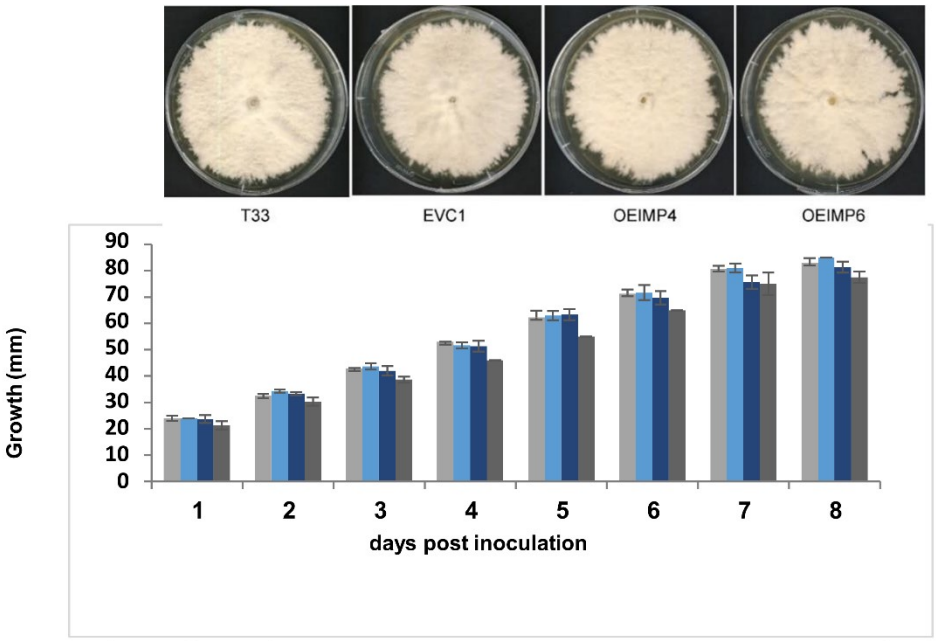
**Overhang for annealing in italics*



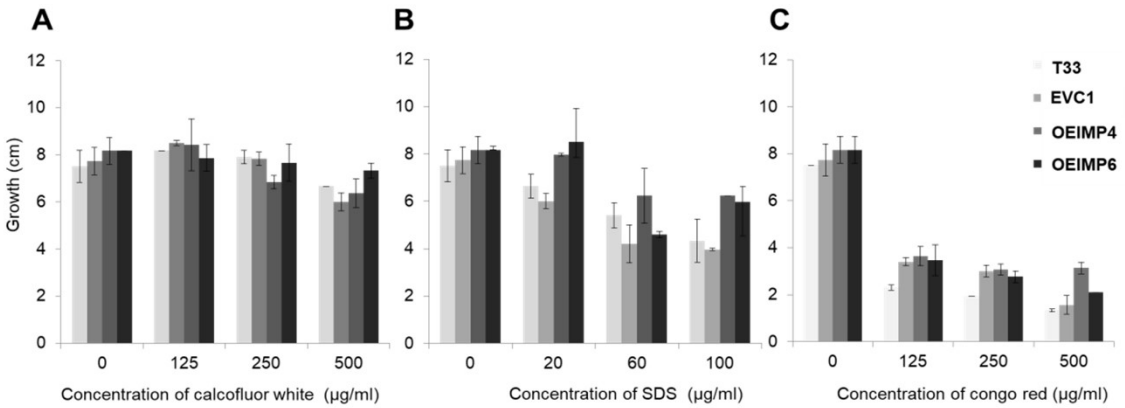
Suppl. Fig. S1: Plasmid construction for generation of *imp1* overexpression strains. (A) Recombination in *S. cerevisiae* was carried out using pRS415 to generate the product fusing the PCR products for the *tef1* promoter (*tef1p*) and the *imp1* gene. (B) Overlapping sequences between two adjacent fragments *tef1p* and *imp1* were used (p1rev and p2for). (C) Fusion product *tef1p::imp1* was cloned in pRS415 to generate pRSTEFPIMP. (D) The *tef1p::imp1* region was excised and cloned into pSK containing the selection marker *trp1* to arrive at pOEIMP.



Suppl. Fig. S2: Verification of *imp1* overexpression by RT-qPCR. Comparison is given for the empty vector control EVC1 and overexpression transformants OEIMP4 and OEIMP6.



Suppl. Fig. S3: Effect of *imp1* overexpression on colony morphology (top) and growth (below) of *S. commune* T33 (grey bars), empty vector control EVC1 (blue), and overexpression transformants OEIMP4 (dark blue) and OEIMP6 (dark grey); n=3.



Suppl. Fig. S4: Susceptibility towards calcofluor white (A), SDS (B), and Congo red (C). Comparison is given in triplicates for the wildtype parental *S. commune* T33, the empty vector control EVC1, and overexpression transformants OEIMP4 and OEIMP6.

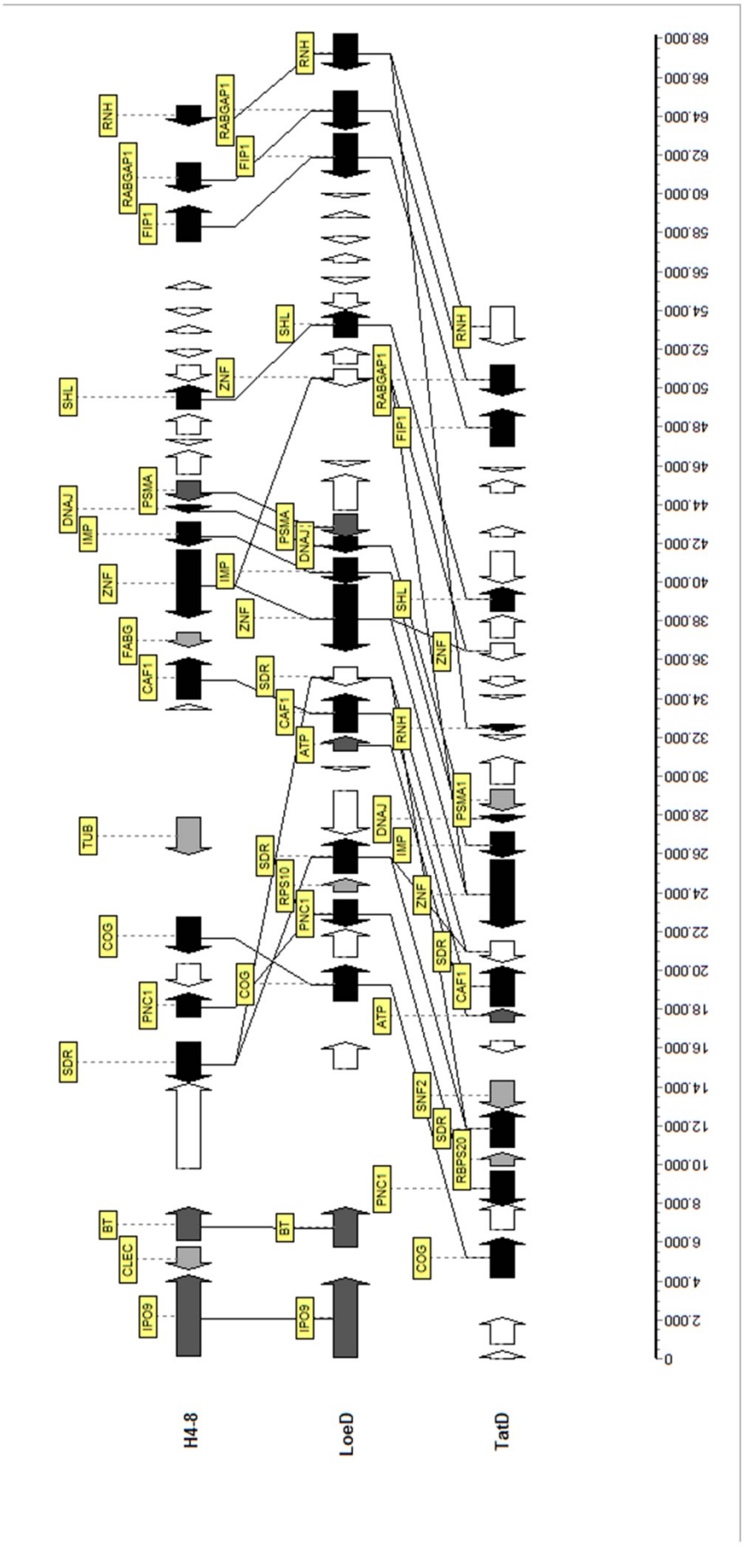
Suppl. Tab. S3: Regulation of genes involved in the inositol signalling cycle. The microarray analysis (Erdmann et al., 2012) is showing comparisons between *S. commune* 12-43 grown on CYM *versus* CYM with seepage water from a former mining site (HSW) and co-isogenic *S. commune* W22 grown on CYM with 0.01 mM cadmium nitrate.

	KOG Annotation		12-43 vs. 12-43 HSW			12-43 vs. W22 Cd		
		Gene Location Scho1	x-fold	p-value	Q	x-fold	p-value	Q
PIS	KOG3240: PI synthase	estExt_fgenesh1_pm.C_20467	-1.45	1.79E-01	1	2.11	1.16E-02	1
	KOG0693: Myo-inositol-1-phospha te synthase	estExt_fgenesh1_pm.C_70303	1.25	4.55E-01	1	2.45	6.76E-03	1
		estExt_fgenesh1_pm.C_70303	2.00	2.81E-03	1	-2.50	2.52E-04	1
		estExt_fgenesh1_pm.C_70303	1.08	6.86E-01	1	-1.50	3.75E-02	1
PI_4K	KOG2381: PI4-kinase	augustus-scaffold_8.g641	2.24	1.21E-01	1	4.61	6.59E-03	1
		e_gw1.2.405.1	1.25	4.86E-01	1	2.81	3.84E-03	1
	KOG0903: PI4-kinase, involved in intracellular trafficking and secretion,	estExt_Genewise1Plus.C_21667	3.82	9.87E-02	0	1.73	4.86E-01	0
PI_5K	KOG0230: PI-4-phosphate 5-kinase and related FYVE finger-containing proteins	estExt_fgenesh1_kg.C_60138	-1.75	6.41E-02	1	1.07	8.11E-01	1
		estExt_Genewise1.C_12180	1.04	8.60E-01	1	3.04	1.67E-04	1
	KOG0229: PI-4-phosphate 5-kinase	estExt_fgenesh1_kg.C_110031	2.41	1.38E-02	1	1.75	9.89E-02	1
PI_3K	KOG0906: PI3-kinase VPS34, involved in signal transduction,	fgenesh1_pm.C_scaffold_5 00027 2	-1.08	8.16E-01	1	1.34	3.56E-01	1
PLC	KOG0169: PI-specific phospholipas e C	estExt_Genewise1.C_170034	1.30	2.09E-01	1	-1.06	7.71E-01	1
		estExt_fgenesh1_pm.C_60287	1.18	4.20E-01	1	1.83	9.07E-03	1
		augustus-scaffold_6.g78	-1.81	2.19E-01	1	-1.00	1.00E+00	1
		augustus-scaffold_3.g897	-4.17	5.43E-02	1	2.59	1.87E-01	0
PKC	KOG2397: Protein kinase C substrate, 80 KD protein, heavy chain	estExt_Genewise1Plus.C_11897	-1.11	7.10E-01	1	2.56	4.35E-03	1
IMK	KOG1620: Inositol polyphosphate multikinase, component of the ARGR transcription regulatory complex,	e_gw1.1.1005.1	1.79	5.90E-01	1	6.94	8.66E-02	1
		estExt_Genewise1Plus.C_10042 7	-15.82	5.24E-03	1	-2.71	2.63E-01	1
	KOG4749: Inositol polyphosphate kinase	e_gw1.2.893.1	-13.45	2.05E-03	1	-6.98	1.46E-02	1
IP_3P	KOG1089: Myotubularin-related PI3-phosphate 3-phosphatase MTM6	estExt_fgenesh1_pm.C_20581	3.88	2.81E-02	1	2.11	2.04E-01	1
IP_4P								
IP_5P	KOG0565: Inositol polyphosphate 5-phosphatase and related proteins	estExt_fgenesh1_pm.C_20547	1.35	7.50E-01	1	7.38	4.55E-02	1
		estExt_Genewise1.C_170073	-1.20	4.45E-01	1	1.13	6.01E-01	1
		augustus-scaffold_5.g421	-9.43	1.26E-01	1	-5.25	2.51E-01	1
		estExt_fgenesh2_pg.C_100071	-8.56	1.58E-04	1	-12.9	2.38E-05	1
IPP	KOG1382: Multiple inositol polyphosphate phosphatase	gw1.1.3820.1	1.86	1.58E-01	1	1.96	1.29E-01	1
		gw1.8.732.1	1.55	2.01E-01	1	2.12	3.59E-02	1
		estExt_Genewise1Plus.C_30697	1.35	4.05E-01	1	-2.21	3.69E-02	1
		e_gw1.7.1022.1	-1.62	5.16E-01	1	-1.10	9.02E-01	1

	KOG Annotation	Gene Location Scho1	12-43 vs. 12-43 HSW			12-43 vs. W22 Cd		
			x-fold	p-value	Q	x-fold	p-value	Q
ITP	KOG0566: Inositol- 1,4,5-triphosphate 5-phosphatase 9.00 (synaptojanin), INP51/INP52/INP53 family	estExt_Genewise1.C_11249	-	4.61E-02	1	-14.53	1.79E-02	1
IMP	KOG2951: Inositol monophosphatase	e_gw1.1.586.1	1.33	4.63E-01	1	1.12	7.67E-01	1
		e_gw1.1.586.1	-1.05	8.93E-01	1	-1.48	2.59E-01	1
		e_gw1.1.586.1	-1.30	2.04E-01	1	-1.20	3.79E-01	1

Suppl. Tab. S4: Intracellular trafficking associated proteins regulated in *imp1* overexpressing transformant pOEIMP4 vs. EVC

ID	x-fold	KOG class and description		BLAST hit, organism, % identity
2598708	2.9	cyto-skeleton	Drebrins and related actin binding proteins	Actin-binding related protein, *Laccaria bicolor*, 70%
2541047	2.7		Kinesin-like protein	Kinesin-like protein, *Armillaria solidipes*, 78%
2674409	2.3		Predicted actin-bundling protein	Protein FRG1, *Hypsizygus marmoreus*, 68%
2681330	2.3		Predicted actin-bundling protein	Ubiquitin-activating enzyme E1-like, *Trametes pubescens*, 63%
2704529	23.8	intra-cellular traf-ficking, secretion, and vesicular transport	Signal recognition particle, subunit Srp19	Signal recognition particle, SRP19 subunit, *Armillaria solidipes*, 61%
2262522	23.7		Protein involved in glucose derepression and pre-vacuolar endosome protein sorting	Snf7-domain-containing protein, *Armillaria solidipes*, 74%
2603193	21.7		Mitochondrial Fe/S cluster exporter, ABC superfamily	Iron-sulfur clusters transporter ATM1, *Coprinopsis cinerea okayama*, 75%
2597192	21.7		Golgi transport complex subunit	Dor1-domain-containing protein, *Armillaria gallica*, 48%
2678633	4.3		Vesicle coat complex AP-3, beta subunit	acyltransferase ChoActase/COT/CP, *Fistulina hepatica*, 60%
2693248	3.5		Signal recognition particle, subunit Srp72	Signal recognition particle subunit SRP72, *Trametes pubescens*, 47%
2460397	3.0		Mitochondrial import inner membrane translocase, subunit TIM8	Mitochondrial import inner membrane translocase *Heterobasidion irregulare*, 68%
2700443	3.0		Guanine nucleotide exchange factor	Sec7 guanine nucleotide exchange factor, *Moniliophthora roreri*, 72%
1185145	2.4		Vacuolar assembly/sorting protein DID2	Vacuolar protein sorting protein 46, *Moniliophthora roreri*, 90%
2698910	2.2		Membrane coat complex Retromer, subunit VPS5/SNX1, Sorting nexins, and related PX domain-containing proteins	PX-domain-containing protein, *Armillaria gallica*, 75% Sorting nexin-41, Hypsizygus marmoreus, 76%
2678145	2.1		Cysteine protease required for autophagy - Apg4p/Aut2p	Thiamine pyrophosphokinase, *Coprinopsis cinerea okayama*, 49%
2007874	2.1		Nuclear transport factor 2	Nuclear transport factor 2, *Neolentinus lepideus*, 75%
2595664	2.1		SNARE protein Syntaxin 1 and related proteins	t-SNARE, *Armillaria solidipes*, 80%
2573839	2.2	poorly characte-rized	Vacuolar sorting protein VPS1, dynamin, and related proteins	Dynamin protein dnm1, *Moniliophthora roreri MCA 2997*, 75%



Suppl. Fig. S5: Chromosomal map of *imp1* surrounding genes in three different strains *S. commune*, H4-8, LoeD, and TatD. White arrows indicate unknown genes, black arrows show that the corresponding genes are present in all three genomes.

5. Discussion

5.1 *S. commune* can survive in contaminated soil without changing the microbial community

A major aim of this study was to investigate if *S. commune* can be used for mycoremediation of contaminated soil. In particular, it was tested if it can survive in the radionuclide contaminated soil of the CEZ. Although *S. commune* is a wood rot fungus, it was reported that several related species can survive in soil (Canet et al. 2001; Morgan et al. 1993; Novotny et al. 2000). In all studies conducted so far, an additional carbon source was added with the fungi into the soil, like hemicellulose with a wheat straw amendment (Morgan et al. 1993). In our study, the survival of S*. commune* was tested without any additional carbon source. For this purpose, *S. commune* was inoculated into the test field in the CEZ and the survival and spread were tested after 6 and 12 months with species-specific primers and quantitative PCR. Additionality, the survival was tested for one year under laboratory conditions in a plate assay. Although *S. commune* grows saprotrophically and has not been described to occur naturally in soil, its survival in the CEZ has been demonstrated. Increased DNA concentrations of *S. commune* could not only be detected in the originally inoculated rows, but also at the sampling site M1, which was about 1 meter away (see Manuscript 1, Figure 1, and Table 1). This leads to the conclusion that the fungus not only survived, but also spread in the contaminated soil. Since the last inoculation of the closer row of plants was 6 months ago, this results in a growth rate of 8 mm per day. This matches the growth rate of 5 -10 mm per day on artificial medium for *S. commune* (Brunsch et al. 2015). This might be relating to the reported finding that fungi grow faster and have longer, unbranched hyphae- the so-called runner hyphae- when growing in soil under stressful conditions e.g. nutrient depletion (Allen 2007; Agerer 1999; Muthukumar et al. 2014). Growth rates of runner hyphal growth are faster than that reported for well-fed hyphae, also for other fungi growing in soil (Camel et al. 1991; Smith, Bruhn, and Anderson 1992). It can be excluded that the detection of *S. commune* DNA in the soil is based on the natural occurrence or spreading by spores since a monokaryotic strain was inoculated.

Moreover, it could be shown that *S. commune* survived for at least one year under laboratory conditions only with CEZ soil without additional nutrients (Manuscript 1). Thus, *S. commune* seems to be so far the only wood-rot fungus that has been demonstrated to survive in soil for several months without an additional carbon source.

At the CEZ, *S. commune* had to cope with increased radiation values of 2-6 µGy/h at ground level and 18-22 µGy per day at a height of one meter measured on the test field. The radiation values are distributed very heterogeneously at ground level due to so-called hot particles, and the radiation distribution in the depth profile is also very heterogeneous (Manuscript 1). A highly dose-relevant hot particle is ^{241}Am. It was distributed heterogeneously on the ground immediately after the reactor accident in the CEZ and thus on the test field (Steinhauser 2018; Walther and Denecke 2013). Consequently, *S. commune* must be able to survive such doses even without a long period of adaptation. It is possible that due to the inhomogeneous distribution of the radiation, the fungus survived by avoiding hot spots. This would offer, at the same time, a longer adjustment time for the resulting hyphae, and thus the possibility of adaptation. Some studies have already been carried out on the adaptation to radiation over a longer period in the CEZ, but none with basidiomycetes (Galván et al. 2014; Klubicova et al. 2012; Kovalchuk et al. 2004). It was reported that several fungi can survive doses up to 1000 Gy/h (Aziz et al. 1997). However, these tests took place under laboratory conditions. *S. commune* had to survive several adverse conditions at the same time in the dry, sandy CEZ soil.

The basidiomycete could not only withstand the harsh conditions, it even was able to compete with other soil fungi under these conditions. With a community analysis at the different sampling points at the test field, we found that neither the inoculation with fungi, nor the planting regime changed the microbial soil community significantly at the phylum level. However, a strong fluctuation in richness and diversity in the bacterial community was observed (Manuscript 2). Since this could not be related to the inoculations, it can be concluded that it is due to the very inhomogeneous contamination of the test field. Sample points that were in the vicinity of strongly radiating hot particles probably had a less species-rich and less diverse

community. This effect could not be observed for fungi, as they spread over longer distances and thus can withstand locally higher contamination. Particularly harsh environmental influences can lead to very limited microbiomes, even to a point where a slight change in conditions such as the inoculation of a new fungus or plant does not longer result in a change of the microbiome composition. The soil of the test field is a sandy podzol that tends to dry out quickly, there is long-term radiation and quite large temperature fluctuations of -30°C to 30°C because of the continental climate, which certainly amounts to adverse conditions (Schulz 2020; https://www.climatestotravel.com/climate/ukraine, 07.07.2021). It is all the more remarkable that *S. commune* survived and was detected in the community analysis.

Thus, *S. commune* is a potentially suitable organism for mycoremediation. Due to its ability to produce laccases for lignin degradation, the fungus is able to change the composition of minerals (Kirtzel et al. 2019). This could lead to an altered bioavailability of metals and radionuclides (Günther et al. 2014). In turn, this may explain the survival in the soil of CEZ, and it provides a case study also implying that other fungi might survive. A study showed that the inoculation of *S. commune* near crops growing in radionuclide contaminated soil leads to a higher amount of harvested potatoes (Vuković et al. 2020). Since the presence of *S. commune* increased the transfer rate of ^{137}Cs into the green of winter rye and the potato tuber, these plant materials might be harvested and safely removed to extract contaminants and thus provide a land management scheme for re-using CEZ soils in the future. Even under natural conditions on the test field in CEZ, the inoculation with *S. commune* led to increased ^{137}Cs values in potato plants (Schulz 2020). Additionally, it was reported that *S. commune* induces the antioxidative response in winter rye shoots grown in radionuclide contaminated soil (Vuković et al. 2020). Coming back to mechanisms for bioremediation, these facts imply that *S. commune* is likely mobilizing radionuclides.

Fungi have a large mycelial network. This can contribute to remediation by transferring radionuclides to result in more homogeneous surroundings for co-occurring organisms like plants. A more even distribution of the contamination may enable agriculture in polluted areas again. This is particularly relevant in the CEZ with the large occurrence of hot particles. We

were able to show that *S. commune* can transport both Cs and Sr (Manuscript 3). Thus, the original disadvantage of metal mobilization can become useful in mycoremediation. Furthermore, it should be investigated, whether *S. commune* inoculated together with mycorrhizal fungi can increase plant metal uptake. After plant harvest, lower resulting contamination might then contribute to soil remediation. Thus, it could be shown that even unusual fungi such as wood-rot mushrooms are suitable for mycoremediation.

5.2 The metal tolerance mechanism of *S. commune* is an avoidance strategy

In order to understand the potential use of *S. commune* as an organism for mycoremediation, the underlying mechanisms by which the fungus reacts to metal stress must be investigated. For this purpose, the level of metal tolerance against Sr, Cs, Zn, and Cd as well as the influence of pH and temperature was checked. This is important because metal toxicity differs with different abiotic factors and environmental conditions change significantly with different habitats. It emerged that the fungus can tolerate significantly higher concentrations than can be found in CEZ or other contaminated areas (Wasser et al. 1991; Nriagu and Pacyna 1988). Overall, it was found that Cs, Zn, and Cd are better tolerated at higher temperatures, regardless of the pH value. In contrast, a change in relation to the pH value could be observed in the case of strontium. As usual, at pH 7.5 the tolerance is highest at the highest tested temperature of 28°C and lowest at the lowest temperature of 16°C. Already at pH 6 the tolerance at 16°C is higher than at the middle temperature. Finally, at pH 4.5, growth is most inhibited at the optimal temperature of 28°C, and at 16°C and a low $SrCl_2$ concentration, growth of *S. commune* was even promoted compared to the control (Manuscript 3 Figure 1 and S2). The previously mentioned growth form of “runner hyphae” can explain the enlarged colony diameter, but *S. commune* also grew with more aerial mycelium at 10 mM and 25 mM $SrCl_2$ (Manuscript 3 Figure S2). Both divalent cations Sr^{2+} and Ca^{2+} can be substituted for each other in cells (Saidak and Marie 2012). Ca is known to be involved as a second messenger on hyphal growth and phosphate storage in *S. commune,* which together could be an explanation for enhanced growth (Murry 2018).

Furthermore, an *S. commune* strain adapted to strontium was produced, which also showed significantly increased tolerance against Cs without a changed morphology. Comparative studies to the wild-type showed that the adapted strain *S. commune* 12-43 Sr transported less Sr and Cs. Also, the uptake of Sr, Cs, and Zn was decreased compared to the wild-type. Whereas the uptake of Cd in the adapted strain is significantly increased. Since the increased tolerance of the adapted strain was accompanied by a lower accumulation of metals, the conclusion that the fungus had developed an avoidance strategy is reasonable. Increased efflux or reduced influx of the metals are likely, however, mechanisms such as extracellular chelation by secreted ligands would also be possible (Bellion et al. 2006). *S. commune* 12-43 Sr seems to respond to Cd by a different mechanism.

Another important investigation to examine the metal tolerance mechanisms of *S. commune* was mRNA sequencing. The comparative analysis of the transcriptome of *S. commune* grown on the two soils compared with artificial medium each with high and low metal content showed that a change in substrate resulted in much more significant differentially expressed genes than a change in metal content. Most of the 264 significant gene regulations were not annotated, but what stood out was that genes that belong to transporters tended to be down-regulated under metal stress. However, genes that were associated with the secretory pathway tended to be up-regulated. This could be already a hint to the metal tolerance mechanism of *S. commune*. This pattern was also found in a transcriptomic analysis of the metal stress response of *Saccharomyces cerevisiae* (Ruotolo et al. 2008). These regulations seemed to be a metal-specific response and lead to enhanced Ni tolerance but higher Cd sensitivity. In our study, contaminated soil was used therefore containing a mixture of metals (the five most abundant bioavailable metals: Al, Mn, Zn, Fe, Cu). Consequently, no statement can be made about the metal specificity. One possible explanation for this expression pattern would be a reduced uptake of metals due to the down-regulated transporters and increased sequestration in vacuoles or export through exocytosis due to the up-regulated secretory-associated genes. The sequestration of metals could already be observed in former studies for *S. commune* (Kirtzel et al. 2019). In this study a sequestering of Cd in vacuoles at the hyphal tip could be

observed too using fluorescence microscopy (Manuscript 3 Figure S4). This was seen in both the wild-type *S. commune* 12-43 and the adapted strain. The same behavior towards Cd seems logical because *S. commune* 12-43 Sr showed no significantly increased Cd tolerance. From a large number of significantly regulated genes, some genes of interest were selected for further investigation. Among other things, two transporters were examined more closely. The first is a general substrate transporter that is associated with metabolism. It was up-regulated under metal stress in both the mRNA sequencing and the subsequent qPCR analysis (Manuscript 3 Figure 5). This corresponds to previous studies, which showed that stress often leads to higher carbohydrate metabolism rates (Chiapello et al. 2015). The second transporter belongs to the group of the Major Facilitator Superfamily (MFS). It was down-regulated in the mRNA-Seq and the qPCR with the wild-type under metal stress, but not in the adapted strain. MFS transporters are rather unspecific and also transport various different toxic compounds and ions (Stergiopoulos et al. Waard 2002). Within the MFS transporters, there is the class of multidrug transporters. They exchange an H^+ for the toxin and can thus counteract an accumulation. This group is assigned an important role in responding to heavy metal stress (Peng et al. 2011). The MFS transporter investigated in our study is not further annotated. Still, a higher regulation under metal stress in the adapted strain could therefore speak for increased export of metals as the acquired resistance mechanism.

Furthermore, the expression of a glutathione S-transferase was examined more closely. Its expression was always up-regulated in the wild-type under metal stress, both in the mRNA-Seq and in the qPCR. Glutathione S-transferases are enzymes that catalyze the binding of glutathione to toxic compounds. The conjugates are then excreted out of the cell or sequestered in the vacuole (Morel et al. 2009). Thus, increased activity is expected in the presence of toxic substances such as heavy metals. Except for Cd, no up-regulation was detected in the adapted strain. This suggests that the cellular stress was significantly reduced by the acquired tolerance mechanism in the presence of Cs, Sr, and Zn.

5.3 Inositol signaling and metal tolerance are connected in *S. commune*

Inositol signaling has been studied intensively for over 30 years (Berridge 1989) and plays an important role in many physiological processes, including metabolic adaptation, vesicle trafficking, and sexual development (Lev et al. 2015; Li et al. 2016; Xie et al. 2017). Since the genome of *S. commune* has been fully published (Ohm et al. 2010) and the fungus is suitable for transformation and genetic modifications (Munoz-Rivas et al. 1986; de Jong et al. 2006), several signaling pathways including inositol signaling have already been researched in it (Raudaskoski and Kothe 2010; Knabe et al. 2013; Murry et al. 2019). But so far, the interplay between the response to metals and the inositol cell signaling pathway has hardly been researched in microorganisms. In basidiomycetes, this topic has not yet been explored at all. This thesis examined how metal stress affects the expression of genes associated with the phospho-inositol cycle and vice versa the influence of altered inositol signaling on metal tolerance.

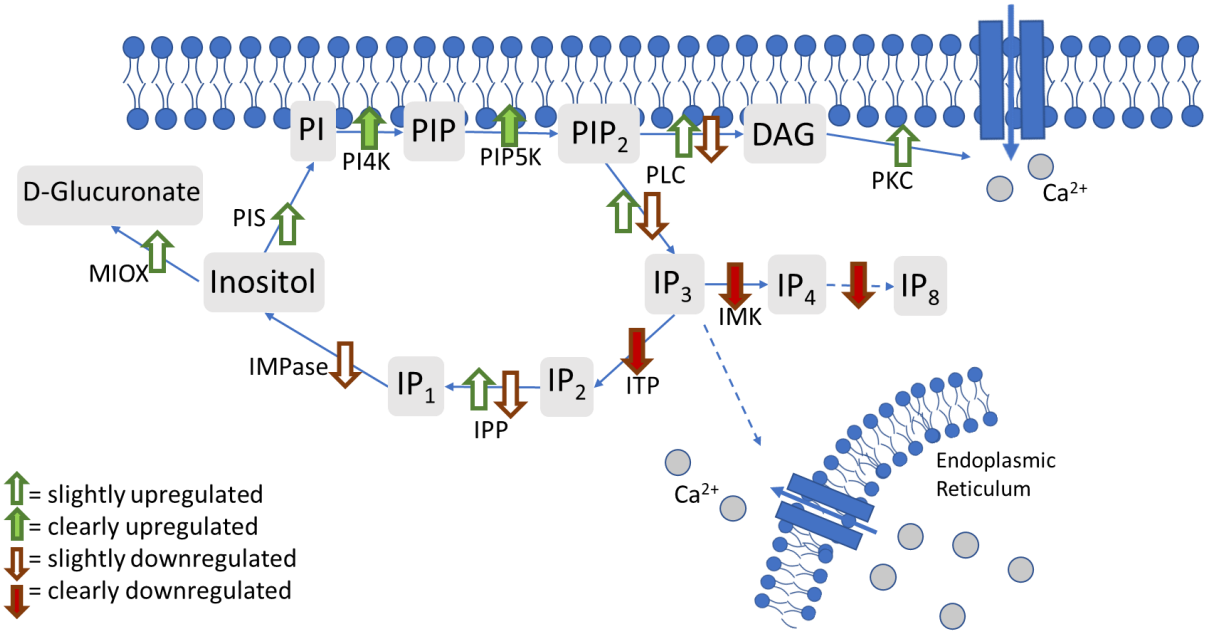


Fig. 4: Schematic representation of the phosphoinositol signaling with gene regulation under metal stress in *S. commune*. Enzyme abbreviations: PLC= phospholipase C; PKC= protein kinase C; IMK= inositol polyphosphate multikinase; ITP= inositol 1,4,5-triphosphate phosphatase; IPP= inositol polyphosphate phosphatase; IMPase= inositol monophosphatase; MIOX= myo-inositol oxygenase; PIS= phosphatidylinositol synthase; PI4K= Phosphatidylinositol 4-kinase; PI5K= phosphatidylinositol 5-kinase.

The expression analysis was done with a microarray analysis (GEO omnibus acc. no. GSE172373). The mRNA sequencing and subsequent qPCR analysis showed that a myo-inositol oxygenase (XM_003027475.1) and a hypothetical inositol polyphosphate phosphatase (XM_003036036.1) were significantly regulated under metal stress. The qPCR analysis was able to confirm the direction of regulation of the mRNA sequencing. The examination of all genes regulated under metal stress, which transcribe for enzymes of inositol signaling, showed that kinases tended to be up-regulated and phosphatases tended to be down-regulated (see Fig. 4 and Manuscript 4 Table S3). This would lead to a shift in the inositol cycle from myo-inositol towards phosphorylated inositol compounds such as PIP_2. Chaouch and Noctor 2010 found that in *Arabidopsis thaliana* oxidatively induced cell death caused by H_2O_2 leads to a reduction in the myo-inositol level. Here the thesis is made that the concentration of myo-inositol in the cell plays a key role in the decision whether oxidative stress leads to a defense mechanism. In the brown algae *Ectocarpus siliculosus* repression of myo-inositol and cross-talk with oxidative stress were found under Cu stress too (Ritter et al. 2014). This supports our results, because the toxic effect of metals comes, among other things, from the formation of oxidative stress (Valko et al. 2005). The up-regulated inositol polyphosphate phosphatase, which has been investigated in more detail by qPCR, was also already associated with the response to oxidative stress in earlier studies (Kaye et al. 2011; Kilaparty et al. 2016). However, this cross-talk has never been proven in fungi before.

A clear down-regulation of inositol multikinases under metal stress could be observed (see Fig. 4 and Manuscript 3, Table S3). This likely leads to lower levels of highly phosphorylated inositol phosphates and pyrophosphates. Since pyrophosphates play a crucial role in regulating phosphate homeostasis and cell death, the reduced level is likely to have an impact on the behavior of *S. commune* (Saiardi 2012; Saiardi et al. 2005).

It could also be shown that the intracellular calcium concentration in *S. commune* was significantly reduced when it was grown with different metals. The calcium concentration is reduced from 7-fold in the presence of $SrCl_2$ to 4-fold in the presence of $CdCl_2$ (Manuscript 4, Table 1). One possible reason for this is the uptake of metals instead of calcium through ion

channels (Zhang et al. 2018), or the changed inositol signaling and thus reduced Ca^{2+} influx. The second, however, seems rather unlikely, since under the influence of metals both the enzymes that lead to the second messenger Diacylglycerol (DAG) were up-regulated and also the protein kinase C (PKC), which leads to the activation of L-type Ca^{2+} channels (Yu 2004) and thus to increased calcium influx. Overall, a lower Ca concentration will lead to changes in physiological processes in the cell, because Ca^{2+} is an important second messenger, which is involved in a variety of processes (Berridge 1989). It has also been reported that increased Ca concentrations have a protective function against oxidative stress and lead to increased Cd tolerance (Huang et al. 2017). Therefore, the greatly reduced Ca levels in *S. commune* probably make it more susceptible to the oxidative stress that is caused by the increased metal concentrations and also has an influence on inositol signaling.

In order to investigate whether altered inositol signaling leads to a change in metal tolerance, a growth test with an inositol monophosphatase overexpression mutant (impOE4) from *S. commune* was compared to its empty vector control (EVC1) and two wild-types (T33 and 12-43). The mutant grew significantly better on cadmium compared to the wild-types and the strain carrying the empty vector control. However, its growth was significantly reduced in the presence of Cs and Zn (Manuscript 4 Figure 3). This suggests that higher IMPase levels, which lead to a shift in the inositol cycle towards myo-inositol, lead to a reduced Zn and Cs tolerance. This finding is in agreement with the results in *Arabidopsis thaliana* and *Ectocarpus siliculosus* (Chaouch and Noctor 2010; Ritter et al. 2014). Since the overexpression of the IMPase in *S. commune* led to an increased Cd tolerance, another mechanism must be present here. No statement can be made about Sr on the basis of the colony diameter, since T33 and the two resulting strains EVC1 and impOE4 grew with significantly less aerial mycelium in the form of "runner hyphae".

6. Conclusion and Outlook

Fungi are almost omnipresent in the environment and play an important role in environmental interactions. With increasing environmental pollution, their role in remediation of contaminated soils is also increasing.

Within this work, we could prove that *S. commune* is a potential candidate for bioremediation. Although it is a natural wood decomposer, it can survive in the habitat soil for at least one year without an additional source of carbon and thus outperform other wood-rotting fungi. There, it succeeded in asserting itself against the natural microbiological community and even spread with relatively great speed. In addition, the fungus can tolerate long-term radiation, high metal concentrations, and fluctuating abiotic conditions.

The mechanism behind this tolerance could be examined more closely using a newly created metal-adapted strain. Because it showed a lower transport and uptake rate, the acquired or strengthened mechanism is most likely an avoidance strategy. Since a potentially metal exporting MFS transporter is higher expressed in the adapted strain, enhanced efflux seems to be likely for the increased tolerance. A lower expression of a glutathione S-transferase under metal stress in the adapted strain indicates lower oxidative stress due to the improved tolerance. In order to be sure which particular tolerance mechanism is the basis or whether several mechanisms complement each other, further studies are necessary. Verification of the increased efflux is also sensibly combined with a detailed examination of the responsible transporter.

Since the mRNA sequencing showed two significantly differentially expressed genes, which are associated with inositol signaling, it was investigated whether there is a connection between metal stress and this pathway. On the one hand, it could be demonstrated that metal stress influences the phospho-inositol cycle and shifts it in the direction of phosphorylated inositol compounds, but probably inhibits the formation of pyrophosphates. On the other hand, altered inositol signaling also affects metal tolerance. If the inositol cycle is shifted in favor of myo-inositol due to IMPase overexpression, *S. commune* becomes more sensitive to Zn and Cs, but more resistant to Cd. The fact that *S. commune* seems to have a different tolerance

mechanism for Cd than for the other tested metals can also be seen from the different behavior of the adapted strain at the Cd uptake. No statement can yet be made about which cellular mechanism *S. commune* uses as a response to Cd stress and how this is exactly related to inositol signaling. Further studies are required to investigate the complexity of linking different pathways.

Overall, *S. commune* seems to be a very suitable candidate for mycoremediation, even if it probably contributes to the mobilization of metals in soil. We were able to prove that the wild-type transports metals along its hyphae and thus contributes to the translocation of mobile metals. This could be useful to make very heterogeneously contaminated soils as in the CEZ usable again for agriculture.

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Acknowledgment

I would like to express my deep gratitude to Prof. Dr. Erika Kothe. She gave me the freedom to implement my ideas within my research and still lead me through my way by inspiring discussions and ideas. Thank you for your support from the beginning of the project to the end! I am also grateful for the opportunity to participate in interesting and informative national and international conferences.

My thanks also go to Dr. Katrin Krause, who, despite her busy schedule, always took time for me for advice, discussions, and finding solutions to upcoming problems.

I am grateful to all the members of the BioVeStRa project for the successful collaboration and the enriching project meetings. In particular, I would like to thank Dr. Georg Steinhauser, Dr. Dharmendra Kumar Gupta, Dr. Wolfgang Schulz, and Karl-Heinz Iwannek, who went with me on an excursion to the Chernobyl Exclusion Zone and made this possible in the first place.

I would like to thank all my dear colleagues and friends Nina Carl, Marie Harpke, Kevin Lenk, Marlene Höller, Flavio Silva Costa, Berit Porsche, Dr. Oluwatosin Abdulsalam, Sebastian Pietschmann, Dr. Reyna Murry, Manuela Östreicher, Jessica Pötschner, Peggy Brand-Schön and Petra Mitscherlich. I thank you for the many encouraging conversations, the new perspectives that you were able to convey to me, and all the wonderful hours that we spent together. The years of the Ph.D. flew by with you and it was a wonderful time.

Finally, I would like to express my gratitude to my family and my beloved husband Markus from the bottom of my heart. You always believed in me, supported me, and motivated me in the ups and downs of my Ph.D. project. I can't imagine doing this without you guys. Thank you!

Author contribution

Manuscript 1: Survival of the basidiomycete *Schizophyllum commune* in soil under hostile environmental conditions in the Chernobyl exclusion zone

Own contribution: 40 %

The soil plate experiment, soil sampling, DNA extraction, and quantification by qPCR were done by me with the subsequent analysis. I wrote the original draft and implemented corrections by coauthors and reviewers. Anne Wollenberger and Falk Lehmann performed the large-scale cultivation of *S. commune*. Additionally, Anne Wollenberger was involved in the experiment with agar and soil columns and she wrote this part of the original draft. Sina Großmann analyzed the soil columns and Alix Günther supervised this experiment. Georg Steinhauser was the supervisor of the CEZ field trip and was involved in conceptualization. Dharmendra Kumar Gupta, Karl-Heinz Iwannek, Ihor Chyzhevskyi, and Sergiy Dubchak were involved at the field site and Sergiy Dubchak were moreover involved in project administration. Serhii Kirieiev provided laboratory rooms at the CEZ. Wolfgang Schulz analyzed and validated the radioanalytics and wrote this part of the original draft. Clemens Walther, Johannes Raff, and Erika Kothe did the conceptualization, administration, supervision, and acquisition of financial support. Erika Kothe was beyond that also involved in writing the original draft and editing.

Manuscript 2: Microbial community analysis in the radionuclide contaminated Chernobyl exclusion zone

Own contribution: 60 %

The soil sampling and DNA extraction were done by me and the bioinformatic evaluation of the sequence data was done by Sebastian Pietschmann. We both did the visualization of the data. I wrote the manuscript for this research article. The planning of the experiments was carried out by me in consultation with Erika Kothe.

Manuscript 3: Metal transport in hyphae of the basidiomycete *Schizophyllum commune*

Own contribution: 60 %

The experimental design and procedure were planned by me in consultation with Katrin Krause and Erika Kothe. I performed the adaptation experiments, staining and fluorescence microscopy, and all experiments belonging to the expression analysis. The growth experiment with influencing factors on metal tolerance was done by my Master student Jenny Shrestha and the transport experiment by Martin Richter under my supervision. I evaluated all data and wrote the original manuscript. Reviewing and editing of the manuscript was done by me, Katrin Krause, Thorsten Schäfer, and Erika Kothe.

Manuscript 4: Inositol signaling in the basidiomycete fungus *Schizophyllum commune*

Own contribution: 35 %

The investigation of metal inositol cycle interactions was done by me. Furthermore, I performed the cell wall integrity growth experiment and the fluorescence microscopy experiment during my master thesis under the supervision of Reyna Murry. Reyna Murry did the phylogeny and created the imp1 overexpression mutant. Furthermore, Reyna Murry together with Jessica Pötschner carried out the cell membrane protein isolation, and the Proteome analysis was done by Thomas Krüger. Writing of the original draft was done by Reyna Murry and me and reviewing and editing by me, Katrin Krause, Thomas Krüger, Olaf Kniemeyer, and Erika Kothe.

Declaration of Honor

I, Lea Traxler, hereby confirm that I myself wrote the present dissertation with the title "The potential of *Schizophyllum commune* for mycoremediation at the Chernobyl exclusion zone". I have not copied any text from a third party or my own examination papers without labeling and all sources, resources, and personal communications I have used have been indicated in the paper. I am also familiar with the current doctoral regulations of the faculty for biological science. I did not use the help of a commercial doctoral consultant and no other third party received any pecuniary benefits from me for the work related to the content of the submitted dissertation. The names of the people who assisted me in the selection and analysis of materials and in writing the manuscripts have been given. I have not submitted this or any other dissertation as an examination paper for a state or other scientific examination.

Ehrenwörtliche Erklärung

Hiermit bestätige ich, Lea Traxler, dass ich die vorliegende Dissertation mit dem Titel "The potential of *Schizophyllum commune* for mycoremediation at the Chernobyl exclusion zone" selbst angefertigt habe. Ich habe keine Textabschnitte eines Dritten oder meiner eigenen Prüfungsarbeiten ohne Kennzeichnung übernommen und alle von mir benutzten Quellen, Hilfsmittel und persönlichen Mitteilungen wurden in der Arbeit angegeben. Des Weiteren ist mir die geltende Promotionsordnung der Fakultät für Biowissenschaften bekannt. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen und auch sonst haben Dritte keine geldwerte Leistung von mir für die Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Namen der Personen, die mich bei der Auswahl und Analyse der Materialien und beim Schreiben der Manuskripte unterstützt haben, wurden genannt. Ich habe weder diese noch eine andere Dissertation früher als Prüfungsarbeit für eine staatliche oder andere wissenschaftlich Prüfung eingereicht.

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Place, date Signature

Curriculum vitae

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Since Aug. 2017	Doctoral candidate at the Institute of Microbiology- Microbial Communication of the Friedrich Schiller University
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Publications

Murry, R., Traxler, L., Pötschner, J., Krüger, T., Kniemeyer, O., Krause, K., Kothe, E. (2021) Inositol signaling in the basidiomycete fungus *Schizophyllum commune.* J Fungi 7, 470.

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Presentations

Talks

Traxler, L., (2020). The white-rot fungus *Schizophyllum commune*: a remediation study in the Chernobyl Exclusion Zone. Bio-Geo Colloquium, Jena, Germany.

Traxler, L., (2019). Studies on the mycoremediation ability in *Schizophyllum commune*. Symposium on Remediation, Jena, Germany.

Traxler, L., (2018). Investigation of potential for radionuclide protection by *Schizophyllum commune*. Symposium on Remediation, Jena, Germany.

Poster

Traxler, L., and Kothe, E. (2019). Interactions in contaminated environment: Investigation of *Schizophyllum commune* in Chernobyl soil. 18th Congress of European Mycologists, Warsaw, Poland.

Traxler, L., Kirtzel, J., Krause, K., and Kothe, E. (2018). Investigation of potential for radionuclide protection by *Schizophyllum commune*. 11th International Mycological Congress, San Juan, Puerto Rico.