Multimetal bioremediation and biomining by a combination of new aquatic strains of *Mucor hiemalis*

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Here we describe a unique microbial biotechnology for simultaneous bioremediation and biomining of twelve ionic metals overcoming the obstacles of multimetal toxicity to microbes. After a thorough search of key microorganisms in microbiomes of many sulfidic springs in Bavaria (Germany) over an area of 200 km², we found three new strains EH8, EH10 and EH11 of *Mucor hiemalis* physiologically compatible and capable of multimetal-remediation and enrichment. We combined the multimetal-resistance, hyper-accumulation and elicitation power of EH8, EH10 and EH11 to develop a novel biotechnology for simultaneous removal, fractionation and enrichment of metal ions. As a first step we showed the intracellular fixing and deposition of mercury as nanospheres in EH8’s sporangiospores. Scanning Electron Microscopy-Energy-Dispersive X-Ray analysis revealed binding and precipitation of other applied metal ions as spherical nano-particles (~50–100 nm) at the outer electro-negative cellwall-surface of EH8, EH10 and EH11 sporangiospores. Microbiomes, germinated spores and dead insoluble cellwalls of these strains removed >81–99% of applied Al, Cd, Co, Cr, Cu, Hg, Ni, Pb, U, and Zn simultaneously and furthermore enriched precious Ag, Au and Ti from water all within 48 h, demonstrating the potential of new biotechnologies for safe-guarding our environment from metal pollution and concentrating precious diluted, ionic metals.

Increasing global demand and use of various metals/metalloids leads to their accumulation in the environment and thereby simultaneously increases the risk of multimetal-toxicity to various organisms. In higher organisms accumulated heavy metals can damage nerves, liver, bones, etc., as well as block functional groups of enzymes and cause cancer. This metal accumulation is mainly due to various industrial processes such as metal production, mining, electroplating, nuclear power generation, municipality waste incineration and by manufacture of a myriad of metal-containing electronic components. Toxic metals/metalloids may even be mixed with recalcitrant and/or radioactive organic pollutants, e.g. Cu and biphenyl, which can increase the severity of metal toxicity and problems of remediation. Controlling this pollution now represents a large challenge for our society. The removal and recycling of toxic metals or metalloids, particularly from water phase, deserves our special attention because metal-contaminated materials like soil, sediment and gaseous emissions can be washed-off with water, so that ultimately metal contaminants are highly concentrated in, e.g., waste water (nuclear, metallurgical, mining) and landfill leachates. Several technologies have been described previously for the removal of toxic metals or metalloids from water, e.g. flocculation, adsorption, bioaccumulation by live microbial cells, immobilization, and reverse osmosis. Existing technologies for metal removal from aqueous phase are either too costly, e.g. by reverse osmosis, or they create excessive additional waste.

In contrast to *ex-situ* physical-chemical methods, *in-situ* natural enrichments of metal ions may take place due to physical-chemical and/or biological processes. Natural hydrothermal conditions, e.g. at temperatures above 60 °C, can enhance oxidative-reductive complexation and precipitation processes of metal ions in water, but have high energy requirements. Therefore, there is an increasing interest in biotechnology for the development of low-cost, low energy-consumption and effective bioremediation and biomining. *Ex-situ* or *in-situ* metal/metalloid removal by microbial biosorption and enzyme-mediated metal precipitation at cell surfaces could be cheap alternatives for treating metal-contaminated wastewater and/or water purification. So far, only a limited number of natural microorganisms have been found to show biosorption properties for a few toxic metals/metalloids, whereby they required very stringent culture conditions, e.g. concerning C- and N-source and

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an optimum pH12. Therefore, genetically modified microorganisms (GMO), e.g. Ralstonia eutropha, Escherichia coli and Saccharomyces cerevisiae, were developed to improve microbial hyper metal-binding capacity/accumulation by proteins13, however their usage faces serious legal restrictions in field applications due to the risk of uncontrolled GMO release and horizontal gene transfer. Microbial volatilization of metals/metalloids from such metal wastes creates additional safety problems during handling. The key obstacle in the development of suitable microbial biotechnology for multimetal bioremediation and biomining is the lack of knowledge about avoiding extreme multimetal toxicity to microorganisms. In this context it is worth mentioning that microbial removal of toxic metal mixes of nickel and cadmium from water phase was very difficult because of high multimetal toxicity to Clostridium thermoacetica even at a concentration of only 1 mM14. In addition, microbial tolerance to toxic metals differs, e.g. the following order of decreasing tolerance of microbes to cadmium, whereby the Cd toxicity increased multi-fold at pH 8–9, was found: actinomyces > eubacteria > gram-negative bacteria > gram-positive bacteria15. As such large differences in toxic metal removal exist, for example 90% of applied cadmium was shown to be removed by a new strain of Pseudomonas aeruginosa in a defined aerobic culture16, whereas only 12% of applied cadmium was removed by another Pseudomonas aeruginosa strain ATCC 1488617. Even genetically modified E. coli with a surface display of metal trapping peptides was shown to mainly bind Cd17, and to lesser extents Cu18 and Zn19, highlighting the contemporary limitations of bacterial toxic multimetal removal.

An alternative biotechnology for the removal of toxic metals could be the use of fungi18 and fungal microorganisms (biofilms)2. The terrestrial fungus Penicillium ochrochloron has been used as a biotrap for the removal of Cu20 and other toxic metal ions from aqueous solutions and surrogate waste waters. Some terrestrial fungal biomass have been chemically pre-treated to improve their metal remediation function21,22. Several strains of the yeast Rhodotorula was shown to be more resistant to Cd than to the heavy metals Ag, Co, Hg and Ni. However, there was no correlation between class of soil fungus and tolerance to cadmium15. Due to the high severity of multimetal toxicity in fungi, until now, as far as known, a maximum of 2–5 toxic metals like Pb, Cd, Cr, Cu, Mn, Ni, Zn were effectively removed at a time by a single terrestrial fungus, e.g. Aspergillus fumigatus, Aspergillus niger, Aspergillus terreus, Macrophomina phaseolina, Penicillium sp., Rhizopus stolonifer, Trichoderma viridae, Trichoderma longibrachiatum22–25. Although the terrestrial fungi Rhizopus and Trichoderma showed high resistance to a range of heavy metals, such as Cd, Cu, Pb and As, they were not assayed in the same experiment26. Viable cells of the yeast Saccharomyces cerevisiae removed five metals Cu, Cr, Cd, Ni and Zn from electroplating effluents, but only after glucose-pretreatment27.

In contrast to terrestrial fungi, aquatic fungi and their natural microorganisms at hydro-terrestrial interfaces have exhibited high metal enrichments and resistance28,29 and protected the unique natural consortia by enriching toxic multimetals from competitive successions of other organisms, e.g. by, micro- and macro algae. In co-presence of harmful multimetals and organic toxins, some fungi may be especially useful because they possess an additional repertoire of enzymes for the detoxification of organic toxins1. In particular the aquatic filamentous fungus Mucor hiemalis EH5 was shown to remove various organic pollutants, especially in fungal dual cultures28, as well as cyanobacterial toxins30,31, furthermore its natural microbe enriched some metal ions28. The strains of aquatic M. hiemalis could be useful for outdoor metal bioremediation under extreme natural conditions, as EH5 was found to thrive in situ in hostile conditions, i.e. cold sulfidic, reducing and/or nearly anoxic aquatic milieu in presence of diverse metals in the natural microorganisms of sulfidic springs28. Alternatively to using live fungal mycelium biomass31 and germinated spores32 for intracellular accumulation and/or biosorption and precipitation, fungal hyphal cell walls can be used, e.g. for chromium removal by terrestrial M. hiemalis cell walls33 or for lead removal by Penicillium chrysogenum cell walls34. So far, few individual live fungi or dead fungal cell walls have been used for the removal of only a few toxic metals from water11,13, as the live fungal mix (species/strain) may be mutually antagonistic during growth. This could be the reason why the successful use of fungal species/strain combinations for biotechnological applications was, until now, lacking.

Through our continued search for biotechnologically relevant fungi, we recently succeeded in isolating the novel aquatic strains of Mucor hiemalis EH8 and EH11 from the microorganisms of cold sulfidic spring waters with extraordinary metal/metalloid bioaccumulation capacity22–25. They were found to be crucial in the fungal-bacterial microbiomes of sulfidic-sulfurous springs for the hyper-accumulation of metals. The purpose of our study was to show how to develop suitable biotechnology for multimetal remediation, fractionation and enrichment based on; 1. Natural observations, ecological considerations, microbial biodiversity and successions; 2. Identification of key aquatic fungal strains (Mucor hiemalis EH8, EH 10 and EH11) in microorganisms of sulfidic spring water; and 3. Inhibition/tolerance assays and use of compatible strains (EH8, EH10 and EH11), e.g. as (a) Activated live sporangiospores, (b) live microorganisms and (c) Purified cell walls from solvent-killed single, and mixed-grown fungal strains25–29 for the simultaneous removal of diverse toxic metals and enrichment of precious metals. Here we describe in detail the testing of physiological tolerances of EH8, EH10 and EH11 in co-cultures, the isolation and preparation of mixed fungal materials (microorganisms, germinated spores and dead insoluble cell walls), the quantitative kinetics of simultaneous metal removal, the localization of metal precipitation by scanning electron microscopy (SEM) and the analysis of composition of metal precipitates by energy-dispersive x-ray (EDX) micro-analysis. This is the first report about the application of triple fungal co-cultures using EH8, EH10 and EH11 for simultaneous multimetal remediation, fractionation and hyper-enrichment (e.g. for biomining), with up to 99% efficiency for some precious metals, from water phase.

Results

Hydro-geochemistry of sulfidic springs and microbial succession. Table 1 shows the chemical and physical parameters of the sulfidic springs Marching, Quarzitwerk and Künzing, from which the M. hiemalis strains EH8, EH10 and EH11 were isolated, respectively. In an area of about 200 km2 we investigated the multimetal enrichment, microbial diversity and succession of microorganisms in microbiomes (biofilms) of 18
Adaptation and evolution of fungal strains that hyper-accumulate toxic metals. Several strains of *M. hiemalis* were found in the special habitats of sulfidic springs and showed unusual hyper-accumulation capacity for different metals, similar to that of their respective microorganisms (Table S1). Their hyper-metal accumulation properties could be due to adaptive pressure and acceleration of evolution triggered by extreme sulfidic spring water environments (see supplement). We have already reported the adaptation of EH8 to mercury stress and its ecological functions. Several strains of *M. hiemalis* from cold sulfidic spring water microorganisms were isolated and assayed for their physiological compatibility as well as for their metal removal functions (see below).

**Table 1.** Chemical and physical features of Marching, Quarzitwerk (Murnauer Moos) and Künzing spring waters as compared to control spring Teugn (September 2003). The contrasting parameters of these three springs as compared to the control spring Teugn are highlighted in bold font. Abbreviations are: ‘disappeared in the year 2004’, *DOC*: dissolved organic carbon, ‘Carle’.

| Component Parameters/Springs       | Unit     | Marching | Quarzitwerk | Künzing | Teugn |
|-----------------------------------|----------|----------|-------------|---------|-------|
| Spring discharge                  | L min⁻¹  | 120      | 180         | 210     | 150–200 |
| Temperature                       | °C       | 10.2–10.6| 10.1–11.4   | 18.9–19.0| 12.4–12.7 |
| Electrical Conductivity           | μS cm⁻¹ | 623–672  | 867–966     | 1310–1320| 665–697 |
| pH                                |          | 5.9–6.5  | 5.1–6.3     | 7.3–7.5 | 4.7–6.4 |
| Redox Potential (Eₐ)             | mV       | –185 to –173|--106 to –97 | –253 to –241| –215 to –192 |
| Oxygen                            | mg l⁻¹   | 1.3–1.8  | 0.4–1.1     | <0.1    | 0.7–1.2 |
| H₂S                               | mg l⁻¹   | <1       | n. m.       | <1      | n. m.  |
| Cations and total metal ions:     |          |          |             |         |       |
| Na⁺                               | mg l⁻¹   | 6.0–8.2  | 5.5–5.7     | 164.4–398.5| 50.7–54.7 |
| K⁺                                | mg l⁻¹   | 0.7–1.1  | 0.70–1.1    | 10.1–10.5| 6.5–6.9 |
| Mg²⁺                              | mg l⁻¹   | 31.3–31.5| 17.7–17.9   | 8.3–9.4 | 22.7–22.9 |
| Ca²⁺                              | mg l⁻¹   | 81.2–85.9| 109.4–120.5| 12.6–47.3| 72.7–74.2 |
| Mn²⁺                              | mg l⁻¹   | 4.5–4.9  | 44.8–270.2  | 2.5–2.7 | 7.6–9.5 |
| Ba²⁺                              | µg l⁻¹   | 15.5–20.4| 16.5–32.4   | 25.8–45.5| 47.0–58.2 |
| Total Co                          | µg l⁻¹   | <1       | <1          | <1      | 0–16.0 |
| Total Ca²⁺                        | µg l⁻¹   | 3.2–6.0  | <0.7–2.7    | 0–14.4  | 1.1–7.6 |
| Total Fe                          | µg l⁻¹   | 1.9–7.8  | 8–16.7      | 15.6–20.2| 9.8–16.7 |
| Li⁺                               | µg l⁻¹   | 6.2–7.9  | 2.3–3.0     | 202.0–279.4| 418.4–49.5 |
| Sr²⁺                              | µg l⁻¹   | 78.9–117.2| 213.1–333.8| 286.1–1005.4| 78.9–117.2 |
| Zn²⁺                              | µg l⁻¹   | 367.1–437.6| 163.2–237.2| n. d.   | 265.2–288.4 |
| Anions:                           |          |          |             |         |       |
| SO₄²⁻                             | µg l⁻¹   | 36.0–360.5| 434.7–434.9 | 572.00  | 420.6–420.9 |
| HCO₃⁻                             | µg l⁻¹   | 1.1–7.8  | 6.4–8.9     | n. d.   | 0.4–1.1 |
| Gas Bubbles:                      |          |          |             |         |       |
| CH₄ (emission)³                   | m³ d⁻¹   | n. d.    | —           | 7       | —      |

different sulfidic springs by metal screening assays, phase contrast and electron microscopy, molecular biological techniques and morphological comparison. Three strains of *Mucor hiemalis* (EH8, EH10 and EH11) were found to be crucial in their respective natural microbiomes for accumulation of diverse toxic metals, as the metal removal or enrichments by pure cultures matched the values for their respective natural microbiomes (Table S1). Bryophyta and macrozoobenthos typical of sulfidic springs were absent in the microbiomes of these springs, but *Archaea, Bacteria*, ciliates and diatoms were detected.

**Fungal inhibition/toxicity assay.** In fungal inhibition/toxicity assays the strains EH8, EH10 and EH11 were not mutually antagonistic (Fig. 2). No visible demarcation line between the dual culture mycelium fronts of EH8 and EH10/EH11 was found, suggesting they are physiologically tolerant to each other (Fig. 2A.1). Other...
Dual cultures of aquatic *Mucor hiemalis*, e.g. EH5 and EH10, and EH10 and EH12, reacted antagonistically to one another, as shown by e.g. formation of demarcation lines between mycelium fronts, release of oily drops at the hyphal tips and color changes in response to the presence of antagonistic strains (Fig. 2A.2–A.3). Purified viable germinating sporangiospores of aquatic *M. hiemalis* strains showed, as expected, strong presence of chitin that could be recognized and visualized as tiny anti-chitin stain spheres on the cell walls, as detected by fluorescence microscopy of anti-chitin fluorescein-labeled antibody (Fig. S2).

**Zeta-potential of cell surface, hyper-metal accumulation and precipitation.** Acid-base titration of the aquatic *M. hiemalis* sporangiospores did not show any distinct pH-values in the investigated pH range suggesting no domination of weak acids or bases (Fig. 2A). The zeta-potential of spore cell surfaces varied depending...
on nutrient/mineral contents of the incubation medium. Surprisingly, a groundwater-like environment with low mineral and nutrient contents was shown to increase the electro-negativity of the sporangiospores in the pH range 6–10, if they were pre-germinated in groundwater (Fig. 2A). Contrastingly, under a mineral-rich medium with high C- or N-contents the zeta potential of sporangiospores' surfaces lost ca. 10% of the electro-negativity at pH 7, mainly due to the favored pre-occupation of some electronegative sites on the spore surfaces by the metal cations supplemented to the fungal growth medium. The surface potentials of the spores showed rapidly increasing electro-negativity ($\sim -100 \text{ mV}$ down to $-480 \text{ mV}$) with increasing pH, indicating strong electrical attraction of aquatic M. hiemalis spore cell walls towards the positively charged metal cations.

Production of spherical metal nano- and micro-particles by activated and elicited sporangiospores. When a mixture of metal ions came into contact with cell surfaces of the activated sporangiospores of EH8, EH10 and EH11, metal spheres were readily produced. SEM showed the adsorption and precipitation of approx. 50–100 nm-sized spherical metal nanoparticles on the cell walls following 48 h incubation in metal salt solutions (pH $\sim 7$). (C) Zeta-potential of aquatic M. hiemalis sporangiospores after germination (1–3 cell stages) depending on nutrient conditions of incubation medium (red circle, C-limited medium; green triangle, C- and N-enriched medium; downward blue triangle, N-limited medium; black square, groundwater control) after 48 h incubation at approx. 30 °C. (D) Removal of metals by dead insoluble cell walls, live spore mix and live microbiomes (Fungal Bf) of strains EH8, EH10 and EH11 in comparison to the control terrestrial fungus DSM 2655. Horizontal bar in B2 and 2B2E indicates scale of 500 nm.

Figure 2. Inhibition/toxicity tests of M. hiemalis strains, detection of chitin, metal removal capacity and surface potential. (A1-3) Inhibition/toxicity tests of aquatic fungal strains, A1. Non-existence of demarcation lines between mycelial fronts showing absence of antagonistic inhibitory reactions among EH8, EH10 and EH11 when they were challenged against each other or grown together in the same plate, A2. Demarcation lines and discolouration indicating antagonistic reactions between EH5 and EH10, and A3. Demarcation lines and oily droplet formations illustrating antagonistic reactions between EH10 and EH12. (B-D) Relationship between metal binding and zeta-potential of the sporangiospore's cell surface. (B) EDX detection of metals bound to the surfaces of sporangiospores. B1. EDX-detection of Al, Pb, Cd, Cr and P at a spot (red rectangle) on the outer surface of the sporangiospores (B1), B2 and B2E (enlarged). Formation of ca. 50–100 nanometer-sized particles (nanospheres; see white arrows) at the outer cell surfaces of sporangiospores following 48 h incubation in metal salt solutions (pH $\sim 7$). (C) Zeta-potential of aquatic M. hiemalis sporangiospores after germination (1–3 cell stages) depending on nutrient conditions of incubation medium (red circle, C-limited medium; green triangle, C- and N-enriched medium; downward blue triangle, N-limited medium; black square, groundwater control) after 48 h incubation at approx. 30 °C. (D) Removal of metals by dead insoluble cell walls, live spore mix and live microbiomes (Fungal Bf) of strains EH8, EH10 and EH11 in comparison to the control terrestrial fungus DSM 2655. Horizontal bar in B2 and 2B2E indicates scale of 500 nm.
dead mixed insoluble cell walls, the spore mix and the mixed microbiomes of EH8, EH10 and EH11 is shown in Fig. 2D. Results showed a similar metal removal capacity of the microbiomes, the spore mix and the mixed cell walls.

**Removal of metal ions by mixed grown EH8, EH10 and EH11.** The first use of activated EH8 sporangiospores eliminated Hg(II), as well as some other metal ions, from water by intracellular accumulation (Table S1). Further results of metal removal by the microbiomes and corresponding fungi are summarized in Table S1. Metal resistance and accumulation assays showed that EH8 of the Marching spring microbiome interfacing moss leaves (Fig. 1A) hyper-accumulated Al (90%), Cr(III) (99%), Ni (86%) and U (89%) at the cell surfaces as well as Hg (99%) intracellularly. EH10 (Fig. 1B) from the Quarzitwerk spring microbiome additionally hyper accumulated Cd (91%), Cu (85%), Pb (93%), and Zn (71%) (Table S1). In contrast, anaerobic EH11 with its unusual spring-like hyphal adaptation (Fig. 1C.3,C.5) from the microbiome of methane-emitting salty sulfidic Künzing spring hyper-accumulated Al (98%), Cr(III) (89%), Cu (87%), Pb (97%), Ni (82%) and Zn (83%)29. If EH8, EH10 and EH11 were grown together, they became elicited and additionally enriched precious metal ions, e.g. Ag, Au and Ti. Therefore, the great potential of these mutually tolerant strains, EH8, EH10 and EH11, was demonstrated for the development of low-cost biotechnology for multimetal bioremediation and/or biomining.

**In situ generation of diverse element particles by microbiomes.** We observed precipitation of diverse nanometer- to micrometer-sized mineral particles, e.g. sphalerite (zinc sulfide), sulfur, iron colloids and iron sulfide, in microbiomes of sulfidic springs (Fig. 3). The precipitation of sphalerite in some microbiomes was also reported by Labrenz et al.36.

**Kinetics of metal removal by elicitation of EH8, EH10 and EH11.** The kinetics of metal removal depending on incubation time by fungal insoluble dead cell wall mix, spore mix and microbiome mix (EH8, EH10 and EH11) was compared with that of the control fungus (terrestrial *M. hiemalis* strain DSM2655, Fig. 4, Table 2). The hyper-accumulation of metals from contaminated water by fungal materials (see above) took place in two phases: an exponential phase (duration: ≤10 hours) and a slow phase (remaining duration: ≥38 hours) (Fig. 4B–F). In the exponential phase, more than 50% of metals applied were removed; the remaining metals were removed in the slow phase. It was demonstrated that the mixed dead cellwalls from EH8, EH10 and EH11 when compared to their live spore mix and live microbiome mix were similarly effective with respect to (1) a wide range of metals removed (Al, Cd, Co, Cu, Cr, Ni, Pb, Zn, Ag, Au, U, Ti) and (2) rate of metal accumulation from water. We demonstrated the high efficiency of metal removal and enrichment by mixed fungal dead cell walls.
by applying a metal mix of Al, Cd, Co, Cu, Cr, Ni, Pb, Zn, Ag, Au, and U, each at a concentration of 1,000 µg/l.

A universal three-parameter rational function with $y = \frac{(a + b \cdot x)}{1 + c \cdot x}$, whereby $y$ = concentration of metal (µg/l) and $x$ = duration of incubation (h) was shown to fit the metal remediation kinetics of all the metals applied optimally depending on incubation duration (h) (Table 2). Kinetics of residual mercury in water was inversely proportional to the amount of intracellular Hg fixed by EH8 independent of concentrations applied (1 mg.L$^{-1}$, 50 mg.L$^{-1}$), apparently due to high mercury tolerance and detoxification activity (Fig. 4B,C).

**Production of drinking water from metal-contaminated water by using successive fractionation and remediation biotechnology.** The successive removal of various toxic metals by toxin-free insoluble cell walls of mixed grown EH8, EH10 and EH11 from water contaminated with 1,000 µg L$^{-1}$ (Ag, Al, Cd, Co, Cr, Ni, Pb) is demonstrated for each metal in Fig. 5. Within 48 h the initial concentrations (each 1,000 µg/l) of aluminium and chromium decreased below the permitted threshold values for drinking water in the first step of fractionation e.g. following treatment of contaminated water with the insoluble mixed cell walls (Fig. 5A). After separation of cell wall materials in the first step, a re-treatment of water containing the residual metals Ni, Pb, U and Zn) by activated cell walls (CW, D), by activated spore mix (Sp, E) and by mixed microbiome (biofilm, Bf) grown on expanded clay spheres (F) of EH8, EH10 and EH11 (Fig. C.2) as well as enrichment of Au, Ag and Ti (Table 3). The fitted curve parameters with statistical significance are given in Table 2. Standard deviations of measurements ($n = 3$) at each data point were less than 5%.

**Figure 4.** Kinetics of simultaneous metal ion removal. (A) Intracellular mercury accumulation (arrows) and deposition of metallic mercury nanospheres (M) by reduction of ionic mercury. (B) Similar kinetics of residual Hg after treatment of EH8’s activated sporangiospores with low (1 mg.L$^{-1}$) and high (50 mg.L$^{-1}$) concentrations. (C) Similar kinetics of intracellular-fixed Hg at low (1 mg.L$^{-1}$) and high (50 mg.L$^{-1}$) concentrations. (D-F) Simultaneous removal of a metal mixture (>81–99% of Al, Cd, Co, Cr, Cu, Hg, Ni, Pb, U and Zn) by activated cell walls (CW, D), by activated spore mix (Sp, E) and by mixed microbiome (biofilm, Bf) grown on expanded clay spheres (F) of EH8, EH10 and EH11 (Fig. C.2) as well as enrichment of Au, Ag and Ti (Table 3). The fitted curve parameters with statistical significance are given in Table 2. Standard deviations of measurements ($n = 3$) at each data point were less than 5%.
Clay spheres will eliminate nearly all Hg(II) (99%), as well as some other metal ions as "fraction 1" from water by elimination by a combination of EH8, EH10 and EH11. Each row in the table represents an experimental (CW), spore mixtures (Sp) and cultivated fungal microbiomes (biofilms, Mb) describing highly efficient metal elimination by the generalized function y = (a + b*x)/(1 + c*x) depending on incubation duration (x, 0–48 h). Standard deviations of measurements at each data point were less than 5%. The asterisk mark (*) indicates the highest elimination of the respective metal ion from the aqueous phase.

| Metal | Parameter | p ≤ | r² | Parameter | p ≤ | r² | Parameter | p ≤ | r² |
|-------|-----------|-----|----|-----------|-----|----|-----------|-----|----|
| Al    | a = 1008.4159 b = −3.2817 c = 0.5618 | 0.0001 | 0.99 | a = 1011.2979 b = −5.3275 c = 0.5561 | 0.0001 | 0.98* | a = 1011.5696 b = −5.5922 c = 0.5520 | 0.0001 | 0.9498 |
| Cd    | a = 991.8978 b = −15.4006 c = 0.2119 | 0.0001 | 0.98* | a = 994.2403 b = −7.9289 c = 0.2869 | 0.0001 | 0.99 | a = 997.5382 b = 581.3458 c = 1.1636 | 0.0001 | 0.96 |
| Co    | a = 947.0998 b = −1.0934 c = 0.1012 | 0.0001 | 0.96* | a = 968.7509 b = 5.3617 c = 0.1183 | 0.0001 | 0.97 | a = 944.6699 b = 0.9920 c = 0.0920 | 0.0001 | 0.99 |
| Cu    | a = 947.9398 b = 1.6486 c = 0.1453 | 0.0001 | 0.98 | a = 938.9100 b = −0.0537 c = 0.1229 | 0.0001 | 0.99 | a = 945.6611 b = −3.9292 c = 0.1349 | 0.0001 | 0.96 |
| Cr    | a = 1007.0131 b = −15.9902 c = 0.4115 | 0.0001 | 0.99 | a = 1006.7962 b = −15.4942 c = 0.3879 | 0.0001 | 1.00* | a = 1008.5085 b = −12.7635 c = 0.4033 | 0.0001 | 0.99* |
| Ni    | a = 945.3009 b = 2.8120 c = 0.0958 | 0.0001 | 0.98 | a = 947.2866 b = 0.7131 c = 0.1030 | 0.0001 | 0.98* | a = 951.5460 b = 2.0655 c = 0.1068 | 0.0001 | 0.98 |
| Pb    | a = 959.8348 b = 9.0128 c = 0.2611 | 0.0001 | 0.93* | a = 959.4407 b = 10.7195 c = 0.2548 | 0.0001 | 0.93 | a = 959.8426 b = 10.8841 c = 0.2584 | 0.0001 | 0.93* |
| Zn    | a = 1001.0351 b = 301.2079 c = 1.1359 | 0.0001 | 0.99 | a = 1001.7014 b = 336.9611 c = 1.2772 | 0.0001 | 0.97 | a = 1000.6658 b = 246.6060 c = 0.9843 | 0.0001 | 0.99* |
| Ag    | a = 906.8907 b = −8.8880 c = 0.0487 | 0.0001 | 0.96 | a = 913.6193 b = −5.8345 c = 0.0575 | 0.0001 | 0.96* | a = 907.2963 b = −7.9693 c = 0.0534 | 0.0001 | 0.96 |
| Au    | a = 906.4437 b = 9.8921 c = 0.0590 | 0.0001 | 0.94* | a = 924.8630 b = 23.8750 c = 0.0821 | 0.0001 | 0.94 | a = 913.7804 b = 18.5878 c = 0.0756 | 0.0001 | 0.93 |
| U     | a = 1005.4774 b = −20.8824 c = 0.4439 | 0.0001 | 0.99* | a = 1006.6880 b = 34.1413 c = 0.4597 | 0.0001 | 0.99 | a = 1004.7597 b = 19.3687 c = 0.3862 | 0.0001 | 0.97 |
| Ti    | a = 922.5146 b = 4.1861 c = 0.0788 | 0.0001 | 0.97* | a = 936.1490 b = 14.6439 c = 0.0994 | 0.0001 | 0.97 | a = 942.3917 b = 15.7809 c = 0.1026 | 0.0001 | 0.97 |

Table 2. Characteristic parameters of the generalized peak-fitting functions of insoluble dead cell walls (CW), spore mixtures (Sp) and cultivated fungal microbiomes (biofilms, Mb) describing highly efficient metal elimination by a combination of EH8, EH10 and EH11. Each row in the table represents an experimental subgroup, whereby a single ionic metal per subgroup was applied. The three parameters (a, b, c), the significance levels (p-values) by Student's t-tests and the correlation coefficients (r²-values) are given for each ionic metal applied in a mixture (1,000 µg/l per metal) after fitting the residual concentrations (y) of each metal ion in water by the generalized function y = (a + b*x)/(1 + c*x) depending on incubation duration (x, 0–48 h). Standard deviations of measurements at each data point were less than 5%. The asterisk mark (*) indicates the highest elimination of the respective metal ion from the aqueous phase.

Cd and Co with the same amount of mixed cell walls removed all of them, keeping the residual concentrations below the permitted levels of metals in drinking water according to the German Water Ordinance37 (Fig. 5B). The optimum metal removal by fungal cell walls occurred at neutral pH, although at pH below 7 or higher than 7 the percentage of metal removal decreased only slightly (Fig. 5C).

Fractionation and biominning of metal ions by successive use of EH8, EH10, EH11 and mixed grown EH8-EH10-EH11. Figure 6A–E show the concept of fractionation and biominning of metal ions based on experimental results. The first use of activated EH8 sparganiospores or microorganisms on expanded clay spheres will eliminate nearly all Hg(II) (99%), as well as some other metal ions as "fraction 1" from water by intracellular accumulation (Table S1). EH8 (Fig. 1A,B) of Marching spring (sparganiospores, microbiomes, cell walls) can retain Al (90%), Cr(III) (99%), Ni (86%), U (89%) as "fraction 2". EH10 (Fig. 1B) from Quarzitwerk spring biofilm will remove Cd (91%), Cu (85%), Pb (93%), and Zn (71%) as "fraction 3". In contrast, aerobically cultivated anaerobic EH11 with its unusual spring-like hyphal adaptation (Fig. 1C, 3–5) from the microbiome of methane-emitting salty sulfidic Künzing spring can adsorb Al, Cd, Co, Cr, Cu, Pb, Ni and Zn as "fraction 4".

The characteristic ecological data and the glutathione S-transferase activities of the springs’ key organisms (EH8, EH10 and EH11), microorganisms and the terrestrial soil MHH (DSM 2655) are shown for the comparison of the biosorption properties of single strain, mixed grown aquatic strains and microorganisms (Table 3). In contrast to the biomass-mixture of separately grown (non-elicited) spores, the biomass-mixtures of activated and mixed-grown (elicited) sparganiospores, cellwalls and microorganisms of EH8, EH10 and EH11 can fractionate and even enrich precious metal ions, e. g. U, Ag, Au, Ti (Tables 2, 3; Figs 2D, 4 and 6D).
### A. Ecological Data

| Aquatic Fungi (Sulfidic-Sulfurous Springs) | Mh EH10 | Mh EH8 | Mh EH11 |
|-------------------------------------------|---------|--------|--------|
| Regional Occurrence/Spring                | Vicinity of Alps (Murnauer Moor) | West of Regensburg (Danube River Side) | West of Passau (Danube River Side) |
| Water Age (years)                         | 114     | 140    | >20,000|
| Start of microbiome formation (2003)      | May     | March  | Whole Year |
| Water temperature (°C)                    | 10.5    | 10.4   | 19.0   |
| Hydrological Type                         | Sulfide-Zinc-Hydrogen-carbonate (+Ca, +Mn) | Sulfide-Zinc-Hydrogen-Carbonate | Sulfide-Methane-Salt-Hydrogencarbonate |
| Sulfide content (mg/L)                    | 1.2     | 0.5    | 0.8    |
| Mineral content (mg/L)                    | 728     | 537    | 1249   |
| Redoxpotential (Eh, mV)                   | −106 to −97 | −185 to −173 | −253 to −241 |
| Water quality (Diatom-Saproby)            | 2.2     | 1.9    | 1.9    |
| Trophy (Diatom-index)                     | 2.1     | 1.7    | 2.2    |

### B. Biomarker (GST) Activity and Metal Biosorption Features

#### GST-Activity (nmol.min⁻¹.mg⁻¹)

| Microbiomes | Cytosolic | Microsomal | Cytosolic | Microsomal | Cytosolic | Microsomal |
|-------------|-----------|------------|-----------|------------|-----------|------------|
| IDNB        | 210.95    | 9,109.62   | 15,486.36 | 5,161.41   | --        | --         |
| CDNB        | 1,347.78  | 7,513.11   | 17,896.42 | 4,328.00   | --        | --         |
| DCNB        | 24,256.05 | 14,789.03  | 13,803.01 | 4,364.44   | --        | --         |
| EPNP        | 21,171.05 | 259,739.64 | 410,556.21| 74,182.73  | --        | --         |
| Fluorodifen | 10,229.79 | 54,066.00  | 21,178.58 | 55,418.01  | --        | --         |

| Fungi        | IDNB (Mhh: 159.69/171.28)² | CDNB (Mhh: 153.03/188.77)² | DCNB (Mhh: 138.27/46.35)² | EPNP (Mhh: 4,701.20/6,303.42)² | Fluorodifen (Mhh: 315.94/635.42)² |
|--------------|--------------------------|---------------------------|-----------------------------|-------------------------------|----------------------------------|
|              | 387.88                   | 372.90                    | 327.90                      | 8,473.16                      | 1,294.71                       |
|              | 676.92                   | 239.52                    | 239.52                      | 10,060.22                     | 1,313.52                       |
|              | 525.95                   | 305.46                    | 305.46                      | 15,342.55                     | 680.00                          |
|              |                          |                            |                             | 2,856.84                      | 81.0                            |

#### Effective Metal Sorption (%)

| Metal | EH10 | Nm# | EH8 | Nm# | EH11 | Nm# |
|-------|------|-----|-----|-----|-----|-----|
| Cd (Mhh**: 53.0%) | 92.0 | 93.8 | 6.0 | 95.5 | 81.0 | 96.6 |
| Cr (Mhh**: 90.5%) | 23.0 | 91.6 | 0.0 | 99.3 | 81.0 | 99.5 |
| Cu (Mhh**: 0.0%) | 38.0 | 75.1 | 99.0 | 91.9 | 91.0 | 95.3 |
| Pb (Mhh**: 96.0%) | 89.0 | 90.8 | 20.0 | 93.6 | 83.0 | 94.8 |
| Hg (Mhh**: 0.0%) | 92.0 | 82.4 | 18.0 | 82.8 | 97.0 | 96.6 |
| Al (Mhh**: 0.0%) | 0.0  | 0.0  | 99.0 | 97.5 | 0.0  | 0.0  |
| Ni (Mhh**: 4.2%) | 18.0 | 79.1 | 90.0 | 98.8 | 99.0 | 90.7 |
| Zn (Mhh**: 24.8%) | 0.0  | 91.8 | 86.0 | 95.1 | 82.0 | 99.7 |
| U (Mhh**: 0.0%) | 71.0 | 73.9 | 46.0 | 48.1 | 86.0 | 96.4 |

**Table 3.** Ecological, biomarker (GST) and biosorption data of the strains EH8, EH10 and EH11. The mean percentages of metal ion removal from water were calculated after measurements following applications of metal mixes with 1,000 µg/l per each metal to natural microbiomes (Nm, biofilms) and corresponding fungal cultures (F). The significant matched values of microbiomes and fungus of selected springs are shown in bold font, whereas contrasting data are given in italics. Standard deviations of measurements (n = 3) at each data point were less than 5%. Abbreviations are marked: *Cytosolic/microsomal GST activity, **Mhh: *Mucor hiemalis f. hiemailis* (DSM 2655, terrestrial strain) for comparison, # mostly intracellular accumulation, - not measured due to low amount of available materials, #Matched values are in bold font; Sorptions by Nm (natural microbiome with key fungus) > sorption by respective key fungus shown in italics are apparently due to other microbiome’s components, being not related to respective key fungus.
Harrison et al. suggested a multifactorial model by which biofilm populations can endure metal toxicity by a process of biodiversification. In contrast to the overall microbial-community role for the metal precipitation, it was shown by our previous studies that if a key microorganism for metal removal exists in microbiomes, it can remove toxic metals simultaneously with high efficiency which is comparable to the microbiome itself (Table S1).

Adaptation and evolution of some key *M. hiemalis* microorganisms in hostile environments of cold sulfidic springs are sufficient to confer special metal resistance, hyper-metal accumulation and detoxification power that can give the whole microbiome selective advantages for survival and facilitate their microbial community members to select favourable successions in extreme microbial habitats (Tables S1, 2).

Hyper-metal accumulating *M. hiemalis* strains EH8, EH10 and EH11 from sulfidic spring water were tested for metal accumulation after inhibition/toxicity assays. Using this assay, they were found to be physiologically tolerant to each other without showing any antagonistic inhibitory effects, and hence used in combination for the development of new metal remediation biotechnology. For fungal metal binding and remediation, the cell wall...
Figure 6. A new emerging biotechnology to enrich precious metals from diluted solutions. If grown together, the elicitation of EH18, EH10 and EH11 by interactions induces changes in chitinised cell walls (see supplement; A–C) capable of enriching precious metal ions like titanium, silver and gold (D), not following mathematical set theory for addition. (A) SEM of mixed grown activated germinating spores showing outer cell wall surface with bound nanometer-sized metal particles. (B) Stereo microscopic image showing elicited spores’ fungal microbiome grown on expanded clay spheres and (C) Solvent-killed and –purified insoluble dead cells/cell walls of mixed grown germinating activated sporangiospores, also to concentrate the precious metals as described. (E) Schematic set up for ex situ fractionation, bioremediation and biomining of ionic metals, especially precious metals, from multimetal-containing water phase.

components and their surfaces could play an important role. The cell wall of *M. hiemalis* consists of chitin (ca. 11%, Fig. S2), chitosan (32%), protein (5%) and phosphorus (1%)11, which are considered important for the determination of biotechnologically relevant features during growth and cell integrity39. In *M. hiemalis* MP/92/3/4 cell walls, the ratio of chitosan to chitin was ~1: 0.84 (17.39% chitosan: 14.57% chitin, re-calculated), when the fungus was grown in adequate inorganic-N (sodium nitrate), whereas it decreased to ~1:1.87 (8.7% chitosan: 16.3% chitin, re-calculated) when the fungus was grown in organic-N (peptone). Due to this ratio change with a concomitant decrease (about 21.8%, re-calculated) of Cr(III) biosorption, chitosan of *M. hiemalis* MP/92/3/4 cell walls is suggested to be involved in the biosorption of Cr(III)11. However, the increment of chitosan by ~8.7% (re-calculated) is not in line with ~1.74% (re-calculated) decrease of chitin in the cell walls of inorganic-N grown *M. hiemalis* cells in comparison to the organic N-grown cells. Thus, chitosan cannot alone be responsible for the increment of biosorption by 27.91%, suggesting involvement of other functional groups, e.g. phosphate-group, in metal binding. In accordance with this interpretation, we found a strong P peak in aquatic *M. hiemalis* cell walls (Fig. 2B), where e. g. Al, Pb, Cd and Cr are adsorbed and precipitated as nanoparticles (Fig. 2B). The cell wall chemical composition of terrestrial *M. hiemalis* was variable, probably due to the influence of nutrition conditions, e.g. type of N-source11. Similarly, we also observed changes of cellular surface potential of aquatic *M. hiemalis* by varying nutritional conditions of growth medium (C-limitation; N-limitation; C-, and N-enriched medium; groundwater) (Fig. 2C). The zeta-potential of pure chitin showed enhancement of electro-negativity with increasing pH after pH ~ 5.7 and reached the highest electronegativity ~ −260 mV at pH ~ 10.8 (data not shown). In contrast, the zeta potential of aquatic *M. hiemalis* spore surfaces at the lowest point is nearly double negative when compared to the zeta-potential of pure chitin, implying the involvement of other electronegative groups in the cell walls of aquatic *M. hiemalis* with twice the capacity for metal binding. The strong presence of a phosphorus peak at the location of precipitates of Al, Cd, Cr and Pb indicated high concentration of electronegative phosphate ligands for the complexation with positively charged metal cations at cell walls in aquatic *M. hiemalis* (Fig. 2B). Similarly, the deposition of Al and U cations into polyphosphate granules was also observed in the cell walls of *Anabaena cylindrica*40 and in *Citrobacter sp*41. The metal precipitation at the cell walls of germinated spores could be catalyzed by acid phosphatase activity by increasing release of phosphate anion ligand for the complexation with metal cation and its precipitation as inorganic insoluble metal phosphate2. The concentration of the phosphate ligand may also exceed the solubility product of the metal phosphate in proximity to nucleation sites on the cell surface41. Similarly, it is possible in *M. hiemalis* by sequential biosorption and precipitation of Cr(III)41. Additionally, the functional groups of chitin, chitosan and other cell wall components as well as changes of molecular configurations and associated high electro-negativity of aquatic *M. hiemalis* cell surface might be involved in the cellular metal precipitation process (Fig. 2B–D), apparently via regulation of cell wall integrity and signalling pathways39. The binding of positively charged heavy metal cations to the fungal cell walls was previously suggested to be due to the electrostatic attractions42. Electro-negativity of the cell surface apparently results
from the occurrence of negatively charged (dissociated) functional groups like carboxylic-, amino-, hydroxyl- and phosphate groups42,43. Hydroxyl-, amino-, acetyl- and carbonyl groups can be also found on chitin and chitosan of fungal cell walls, but additionally the presence of carboxyl groups on the fungal cell surface was suggested to be important for the biosorption of Hg\(^{2+}\) (85.34%) and Co\(^{2+}\) (73.05%)44.

EDX microanalysis demonstrated that the removed metals were deposited as nanoparticles (approx. 50–100 nm) in clusters on the surface of the aquatic M. hiemalis cell walls (Fig. 2B). Nanometer- to micrometer-sized particles were deposited in the natural microorganisms of the sulfidic springs, probably due to aggregations induced by microbial interactions with metal cations46 (Fig. 3). Deposition of nanosized sphalerites (zinc sulfide) was shown to be mediated by the microbial community46. It was reported that microbes could change the orientation of metal ions in 3D space for the formation of nanospheres in the microbial community45. Here we demonstrate the use of a unique combination of EH8, EH10 and EH11 in producing diverse metal nanoparticles or nano-alloys, not only important for bioremediation, but also essential for e. g. biomedical (e.g. nano-contrasting substances in medical diagnosis), industrial (e.g. nano-catalysts) or biomining (e.g. selective enrichment) applications. Our discovery of this superior biotechnology for multiple nanometal particle and nano-alloy production is noteworthy as it supersedes biotechnologies focussed on single specialised fungi capable of producing only one particular metal nanoparticle46. For survival and additional resistance, the fungal microorganisms and microbiomes might have evolved the ability to interact their electronegative cell surfaces with toxic metal cations to produce non-toxic aggregates of nanospheres. SEM-EDX analysis showed intracellular accumulation of highly toxic ionic Hg and subsequent chemical reduction to metallic nanospheres by EH8's live (germinating elongating) sporangiospores. Fixing of intracellular Hg was inversely proportional to the diminishing residual Hg in water phase with time, following a generalized kinetics function of intracellular Hg accumulation independent of applied concentrations (Fig. 4A–C)

All of the three fungal materials (mixed microbiomes, spor mix, dead mixed insoluble cells/cell walls) of combinations of EH8, EH10 and EH11 share a mutual mechanism of metal biosorption. They mainly use cell surfaces for simultaneous hyper-acummulation and precipitation of twelve tested metal ions (Fig. 5D), apparently due to appearance of extra special chemical and physical properties in their cell walls by elicitation that could not be revealed by the toxicity/inhibitory tests (Fig. 1). As the cell wall surfaces play a great role, the dead cells/cell wall skeletons irrespective of preparation method (autoclaving/sterilization, solvent killing) mainly contribute to the removal of most of the metals. However, calculations using curve-fitting point to preferential binding of Cd, Co, Pb, Au, U and Ti by solvent killed and solvent washed dead cells (cell wall skeletons) in juxtaposition to other metal ions (Table 2), supporting the view that killing of fungus increases biosorption of some metals47. Our calculations also showed preferential fixing of Al, Cr, Ni and Ag by the live germination-activated spores. Contrastingly, the fixing of Cu, Pb and Zn beside Al was rather preferred by the live fungal microbiomes (Table 2). However, high metal removal values of all of the tested materials showed maximum 31% variations among them depending on metal ion and material used. Noteworthy, the three metals Au, Ag and Ti were fractionated and enriched only by the above mentioned growth combination of EH8, EH10 and EH11 by elicitation, i.e. they were not hyper-acummulated by any individual EH8 or EH10 or EH11 or by any combination not grown together. The underlying mechanism of gaining additional metal removal/enrichment functions by their combination of EH8, EH10 and EH11 can be mainly explained on the basis of elicitation/adaptation of cell wall composition and functions, whereby increment of surface display of pertinent functional (electronegative) groups and orientation via molecular, biochemical and physiological interactions can be assumed to take place during cell wall biosynthesis of germinating/elongating EH8, EH10 and EH11 sporangiospores in a mix. Thus, such changes apparently result in changes of ionic interactions of electronegative functional groups of cell surface with metal cations, complication reactions, chemical reduction and acceleration of metal precipitation processes. Analogously, Kotrba et al. showed four times more binding of Cd\(^{2+}\), but Cu\(^{2+}\) and Zn\(^{2+}\) in less amounts, to cell surface by genetically modifying the surface display of short metal binding peptides on Escherichia coli48. On the contrary, our fungal technology using EH8, EH10 and EH11 covers a wide range of metals for binding.

Inhibitory concentrations of copper or cobalt in the growth medium can also alter the cell wall's metal binding properties of Cunninghamella blakesleean46. The diverse metal removal efficiency of mixed cell walls (EH8, EH10 and EH11) in the range of 67% to >99% of initial concentrations (Fig. 2C) was similar to that of the corresponding spor mix and microbiome, but much higher than that of the terrestrial control fungus M. hiemalis strain DSM2655, which removed only Cd (53%), Cr (90.5%), Pb (96%) and Zn (24.8%). The maximum biosorption capacity of Cr(III) by the cell wall of terrestrial M. hiemalis MP/923/4 was given as 132 mg/g dry-weight, whereas the whole cells of terrestrial M. hiemalis MP/923/4 reached a maximum of 21 mg/g dry-weight49. On the contrary, our fungal technology using EH8, EH10 and EH11 covers a wide range of metals for binding.

The future. Naturally occurring EH8, EH10 and EH11 simultaneously removed nine toxic metals (Al, Cd, Co, Cu, Cr, Ni, Pb, Zn) and thereby also enriched four precious metals (U, Ti, Ag, Au) (Fig. 6A–E), a result that was elusive to date. For the first time it was demonstrated that if the physiologically tolerant (compatible) fungi were grown together, their respective functions became biologically elicited (enhanced) and acquired additional properties of Ti, Ag and Au enrichment, not following the mathematical union of the three sets of metal accumulations by EH8, EH10 and EH11 individually. This mixed fungal technology may help in promoting cheap biotechnology for bioremediation, biofractionation and biomining of metals. A new bioremediation technology with its wide coverage of toxic metals at high concentrations without any genetic modifications or pre-treatment may have high competitive advantages in securing a significant part of the multi-billion dollar remediation and biomining market. Landfill leachate, mine drainage and waste water with toxic metal mixes can also be decontaminated by using column fillings of mixed grown EH8, EH10 and EH11. A step-wise treatment of multimetal-contaminated water using insoluble EH8, EH10 and EH11 cell wall mix can even deliver drinking water (Fig. 5A,B), which is just one of the application areas in which the biotechnological potential of aquatic M.
**Phanerochaete chrysosporium** remains to be exploited. Even toxic Hg could be removed by live EH8 sporangiospores. In contrast, most of the other microorganisms have so far failed to remediate a myriad of toxic metals simultaneously due to the extreme synergistic multimetal toxicity. As for example, simultaneous presence of Ni and Cd in solutions exerts toxicity to *Clostridium thermoaceticum* even at the low concentration of 1 mM.

Some merits of this new biotechnology are worth mentioning:

1. Possibility of ex-situ and in-situ applications
2. Possible regeneration and recycling of biosorbents, e.g. by washing with 5 mM EDTA
3. Minimization of microbial waste by burning
4. Possibility of multimetal nanospheres production for industrial and medical use, nearly independent of pH, e.g. only 10% efficiency loss was detected at pH 4 (Fig. B2B, B2E)
5. Independence of growth from temperature variations in a wide range, enabling even an in situ
treatment of a metal contaminated aquifer using fungal microbiomes on expanded clay down to near freezing temperature,
6. High safety assurance while using toxin-free fungal cell wall mixes for water purification,
7. Cheap biotechnology: even extracted fungal cell walls exhibited 2–3 times superior binding capacity for
ionic metals compared to other materials used, thereby about 30% less cell wall biomass is required for
the same efficiency of metal removal by using spore mixes or microbiomes. A calculation by Holán and Vole-
sky showed at least double the metal removal capacity of insoluble fungal cell walls compared to marine
algae on the basis of dry-weight.
8. Biological elicitation by the fungal strains' mixed growth enhanced their ability to enrich precious metal
ions more than the union of three sets of metal enrichments by the strain EH8, EH10 and EH11 individually,
ally, implying the biological set union has a system response greater than the corresponding mathematical
set union.
9. In combination with *Phanerochaete chrysosporium*, aquatic *M. hiemalis* strains might also simultaneously
remove many recalcitrant organic pollutants from their mixed contaminations with multiple metals in
waste water.

Last but not least, the presented results may inspire us to use these new strains EH8, EH10 and EH11 to con-
centrate and extract precious metals from oceans and seas at a low-cost, which was once tried in vain by the great
German chemist F. Haber. Later Necker also dreamt of extracting gold, silver and uranium from seas and
oceans using emerging technology, but it was also found to be economically infeasible because of low concentra-
tions (e.g. ~10 ng L⁻¹ gold and silver). A feasible low-cost method for biotechnological extraction of gold has
awaited a break-through since the discovery that deposition of gold in ores was due to microbial activity. Now
we are on the verge of an economically feasible emerging biotechnology, simply showing nature's power in special
microorganisms and microbiomes of selected sulfidic springs to remediate multimetal-contaminated water and
even to extract and concentrate some precious ionic metals from extremely diluted solutions present in lakes and
oceans (Fig. 6). The use of insoluble fungal dead cell-wall fractions avoids the eco-toxicological risk and conflict
arising from current legislation restricting the use of live fungi, microbiomes and/or genetically modified/engi-
neered microorganisms for drinking water purification, bioremediation and/or biomining in the fields, as there is a risk of toxin release by live fungus. However, in presence of recalcitrant highly toxic organic substances mixed
with toxic metals a combination of aquatic *M. hiemalis* with non-pathogenic *P. chrysosporium* could be used.

The ability of mixed grown EH8, EH10 and EH11 to fractionate and enrich precious metal ions like U, Au, Ag
and Ti from the aqueous phase opens a new possibility of simultaneous water purification, biofractionation
and biomining of precious metals, even from highly diluted concentrations present in marine water. Thus, our results
may be useful for the development of an efficient large-scale low-cost fungal biotechnology for the treatment of
multimetal-contaminated industrial waste water, land-fill leachate and ground water and/or for biomining of
valuable metals.

**Methods**

**Heavy metal stock solutions.** Heavy metal salts or solutions used were of analytical grade and
obtained from commercial sources (Sigma-Aldrich-Fluka, Merck): Al(NO₃)₃.9H₂O, AgNO₃, AuCl₃aq.,
Cd(NO₃)₂.4H₂O, Co(NO₃)₂.6H₂O, Cr(NO₃)₃.9H₂O, Cu(NO₃)₂.3H₂O, HgCl₂, Ni(NO₃)₂.6H₂O, Pb(NO₃)₂, TiCl₃,
U(CH₃COO)₂.2H₂O and Zn(NO₃)₂.6H₂O. Stock solutions of required concentrations were prepared in ultra-pure
de-ionized water below the solubility limit of the metal salts.

**Toxicity/inhibition assay.** The tests of strain toxicity/inhibition i.e. strain compatibility based on strain
morphology were carried out on malt-extract agar plates by inoculations of mycelium pieces obtained from commercial sources (Sigma-Aldrich-Fluka, Merck): Al(NO₃)₃.9H₂O, AgNO₃, AuCl₃aq.,
Cd(NO₃)₂.4H₂O, Co(NO₃)₂.6H₂O, Cr(NO₃)₃.9H₂O, Cu(NO₃)₂.3H₂O, HgCl₂, Ni(NO₃)₂.6H₂O, Pb(NO₃)₂, TiCl₃,
U(CH₃COO)₂.2H₂O and Zn(NO₃)₂.6H₂O. Stock solutions of required concentrations were prepared in ultra-pure
de-ionized water below the solubility limit of the metal salts.

**Zeta-Potential of fungal spore surfaces.** The surface charge (relative zeta-potential) of fungal spores at
different pH was determined by measuring streaming current potential shift in the diffuse layer of fungal
spore surfaces (electrostatic double layer model) after induction of a water stream by placing a current over the
surface. For this purpose, the measuring electrode was fixed on a surface containing the fungal spores and the
potential shift was detected using a particle charge detector Mütek PCD 03-pH (Mütek, Herrsching, Germany).
Sporangiospores (2–5 ml spore suspensions) of aquatic *M. hiemalis* germinated in different nutrient liquid media
(low-C; low-N; C-, and N-enriched media; water control) were diluted to give final mean concentrations of
about 4.4 × 10⁹ spores in 20 ml. The initial pH values of *M. hiemalis* spore dilutions were between 7.6 and 8.3. The
Preparation of microorganisms, spore mix and dead insoluble mixed fungal cell walls. All the three strains, EH8, EH10 and EH11, were able to grow under aerobic conditions on malt extract-agar plates, although EH11 was also found to live under strictly anaerobic conditions in liquid cultures. The germinated spores of the strains were used as 1:1:1-mixture, as they were found to be physiologically compatible when challenged separately and against each other. Briefly, fungal sporangiospores (see results) from five malt extract-agar (2.1, wt/wt) plates of each strain were collected and enriched by filtration and centrifugation technique. The fungal spores were counted using a hemacytometer (Sigma) and image processing software (ProImage v.3.01, MicroMotion). Germination of sporangiospores \( (5 \times 10^7, \text{EH8/EH10/EH11} = 1/1/1) \) was induced in 50 mL C-, N-enriched medium\(^5\) or on expanded clay spheres at 30 °C under shaking at 120 rpm. Active germinated spores (1–3 cell stages) were purified by repeated washing with PBS (pH 7.4) and centrifugation cycles, and then absorptions \( (A_{270\text{nm}}, A_{650\text{nm}}) \) of diluted suspensions were determined. Calibration curves for spore biomass (fresh- or dry-weight, mg) were obtained by standard additions of spore suspensions to N-(4-aminophthalaldehyde) and B-, C- and/or molecular biological sequencing\(^5,28\).

Material Preparation. Active germinated spores (5 × 10^7, EH8/EH10/EH11 = 1/1/1) were separated by centrifugation, fixed with 1% glutaric aldehyde (15 min), then treated with 2% OsO4 for 2 h and dehydrated in an increasing ethanol gradient (50%, 80%, 100%) in water. The uncut samples were sprayed with nano gold particles prior to observations with scanning electron microscopy (SEM, model JSM 630 F, Jeol) at 5–15 kV in the secondary electron mode. The sites of metal accumulation by microbiomes depending on pH was studied in the pH range 3–7. As some metal salts might precipitate under alkaline conditions due to the formation of insoluble hydroxides, the data taken at pH higher than 7.5 are not taken into account. The fungal spores or microbiomes were incubated in water of selected pH (spores) were separated by centrifugation, fixed with 1% glutaric aldehyde (15 min), then treated with 2% OsO4 for 2 h and dehydrated in an increasing ethanol gradient (50%, 80%, 100%) in water. The uncut samples were sprayed with nano gold particles prior to observations with scanning electron microscopy (SEM, model JSM 630 F, Jeol) at 5–15 kV in the secondary electron mode. The sites of metal accumulation by microbiomes depending on pH was studied in the pH range 3–7. As some metal salts might precipitate under alkaline conditions due to the formation of insoluble hydroxides, the data taken at pH higher than 7.5 are not taken into account. The fungal spores or microbiomes were incubated in water of selected pH containing heavy metals of various concentrations for 48 h. The pH of incubation water was monitored before and after experiment.

Glutathione-S-transferase activity. The glutathione S-transferase activity of aquatic fungi was determined as described\(^28\).

Assay of pH-stability of metal sorptions by fungal materials. The capacity of heavy metal adsorption and accumulation by microbiomes depending on pH was studied in the pH range 3–7. As some metal salts might precipitate under alkaline conditions due to the formation of insoluble hydroxides, the data taken at pH higher than 7.5 are not taken into account. The fungal spores or microbiomes were incubated in water of selected pH containing heavy metals of various concentrations for 48 h. The pH of incubation water was monitored before and after experiment.

Statistical analysis. The statistical analysis of data was performed using the Student’s t-test and the Mann-Whitney U test\(^27\). The data for the curves were fitted by using the software Sigma Plot 8.0 for Windows (SPSS Inc., United States).

Identification. All the strains were isolated and identified by comparison of morphology, mating behavior and/or molecular biological sequencing\(^5,28\).
**Strains.** Strains EH8, EH10 and EH11 have been deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) as strains DSM 16290, DSM 16291 and DSM 16292, respectively. The ITS1-5.8S – ITS2 sequences of plus- and minus-strands of aquatic *Mucor hiemalis* have been deposited at the Genbank under the accession numbers GU183689 and GU183690, respectively.

**Data Availability**

All data concerning the manuscript have been presented.

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Author Contributions
E.H. designed the experiments, supervised the experimental work and also conducted experiments for Fig. 1B, Fig. 2C, Fig. S2, calculated plots and re-drew graphics for Fig. 3 (zoomed view), Fig. 4.B, Fig. 4.C, Fig. 6E; performed statistical analysis of Table 2, interpreted and discussed all the results as well as wrote the whole manuscript. J.F. performed all other experiments under the supervision of E. H., interpreted and discussed all the results as well as wrote the whole manuscript. J.F. performed statistical analysis of Table 2, interpreted and discussed all the results as well as wrote the whole manuscript. J.F. performed all other experiments under the supervision of E. H., interpreted and discussed all results.

Additional Information
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