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Fungal Arsenic Tolerance and Bioaccumulation: Local Strains from Polluted Water vs. Allochthonous Strains

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Abstract: The paper deals with the possible use of fungi to decontaminate polluted waters. Specifically, the focus is the selection of the most promising fungal strain capable of bioaccumulating arsenic, which is a globally widespread environmental contaminant. To this aim, allochthonous fungal strains from the ColD UNIGE JRU MIRRI strains collection were selected. Their capability to survive and accumulate this kind of pollutant was evaluated and compared with that of an autochthonous fungi set directly isolated from the arsenic polluted water. A preliminary screening at various concentrations of arsenic (0, 200, 400, 800, 1600 μg L^{-1}) revealed that the best performing strains were *Aspergillus niger* and *Penicillium expansum* among the autochthonous strains and *Aspergillus niger* and *Penicillium expansum* among the allochthonous strains. Moreover, all the strains were subjected to bioaccumulation tests at a 1600 μg L^{-1} concentration. Local and allochthonous fungal strains showed different behaviors: the allochthonous strains grew rapidly and in a sustained way but without expressing any bioaccumulation activity. On the contrary, the indigenous fungi, despite a moderate growth, showed a good bioaccumulation capacity (in particular, *Aspergillus niger*). The results highlight the importance of employing native strains isolated from contaminated matrices to make a mycoremediation protocol more efficient.

Keywords: fungi; mycoremediation; environmental contaminants

Citation: Canonica, L.; Cecchi, G.; Capra, V.; Di Piazza, S.; Girelli, A.; Zappatore, S.; Zotti, M. Fungal Arsenic Tolerance and Bioaccumulation: Local Strains from Polluted Water vs. Allochthonous Strains. *Environments* **2024**, *11*, 23. https://doi.org/10.3390/environments11010023

Academic Editors: Zacharias Frontistis, Athanasia Petala and Adamantia A. Kampioti

Received: 29 December 2023
Accepted: 19 January 2024
Published: 22 January 2024

1. Introduction

Arsenic is a naturally occurring metalloid within minerals, such as pyrite and realgar, which can be released during volcanic meteorological (rock erosion) processes. It can also be derived from anthropic activities related to mining, industrial, and agricultural activities.

Combustion of fossil fuel, wood preservation, fertilizers and pesticides, sewage sludge and electronics are the main sources of anthropogenic arsenic pollution [1,2]. Many factors affect the arsenic environmental fate, such as the drainage of the metalloid into drinking and groundwater [3].

It is a non-biodegradable element, and it can be found in organic form (if associated with carbon or hydrogen) or in inorganic form (combined with oxygen, sulphur, and chlorine). In toxicology, arsenic is regarded as a heavy metal [4,5].

Currently, arsenic environmental pollution is widespread globally, mainly in Bangladesh, China, India, and various parts of America, where soil and water contamination are serious problems [6,7]. The alarming situation is becoming relevant due to the effects on human health related to the consumption of food and water contaminated by arsenic. For this reason, the World Health Organization has established that the maximum concentration limit of arsenic in drinking water should not exceed 10 μg L^{-1}. The main routes of

human exposure to arsenic are the ingestion of contaminated drinking water and the consumption of food crops grown in polluted soils or irrigated with arsenic-polluted water [5]. The ingestion of arsenic through polluted water increases the risk of liver cancer to the lungs, skin, bladder, and kidneys and other numerous acute and chronic diseases [8,9]. According to numerous epidemiological studies, the International Agency for Research on Cancer (IARC) classified inorganic arsenic as a definite human carcinogen. In addition, the genotoxicity of arsenic and its ability to cause oxidative stress at the cellular level have also been confirmed [10–12]. In recent years, several types of advanced purification techniques have been developed to decontaminate polluted matrices. These methods include chemical techniques such as the use of oxidants, physical methods such as microfiltration membranes, and hybrid chemicalphysical methods such as flocculation and coagulation, ion exchange and electrokinetic methodologies [5,13]. However, these methods are expensive and can produce sludges, which are difficult to dispose of, with a strong impact on microbial and geochemical environmental processes [14,15]. For these reasons, the research area of bioremediation is becoming increasingly important [1,16]. The clean innovative remediation methodology involves the use of organisms able to tolerate and bioaccumulate toxic pollutants. The living species studied in this field encompass bacteria, plants, and fungi. Many of these organisms show good tolerance and bioaccumulation of heavy metals and metalloids, such as arsenic [17,18]. In particular, fungi are gaining interest due to their ecological and metabolic characteristics [19,20]. They are ubiquitous, well adapted to various environmental conditions, including extreme ones: differently from other organisms, limiting factors, such as pH and toxic concentrations, appear to not affect the growth of fungi. Regarding metals, fungi are able to passively bioadsorb on their cellular wall or actively bioaccumulate toxic elements within their cells [13].

In addition, thanks to their rapid growth and low-specificity exoenzyme production stimulated by nutrient deficiency, fungi meet the essential requirements for their application in the field of bioremediation [21,22].

This study aims at testing whether strains isolated from a polluted matrix (in our case, arsenic-contaminated water and therefore defined as autochthonous, native, or local fungi) can perform better than allochthonous strains, i.e., selected from our collection named ColD UNIGE JRU MIRRI-IT, Laboratory of Mycology, University of Genoa (ColD). The tolerance and bioaccumulation capacities of both these kinds of strains have been evaluated to understand the main differences and in view of possible applications in mycoremediation protocols.

2. Materials and Methods

2.1. Collection of Soil Samples

Water samples were collected from groundwater taken at 7–10 m depths from a contaminated site used for steelmaking activities. Industrial residues were used as filling materials for morphologically levelling the soil of the investigated areas. These materials released some heavy metals into the underlying groundwater, with arsenic reaching concentrations of several hundred $\mu g\ L^{-1}$. The water samples collected were taken to the laboratory for further analysis.

The samples were analysed from a physicochemical perspective. The total metal concentration was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). High concentrations of arsenic (As) were detected.

2.2. Isolation and Identification of Indigenous Fungal Strains and Selection of Fungal Strains from Our Collection

The water samples were serially diluted up to obtain two different concentrations, 1600 $\mu g\ L^{-1}$ and 16 $\mu g\ L^{-1}$, using standard pour plate technique (dilution 1:100). A 1 mL volume of polluted water was plated onto the fungal media Malt Extract Agar (MEA) and Rose Bengal (RB). The plates were incubated at 24 °C for 7 days for fungal growth and monitored for further 7 days. Three replicates were performed for each type of medium.

Isolation of fungal strains was carried out on Malt Extract Agar (MEA). The pure isolates were stored in cryovials containing nutrient broth and glycerol (15%) at −20 °C and deposited in the ColD collection. Fungal identification was performed by means of morphological and molecular polyphasic approaches. The amplification of the β-tubulin gene was carried out with Bt2a and Bt2b primers [23] and with ITS1F and ITS4 primers for the amplification of ITS region [24].

Four fungal strains were selected from ColD to be tested and compared with the indigenous strains. The fungi from ColD are reported in the literature [25,26] as being able to clean up heavy metal contaminated matrices. Specifically, *Penicillium* Link and *Aspergillus* P. Micheli were demonstrated to tolerate and remove arsenic from liquid medium [27,28].

2.3. Tolerance and Bioaccumulation Tests

To screen the growth capacity of the fungi, an MEA medium was prepared by adding polluted water with four different concentrations of arsenic: 200, 400, 800, and 1600 μg L^{-1}. Starting from the initial 1600 μg L^{-1} concentration, serial dilutions were carried out to achieve a volume of 250 mL for the three desired concentrations (200, 400, and 800 μg L^{-1}). Fungal controls were cultivated on plates by adding distilled deionized water. The media were sterilized by autoclaving (121 °C, 20 min) and then inoculated with 1 mL of fungal solution, prepared using conidia of each fungal strain diluted in a suspension of 5 mL of Tween80 (polysorbitan 80). Conidia from seven days old colonies were used for the arsenic tolerance tests.

The screening test was conducted in three replicates for each concentration. Nine plates for each treatment and control were prepared.

Then, the metal tolerance and the bioaccumulation capacity were tested by preparing an MEA media added with polluted water containing the initial arsenic concentration of 1600 μg L^{-1} and poured onto plates (diameter 90 mm). Sterilized microporous cellulose acetate membranes (BioRad, Hercules, CA, USA) were placed on each Petri dish and inoculated with 1 mL fungal solution (previously diluted in 45 mL distilled deionized water). After 14 days of incubation at 24 °C in the dark, fungal biomass was gathered by means of a sterile plastic scraper and dried in oven at 40 °C to obtain dry biomass. The latter was weighed and chemically analysed.

To quantify and, in turn, compare the metal tolerance, several indexes were proposed in the literature. Here, we report on the ones adopted for our research.

The first metal tolerance index TI_d consists of estimating the diameters of fungal colonies. The diameters of colonies were measured after seven days of incubation in the dark at 24 °C.

More specifically, TI_d is defined as the ratio between the mean diameter of colonies under testing and the averaged diameter of colonies used as control [29,30].

$$TI_d = \frac{d_{cut}}{d_c} \quad (1)$$

where

d_{cut} is the mean diameter of the colonies under test, viz., the colonies of which we want to evaluate the tolerance capabilities;

d_c is the mean diameter of the colonies used as control.

Another method to evaluate the metal tolerance is based upon fungal biomass dry weights. In this case, the tolerance index TI_w is defined as the ratio between dry weight of treated mycelium and dry weight of control mycelium [31,32]. In detail,

$$TI_w = \frac{W_{cut}}{W_c} \quad (2)$$

where

W_{cut} is the dry weight of the mycelium of colonies under testing, viz., the colonies of which we want to evaluate the tolerance capabilities;

W_c is the dry weight of the colonies used as control.

The bioconcentration factor (BCF) was estimated as the ratio of the concentration of arsenic present in the dry biomass and the contaminant concentration present in the plates before the fungal inoculation (in our case 1600 μg L^{-1}), as suggested by Rosa et al. [32–34]. The metalloid concentration was evaluated by means of an ICP-MS.

The experiments were performed in completely randomized design with 3 replicates and the mean values, standard deviations, and 95% confidence intervals were also estimated.

3. Results and Discussion

3.1. Chemical Analysis

The water samples from the polluted area were chemically analysed and the metal(loid) concentrations measured. The related data are reported in Table 1. The concentrations of cadmium, copper, lead, mercury, nickel, and total chromium were within the legal limits. Chromium (VI) exceeded the limits by more than five times; the concentration of total arsenic was 1600 μg L^{-1}, thus being the most abundant element in the ionomic compositions of the samples. The concentration exceeds 100 times the legal threshold limit. Numerous studies investigated the effects of arsenic on human health, and a correlation appears to exist between arsenic concentration and cancer incidence in populations with high exposure to the metalloid [19,35,36].

Table 1. Chemical analysis of water samples collected from polluted area.

Heavy Metal	M.U.	Result	Confidence Interval	Legal Limit Value	Analytic Method
arsenic (total As)	μg L^{-1}	1607	±161	10	EPA 6020B 2014
cadmium (Cd)	μg L^{-1}	1	±0.1	5	EPA 6020B 2014
chromium (VI)	μg L^{-1}	26.4	±3.6	5	EPA 7199 1996
copper (Cu)	μg L^{-1}	5	±0.5	1000	EPA 6020B 2014
lead (Pb)	μg L^{-1}	1	±0.04	10	EPA 6020B 2014
mercury (Hg)	μg L^{-1}	<0.10		1	EPA 6020B 2014
nickel (Ni)	μg L^{-1}	5	±0.3	20	EPA 6020B 2014
total chromium	μg L^{-1}	31	±2	50	EPA 6020B 2014
zinc (Zn)	μg L^{-1}	169	±9	3000	EPA 6020B 2014

Concentrations greater than 0.35 mg L^{-1} appear to cause a high incidence of vascular disease [36]. The Environmental Protection Agency (EPA) calculated a unit risk of skin cancer from consuming arsenic-contaminated drinking water up to the maximum contaminant level (MCL) of 50 μg L^{-1} [37].

3.2. Mycological Analysis of Polluted Water Samples and Selection of Fungal Strains from the ColD Collection

The presence of fungal strains able to survive in high quantity of detected arsenic demonstrates the possibility of exploiting fungi as mycoremediation agents.

The mycological analysis of contaminated water allowed us to identify 171 CFUs per mL belonging to 6 main morphotypes. The most common strains belonged to the genera *Aspergillus* P. Micheli (82%), followed by *Penicillium* Link (15%) and *Mucor* Fresen (3%).

The most representative strain of *Penicillium* spp. was *P. expansum* Link, and the most abundant species of *Aspergillus* strains was *A. niger* Tiegh. A similar distribution of genera was also observed in the work of Oliveira, where *Penicillium* and *Aspergillus* were found to be the most representative genera in groundwater (37% and 25%, respectively) [38]. In terrestrial and aquatic environments, Lategan et al. also detected fungal species mainly ascribable to the genera *Penicillium* and *Aspergillus* [39].

As far as allochthonous fungi, four strains were selected from the CoLD, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries; *Penicillium expansum* Link; *Pleurotus ostreatus* (Jacq.) P. Kumm.; and *Aspergillus niger* Tiegh, based on the research results present in the literature.

Specifically, *Cladosporium cladosporioides* achieved excellent results in the work by Garza-González et al.: the fungus was able to remove 491.85 mg of Cr (VI) from 1 g of biomass after 288 h of experimentation [40].

Studying the potential absorption of Cu (II) from fungal biomass highlighted the capacity of *C. cladosporioides* to accumulate 97% Cu (II) dissolved in solution after 120 h of stirring [41]. The biosorption properties were also examined in a comparative study of two strains of *C. cladosporioides*. The fungal strains were able to bioabsorb metals, in particular, gold, silver, and cadmium [42]. Bioremoval of arsenic (V) and mercury from aqueous solutions were also investigated by J. F. Cárdenas-González [43].

Some fungal strains of *Penicillium* genus have paramount potential for bioremediation and detoxification of metals from polluted matrices. [22,44]. In the study by Martins et al. [45], eight species of *Penicillium* were tested on various metals present in aqueous residues, such as cadmium, copper, lead, and nickel. Four strains of species *P. citrinum* Thom, *P. brasilianum* Bat., *P. funiculosum* Thom, and *P. janczewskii* K.W. Zaleski showed the ability to remove heavy metals, especially lead. Another example is reported in the paper by Mohammadian et al. [25], where *Penicillium simplicissimum* (Oudem.) Thom was isolated from lead–zinc polluted soils and tested on Cd, Pb, and Cu.

Even macrofungi can be exploited in the field of bioremediation of pollutants. The genus *Pleurotus* was fruitfully tested on different pollutants, from solid waste to drugs and chlorinated pesticides [46]. In addition to the genera *Trametes* Fr., *Phanerochaete* P. Karst., and *Bjerkandera* P. Karst., *Pleurotus* showed good potential in degrading polycyclic aromatic hydrocarbons. This characteristic is due to the ligninolytic enzymes [47]. In particular, *Pleurotus ostreatus* (Jacq.) P. Kumm., an edible fungus commonly called oyster mushroom, was studied in the presence of heavy metals and showed good removal rates of Cd and Cr from a liquid culture [48]. The *P. ostreatus* capacity to degrade and bioadsorb heavy metals and phenolic compounds was investigated by Bhatnagar et al. [26].

3.3. Tolerance and Bioaccumulation Tests

The fungal capability to grow on polluted medium was investigated; screening and tolerance tests were conducted. Among autochthonous fungi, *Penicillium expansum* and *Aspergillus niger* were selected, as they were the most abundant species in our water samples. The selected strains grew in the media at every arsenic concentration.

Regarding the growth capacity, the local fungi isolated from polluted water showed no significant difference between the control and treatments. Concerning the fungal species selected from the laboratory collection, *Penicillium expansum* and *Aspergillus niger* grew faster than *Cladosporium cladosporioides* and *Pleurotus ostreatus*. For this reason, these two last species were not tested further. Table 2 shows the results of our investigation: the number of "pluses" indicates the growth rate at the different levels of arsenic.

Table 2. Screening of tested fungal strains at different arsenic concentrations (0, 200, 400, 800, and 1600 μg/L). The number of plus signs indicates the level of fungal growth.

Fungal Strains	**Treatments (As μg L^{-1})**				
	0	200	400	800	1600
Autochthonous fungi (i)					
Aspergillus niger (i)	+++	+++	+++	+++	+++
Penicillium expansum (i)	+++	+++	+++	+++	+++
Allochthonous fungi (a)					
Cladosporium cladosporioides (a)	+	+	+	+	+
Penicillium expansum (a)	+++	+++	+++	+++	+++
Pleurotus ostreatus (a)	++	++	++	++	++
Aspergillus niger (a)	+++	+++	+++	+++	+++

The tolerance index TI_d was computed for all the Petri dishes in order to estimate the tolerance of both the test and control colonies. The diameters of the colonies are benchmarks for the assessment of the health status of the fungus under study; this allowed us to measure possible variations in the growth rate.

In our case, the TI_d values range from 0.8 to 1, thus showing a high level of tolerance to all the arsenic concentrations.

Then, we selected the most arsenic-tolerant strains to estimate their bioaccumulation capacity. To this aim, we evaluated the Ti_w and BCF indices of the colonies developed on the MEA medium at 1600 µg L^{-1} arsenic concentration.

The Ti_w index allows an indirect assessment of the metabolic response of the fungus in the presence of arsenic.

Regarding the dry weights and Ti_w index, the differences between the test and control fungal biomasses are well evident.

The indigenous fungal isolates of *Penicillium expansum* (i) and *Aspergillus niger* (i) showed that the dry weights of the test colonies were lower than control colony weights (Table 3). Conversely, for the selected allochthonous species, the biomasses of *Aspergillus niger* (a) and *Penicillium expansum* (a) significantly increased with respect to the fungal control biomasses (Figure 1). Concerning the tolerance index Ti_w, both the indigenous species showed values less than 1, whereas the selected species from ColD had values higher than 1 (Table 3). These results show that the selected fungi *Penicillium expansum* (a) and *Aspergillus niger* (a) tend to increase their metabolic rates when subjected to the stress associated with the presence of high concentrations of arsenic added to the culture media.

Table 3. Mean values of dry weights of fungal colonies in the tolerance tests and related standard deviations.

Fungal Strains	Control (g.)	Test (g.)	TIw
Aspergillus niger (i)	2.485 ± 1.167	1.641 ± 0.080	0.66
Penicillium expansum (i)	1.753 ± 0.401	1.105 ± 0.098	0.63
Penicillium expansum (a)	1.661 ± 0.046	2.387 ± 0.182	1.44
Aspergillus niger (a)	3.391 ± 0.139	5.014 ± 0.298	1.48

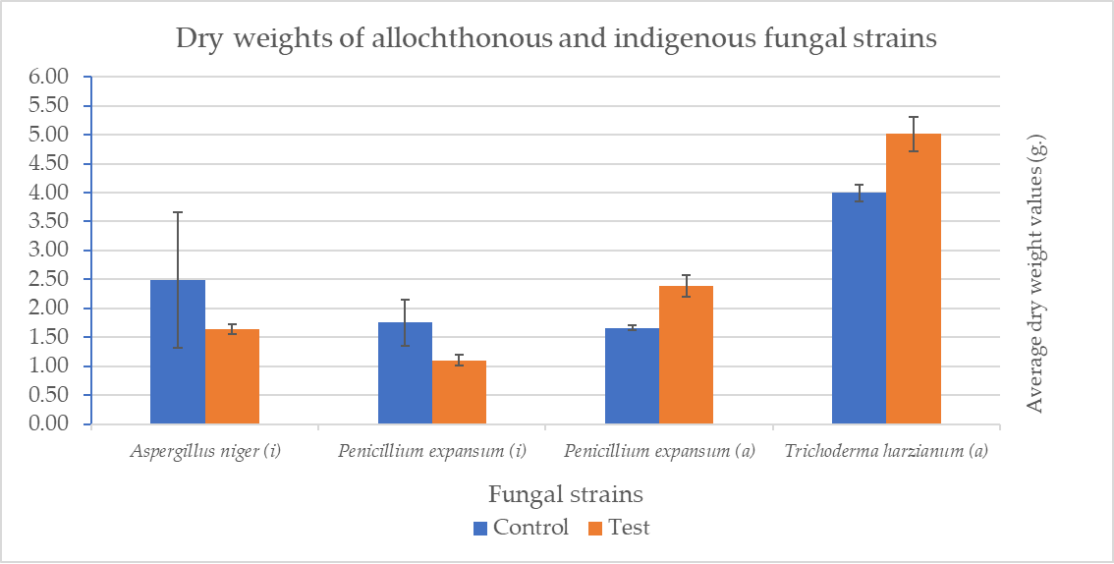

Figure 1. Mean values of dry weights of fungal strains tested.

The bioconcentration factor (BCF) is a measure of the amount of metal removed from the culture media in relation to the biomass for each strain. The BCF is an index that evaluates the bioaccumulation capacity, providing an additional indication of the efficiency

of the fungus in bioconcentrating metals. Indeed, fungi may have different bioconcentration capacities under the same growth conditions. In other works, this parameter was used to select the best performing fungi for bioremediation applications [33]. BCF values larger than 1 give us an indication of the fungal strain's capacity to accumulate metal ions.

Regarding the bioconcentration factor of the fungal isolates, *Aspergillus niger* (i) bioconcentrated more arsenic than *Penicillium expansum* (i), whereas the selected species (a) showed no bioaccumulation ability (Table 4).

Table 4. Bioconcentration Factor (BCF) values of fungal strains tested.

	BCF	
Fungal Strains	**Control**	**Test**
Penicillium expansum (a)	0	<0.001
Aspergillus niger (a)	0	<0.001
Penicillium expansum (i)	0	1.16
Aspergillus niger (i)	0	2.13

As a result, the local fungi isolated from contaminated water were able to tolerate and bioconcentrate the arsenic added to the culture medium, as suggested by the BCF indices. Conversely, the selected allochthonous fungal strains successfully grew on polluted media, without expressing any bioaccumulation capacity.

The fungal genera *Aspergillus* and *Penicillium* were examined in a number of studies about bioremediation.

In the study by Valix and Loon, the heavy metal (Ni, Co, Fe, Mg, and Mn) bioleaching abilities of *Penicillium simplicissimum* and *Aspergillus niger* were investigated [49]. Both the species showed good tolerance in the presence of metals, and *P. simplicissimum* also at the very high concentrations (8000 ppm). The biosorption ability of *Aspergillus niger* was examined in various studies. K. Tsekova et al. [50] achieved encouraging results: the biomass beads removed from 61.5% to 96.2% of metals in the presence of copper, nickel, cadmium, lead, and manganese.

Cárdenas-González et al., Čerňanský et al., and Kapoor et al. obtained promising results concerning the bioabsorption rates [43,51,52]. These outcomes could be related to the production of organic acids, such as gluconic, citric, and malic acids, involved in bioleaching metals. These molecules are produced and released by *Aspergillus niger*, even in great quantities [53].

Regarding arsenic, good removal rates were recorded by Mukherjee, who observed 70–76% removal by *Aspergillus flavus* and *A. niger* at 100 ppm arsenic present in solution [28]. These fungal strains were previously isolated from polluted sites of Kolkata.

Arsenic bioremoval by means of fungal strains belonging to the *Penicillium* Link 1809 genus was also studied. Visoottiviseth and Panviroj, reported that *Penicillium* sp. was able to uptake arsenic, reaching 30% arsenate removal and 40% arsenate after five-day incubation in liquid solution [27]. Shrivastava K. et al. showed that *Penicillium polonicum* expressed an excellent accumulation capacity, reaching up to 89,92% removal of arsenate from medium [54].

The genus *Penicillium* also achieved good results with other heavy metals. The study conducted by Chen et al. demonstrates the ability of *Penicillium simplicissimum* to reduce the phytotoxicity of Cd and Pb [55]. Lead removal was efficient for four different fungi, all belonging to the genus *Penicillium* [45,56].

In our study, *Penicillium* and *Aspergillus* strains, isolated from arsenic-polluted water, successfully grew and showed arsenic bioaccumulating activity. To the best of our knowledge, our study is the first to assess the behavior of *Penicillium expansum* in the presence of arsenic. Allochthonous fungi developed well, without expressing any uptake capacities. These fungi appear to be able to live in the presence of heavy metals at high concentrations in the hostile environmental conditions taken into account in our investigation. This result

further confirms the advantage of exploiting native fungal strains in heavy metal bioremoval protocols and agrees with the observations of Bharagava and Chowdhary and Khan et al. [16,57].

Monitoring and data collection are crucial for the development of efficient applications, especially in the context of in situ bioremediation. Identifying effective strains capable of degrading and bioaccumulating is essential for mycoremediation applications. For instance, mycoremediation of water contaminated with metals is a feasible application. Fungi can be inoculated into contaminated water, and as the mycelial mass grows, it accumulates metals and decontaminates the water. Through simple filtration, the decontaminated water can then be separated from the fungal mass.

In other cases, it is possible to introduce special filters with fungal inoculum into the contaminated water, separated physically but not chemically from the surrounding water. Once the fungus develops within the filter, it is sufficient to remove it, resulting in decontaminated water.

4. Conclusions

This work deals with allochthonous and native fungi isolated from arsenic-rich groundwater. The selection of fungal strains plays a crucial role. Indigenous fungi, isolated from contaminated environmental matrices, represent the essential tool to act efficiently with low environmental impacts. Indeed, local strains are metabolically adapted to the environment, without threatening the local biodiversity.

The main purpose of this work was to evaluate the adaptive and bioaccumulation abilities of the strains isolated from polluted groundwater and the strains selected from the ColD collection. Screening assays allowed us to evaluate their tolerance capacities. The best performing strains were selected for bioaccumulation tests. The results showed that all the tested fungal strains tolerate arsenic, even though only the native fungi show bioaccumulation capacities. This suggests that the exploitation of native fungi is preferable in mycoremediation protocols.

Future developments in this work and in this field will aim to investigate the best environmental conditions to support fungal bioaccumulation. Factors such as temperature, pH, oxygen availability and nutrients can significantly affect the metabolic rhythms of fungi. These parameters serve as benchmarks for an efficient utilization of fungi in mycoremediation protocols. These methods are particularly applicable in the case of waters, as it is easy to separate the bioremediating fungal mass from the water matrix once decontaminated.

Author Contributions: Conceptualization, G.C., S.D.P., A.G. and M.Z.; methodology, G.C., S.D.P., A.G., S.Z. and M.Z.; statistical analysis and software, S.Z.; validation, G.C., S.D.P. and M.Z.; formal analysis, L.C. and G.C.; investigation, V.C., L.C. and G.C.; data curation, L.C. and G.C.; writing—original draft preparation, L.C.; writing—review and editing, L.C., S.Z. and M.Z.; supervision, M.Z. and S.Z.; funding acquisition, M.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This study was partly financed by an internal grant from the University of Genoa named BIPE 2021 Mission—"Promoting Competitiveness".

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available on request from the corresponding author, M.Z. The data are not publicly available due to privacy reasons.

Acknowledgments: The authors would like to thank all students and colleagues involved in the BIPE 2021 project.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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