

Highly Heavy Metals Tolerant Fungi Isolated From the Sand of Polluted Beaches in the Area of Annaba - East of Algeria

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ABSTRACT

Heavy metals are chemical compounds difficult to remediate through conventional technologies. The use of microorganisms such as fungi provides an alternative pathway for the removal of these pollutants from contaminated environments. For the first time in Algeria, this investigation concerns the isolation, from the sand of a polluted beach, of strains of metal tolerant fungi towards Copper, Lead and Zinc. The isolation was achieved on Potatoes Dextrose Agar, and the identification was followed by screening, tolerance index, minimum inhibitory concentration and assessment of the amount of uptake of each strain against the three heavy metals on Czapek Yeast Agar. The screening of the resistant fungal strains showed a high tolerance of *Aspergillus clavatus*, *Aspergillus terreus*, *Fusarium oxysporium*, *Penicillium chrysogenum* and *Trichoderma viride* to the three heavy metals. In some cases, the values of the minimum inhibitory concentration were up to 16000 mg l⁻¹. The amount of metal uptake was over expectation showing the highest uptake for Copper ions of 1.6 mg g⁻¹ by *Aspergillus terreus*, and 3.3 mg g⁻¹ for Lead ions by *Penicillium chrysogenum*. The greatest uptake for Zinc ions was 4.8 mg g⁻¹ by *Penicillium chrysogenum*. The Tolerance Index showed a different statistical tolerance pattern of *Aspergillus clavatus* for Lead and *Aspergillus terreus* for Copper (P≤0.05). There was no statistical difference in the capacity to remove Lead by *Aspergillus terreus*, *Fusarium oxysporium* and *Penicillium chrysogenum* (P≤0.05).

KEYWORDS: Heavy metals; Pollution, Fungi, Tolerance Index; Uptake; Screening; Annaba; Algeria.

1. INTRODUCTION

If compared to water, heavy metals have a relatively high density [1]. They are classified as essential metals (Copper, Manganese, Zinc, and Iron) toxic at high levels, and nonessential metals (Cadmium, Lead, Mercury, and Nickel) [2]. In the environment, sources of heavy metals are natural or artificial processes through the anthropogenic impacts including discharges from the various industrial activities such as mining, smelting works, electroplating industry, as well as from the discharges of pesticides and phosphate fertilizers used in the agricultural sector [3,4,5]. Industrial wastewaters contain high concentrations of heavy metals which are transferred to animals and to human through the food chain [6]. Heavy metals are difficult to remediate because the natural environmental compartments (soil and water) are not able to totally eliminate these toxic elements [7]. The chemical treatment methods have many disadvantages including high costs, high energy and/or reagents requirements, and often lead to the formation of toxic complex products that necessitate waste products disposal. Restoring heavy metals through efficient and economical procedures requires the use of different complex options of metal-separating methods.

To some extent, the bioaccumulation of heavy metals by organisms, mainly microorganisms, can be successful [8]. A long-term exposure of microorganisms to high metal concentrations induces the development of resistance processes. Therefore, if isolated from contaminated wastewaters and soils with high concentrations of heavy metals, these microorganisms may represent a biological source for the removal of these metals [9].

In various ecosystems, the fungi can exhibit a rapid growth as well as the ability to uptake heavy metals [10]. This uptake of metals by the fungal biomass properties is divided into bioaccumulation (energy-dependent processes) and significant biosorption (binding of metals to the wall cell without energy) [11]. Fungi have also the ability to alter the chemical status of the metal ions through various processes such as

reduction, bioaccumulation, mobilization and immobilization [12]. They, therefore, can clean the environment, protects the biodiversity from the metals, and allow subsequent reuse [10]. It has been demonstrated that fungal strains, isolated from differently polluted areas, showed an important tolerance towards toxic metals and can provide a tool for the elimination heavy metal from polluted soils [9;13].

In Algeria, no attempts of isolation of heavy metals tolerant fungal strains were investigated. The present study is the first of its type in Algeria, and was conducted in order to isolate resistant fungal strains of five filamentous fungi; *Aspergillus clavatus*, *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium chrysogenum* and *Trichoderma viride* from a polluted beach in order to assess their tolerance to high concentrations of Copper, Lead and Zinc.

2. MATERIALS AND METHODS

2.1. Samplings

This investigation was carried out during three separated periods; December 2010, March and June 2011. Randomly, sand samples were collected from 3 stations located in two separated beaches in the littoral of Annaba city. Station 1 (S1) (Lat: 36° 51' 50" N ; Long: 7° 46' 43" E) and Station 2 (S2) (Lat: 36° 52' 30" N ; 7° 47' 57" E) are situated in the eastern part of Annaba city at Sidi Salem Beach which is directly under the influence of the urban sewage and the nearby industrial wastewaters and discharges of Ferial Fertilizers Complex. This beach also includes the estuaries of Meboudja and Seybouse wadis known as highly heavy metal contaminated outlets for the discharges of El Hadjar steel factory and the nearby industrial area [14]. Station 3 (S3) (Lat: 36° 57' 23" N ; Long : 7° 46' 50" E) is located in the northern part of Annaba city at Ain Achir beach which is fairly distant from the continental influence, but mostly under the Modified Atlantic Water current intrusion (**Fig.1**)

During the samplings operations, precautions measures were taken to avoid an eventual contamination from the surface of the sandy bottom. At each station, four slightly separated core samplings were carefully taken at a depth of -5 cm from the top of the bottom. For each station, the 4 core samples were added together in a sterilized glass bottle then transferred to the laboratory in a cool box at 4°C.

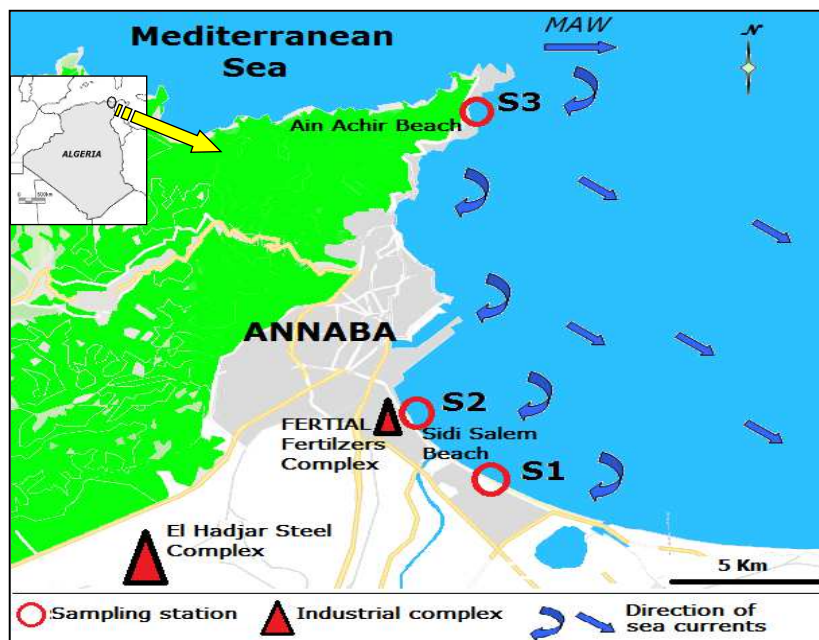


Figure 1: Sampling stations and main local sea currents (MAW = Modified Atlantic Water current).

2.2. Heavy metals analysis of sand samples

Assessments of the concentrations of Copper, Lead and Zinc in the sand samples were carried out within the following 24 hours as indicated in the literature [15]. For each sample from each station, 1g of sand was mixed in a 50 ml flask with 10 ml from a solution of HNO₃: HClO₄ (1:2), then heated for half an

hour. After filtration through a Whatman filter paper (N°1), the volume was completed with distilled water up to 50 ml. The amounts of Cu, Pb and Zn in the digested sand samples were assessed by atomic absorption spectrophotometer as indicated in the literature [16]. The analysis was carried out in triplicate.

2.3. Isolation of strains

Within the 08 hours following the sand samplings, isolation of the fungi was made through the serial dilution method on Potato Dextrose Agar (PDA). The plates were incubated at $25\pm 2^{\circ}\text{C}$ for 7 days. The colonies were purified individually on PDA plates, and then incubated at $25\pm 2^{\circ}\text{C}$ for at least 7 days [17].

2.4. Identification of isolated fungus

All the fungal isolates on PDA were identified on the basis of the colony characteristics; the macroscopic (morphology, colour, shape and appearance of the colony) and the microscopic characteristics (septation and appearance of the mycelium, sporangiophore position, columella shape, diameter, texture of the conidia, and the spore shape). Various keys of identification were used [18,19,20].

2.5. Screening for heavy metals- tolerant fungi

Small agar plugs (5 mm) from young purified fungal of 7 days culture were screened for Copper (Cu^{2+}), Lead (Pb^{2+}) and Zinc (Zn^{2+}) tolerance in order to select the heavy metal tolerant strains. Concentrations of 200, 400, 600, 800 and 1000 mg l^{-1} of CuSO_4 , $\text{Pb}(\text{NO}_3)_2$, ZnSO_4 were separately added to the CYA medium. The pH of the solid medium was adjusted to 6 with 1 M solution of Sodium Hydroxide before autoclaving [17]. The plates were incubated at $25\pm 2^{\circ}\text{C}$ for 7 days. In parallel, cultures without heavy metals were carried out as a control bench [15,16].

2.6. Heavy metals Tolerance Index (TI) of fungi

The TI is an indicator of the response of an organism to a metal stress [21], taking into account that the higher the TI, the greater the resistance [22]. The TI was estimated as indicated in the literature [16]. For each plate on CYA and after 7 days of culture at $25\pm 2^{\circ}\text{C}$, the diameter of the colony extension, from the point of inoculation, was measured for both colonies incubated in medium with heavy metals and colonies incubated in medium without metals (batch control). Measurements were carried out in triplicate. The mean value was respectively calculated for each strain and the TI was calculated as follows:

$$TI = \frac{Dt}{Du}$$

With : Dt = diameter of the radial extension (cm) of the treated colony
 Du = radial extension (cm) of the untreated colony (control batch).

2.7. Determination of the Minimum Inhibitory Concentration (MIC)

The MIC of the isolates is defined as the lowest concentration of metals that inhibits the visible growth of the isolates. The three heavy metal ions were added separately to CYA medium at concentrations ranging from 200 mg l^{-1} up to the resistance level of 1600 mg l^{-1} with intervals of 200 mg l^{-1} . The metal ions added plates were inoculated with 5mm agar plugs from young fungal cultures of 7 days grown on a normal CYA medium. They were then incubated at $25\pm 2^{\circ}\text{C}$ for 7 days. The MIC of the fungi is considered after at least 7 days of growth [16, 22].

2.8. Removal of heavy metals by fungal isolates from liquid media

The tolerant fungal isolates were evaluated for uptake of heavy metals in a CYA broth medium containing concentrations varying from 200 mg l^{-1} to 1600 mg l^{-1} individually for Copper, Lead and Zinc. Flasks of 200 ml were inoculated with 5mm Agar plugs from young fungal cultures of 7 days, and then incubated on a shaker at 150 rpm, and at $25\pm 2^{\circ}\text{C}$ during 7 days. The Control batches of flasks without heavy metals were processed similarly.

After 7 days, the fungal growth was harvested. After centrifugation at 9000 rpm/s and filtration through a Whatman filter paper, each harvested fungal biomass was washed with double distilled water 2 to 3 times and dried in hot air oven at $70\pm 2^{\circ}\text{C}$ for 3 hours. The digestion of the dried fungal biomass was made with Nitric acid and Perchloric acid (3:1 ratio). The digested fungal biomass was filtered again through a Whatman filter paper and the volume was completed up to 50 ml in a volumetric flask. The heavy metal uptake was estimated using Atomic Absorption Spectroscopy (AAS) [23].

2.9. Statistical analysis

The difference in TI among the individual isolates was checked over in triplicate through statistical tests; one-way ANOVA and Post-Hoc multiple-comparisons (*Tukey test*) using the software SPSS 20. The difference is considered significant when $P < 0.05$.

3. RESULTS

3.1. Heavy metals analysis of sand samples

All the measured data for the three heavy metals were compared with the standard limit values of the Act N° 220/2004 of the National Council of the Slovak Republic. The extremely high and above-limit values of Copper were found in the sand of the polluted beach at **S1**, while Lead and Zinc were moderate at **S2**. Copper was found as the main pollutant at **S1**. On the other hand, the limit values for Lead and Zinc were exceedingly high at **S2**, whereas the value of Lead was higher than those of Copper and Zinc at **S3** (**Tab.1**).

Table 1: Heavy metal contents in the sand samples (mg/kg).

Sampling Stations	Copper	Lead	Zinc
S1	1.2	0.99	0.4
S2	94.5	68.4	7.8
S3	0.18	0.31	0.09

3.2. Isolation of strains

25 species belonging to 16 genera were isolated from S2 and S3. Besides, 38 species belonging to 19 genera were isolated from S1 that exhibited the highest fungal diversity. *Aspergillus clavatus* was only found in the sand of the S1.

3.3. Screening for heavy metals tolerant fungi

Most of the isolates showed a high resistance against one metal at least. Isolates of *Aspergillus clavatus*, *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium chrysogenum*, and *Trichoderma viride* showed a high resistance against Copper, Lead and Zinc at a concentration of 1000 mg l^{-1} , with a clear difference in their degree of growth in the presence of metals.

3.4. Heavy metals Tolerance Index of fungi

Investigated at various concentrations of Cu^{2+} , Pb^{2+} and Zn^{2+} , the TI of *Aspergillus clavatus*, *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium chrysogenum*, and *Trichoderma viride* revealed different patterns for each metal. Copper and Zinc were tolerated by *A.terreus*, *A.clavatus*, *F.oxysporum*, *T.viride*, and *P.chrysogenum*, while Lead is only tolerated by *A.clavatus*, *A.terreus*, *T.viride*, and *F.oxysporum* (**Fig.2**).

The results from the mean values ($n=3$) demonstrated that the TI showed different statistical tolerance patterns of *A.clavatus* for Lead and *A.terreus* for Copper ($P \leq 0.05$). On the other hand, there was no statistical difference in the capacity to remove Lead for *A.terreus*, *F.oxysporum* and *P.chrysogenum* ($P < 0.05$).

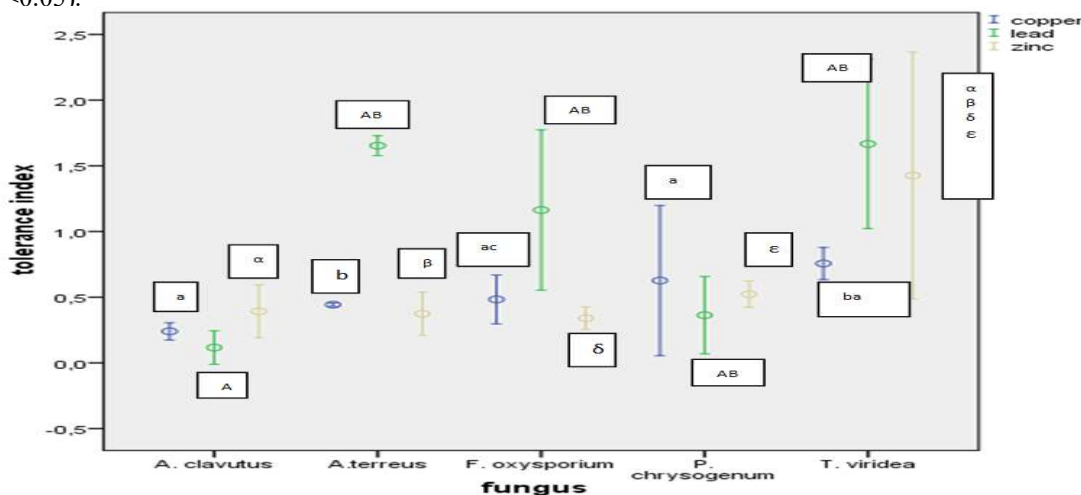


Figure 2: Tolerance Index of the fungal strains at different concentration of Copper, Lead, Zinc ions on CYA at 25 °C after 7 days. The different letters show the significant difference ($P \leq 0.05$)

3.5. Determination of the Minimum Inhibitory Concentration (MIC)

Depending on the fungal isolates, the data of the MIC revealed various resistance levels towards the heavy metals:

- *A.terreus* and *P.chrysogenum* strains showed more resistance against Copper with a MIC of 1600 mg l^{-1} higher than all other tested fungal strains (Fig.3).
- *A.terreus* and *T.viride* were the most Lead resistant strains with a MIC up to 1600 mg l^{-1} . *T.viride* and *F.oxysporum* exhibited a better growth at 400 mg l^{-1} than at 200 mg l^{-1} (Fig.4).
- *A.terreus* and *P.chrysogenum* tolerated Zinc concentrations up to 1600 mg l^{-1} (Fig.5).
- All fungal strains exhibited a better growth at lower concentrations of heavy metals but it became reduced in the presence of higher concentrations excepting for *P.chrysogenum* and *T.viride* against Copper.

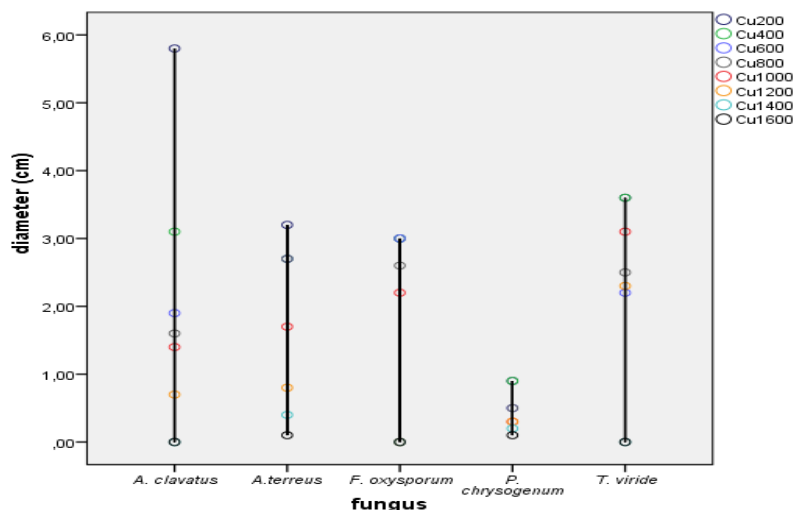


Figure 3: MIC of the fungal strains at different concentration of Copper ions on CYA at 25 °C for 7 days.

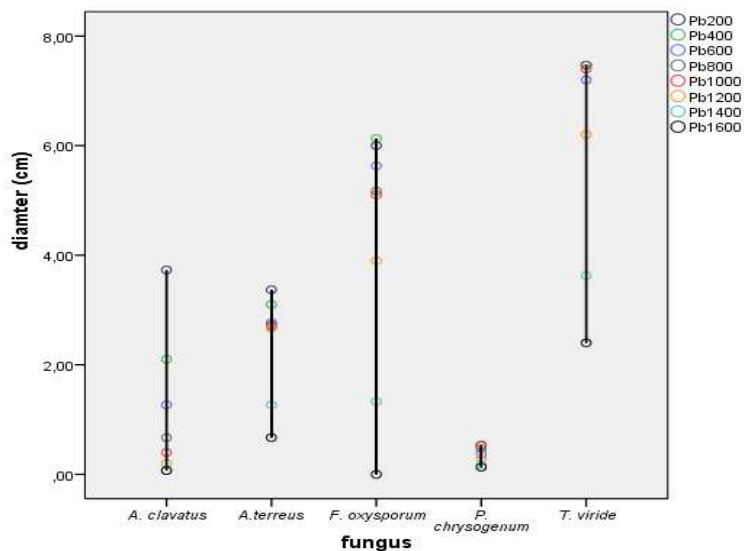


Figure 4: MIC of the fungal strains at different concentration of Lead ions on CYA at 25 °C for 7 days.

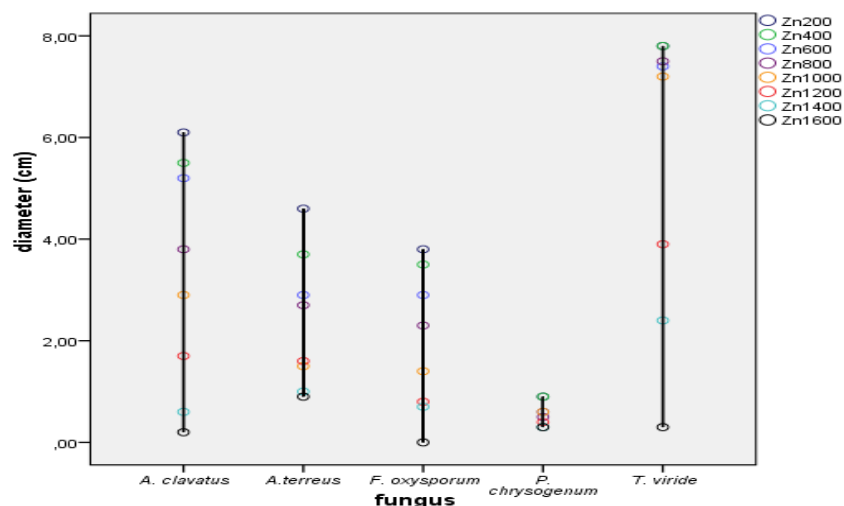


Figure 5: MIC of the fungal strains at different concentration of Zinc ions on CYA at 25 °C for 7 days.

3.6. Heavy metal Uptake by the fungal strain

The highest recorded uptake of heavy metals were 1.6 mgg^{-1} of Cu^{2+} for *A.terreus* at 400 mg l^{-1} , 3.3 mgg^{-1} of Pb^{2+} for *P.chrysogenum* at 800 mg l^{-1} , and 4.8 mgg^{-1} for Zn^{2+} for *P.chrysogenum* at 1200 mg l^{-1} . It was also noticed that the heavy metal accumulation increased as the amendment concentrations increased until reaching the highest levels of accumulation. For all the tested strains, the uptake amount of 1600 mg l^{-1} was recorded for the three heavy metals.

Over time, the removal of Lead showed a similar profile to that for Zinc, except that the absolute amount of lead removed was in an order of magnitude less than for zinc. The amount of Cu^{2+} , Pb^{2+} and Zn^{2+} removed (absorbed and adsorbed) increased in response to the augmented heavy metals concentrations (Fig.6,7,8).

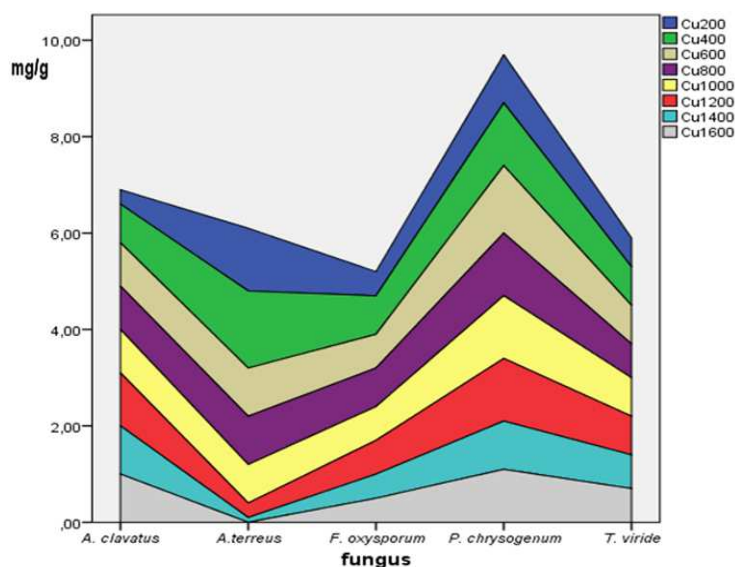


Figure 6: Uptake amount of Copper ions by the five fungal strains on CYA at 25 °C for 7 days.

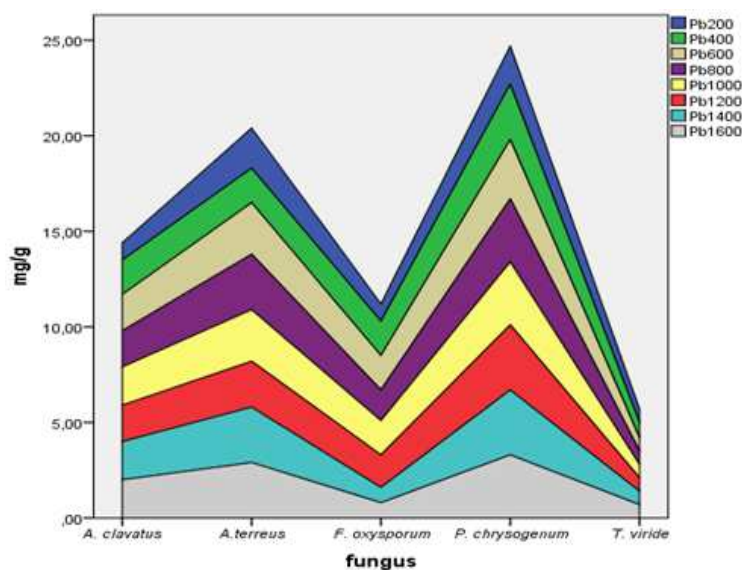


Figure 7: Uptake amount of Lead ions by the five fungal strains on CYA at 25 °C during 7 days.

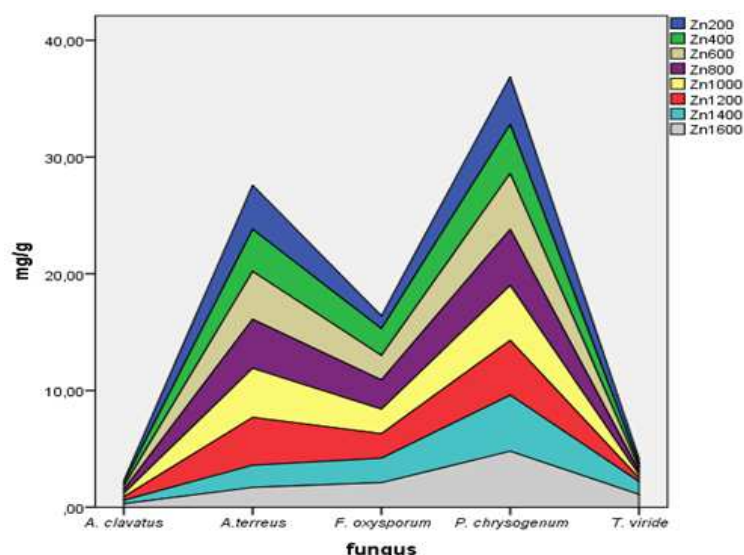


Figure 8: Uptake amount of Zinc ions by the five fungal strains on CYA at 25°C during 7 days.

4. DISCUSSION

The results of the sand analysis suggest that the two investigated areas, Sidi Salem Beach and Ain Achir beach are contaminated by the pollutant flows from the local urban and industrial wastewaters and discharges despite the fact that **S1** is under the direct influence of the MAW. It is evident that the sources of water and littoral pollution involve most of the entropic activities including the dumping of domestic wastes, sewage, agricultural wastes and industrial effluents into water bodies [23].

Various genera of fungi were isolated from metal-polluted environments [24], showing the ability to resist and to grow in the presence of toxic concentrations of heavy metals [25-26]. During this survey, the fungal strains isolated from the sandy contaminated environments exhibited a multi-tolerance behaviour. This selection is probably driven either by the most toxic elements or by more different metals acting synergistically [27]. The fungal biosorption of heavy metals varies from species to species but is also based on other factors [16]. On the other hand, despite the fact that it was isolated at **S3**, a distant site from the polluting outlets of the industrial area (Meboudja wadi and Seybouse wadi), *A. clavatus* showed a high tolerance to elevated concentrations of heavy metals. This suggests that some changes of the

dominant sea currents in the direction of the North-West side could be responsible for the dissemination of pollutants, including heavy metals, towards Ain Achir beach.

The results of this survey also revealed that most of the isolates were multi-resistant to Cu, Pb and Zn with the levels of resistance depending on the tested isolate. Species from the same genus did not have the same degree of tolerance as *A. terreus* showed more tolerance than *A. clavatus*. This difference in the metal tolerance may be due to the presence of one or more mechanisms of resistance and tolerance exhibited by each fungal strain [27]. Some studies [17,20] indicated that fungi species from various genera, and particularly of *Fusarium*, were isolated from contaminated soils, and were showing the ability to tolerate the presence of different heavy metals. Indeed, species of the genus *Aspergillus* and other tolerant fungal strains are also indicated having an elevated resistance to high levels of heavy metals concentrations [17, 28, 29, 30].

On the other hand, the toxic effect of each heavy metal increases with its rising concentration in the growth medium [31]. Despite the fact that Copper is an antifungal agent, the growth of *Aspergillus flavus* in the presence of high concentrations of Copper was reported in the literature [32]. The mechanism of tolerance to Copper is linked to its attachment to the surface absorption sites [33]. Zinc is a micronutrient and if adsorbed at high concentrations, the cells use a pathway to inhibit its uptake [34]. However, Lead has no metabolic relevance in fungi, and its uptake occurs through both intracellular and extracellular processes [4].

The term adaptation speed is an important armor that prompts one fungus more powerful than other fungi with higher MIC property. However, accumulation of excessive levels of these metals could be cytotoxic to the fungi [29].

Recent study, reporting on the strategies and uptake patterns for specific metal accumulations, indicated that these mechanisms may occur through the oxidation of organic acids tending with the uptake of some toxic metals [22,35]. Toxic metals such as Lead can destabilize the membrane structure and induce stress, generating the secretion of mucilaginous binding molecules that have a high affinity for metals [36] which physicochemical properties facilitate their uptake [37].

The highest uptake of Copper showed in this survey by *P. chrysogenum* indicated that having more binding sites on the cell wall, this fungus has also a biosorbent potential to remove Lead from the polluted environment. At 1600 mg l⁻¹, the uptake of heavy metal by the dead fungal strains can be explained by the uptake that occurs in the dead fungal cells because of the physic-chemical interactions between the metal ions and the negatively charged groups on the surfaces of dead cells [38]. On the other hand, it must be mentioned that during this work, most of the fungal strains exhibited changes in their morphology or in their medium with the formation of colourful mycelia due to the presence of heavy metals (mainly Zinc) as reported in the literature [39].

Finally, through the results of this investigation, it is evident that Mother Nature offers an alternative way to resolve some issues of the environmental pollution by heavy metals. The natural fungal strains, exhibiting a high tolerance against heavy metals such as Cooper, Lead and Zinc, can be used for the purification of contaminated environments. This latter point disserves a particular interest of further studies.

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