THE DEVELOPMENT OF A MICROBIAL METAL – RESISTANT CONSORTIUM FOR METAL BIOREMEDIATION IN A SIMULATED FLUVIAL SYSTEM

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ABSTRACT
Our main goal was to enrich the microbial population which develops when a pollution event changes the equilibrium of a fluvial system and thereafter investigate the effectiveness of this adapted bacterial consortium in a simulated bio-remediation process. The contaminated soil of a highly heavy metal polluted mining deposit was mixed with non-contaminated fluvial sediment in a ratio of 1:3. After the addition of fresh fluvial water, the system was allowed to equilibrate for one month. Then, a research was carried out on experimental microcosms in order to test the effects of the surviving microorganisms on toxic heavy metals (Cu, Fe, Zn) that are polluting surface sediments. The adapted microbial population was first enriched in aerobic conditions with a high concentration of heavy metals (up to 300 mg/l Cu; 400 mg/l Fe; 600 mg/l Zn) and afterwards the consortium obtained was applied in a microcosm to test its effect on the decontamination of heavy metals. The study has demonstrated that some microorganisms can survive the contamination. However, with regard to the tested metals, the microorganisms did not influence the status of the metals during the period of the analysis.

Key words: microbial consortium, mining contamination, fluvial system, remediation.

INTRODUCTION

The soils and waters of the mining areas, present a high content of toxic heavy metals. In order to avoid environmental risks, mining wastes need to be disposed in specially designed and safe places (LACAL ET AL., 2003). The water decontamination in the vicinity of plants constituted a challenge for a long period of time (UMRANIA, 2006), due to the fact that the industrial use of heavy metals led to their alarming increase in the environment (PARDO, ET AL., 2003). Extracting the metals from the polluted environment is paramount in protecting the environment and the human society (VULLO, ET AL., 2008).
The concentration of metals in the sediments of rivers is subject to changes due to the deposition-remobilization processes. Approximately 99% of pollutants are stored in sediments and less that 1% are, in fact, dissolved in water. Nonetheless, the composition of sediments is very important in their capacity of adsorbing and retaining contaminants (FILGUEIRAS ET AL., 2004).
Microorganisms and heavy metals can have different relationships: enzymatic production, oxidation or reduction of heavy metal, precipitation and solubilization are important mechanisms that are not so well known but that have great potentialities for future application of large scale bio-remediation (VALLS AND DE LORENZO, 2002). Studying the native microbial consortium and its remediation capacity is important in order to find the more efficient organisms and group of organisms that can be involved in some natural remediation of

polluted areas (RUIZ, ET AL., 2008). Numerous studies demonstrated the efficiency of bacteria in the extraction of metals; biological processes allow achieving their solubilization or immobilization (PARK, ET AL., 2008).
This study investigates the capacity of a naturally adapted microbial consortium to survive in a contaminated fluvial system and its effectiveness in contributing to the metals' solubilization.

MATERIALS AND METHODS

Collecting the sample

The water and sediment samples subjected to the study were collected after a 30 days' time frame during which a situation of mining contamination of a fluvial system was simulated. The study implied the use of discharged materials pertaining to a mining deposit which has as basis rocks: Galena (PbS), Spharelite (ZnFe)S, Bornite (Cu_5FeS_4), Pyrite (FeS_2) and Chalcopyrite ($CuFeS_2$). The mining deposit with these characteristics is located in the city of Zlatna, Romania and represents the former slag warehouse of the S.C. Ampelum S.A. plant, which ceased activity more than 10 years ago. The mining material was mixed with carbonate fluvial sediment coming from Reno River (North Italy), collected from the overflow in Sala Bolognese, Bologna (Italy). Both sediments were mixed and about 25 l of water from a non – polluted river was added. This *in vitro* system was allowed to equilibrate for one month and the resulting sediments were used for enrichment experiments, as described below.

Obtaining a microbial consortium and enriching the bacteria with tolerance to a high concentration of heavy metals

In order to obtain a microbial consortium able to tolerate heavy metals, aliquots of equilibrated sediments were enriched for 20 days in Nutrient Broth, modified by Cu, Fe and Zn ($CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$). The concentration of heavy metals in the medium was progressively increased by periodically extracting samples and refreshing the consortium.
The enrichment was realized at room temperature, in aerobiosis, and with an agitation of 98 rpm. A sterile 200 ml Erlenmeyer flask was used, and 90 ml of modified Nutrient Broth (NB) was added.
Every 5 days, 10 ml of the supernatant was transferred in fresh NB medium + metals at increasing concentrations, starting from Cu 80 mg/l, Fe 200 mg/l and Zn 150 mg/l and ending with Cu 900 mg/l, Fe 400 mg/l and Zn 600 mg/l. These last concentrations were chosen to stimulate the ambient conditions resulting from the artificial system with the mixed sediments (polluted and nonpolluted) obtained after the one month adaptation period.

Applying the consortium in an aerobic ex-situ bioremediation system

A simulated micro-remediation experiment was carried out using *in vitro* conditions and lasted 30 days.

Preparing the microbial consortium to be used as inoculum

Aliquots of 10 ml from the enrichment experiment were cultured in NB at room temperature, for 24h and then centrifuged at 6000 rpm for 15 min. The pellet was re-suspended in 20 ml of Saline solution (0.9% NaCl) in order to obtain an inoculum with a final concentration of

10^7CFU ml^{-1}. The remaining pellet was suspended in protective medium adding 15% glycerol to the Nutrient Broth, immediately frozen and stored at -80ºC until further analyses.

Testing the inoculum in batch bioremediation system

The adapted inoculum resulting from the previous enrichment treatment was tested in a simulated process of aerobic bioremediation.

Two microcosms were prepared as batch in glass flasks of 500 ml filled with 300g of the mixed sediments and 200 ml of water which were collected after 30 days from the first experimental system. Microcosm (1) also contained the obtained microbial consortium, whereas microcosm (2) was used as control.

Both systems were incubated with an agitation of 115 rpm at room temperature. Remediation experiment lasted 30 days during which aliquots of 10 ml of water and 20 g of sediment were periodically collected for heavy metal determination.

Chemical analysis of the microcosm

10 ml water was filtered with Watman No. 42 filters. The pH was gauged with potentiometric pH-meter (Crison) every 5 days, and the concentration of heavy metals was analyzed with Inductive Coupled Plasma - Optical Emission Spectroscopy (ICP-OES – Ametek, Spectro). The tested sediments were collected at 10 days' interval and dried at room temperature. pH was measured in ratio 1:2.5 (sediment: distilled water) with potentiometric pH-meter (Crison). The total content of metals in the sediments was determined by digestion in a microwave owen (Millestone) with 6 mL HCl and 2 mL HNO_3. The Teflon recipients were placed in the Milestone 1200 device, for 2 hours, and then the samples were brought at volume with Milli-Q water, filtered with Watman No. 42 filters and analyzed with an Inductive Coupled Plasma - Optical Emission Spectroscopy (ICP-OES).

RESULTS

Numerous studies and researches highlighted the bioremediation capacity of microorganisms and fungi isolated from contaminated environments. Thus, microorganisms pertaining to species that have the capacity to reduce the sulfate to sulfide, called sulfate-reducing bacteria, and afterwards the sulfide dissolves metals forming insoluble precipitates, are very important in the bioremediation process, and used in vast areas (UMRANIA, 2006, KUMAR AND NAGENDARN, 2007, MARTINS, 2009).

Table 1 displays the concentrations of copper and zinc from mining material, fluvial sediments and river water used as starting materials at the beginning of the *in vitro* experiment that lasted for one month.

Table1. Starting materials used for the *in vitro* experiment

	Copper	**Zinc**
Mining material (mg/kg)	11363.21	6841.56
Fluvial sediment (mg/kg)	19.35	33.22
River water (µg/l)	20	40

The bacterial inoculum tested in this study contained bacteria, of different shapes and size (cocci, motile and nonmotile bacilli, occurring both in solitary and in pairs or chains, some were also spore-forming), as revealed by microscopic observations. Aiming to identify at species' level the microorganisms present in this consortium, the inoculum was cultured in Nutrient Agar and the colonies were isolated for DNA extraction and sequencing of 16SrDNA gene (study in progress). For this study, we only tested the putative remediation activity of this consortium without any preliminary investigation on its species composition.
The values obtained for the water and sediments subjected to the remediation process were analyzed.
Copper (Cu) and Zinc (Zn) concentrations were determined during the experiment by taking samples from both microcosms at regular intervals, i.e. every 5 days for water samples and every 10 days for sediments sample. The results are highlighted in *Figures 1* and *2*.
Figure 1- shows the variations of the Cu concentrations, both in the control batch and in the treated one. The water samples in the T0-T10 time interval show a decrease of the Cu concentration in both cases. After this period, an increase of the concentrations follows, and at the end of the experiment the concentrations had slightly lower values than their initial ones. The Cu values from sediments show a slight increase in the first stage followed by a decrease for the treated batch until the end of experiment, whilst the control batch shows a little increase.

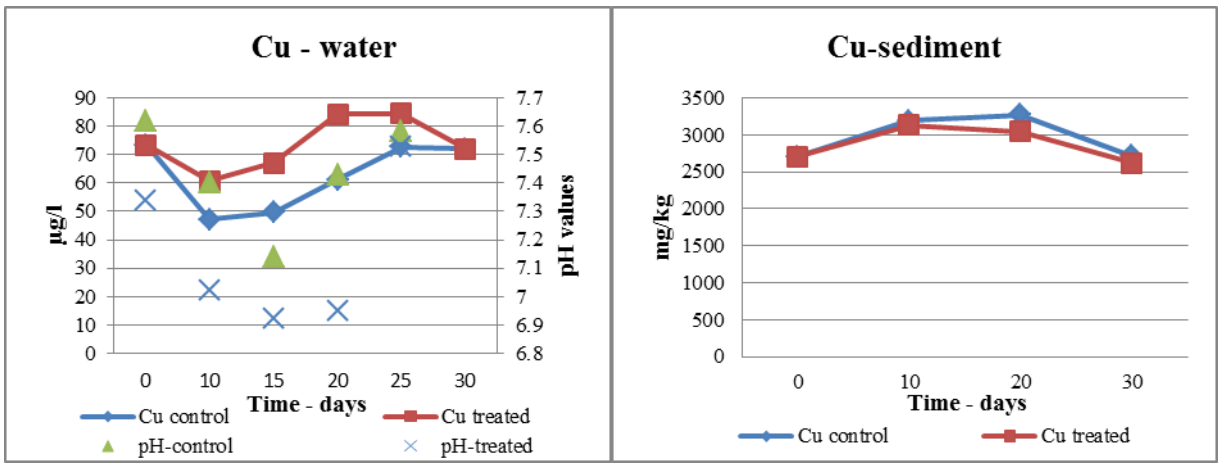

Figure 1. Copper concentration in water and sediments sample

Figure 2- shows the Zn concentrations obtained as a result of this experiment. For the water samples, zinc had a sudden decrease in the first 10 days, whilst in the sediments the concentration increased in the same period without showing any particular change in pH that is near neutral for the entire duration of the experiment. During the entire length of the process, this metal had approximately the same trends as the Cu in sediment, while in water Cu is increasing during the first 20 days and decreasing at the end of the experiment. This fluctuation could be due to microorganism but the length of the experiment was probably not enough to verify this hypothesis. Further investigation on the mobility of these metals in a neutral pH is also required.

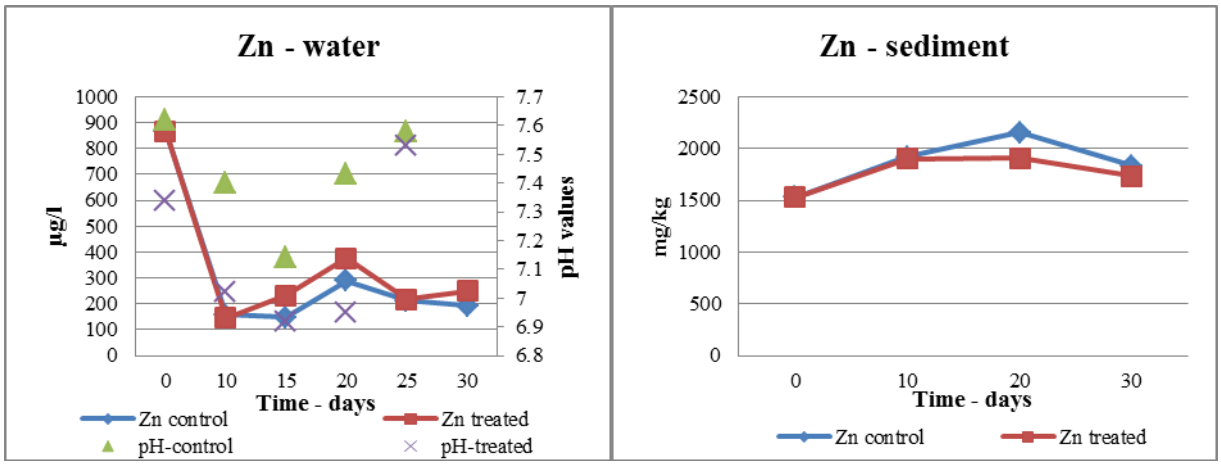

Figure 2. Zinc concentration in water and sediment samples

CONCLUSIONS

This study aimed to demonstrate the survival capacity of an indigenous microbial consortium when a pollution event changes fluvial system characteristics and to assess the possibility for natural bioremediation. The experiment was conducted under conditions as similar as possible to the natural environment, without adding chemical factors that could facilitate the procedure, but bearing the drawback of possibly contaminating water and sediments.

The study has demonstrated that some microorganisms can survive the contamination but further study should investigate on the temporal mutation of this consortium and its potentiality.

With regard to the bio-remediation capacity of the consortium, we can infer that, as far as the tested metals are concerned, the microorganisms did not influence the status of the metals during the length of the experiment.

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