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Mutualistic Interactions during Bioleaching of Marine Contaminated Sediment

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In coastal marine systems at high level of anthropization and socio-economic exploitation, like harbours, sediment represents a sink and a source for contaminants. Indeed, organic and inorganic pollutants coming from the water column are adsorbed and accumulated by sediment, as a consequence of natural processes occurring in all the aquatic systems. Unlike organic contaminants, metals cannot be degraded. Metal contamination in marine sediment is an environmental problem of high magnitude and, as a consequence, there is a need for environmentally friendly strategies. In this contest, biologically mediated leaching (*bioleaching*) may offer a solution. In the present work, bioleaching is applied on metal contaminated sediment coming from a marine harbour. Laboratory scale experiments were performed, at 10 g/L sediment content, in order to deepen the mutualistic interaction between chemolithotrophic Fe/S oxidizing bacteria, commonly applied in bioleaching strategies, and acidophilic heterotrophic Fe-reducing bacteria. Indeed, previous papers have partially investigated this aspect and offer some interesting insights. Here, the mutualism eventually occurring is better investigated, in view of the understanding of the processes involved during bioleaching, where the interaction metal-bacteria-sediment appears to be of central importance. Target metals were those mainly relevant in the sediment sample: arsenic (48 ± 2 ppm), chromium (140 ± 50 ppm), cadmium (1.8 ± 0.5 ppm) and zinc (1030 ± 70 ppm). During the experiment, other investigated responses were pH, microbial abundances and iron speciation. At the end of the experiment, Zn and Cd were the main solubilized metals, while Cr and As were scarcely removed from the sediment. Data about iron speciation also suggested that mutualism between chemolithotrophs and heterotrophs occurred, despite the presence of a complex environmental matrix such as marine sediment.

1. Introduction

Bioleaching is widely applied in commercial extraction of gold, copper, cobalt, nickel and zinc from ores and mineral concentrates (Brierley, 2008) but what is most interesting is that, in the last two decades, bioleaching has been investigated by the scientific community in the bioremediation field as a promising environmentally friendly technique for metal removal in contaminated sediments (Chen and Lin, 2010; Gadd, 2004; Chartier et al., 2001; Brierley and Brierley, 2001). Metal contaminated sediments coming from dredging activities in highly anthropized coastal areas, in fact, represent a high magnitude and widespread environmental problem. On the basis of SedNet estimations, around 100 and 200 Mm^3 of contaminated sediment might be produced yearly in Europe (Bortone et al., 2004). Dumping the untreated sediment at sea has a high environmental impact and is often not possible (Carette, 2011). In this contest, bioleaching may offer a solution as potential *ex-situ* treatment for sediment re-use purposes.
Bioleaching is based on the exploitation of chemolithotrophic Fe/S oxidizing bacteria (e.g., *Acidithiobacillus ferrooxidans*), isolated from acid coal mine drainage, which can lead to the solubilization of metals from the sedimentary matrix through their metabolism products (Rohwerder et al., 2003; Rawlings and Johnson, 2007). A variety of heterotrophic microorganisms have been isolated from the same natural environment and some of them have been reported to favour bioleaching strains (Fournier et al., 1998; Johnson, 1998, 2008) giving new insights for the application of bioleaching in bioremediation field. According with Fournier

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and co-authors (1998) *At. ferrooxidans* pure cultures would fail in iron oxidisation in environment acidification during a bioleaching process for wastewater sludge. Co-culture with iron-oxidising and heterotrophic acidophiles have often displayed more efficient mineral leaching (Hallmann et al., 1992). It has been postulated that heterotrophic bacteria can remove the inhibition by metabolising organic materials, known to thwart *At. ferrooxidans* and *Leptospirillum ferrooxidans* growth (Johnson, 1998; Rohwerder et al., 2003). In the acid mine drainage ecosystem it has also been observed that heterotrophic Fe-reducing bacteria (e.g., *Acidiphilium cryptum*) can re-cycle ferric ions coming from Fe/S oxidizing bacteria metabolism; this interaction between the two bacterial groups has been described as mutualistic (Johnson, 1998) and has been partially investigated during bioleaching of metal contaminated sediment (Beolchini et al., 2009, 2011). Toward a better understanding and exploitation of the microbial diversity in biotechnologies for aquatic sediment clean-up, we have deepened the mutualism eventually occurring among Fe/S oxidizing and Fe-reducing bacteria and how interaction metal-bacteria-sediment may affect the efficiency in metal removal from contaminated marine dredged sediment.

2. Materials and methods

2.1 Sediment samples

Sediment samples were collected from the port of Piombino (42° 55' 54.58'' N, 10° 32' 34.13'' E; Italy) by means of a modified Van Veen grab. After collection, sediment has been sieved to <2 mm to remove gravel, homogenized and then stored at 4°C. Total content in Zn, Pb, Cr, As, Ni and Cd in the sediment was 1030, 200, 140, 48, 29 and 1.8 ppm, respectively, as determined by ICP-AES (US EPA 200.7, 2001), after acid digestion (US EPA 3051, 1996). Metal partitioning was investigated by the procedure described by Quevauviller (1998). Total organic matter and carbonates in the sediment were 65 ± 5 mg/g and 380 ± 10 mg/g, respectively, according with the procedure described by Dell'Anno and co-authors (2002).

Table 1: Metal content in the sediment and their removal percentage during bioleaching experiments. Because metal amount in the residual fraction is assumed to be barely mobilisable in bioleaching operating conditions, removal yields in non-residual fractions are also reported.

	Untreated sediment	**Controls (no inoculum)**	**Autotrophs**	**Autotrophs and heterotrophs**
Zn	1030 ± 70 ppm	38 ± 5 %	36 ± 8 %	38 ± 7 %
in non-residual fractions*	55 %	70 ± 10 %	65 ± 10 %	70 ± 10%
Cr	140 ± 50 ppm	1 ± 0.1 %	0 ± 0 %	0 ± 0 %
in non-residual fractions*	10 %	3 ± 2 %	1 ± 1 %	1 ± 1 %
As	48 ± 2 ppm	1 ± 1 %	0 ± 0 %	0 ± 0 %
in non-residual fractions*	3 %	10 ± 5 %	10 ± 5 %	5 ± 3 %
Cd	1.8 ± 0.5 ppm	1 ± 1 %	16 ± 1 %	16 ± 1 %
in non-residual fractions*	29 %	2 ± 2 %	53 ± 3 %	54 ± 2 %

*: % in non-residual fractions represents the sum of metal content in exchangeable, carbonate, reducible and oxidizable geochemical fractions in the sediment, according with a selective sequential extraction procedure described in Quevauviller (1998).

2.2 Bioleaching experiments

Bioleaching experiments were performed by inoculating sediment slurries with three different autotrophic Fe/S oxidizing strains (i.e., *Acidithiobacillus ferrooxidans* DSMZ 14882T, *At. thiooxidans* DSMZ 14887T and *Leptospirillum ferrooxidans* DSMZ 2705T) and a heterotrophic Fe-reducing strain (i.e. *Acidiphilium cryptum* DSMZ 2389T).

Laboratory scale bioleaching experiments have been set up as follows: 100 mL of a 10g/L slurry of sediment (dry weight) and modified 9K thiobacilli medium (Fe^{2+} 0.9 g/L; i.e. 1/10 of the concentration in the

9K medium; Silverman and Lundgren, 1958) were dispensed in autoclaved 250 mL Pyrex flasks; the initial pH was adjusted to 2 with 5M H_2SO_4; 10 mL of bacterial inoculum were added. Microcosms so established were incubated under continuous mixing at 175 rpm horizontal shaking, at room temperature (25 ± 3°C), for 10 days. Experimental treatment differed for the presence/absence of the heterotrophic strain. Control treatments without bacteria inoculum were also performed. Four replicates were set up.

2.3 Analytical determinations

During the experiments, small aliquots of the slurries were periodically collected from each microcosm for measuring pH, for the enumeration of prokaryotic cells (Total Prokaryotic Number, TPN) and for the analysis of the iron speciation. In particular, pH was measured by the pH meter inoLab Multi 720 (WTW), TPN has been performed in compliance with the procedure by Danovaro (2010), while the concentration of soluble Fe^{3+} was analysed by reacting filtered aliquots of the slurry with KSCN and reading for absorbance at 480 nm wavelength (UV-VIS spectrophotometer JASCO 7850; Vogel, 1989); for the quantification of total soluble Fe aliquots of the supernatant has been oxidized with $KMnO_4$ and then read for absorbance.

Solubilized Zn, Cr, As and Cd in the solution phase were measured at the end of the experiment: subsamples of the suspension were centrifuged, stabilized at pH 2 with HNO_3 and analysed by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES; EPA 200.7, 2001). Total Zn, Cr, As and Cd left in the sediment was determined, after acid digestion (US EPA, 1996), by ICP-AES (US EPA, 2001).

Table 2: Output of the analysis of variance (α = 0.05) carried out on the data set.

Response variable	Source	df	SS	MS	F ratio	P	% of explained variance
Zn	Inoculum	2	0.007	0.003	0.371	n.s.	
	Error	9	0.081	0.009			
	Total	11	0.087				
Cr	Inoculum	2	0.001	0.001	2.351	n.s.	
	Error	9	0.002	0.000			
	Total	11	0.004				
As	Inoculum	2	0.006	0.003	1.732	n.s.	
	Error	9	0.015	0.002			
	Total	11	0.021				
Cd	Inoculum	2	0.743	0.371	339.358	***	100%
	Error	9	0.010	0.001			
	Total	11	0.753				
final pH	Inoculum	2	0.224	0.112	0.476	n.s.	
	Error	9	2.112	0.235			
	Total	11	2.336				
Fe oxidation	Inoculum	2	0.993	0.497	5.842	*	87 %
	Error	9	0.765	0.085			
	Total	11	1.758				
Fe precipitation	Inoculum	2	0.079	0.040	0.589	n.s.	
	Error	9	0.606	0.067			
	Total	11	0.685				

df: degree of freedom; MS: mean square; F: F-ratio; P: significance level.
n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

3. Results and discussion

Main results about metal solubilization during bioleaching experiments are shown in Tab.1. Metals and semimetals of interest in this paper were Zn, Cr, As and Cd, because characterized by high concentrations. Investigated elements can be ordered with decreasing mobility, as follows: Zn>Cd>Cr>As (Tab.1).

After a 10 day treatment, Zn and Cd showed the highest solubilization yields, which corresponded to 65 ÷ 70 % and round 55 % of their content in the non-residual fractions of the sediment, respectively. On the contrary, As and Cr were scarcely mobilized. In general, more mobile metals (i.e., with higher content in non-residual fractions) were characterized by higher solubilization by bioleaching. Nevertheless, the effect of bioleaching strains was metal specific: Zn solubilization in the absence of Fe/S oxidizing bacteria (i.e., c controls without inoculum) were comparable with those obtained in the presence of bacteria, while Cd was barely solubilized in the absence of bioleaching strains (Tab.1). According with the analysis of variance (ANOVA, a = 0.05), Cd solubilization was the only metal to be significantly favoured by the presence of Fe/S oxidizing bacteria (Tab.2). Contrary to what was expected, the presence of the heterotrophic strain *A. cryptum* did not directly affect metal solubilization, even for Cd. Indeed, heterotrophic strains isolated from most extremely acidic environments would favour metal solubilization in metal leaching (Hallmann et al., 1992; Fournier et al., 1998; Rawlings, 2005). Furthermore, former bioleaching experiments, with sediment samples coming from a different marine port, have demonstrated that Cd, Cu and Zn may reach higher solubilization yields during bioleaching with autotrophic and heterotrophic strains, together (Beolchini et al., 2011). This discordance may suggest that metal solubilization in contaminated marine sediment is also affected by the nature of the sediment.

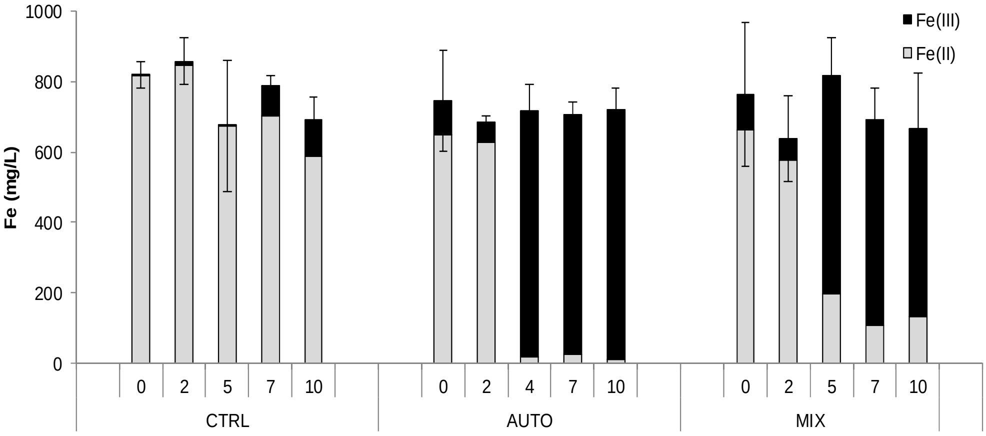

Figure 1: Iron speciation in the solution phase during the experiment. CRTL: controls without bacteria inoculum. AUTO: bioleaching treatments with autotrophic strains (At. ferrooxidans, At. thiooxidans and L. ferooxidans). MIX: bioleaching treatments with autotrophic strains (At. ferrooxidans, At. thiooxidans and L. ferooxidans) and heterotrophic bacteria (A. cryptum)

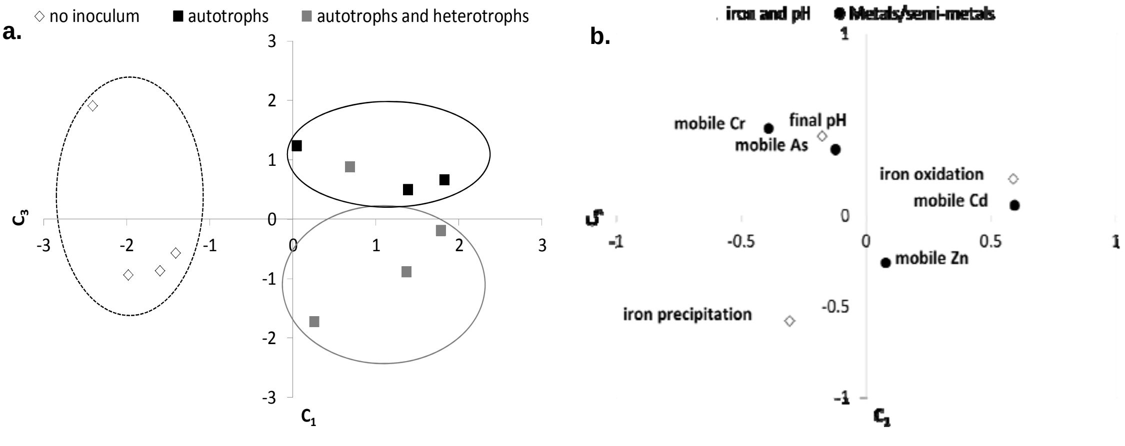

Figure 2: Output of the Principal Component Analysis (PCA). Score (a) and loading (b) plot of the first (36 % variance) and the third (19 % variance) of the dataset of experiments. First principal component on the horizontal axis (C1); third principal component on the horizontal axis (C3)

Data about pH, iron speciation and prokaryotic abundances (TPN) have suggested that strains were active in the presence of sediment (data about TPN not shown) and provide insights about the *A. crytpum* role in bioleaching with contaminated marine sediments. Inoculated flasks were characterized by pH values round

2 ÷ 2.5, all over the treatment, while pH in controls increased to values 3 ÷ 4. A very little iron oxidation occurred in control treatments, while iron was completely oxidized in inoculating microcosms after 4 days. Treatments with *A. cryptum* also showed an amount of ferrous ions in solution, as time elapses (Figure 1). These data support that the Fe-reducing heterotrophic strain (i.e., *A. cryptum*) was active in re-cycling Fe(II) ions, a growth substratum for Fe/S oxidizing bacteria.

A PCA (Principal Component Analysis; SIMCA-P 7.0 software, Umetrics) was performed to identify general patterns in the dataset of the experiments. The first principal component was likely to discriminate between inoculated (any acidophilic consortia) and not inoculated experiments (i.e., controls); on the other hand, the third component seems to discriminate between autotrophic and mixed autotrophic/heterotrophic inocula (Figure 2a). No significant effect was observed for the second component (data not shown). A positive association between iron oxidation and cadmium mobilization is also evident (Figure 2b): treatments with high value in iron oxidation were characterized by higher Cd solubilization. The ANOVA confirmed that the latter response variables were positively affected by the factor “inoculum” (Table 2). A linear regression between iron oxidation and Cd solubilization also showed a positive correlation (r^2 = 0.9436).

4. Conclusions

Results above discussed suggest that, with 10 g/L marine contaminated sediments, heterotrophic Fe-reducing bacteria (i.e., *A. cryptum*) are active in ferric ions reduction and Fe/S oxidizing bacteria in iron oxidation. According with Johnson (1998) this iron re-cycling can be described as a mutualistic interaction between the two bacterial groups. Contrary to what was expected and to what was already observed for other marine contaminated sediments, the presence of heterotrophic Fe-reducing bacteria did not directly affect metal and semi-metals removal. Conversely, Fe/S oxidizing bacteria were determinant for Cd solubilization, which was almost not solubilized in control tests. Cd solubilization was highly related to the iron oxidation. Compared with the available scientific literature, results also suggest that marine sediment clean-up from metal contamination is characterized by metal specific and sediment specific performance.

These consideration should be taken into account in view of a potential application of bioleaching in marine sediment clean-up. The exploitation of Fe/S oxidizing bacteria and their mutualistic interaction with heterotrophic Fe-reducing strains should be finalised at the production and re-cycling of leaching produced. However, further investigation should be performed to investigate marine sediments with different origins and geochemical properties, in order to highlight which main factors affect the bioleaching of metals from marine sediments. Other bioleaching strains could be also investigated. Among the latter, thermophilic strains may have interesting applications in the bioleaching with marine sediments, especially considering that the higher temperature may modify the chemical equilibria and thus affect the entire process.

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