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Bioremediation of contaminated marine sediments can enhance metal mobility due to changes of bacterial diversity

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ABSTRACT

Bioremediation strategies applied to contaminated marine sediments can induce important changes in the mobility and bioavailability of metals with potential detrimental consequences on ecosystem health. In this study we investigated changes of bacterial abundance and diversity (by a combination of molecular fingerprinting and next generation sequencing analyses) during biostimulation experiments carried out on anoxic marine sediments characterized by high metal content. We provide evidence that the addition of organic (lactose and/or acetate) and/or inorganic compounds to contaminated sediments determines a significant increase of bacterial growth coupled with changes in bacterial diversity and assemblage composition. Experimental systems supplied only with organic substrates were characterized by an increase of the relative importance of sulfate reducing bacteria belonging to the families *Desulfobacteraceae* and *Desulfobulbaceae* with a concomitant decrease of taxa affiliated with *Flavobacteriaceae*. An opposite effect was observed in the experimental treatments supplied also with inorganic nutrients. The increase of bacterial metabolism coupled with the increase of bacterial taxa affiliated with *Flavobacteriaceae* were reflected in a significant decrease of Cd and Zn associated with sedimentary organic matter and Pb and As associated with the residual fraction of the sediment. However, independently from the experimental conditions investigated no dissolution of metals occurred, suggesting a role of bacterial assemblages in controlling metal solubilization processes. Overall results of this study have allowed to identify key biogeochemical interactions influencing the metal behavior and provide new insights for a better understanding of the potential consequences of bio-treatments on the metal fate in contaminated marine sediments.

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1. Introduction

Coastal sediments subjected to high anthropogenic pressure can accumulate large amounts of contaminants, with potential detrimental consequences in the provision of ecosystem's goods and services (Micheli et al., 2013). Sediment contamination with metals represents a widespread problem of major concern, due to their persistence and toxicity, even at low concentrations. The toxicity and bioavailability of metals are strictly dependent upon their speciation, which is influenced by different factors, including their partitioning among the geochemical fraction of the sediment (Chapman and Wang, 2001; Hlavay et al., 2004; Okoro and Fatoki, 2012). For such a reason, the determination of total metal concentrations is not sufficient to provide reliable information about their mobility and bioavailability in aquatic benthic ecosystems (Ahlf and Förstner, 2001; Jain, 2004; Prica et al., 2010). Selective extraction techniques allow to investigate the potential mobility of any metal species by analyzing their partitioning among different geochemical fractions of the sediment. Indeed, metals in the residual phase of the sediment, being entrapped within the crystalline lattice of primary and secondary minerals, are thought to be stable. By contrast, metals in non-residual fractions (especially in the exchangeable and carbonate fraction) can be mobilized/solubilized more easily by biotic and abiotic processes (e.g. changes in ionic strength, pH, and oxidation/reduction state), thus becoming more bioavailable for the marine food webs with potential detrimental effects on ecosystem health (Gleyzes et al., 2002; Dell'Anno et al., 2003; Eggleton and Thomas, 2004; Toes et al., 2004; Akcil et al., 2014).

The fate of metals and semi-metals in the sediment depends upon the balance between immobilization (i.e., redox transformations, precipitation, adsorption and intracellular uptake) and mobilization processes (i.e., redox reactions, leaching, volatilization by methylation and chelation/complexation; van Hullebusch et al., 2005; Gadd 2010). The microbial activity can largely affect these processes by influencing the environmental conditions, the metal scavenging capacity (mainly due to the presence of sulfides, iron/manganese oxides and complex organic compounds) and the presence of soluble ligands, with cascade effects on the speciation and fate of metals (Sauvé et al., 2000; Warren and Haack, 2001; Jain, 2004; Prica et al., 2010).

Biological treatments based on the exploitation of the autochthonous microbial assemblages are gaining increasing prominence in the remediation of a variety of environmental matrices such as wastewaters, soils and sediments contaminated with metals (Tabak et al., 2005). A particular attention has been paid at exploring the potential of sulfate reducing bacteria (SRB) in decreasing the mobility of metals and semi-metals by generating sulfides, which are compounds with very low solubility product constants (Evangelou, 1998; Gadd, 2004; Jiang and Fan, 2008). Although sulfate reduction is widespread in anoxic marine sediments, other microbial-mediated redox processes (e.g., using iron, nitrate and manganese as electron donors) occur and may affect the fate of metals and semi-metals (Dell'Anno et al., 2009). Moreover, in surface marine sediments the relevance of such biological processes varies in space and time, due to a wide range of abiotic (e.g. temperature, nutrient availability) and biotic (e.g. mortality rates induced by grazers and/or viruses) factors (Jørgensen, 2000). As a consequence, the development of efficient bio-treatments for contaminated marine sediments requires a comprehensive understanding of the complex biogeochemical interactions occurring along with the identification of the main microbial taxa involved.

Since only a minor fraction of marine bacteria can be identified by culturing (Connon and Giovannoni, 2002), culture-independent procedures based on molecular tools have been used to provide insights into the diversity and functions of bacterial assemblages during bioremediation of contaminated marine sediments (Head et al., 2006; Pandey et al., 2009; Rocchetti et al., 2012). In particular, molecular fingerprinting techniques, such as Terminal Restriction Fragment Length Polymorphism (T-RFLP), Denaturing Gradient Gel Electrophoresis (DGGE) and Automated Ribosomal Intergenic Spacer Analysis (ARISA), along with gene cloning and sequencing have been frequently applied for investigating changes in bacterial diversity and assemblage composition during bioremediation experiments (Pandey et al., 2009, and citations therein). Moreover, next generation sequencing platforms now allow to explore the microbial diversity with an unprecedented detail, representing powerful tools to improve our comprehension of the interactions between bacterial diversity patterns and changes in metal speciation induced by bio-treatments (Sogin et al., 2006; Roesch et al., 2007; dos Santos et al., 2011).

In this study, we have investigated the effect of different biostimulation strategies on the abundance and diversity (by using molecular fingerprinting and next generation sequencing analyses) of bacterial assemblages in relation with metal and semi-metal mobility in contaminated marine sediments. The aim of this study was to identify the interactions between bacterial diversity patterns and changes in metal repartition in order to improve our understanding of the potential consequences of bio-treatments on metal fate. Indeed, this information is needed for a better comprehension of the potential impact associated with changes in metal mobility induced by bio-treatments on ecosystem health.

2. Materials and methods

2.1. Sampling and sample processing

Sediment samples were collected from the port of Piombino (42°55′54.58″ N, 10°32′34.13″ E; Tyrrhenian Sea, Italy) by means of a Van Veen grab. Such sampling site was selected because it is characterized by high contamination levels of metals and organic compounds which would require remediation actions (Cicero et al., 2001; Fonti et al., 2013) and, thus, it can be considered an ideal model for testing the consequences of bio-treatments applicable for the abatement of organic pollutants on the mobility of metals and semi-metals. The sediment samples were reduced and appeared black. Immediately after collection, values of pH and Eh were determined and sediments transferred to anaerobic jars for laboratory experiments of bioremediation. Additional sediment sub-samples

were collected for the analysis of grain size, mineralogical composition, water and total organic matter content, (semi-) metal concentrations and speciation.

2.2. Sediment characteristics

Grain size was determined by the sieving technique, which revealed that sediments were largely dominated by the silt–clay fraction (i.e. <63 μm, ca. 95%). Mineralogical composition was analyzed by X-ray powder diffractometer (XRD; Philips X Pert 1830; Bragg-Brentano (θ/2θ) geometry, Cu-Kα radiation, 40 kV, 30 mA). For the phase identification the database of the Joint Committee on Powder Diffraction Standards (JCPDS) was used. Sediment water content was calculated as the difference between wet and dry weight and expressed as percentage. For the analysis of the total organic matter (TOM), sediment sub-samples ($n = 5$) were treated with an excess of 10% HCl to remove carbonates that may interfere with TOM determination (Buchanan et al., 1971). TOM was determined as the difference between dry weight (60 °C, 24 h) of the sediment and weight of the residue after combustion (450 °C, 2 h; Parker, 1983).

The concentrations of (semi-)metals in the sediment were determined after acid digestion as follows: sub-aliquots of dried sediment (ca. 0.5 g) were transferred in Teflon vessels, added with 5 mL fluoridric acid and 1 mL of "aqua regia" (i.e. $HCl:HNO_3 = 3:1$) and, then, incubated at 150 °C for 90 min. At the end of the incubation, 5 mL of 10% boric acid were added and the extracts were analyzed by inductively coupled plasma-atomic emission spectrometry.

The distribution of metals and arsenic in the different geochemical fractions of the sediment was determined by a procedure of selective sequential extraction (SSE). The protocol applied here is the three-step extraction of the European Measurements and Testing programme (Förstner, 1993; Salomons, 1993). Four different fractions are considered: i) the exchangeable and carbonate bound fractions (hereafter defined as exchangeable fraction), extracted utilizing 0.11 M acetic acid, pH 2.8; ii) iron and manganese oxides fraction (i.e. reducible fraction), extracted with 0.1 M NH_2OH, pH 2; iii) organic and sulfide fraction (i.e. oxidizable fraction), extracted by hydrogen peroxide 30% and treated with ammonium acetate at pH 2, and iv) the residual fraction, that remains in the solid (i.e., the metals in the crystalline lattice of primary and secondary minerals), determined by difference with the total metal content. Details of this protocol are reported in the Appendix of Quevauviller (1998).

2.3. Biostimulation experiments

Biostimulation experiments were performed in 250 mL flasks containing 200 mL of a 250 g/L slurry of sediment (referred to dry weight), previously diluted with 0.2 μm pre-filtered artificial seawater (i.e. 40 g/L sea salts, Sigma–Aldrich S9883 in MilliQ water). Microcosms were added with sodium acetate, lactose and/or inorganic nutrients, according a 2^3 full factorial plan, for a total of 8 experimental treatments (Table 1). All of the treatments were performed in two replicates. Sodium acetate and lactose, both at a final concentration of 20 mM C, were selected as electron donors for stimulating reducing processes in the sediment (Finke et al., 2007; Dell'Anno et al., 2009). The amount of carbon supplied was approximately equal to the total organic carbon concentrations of the sediment samples, with the exception of treatment 6 and 8, where the concentration of C was twice, since lactose and acetate were present together. A similar approach has been previously applied to investigate the effect of biostimulation strategies on anoxic marine sediments contaminated with hydrocarbons and metals (Dell'Anno et al., 2009; Rocchetti et al., 2012). Ammonium sulfate and potassium phosphate were selected as source of inorganic N and P. The final concentrations of inorganic nutrients were defined on the basis of the organic carbon content in the sediment, according to a molar C:N:P ratio of 100:10:1, optimal for sustaining microbial activity (Morgan and Watkinson, 1992; Beolchini et al., 2010).

Table 1 – Experimental plan. The experimental factors investigated are the presence/absence of sodium acetate, inorganic nutrients and lactose, according to a full factorial plan 2^3. CTRL = sediments without any substrate addition.

Treatment	Acetate	Nitrogen + phosphorous	Lactose
1 (CTRL)	–	–	–
2	–	–	+
3	–	+	–
4	–	+	+
5	+	–	–
6	+	–	+
7	+	+	–
8	+	+	+

+: Present.
–: Absent.

Microcosms were closed to avoid oxygen diffusion and then incubated for 60 days in the dark at room temperature (20 °C ± 1). After 30 and 60 days of incubation, sediment aliquots were collected for the analyses of metal partitioning (see above) and of bacterial abundance and diversity.

2.4. Total bacterial abundance

Bacterial cells were detached from sediment samples using pyrophosphate (5 mM final concentration) and ultrasound treatment (3 times for 1 min) to increase the extraction efficiency (Danovaro et al., 2001). For bacterial counts, subsamples were diluted 100 to 500 times, stained with Acridine Orange and filtered on black Nuclepore 0.2 μm filters. Filters were analyzed under epifluorescence microscopy. Ten to 50 fields were viewed at × 1000 magnification and a minimum of 400 bacterial cells were counted. Bacterial counts were normalized to sediment dry weight after desiccation.

2.5. Analysis of bacterial diversity by ARISA

DNA was extracted from the sediment samples using the UltraClean soil DNA isolation kit (MoBio Laboratories), following the manufacture instructions. Previous studies revealed that this kit provides estimates of bacterial diversity comparable to those obtained using other *in situ* lysis procedures (Luna et al., 2006). DNA was amplified using the universal bacterial

primers 16S–1392F (5′-GYACACACCGCCCGT-3′) and 23S-125R (5′-GGGTTBCCCCATTCRG-3′), which amplify the intergenic region ITS1 between the 16S and the 23S rRNA operon plus ca. 282 bases of the 16S and 23S rRNA (Hewson and Fuhrman, 2004). The 23S-125R primer was fluorescently labeled with the fluorochrome HEX (MWGspa Biotech). We used 30 PCR-cycles, consisting of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, preceded by 3 min of denaturation at 94 °C and followed by a final extension of 10 min at 72 °C. To check for eventual contamination of the PCR reagents, negative controls containing the PCR-reaction mixture but without the DNA template were run during each amplification analysis. Positive controls, containing genomic DNA of *Escherichia coli*, were also used. PCR-products were checked on agarose-TBE gel (1%), containing ethidium bromide for DNA staining and visualization. For each sample, two different PCR reactions were run and then pooled together to minimize stochastic PCR biases (Polz and Cavanaugh, 1998). This process was carried out in duplicate, for a total of 4 different PCR reactions for each sample. The two resulting PCR combined products were then purified using the Wizard PCR clean-up system (Promega).

About 5 ng of amplicons were mixed with 14 μl of internal size standard (GS2500-ROX; Applied Biosystems, Foster City, CA) in deionized formamide, then denatured at 94 °C for 2 min and immediately chilled in ice. Automated detection of ARISA fragments were carried out in capillary gel electrophoresis (ABI Prism 3100 Genetic Analyzer, Applied Biosystems) and the ARISA fragments in the range of 390 ÷ 1400 bp were analyzed using Peak Scanner software 1.0 (ABI). Only bacterial ribotypes accounting for at least 0.11% of the total integrated peak height were considered. This value corresponds to the highest number of different peaks which can be discriminated by means of the ARISA method with the primer set and the standard utilized in this study (Danovaro et al., 2006).

2.6. Taxonomic composition of bacterial assemblages by 454 sequencing

To identify changes in the taxonomic composition of bacterial assemblages, 454 sequencing analyses of bacterial 16S rRNA genes were carried out on sediment samples collected before incubation and on selected sediment samples after 30 days of incubation. Such selected samples included: control samples (i.e. without any external supply, corresponding to treatment 1), samples containing lactose and inorganic nutrients (corresponding to treatment 4), samples containing lactose and acetate (corresponding to treatment 6) and samples containing acetate and inorganic nutrients (corresponding to treatment 7). The 16S rRNA genes were amplified using specific primers for Bacteria S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′; Klindworth et al., 2013). PCR reactions were performed in a volume of 50 μl in thermocycler (TGradient, Biometra) using the PrimeSTAR GXL DNA Polymerase (Takara Bio). We used 30 PCR-cycles adopting the same thermal cycling conditions described by Klindworth et al. (2013). To check for eventual contamination of the PCR reagents, negative controls (containing the PCR-reaction mixture but without the DNA template) were run during each amplification analysis. Positive controls, containing genomic DNA of *E. coli*, were also used. PCR-products were checked on agarose-TBE gel (1%), containing ethidium bromide for DNA staining and visualization. Four PCR amplification per sample were run and subsequently pooled together. Bacterial 16S rRNA genes were then sequenced on a 454 FLX Titanium platform.

The analysis of sequences was carried out using the MOTHUR pipeline (Schloss et al., 2009). The binary output (i.e., Standard Flowgram Format, SFF) was used for modeling the original flowgrams, that we used in the data processing stage for cleaning the sequences: this approach allows to obtain high quality data, since sequencing errors and the background noise (signal intensity between 0.5 and 0.7) can be greatly reduced (Quince et al., 2009; Schloss et al., 2011). We also removed any read with more than 8 homopolymers and more than 1 mismatches to the primer; chimeras were detected via chimera.uchime algorithm (Edgar et al., 2011) and then removed. To generate Operating Taxonomic Units (OTUs) we obtained representative sequences and we aligned to the SILVA-based reference alignment (http://www.arb-silva.de/). This alignment was then filtered to obtain the sequences overlapped in the same region and then it was used to construct a column-formatted distances matrix. The latter was then used to cluster the sequences into OTUs, defined by a 0.03 distance, according the average neighbor method. Finally, the distance matrix was used to construct the rarefaction curves (1000 randomization) and to calculate β-diversity indices (Chao et al., 2006). To compare samples with different number of sequences, random re-sampling of an equal number of sequences per sample (i.e. at the lowest values of reads obtained) was carried out by using the sub sample tool (Schloss et al., 2011).

2.7. Statistical analysis

According to the experimental factorial plan, we carried out an analysis of variance (ANOVA) using the JMP Statistical Discovery software (version 10, SAS Institute, Inc.). The parametric assumptions of normality and equal variance were assessed by Shapiro–Wilk test and O'Brien's test, respectively, and when necessary data were appropriately transformed. When significant differences were encountered, ANOVA was followed by Tukey–Kramer HSD post-hoc test and Dunnett's Method ($\alpha = 0.05$).

To test for differences in bacterial assemblage composition (determined by ARISA) during biostimulation experiments, a permutational multivariate analysis of variance (PERMANOVA) was also carried out using the software PRIMER 6.0 (Plymouth Marine Laboratory). PRIMER 6.0 was also used to investigate the similarity among bacterial assemblage composition determined by 454 sequencing by means of cluster analysis (based on Bray–Curtis similarity). Finally, a principal component analysis (PCA) was used to investigate the relationships among biotic and abiotic variables using data of total bacterial abundance, bacterial ribotype richness and evenness and metals in the different geochemical fractions of the sediment. To better highlight the mobilization/immobilization of (semi-)metals as function of their distribution in the different geochemical fractions of the sediment, a

new parameter M has been introduced in the statistical analysis:

$$M = -\Delta_{res} - 0.33\Delta_{oxid} + 0.33\Delta_{redu} + \Delta_{ex/carb}$$

where Δ represents the percentage of variation of the different (semi-)metals associated to each geochemical fraction: residual (Δ_{res}), oxidizable (Δ_{oxid}), reducible (Δ_{redu}) and exchangeable/carbonatic ($\Delta_{ex/carb}$). M values >0 indicate (semi-)metal mobilization. PCA was carried out on standardized data using JMP Statistical Discovery software. Bartlett's test of sphericity provided significant results ($\chi^2 = 77.53$, p-value < 0.001).

3. Results

3.1. Sediment characteristics

The sediment samples collected in the port of Piombino were mainly constituted by quartz, albite and calcite (Table 2). Iron oxides and other silicates were also detected but in a smaller proportion. Sulfide minerals were not detected, thus their contribution to the mineralogical composition can be considered negligible. The sediment samples collected in the port of Piombino contained high concentrations of organic matter, even higher than those previously reported in coastal areas subjected to high human pressure (Dell'Anno et al., 2002) and were also characterized by a high content of metals and As (Table 2).

The sequential extraction procedure revealed that Zn was the most mobile metal, with ca. 45% of its total content in the non-residual fractions (Table 2). Pb and Cd were largely distributed between the oxidizable and the residual fractions, while Cr and As were mainly associated to the residual fraction (i.e., ≥85%; Table 2). The principal scavenging phase for the metals in the oxidizable fraction was likely represented by the organic matter, due to its high content in the investigated sediment.

3.2. Changes in metal and semi-metal partitioning during bioremediation

Changes in metal partitioning due to bio-treatments were already evident after 30 days of incubation (Fig. 1). The major changes were observed for the partitioning of Zn, Pb and Cd, while Cr and As displayed smaller variations, in congruence with their high association to the residual fraction (i.e. 85% and 98%, respectively; Table 2). The observed changes in partitioning were not associated with metal solubilization processes, as the concentrations of all metals and As in the solution phase were below their detection limits (i.e. Cd < 1 μg/L; Cr < 5 μg/L; Zn, Pb and As <10 μg/L).

With the exception of Cr, metals and semimetals investigated in the present study increased in the exchangeable/carbonatic fraction (Fig. 1; range: 32–44%, 0–26%, 14–40% and 2–20% for Zn, Cd, Pb and As, respectively). The increase of Zn and Cd in the exchangeable/carbonatic fraction was associated with a concomitant decrease of these metals in the oxidizable fraction. Significant changes of metals and semi-metals occurred also in the residual fraction with changes more evident for Pb (Fig. 1; Supplementary Materials, Table S1). The analysis of variance (ANOVA) pointed out that each (semi-)metal investigated was influenced by the experimental factors in a metal specific way (Supplementary Materials, Table S1), although a common pattern within treatments was always evident. Zn

Table 2 – Characteristics of the sediment samples. Main geochemical characteristics of the Piombino's sediments. The concentrations and the partitioning of Zn, Cd, Pb, Cr and As among the geochemical fraction of the sediment is also reported.

Mineralogical composition				Quartz Albite Calcium carbonate Potassium Aluminum Sulfate Hydrate Hematite Sodium Hydrogen Zinc Silicate Clinochlore	
Grain size (% silt–clay)				88	
Water (%)				25 ± 1	
Carbonates (mg/g)				380 ± 10	
TOM (mg/g)				65 ± 5	
Element	Total content (ppm)	Exch/Carb	Reducible	Oxidizable	Residual
Zn	1030 ± 70	0.0%	4.1%	39.5%	56.4%
Cd	1.8 ± 0.5	0.0%	0.0%	31.1%	68.9%
Pb	200 ± 20	0.0%	0.5%	21.7%	77.8%
Cr	140 ± 50	0.0%	0.0%	9.4%	90.6%
As	48 ± 2	0.0%	0.0%	1.3%	98.7%
Ni	29 ± 5	n.d	n.d	n.d	n.d
Cu	37 ± 4	n.d	n.d	n.d	n.d
Fe	84 ± 8 × 10^3	n.d	n.d	n.d	n.d

Exch/Carb: exchangeable/carbonatic fraction.
n.d.: Not determined.

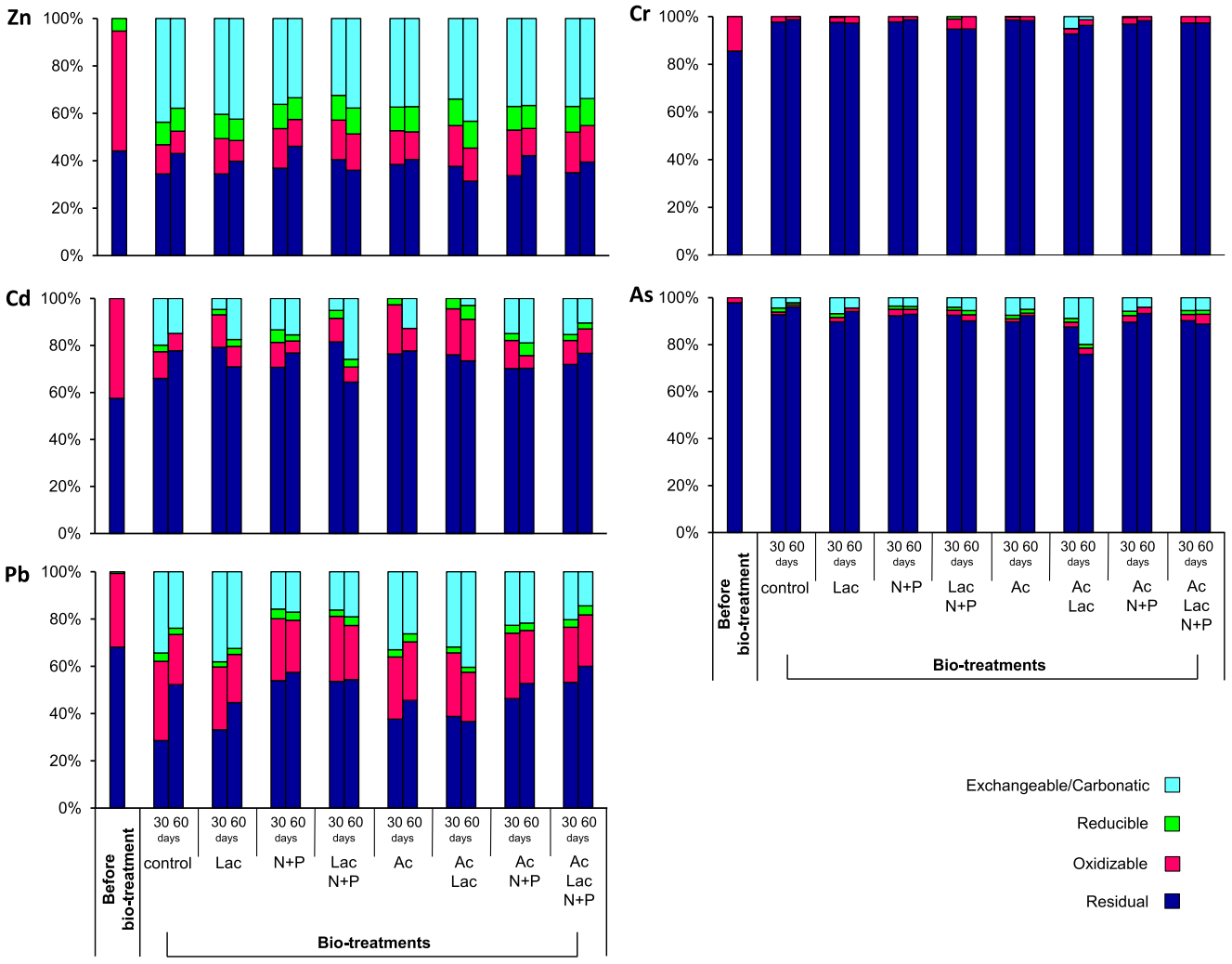

Fig. 1 – Metal partitioning in the sediment before and after biostimulation experiments. Ac: acetate; Lac: lactose; N + P: inorganic nitrogen and phosphorous.

partitioning was unaffected by the factors investigated and its changes were dependent exclusively upon the incubation time (i.e. longer incubation time determined a decrease of Zn in the oxidizable fraction). Similarly, the shift of Cd from the oxidizable towards the exchangeable/carbonatic fraction was also favored by longer incubation time, although less relevant in the presence of acetate. The presence of inorganic nutrients favored the mobilization of Cd (released mainly from the oxidizable fraction; 29% of the variance) and Pb (released mainly from the residual fraction; 58% of the variance). On the contrary, the increase of As in the exchangeable/carbonatic fraction, to the detriment of the residual one, was mainly influenced by the presence of acetate and/or lactose.

According to their predominance diagrams (Supplementary materials, Figure S1), Zn, Cd and Pb can speciate as carbonates (e.g., $Zn_5(OH)_6(CO_3)_2$, $ZnCO_3 \cdot H_20$, $CdCO_3$, $PbCO_3$), which are very stable compounds in our experimental conditions (pH ca. 8.0 and low Eh values). On the contrary, Cr and As do not form carbonates but speciate mainly as anionic compounds.

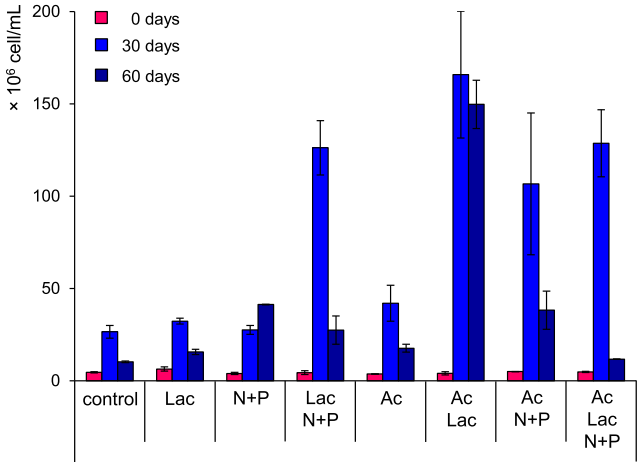

Fig. 2 – Changes of bacterial abundances during bio-treatments. Total bacterial abundances (TPNs) in the sediment after 0, 30 and 60 days of incubation. Ac: acetate; Lac: lactose; N + P: inorganic nitrogen and phosphorous.

3.3. *Bacterial abundance and diversity*

The bacterial abundances increased significantly in all biostimulation experiments (Fig. 2). With the exception of microcosms supplied with inorganic nutrients or lactose plus acetate (treatments 3 and 6, respectively), the highest bacterial abundances were observed after 30 days of incubation, followed by a significant decrease. The analysis of variance indicated that the addition of acetate and/or lactose was the most important factor explaining the increase of the bacterial abundances (Supplementary Materials, Table S2).

ARISA results revealed a decrease in the number of bacterial ribotypes after 30 days of incubation, often followed by an increase in the further 30 days (Fig. 3A). Unlike bacterial abundance, bacterial ribotype richness was mainly influenced by the addition of inorganic nutrients (Supplementary Materials, Table S3). In particular, after 30 days of incubation an important fraction of the variance was explained by the presence of inorganic nutrients, although after 60 days such an effect was also due to the concomitant presence of organic substrates. The biostimulation determined also significant changes in the composition of the bacterial assemblages (Fig. 3B). In particular, inorganic nutrient addition explained the highest fraction of the total variance of the bacterial assemblage composition (Supplementary Materials, Table S4).

The importance of inorganic nutrients in affecting the bacterial assemblage composition and changes in (semi-) metal mobility was also highlighted by PCA. In particular in the score plot reported in Fig. 4A, points splitted into two groups along the axis of the First Principal Component (explaining 38% of the total variance), with microcosms without inorganic nutrients on the right and microcosms containing inorganic nutrients on the left. Moreover, the loading plot (Fig. 4B) highlighted that microcosms added with

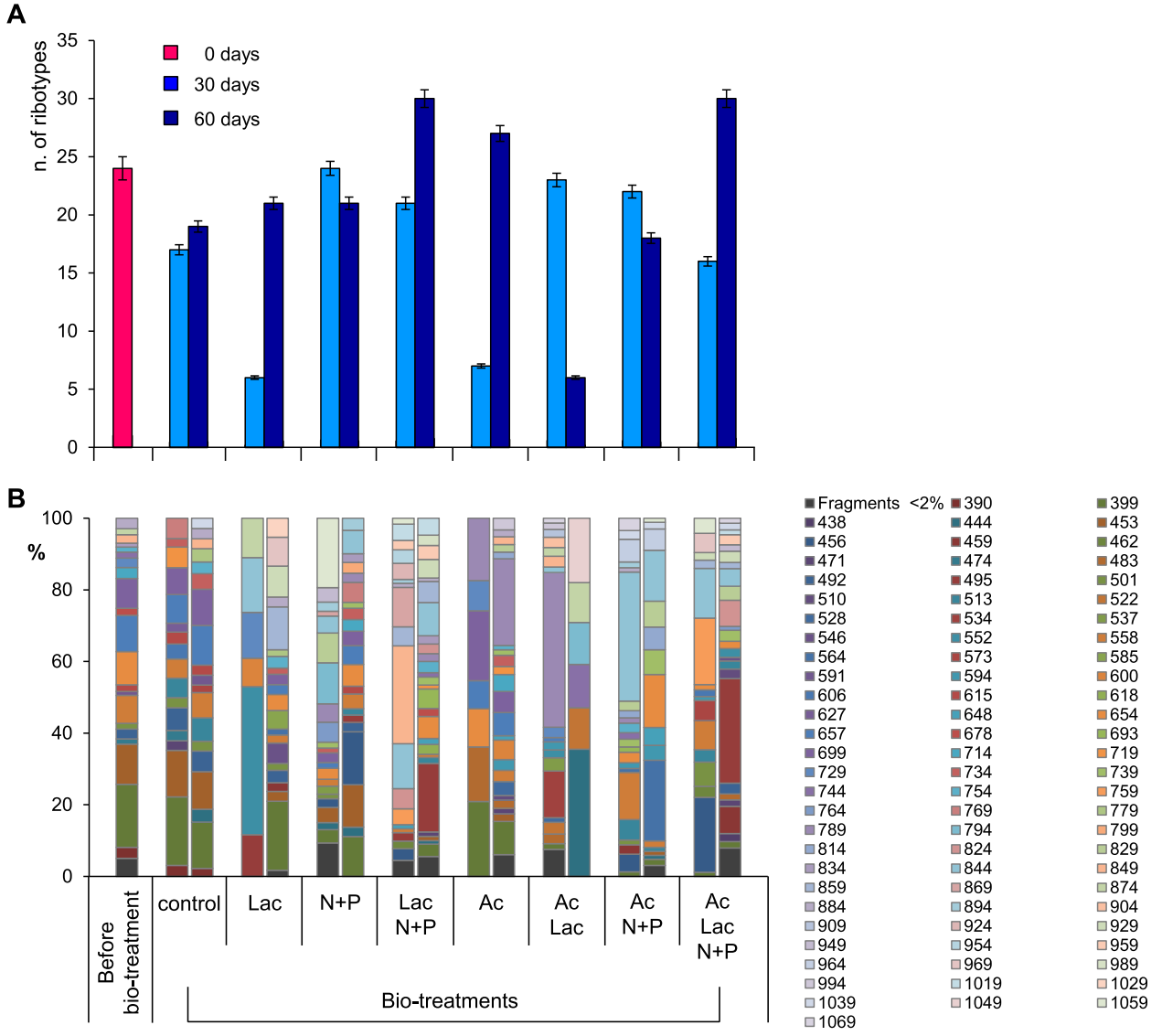

Fig. 3 – Bacterial richness and assemblage composition obtained by ARISA. A) Changes of bacterial ribotype richness during biostimulation experiments; B) bacterial assemblage composition (as relative abundances of the different ribotypes identified) during biostimulation experiments.

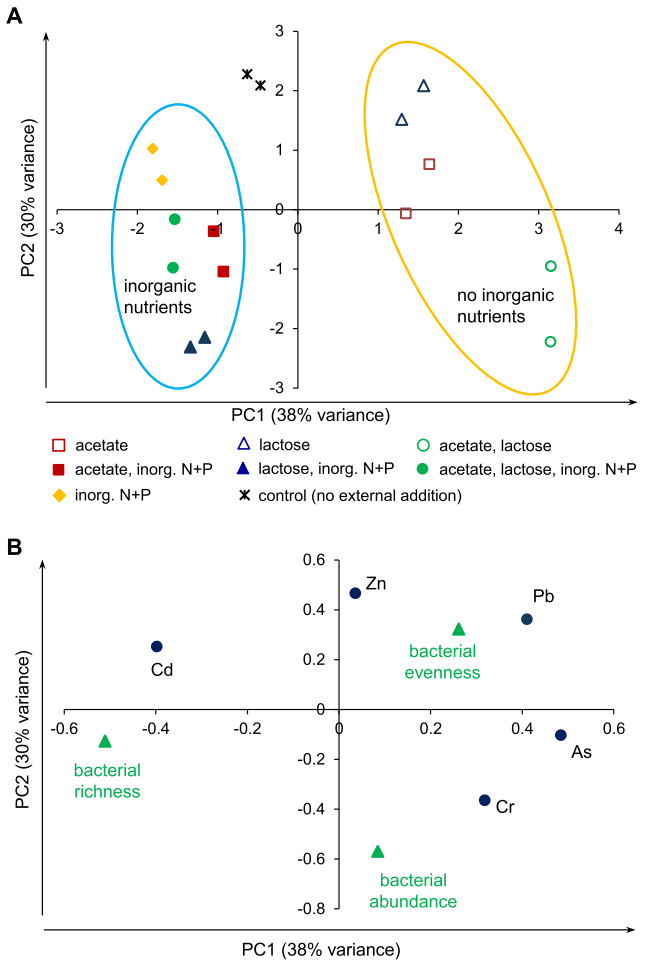

Fig. 4 – Output of the Principal Component Analysis (PCA). A) Score plot of the first and second principal components (PC1, PC2, respectively); B) loading plot of the response variables with the respect to the first and the second principal component.

inorganic nutrients were characterized by a higher bacterial ribotype richness, an increase of the mobility of Cd and a decrease of the mobility of Pb and As, compared with microcosms in which only organic substrates were added.

3.4. *Taxonomic composition of bacterial assemblages just before and after bio-treatments*

The pyrosequencing analysis of bacterial 16S rRNA genes allowed to obtain a total of 78,215 sequences after cleaning (Table 3). After 30 days of incubation, the highest numbers of OTUs were detected in the control samples (i.e. treatment 1), where no external compounds were added, and in the microcosms with acetate plus lactose (Table 3). The expected numbers of OTUs were even higher, as revealed by Chao1 index (Table 3) and the rarefaction curves which did not reach saturation (Supplementary Materials, Figure S2). To compare bacterial assemblage composition among sediments with a different number of sequences all data were normalized to the lowest number of sequences (i.e. 7179 sequences) by random replicated re-sampling. Both at the beginning and after 30 days of incubation, the bacterial assemblage was dominated by OTUs affiliated with the phylum *Proteobacteria* (52–58%). Other phyla were represented by *Bacteroidetes* (6–13%), *Actinobacteria* (3–5%), *Acidobacteria* (2–4%), *Planctomycetes* (2–7%) and *Firmicutes* (3–6%; Fig. 5). Unclassified bacterial taxa contributed for 7–14% to the bacterial assemblages. Bacterial sequences that can be classified at family level accounted for 37–53% of the total assemblages. Clear changes of the main bacterial families identified in the different microcosms were observed (Fig. 6A; Supplementary Materials, Table S5). In particular, after 30 days of incubation in the presence of inorganic nutrients, the relative contribution of sulfate reducing bacteria belonging to the families *Desulfobacteraceae* and *Desulfobulbaceae* decreased (altogether accounting for 5 and 6% in the treatment 4 and 7, respectively) when compared to experimental systems without any substrate addition (10%) or supplied only with organic compounds (12%). Such a decrease was associated with a concomitant increase of members affiliated with *Flavobacteriaceae* (7 and 9% in the treatment 4 and 7, respectively) and *Rhodobacteraceae* (mainly belonging to the *Roseobacter*-clade, 8–11%). A cluster analysis showed that in the presence of inorganic nutrients (treatments 4 and 7) the taxonomic composition of the bacterial assemblages grouped together and that it was more dissimilar to microcosms without any substrate addition (treatment 1) than microcosms supplied only with organic compounds (treatment 6, Fig. 6B). The higher dissimilarity of taxonomic composition in the microcosms containing inorganic nutrients was further confirmed by the values of Smith's Theta and Sørensen's coefficients and the number of the shared OTUs (Supplementary Materials, Table S6).

Further statistical analyses based on PCA highlighted an association between specific bacterial taxa and metal mobility (Fig. 7). Bacterial OTUs belonging to Phycisphaeraceae, *Planctomycetaceae*, *Phyllobacteriaceae* and other families with relative abundance lower than 1% were associated with an increase in the mobility of Cr and As, while an inverse association with Cd mobility was observed. Families of sulfate reducing bacteria (i.e. *Desulfobacteraceae* and *Desulfobulbaceae*) apparently promoted a decrease in Cd mobility (explaining 31.6% of the variance on the second principal component), whereas other bacterial taxa belonging to unclassified families exerted an opposite effect (51.8% of the variance on the first principal component). Bacterial taxa affiliated with different families, such as *Oceanospirillaceae*, *Sinobacteraceae* and *Flavobacteriaceae* and other unclassified bacteria belonging to *Firmicutes*, *Actinobacteriaceae* and *Bacteroidetes*, apparently promoted a decrease in Pb mobility. On the contrary, other unclassified bacteria played an opposite effect.

4. Discussion

Previous studies have provided evidence that bioremediation strategies applied to contaminated marine sediments can determine significant changes in the metal partitioning with potential consequences on their bioavailability and toxicity (Dell'Anno et al., 2009; Rocchetti et al., 2012). However, the biogeochemical interactions among abiotic and biotic

Table 3 – Summary of 454 sequencing analysis carried out on bacterial 16S rRNA genes. Reported are the number of reads before and after cleaning, the number of OTUs and the expected number of OTUs estimated through the Chao1 richness estimator.

	Reads before cleaning	Reads after cleaning	OTUs	Chao1
Sediments before bio-treatments	11,895	7179	923	1532
Sediments without addition	45,638	28,477	1859	2288
Sediments added with inorganic nutrients and lactose	32,536	13,238	597	856
Sediments added with acetate and lactose	25,612	16,174	835	1154
Sediments added with inorganic nutrients and acetate	26,104	13,147	671	1036

processes influencing metal mobility (and their consequences on metal fate) are still largely unknown, but their comprehension is crucial for the definition of ecologically compatible strategies suitable for the *in situ* reclamation of contaminated marine sediments.

Bio-treatments carried out in the present study enhanced the mobility of Zn, Pb, Cd and As as highlighted by the significant increase of such (semi-)metal concentrations in the exchangeable/carbonatic fraction of the sediment (Zn: 330–453 $\mu g\ g^{-1}$, Pb: 28–80 $\mu g\ g^{-1}$, Cd: 0.05–0.47 $\mu g\ g^{-1}$, As: 1–10 $\mu g\ g^{-1}$) when compared to values at the beginning of the experiments (undetectable for all metals). These results suggest that bio-treatments can enhance the probability of adverse biological effects due to metal contamination even when the total metal concentrations in the sediments are below the threshold levels assumed to induce such detrimental effects (Long et al., 1995).

Available information dealing with the effects of biostimulation on metal mobility in the sediment is extremely scant for a proper comparison. Our results agree with previous findings which highlighted an increase of Pb and Zn in the exchangeable/carbonatic fraction during bio-treatments carried out on marine sediments using the same experimental conditions (i.e anoxic conditions, temperature regime) and organic substrate supply (i.e. acetate addition; Dell'Anno et al., 2009). Conversely, similar experimental treatments carried on aquatic sediments collected in hypoaline ecosystems provided opposite results (i.e. a decrease of Zn in the exchangeable/carbonatic fraction; Rocchetti et al., 2012). Such contrasting results could be due to differences in the geochemical characteristics of marine vs hypoaline sediments (e.g. mineralogical composition) and in the microbial assemblages contained therein.

All of the bio-treatments investigated in the present study were effective in stimulating bacterial growth as indicated by the significant increase of the bacterial abundance with time, even in the microcosms where organic substrates were not supplied, indicating that organic compounds in the sediment were degraded and used for sustaining the bacterial metabolism. The organic matter in the sediment represents an important scavenger of metals and semi-metals (van Hullebusch et al., 2005; Warren and Haack, 2001) since at basic pH values, as those characterizing benthic marine ecosystems, the carboxyl, phenolic, alcoholic and carbonyl functional groups dissociate and thereby increase their affinity for metal cations (Guo et al., 2006; Yin et al., 2002). The decrease of metals associated with the oxidizable fraction (i.e. sulfides and organic matter), especially Zn and Cd (from 51% to 9 ÷ 19% and from 42% to 5 ÷ 21%, respectively both in the presence of acetate plus inorganic nutrients) but also Pb and

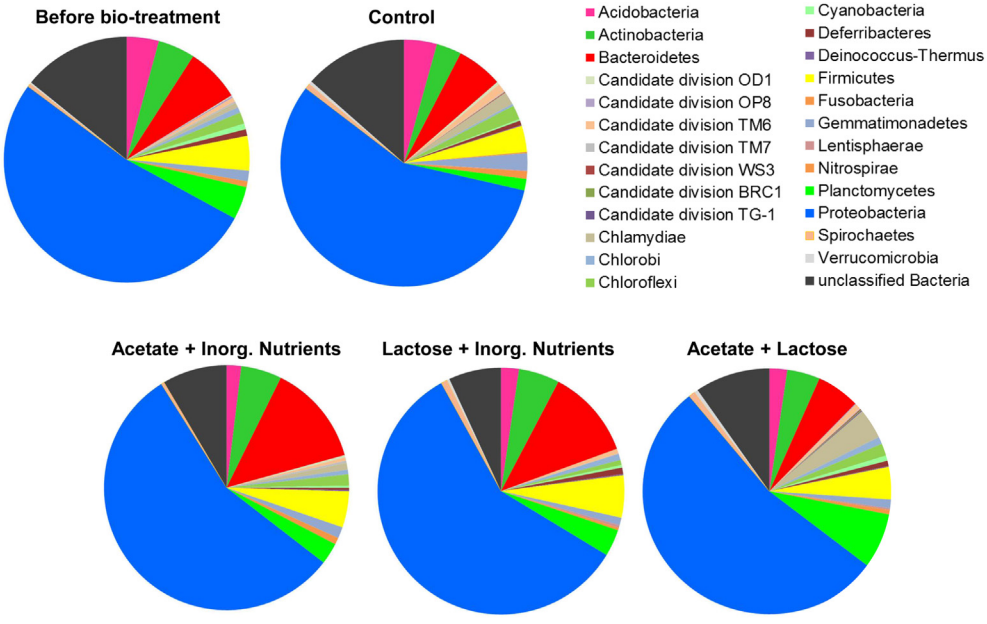

Fig. 5 – Taxonomic composition of bacterial assemblages obtained by 454 sequencing analysis. Taxonomic composition at phylum level of selected samples before and after 30 days of incubation.

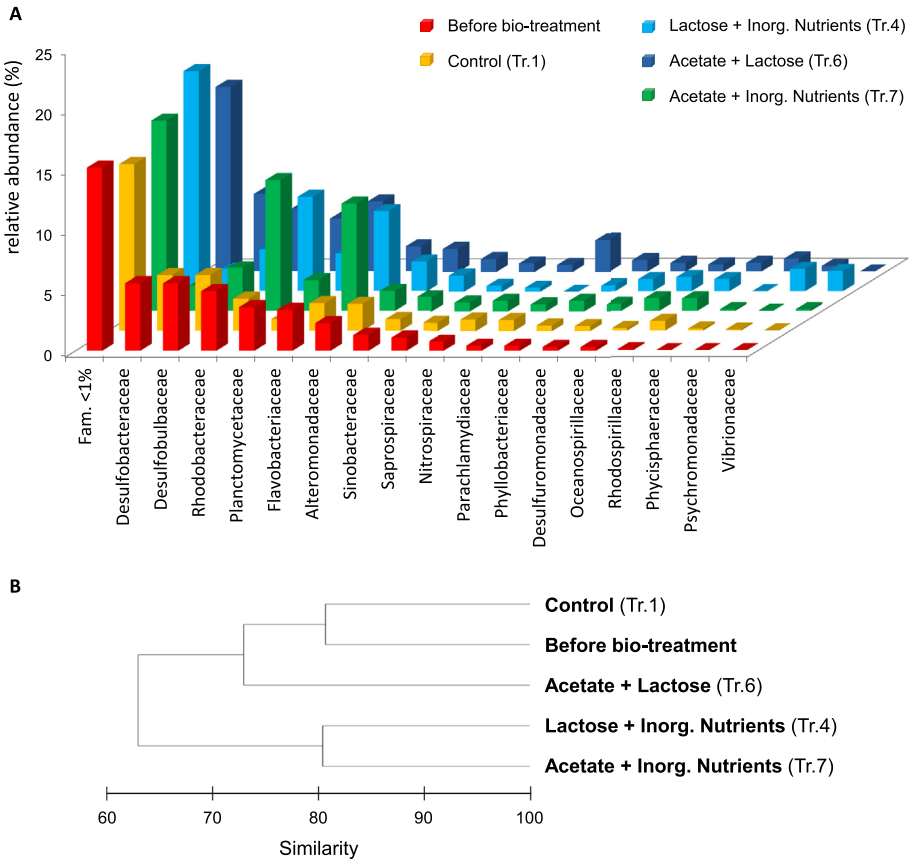

Fig. 6 – Bacterial families detected by 454 sequencing analysis. A) Taxonomic composition at family level before and after 30 days of incubation; B) output of the cluster analysis based on Bray–Curtis similarity of the bacterial assemblage composition. Families contributing for <1% are grouped together and unclassified OTUs are not shown (for a complete list, see Supplementary Materials Table S5).

Cr (from 31% to 21 ÷ 28% in the presence of lactose and from 15% to 1 ÷ 5%, respectively), was reasonably at the detriment of the organic component, since the release of (semi-)metals from sulfides occurs in very acidic and highly oxidative conditions (Licht, 1988). These findings suggest that biodegradation processes of organic matter can significantly enhance the mobility of metals in the sediment. This was also confirmed by pyrosequencing analysis, which revealed the presence of different heterotrophic bacterial taxa able to degrade a variety of complex organic substrates (e.g., belonging to the phyla *Bacteroidetes*, *Planctomycetes* and *Firmicutes*, Bernardet and Yasuyoshi, 2006; Tadonléké, 2007; Fenchel et al., 2012; Fernández-Gómez et al., 2013). Beside this, metals released from the oxidizable fraction (and from the residual fraction with a minor extent) did not move in the solution phase but shifted towards other geochemical fraction of the sediment, suggesting a potential role of the bacterial assemblages also in controlling solubilization processes (e.g., complexation with dissolved organic compounds or chlorides; Gadd, 2010). Our findings indicate that an increase in metal mobility does not necessarily determine a metal solubilization. However, naturally occurring processes of sediment resuspension and bioturbation can favor the release of (semi-)metals from the more mobile fractions of the sediment to the solution phase and/or increase bioaccumulation processes within the benthic food webs (Calmano et al., 1993; Chen et al., 1999; Baumann and Fisher, 2011) with detrimental effects on ecosystem and human health (Hollabaugh, 2007; Pastorelli et al., 2012). Thus, care should be devoted at monitoring the potential changes in the (semi-)metals mobility induced by bio-treatments especially if applied *in situ*.

Our results have highlighted also that during bio-treatments (semi-)metals undergo specific changes depending upon their initial partitioning in the sediment, their intrinsic physical-chemical characteristics and upon changes in bacterial assemblage composition due to the selection of different bacterial taxa. In particular, Zn in the oxidizable fraction significantly decreased with the incubation time (from 51% at the beginning of the experiments to 12 ÷ 19% and 9 ÷ 15% after 30 and 60 days of biostimulation, respectively), but without any significant effect due to the addition of organic and/or inorganic compounds. Zn is known to be one of the most mobile metals, since it is highly sensitive to abiotic and biotic-induced changes (Reddy and DeLaune, 2004) and dissociates from the binding groups of the organic matter (Van Hullebusch et al., 2005). Indeed, we observed an increase of Zn mobility in all of the experimental treatments, including those in which minor changes

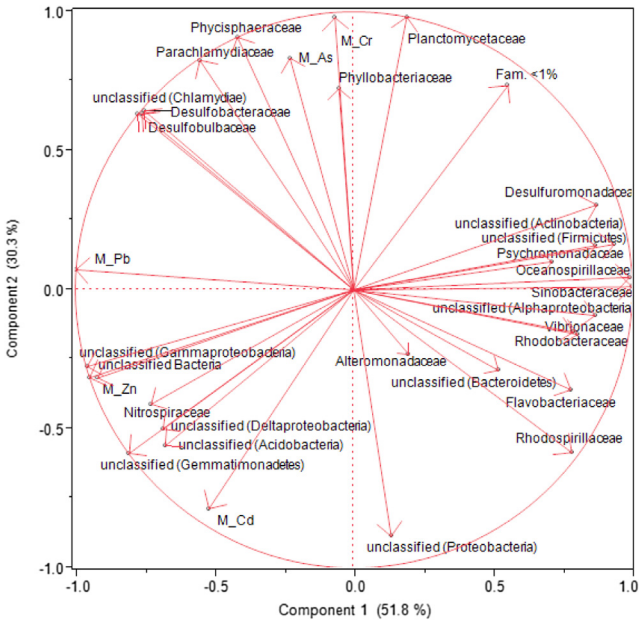

Fig. 7 – Loading plot. Eigenvectors calculated by PCA using the response variables i) taxonomic composition at family level of the bacterial assemblages and ii) (semi-)metal mobility. M: mobility parameter (see details in par. 2.7).

of bacterial abundance and diversity occurred. Conversely, the addition of organic and/or inorganic compounds determined significant changes in Cd partitioning. In particular, in the presence of acetate, a very suitable substrate for sulfate reducing bacteria, we observed the highest Cd concentrations in the oxidizable fraction, whereas the presence of inorganic nutrients promoted a Cd mobilization. Such variations in Cd partitioning were reflected by differences in bacterial diversity and assemblage composition. Indeed, microcosms supplied only with organic substrates were characterized by a strong increase of the relative importance of sulfate reducing bacteria belonging to the families *Desulfobacteraceae* and *Desulfobulbaceae* with a concomitant decrease of members affiliated with *Flavobacteriaceae*, which includes several bacterial taxa able to degrade a variety of complex organic substrates (Bernardet and Yasuyoshi, 2006; Fuchsman et al., 2012). The opposite was observed in the microcosms supplied also with inorganic nutrients. These findings suggest that the extent of partitioning changes of metals was dependent upon an interplay between organic matter decomposition and sulfate reduction processes in which the former caused the dissociation of Cd and the latter favored its precipitation as sulfides. Finally, also bacterial taxa affiliated mainly to unclassified families of Proteobacteria appeared to favor the increase of Cd content in the exchangeable/carbonatic fraction of the sediments.

We observed that Pb behaves in a different way compared to Cd. Indeed, in the absence of inorganic nutrients (where a lower bacterial ribotype richness was observed), Pb in the exchangeable/carbonatic fraction increased (from 0% before biostimulation up to 40%). Pb was also characterized by a significant decrease in the residual fraction of the sediment (from 68% before biostimulation to 36 ÷ 59%) and such an effect was consistent in all of the experimental conditions investigated. Highly insoluble Pb phosphates (e.g. pyromorphite, $Pb_5(PO_4)_3Cl$) are known to be solubilized from the residual fraction of terrestrial soils by phosphate-solubilizing bacteria (Ryan et al., 2001; Tabak et al., 2005; Rajkumar et al., 2009). Phosphate-solubilizing bacteria have been also reported in sediments receiving high terrigenous inputs (Sahu et al., 2007), such as those investigated in this study. At the same time also Pb incorporated in the crystal lattice of silicates, which represent an important component of the sediments investigated in the present study, can undergo bacterial-mediated solubilization (Brehm et al., 2005). Altogether these findings can contribute to explain the decrease of Pb in the residual fraction of the Piombino's sediment, although the identity of the bacterial taxa involved still needs to be clarified. Cd, Zn and Pb, which were characterized by the widest changes in partitioning, shifted towards the exchangeable/carbonatic fraction. These three metals can speciate as carbonates, which are stable compounds in the experimental conditions investigated, as highlighted by the predominance diagrams (Supplementary Materials, Figure S1).

As was almost entirely associated with the residual fraction of the sediment (98% of its total concentration), likely as arsenate (As(V)), one of the main inorganic forms of As in contaminated soils and sediments, that is commonly detected in the residual fraction and can be discriminated from silicon co-precipitates only by specific SSE techniques (Goh and Lim, 2005). Bio-treatments determined the shift of As towards the exchangeable/carbonatic fraction. Nevertheless, As does not speciate as carbonate by itself, but it forms very stable compounds as sulfides. We did not observe an increase of As into the oxidizable fraction, so the bio-precipitation of As sulfides by SRB can be considered as negligible. According to our results, Phycisphaeraceae, *Planctomycetaceae* and rare bacterial groups could be involved in favoring an increase of As mobility as As(III), although chemical reductions due to changes in the environmental conditions cannot be excluded. Finally, as suggested by our simulations, the bio-treatments induced Cr to dissociate from the organic matter and to move towards the residual fraction of the sediment, likely in the form of highly insoluble compounds (e.g. Cr (III) oxides). Such a shift could be favored by bacteria affiliated with Phycisphaeraceae and *Planctomycetaceae*, as well as by other bacteria belonging to families with abundances lower than 1%.

Overall our findings suggest that biological processes, geochemical properties of the sediment (e.g., mineralogical composition, organic matter, carbonate and sulfide content) and intrinsic physico-chemical characteristics of metal species interact generating complex patterns that determine the fate of (semi-)metals in contaminated marine sediments. In particular, changes occurring in benthic bacterial metabolism and diversity due to bio-treatments can influence the mobility of metals in the sediments, primarily by increasing the release of metals associated with organic matter and bound to minerals or highly insoluble compounds. Such effects, however does not necessarily lead to metal dissolution, suggesting a potential opposite role exerted by other bacterial taxa which can favor bio-precipitation processes of metals.

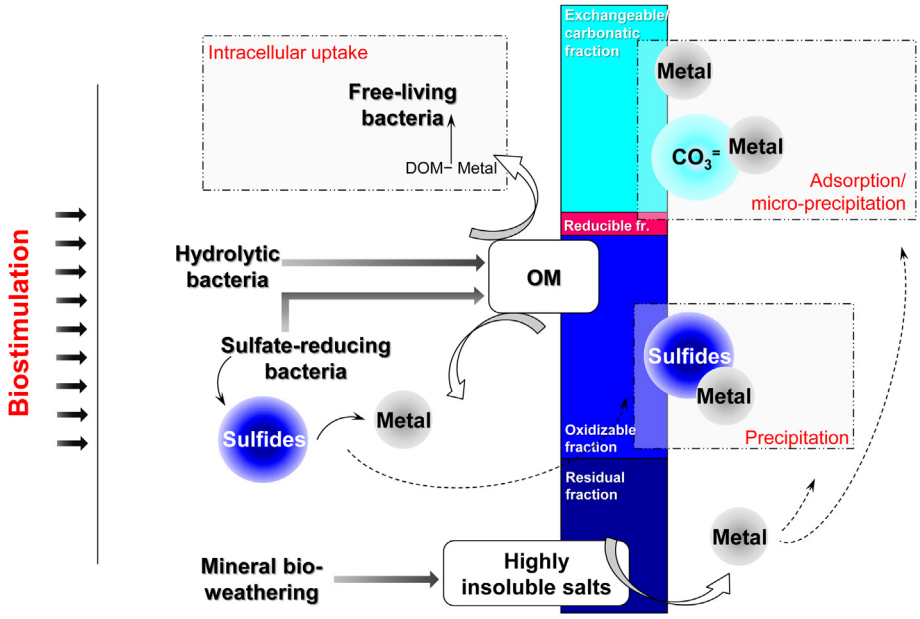

Fig. 8 – Conceptual model showing the main biogeochemical interactions influencing metal fate. During bio-treatments of contaminated marine sediments, the main sediment components involved in the release of (semi-)metals are represented by organic matter (OM) in the oxidizable fraction and high insoluble salts in the residual fraction. Different microbial groups are responsible of this phenomenon. The biodegradation of the organic matter leads to the direct release of metal cations but also to solubilization, mediated by the dissolved organic matter (DOM). Other phenomena could lead metals to pass in the solution phase (chelation with DOM is just an example), but the microbial assemblages play a role also in controlling solubilization processes (e.g., by uptake of DOM). Once (semi-)metals are released, they can undergo precipitation as sulfides, carbonates and phosphates, to adsorption or to further redox transformations. Oxidation/reduction reactions directly involving the transformation of (semi-)metals are not reported because they usually involve the biotransformation of metals (e.g. Cr(VI)) in the intracellular space for detoxification purposes. The relative importance among each one of the biogeochemical processes involved is dependent upon the type of bio-treatment and the microbial diversity present in the sediment.

5. Conclusions

Our results pointed out that the bioremediation of autochthonous microbial assemblages in contaminated anoxic marine sediments modifies the partitioning of metals and semi-metals. This should be carefully taken into account if bio-treatments are applied *in situ* since an enhanced mobility of metals can increase their bioavailability and toxicity. The natural proclivity of metals to speciate as compounds stable and abundant in marine sediments (e.g., carbonates), their affinity for organic matter and/or minerals and the presence of different bacterial taxa with different functions (e.g., decomposers of complex organic substrates, sulfate reducers) are key factors influencing the metal fate during bioremediation of contaminated marine sediments. However, the extent of these changes is dependent upon the (semi-)metals considered, being intrinsically linked to their initial partitioning in the geochemical fractions of the sediment and their physical-chemical characteristics.

Overall, our findings allow to define a new conceptual model in which the interactions among the main abiotic and biotic processes are showed for the first time in explicit form (Fig. 8). Despite no definitive conclusions can be drawn on the complex biogeochemical processes and interactions dictating changes of metals and semi-metals due to bio-treatments, such simplified model provides new insights for a better understanding of the potential consequences of bioremediation strategies on metal fate in contaminated marine sediments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.10.035.

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