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Exploring bacterial community composition in Mediterranean deep-sea sediments and their role in heavy metal accumulation

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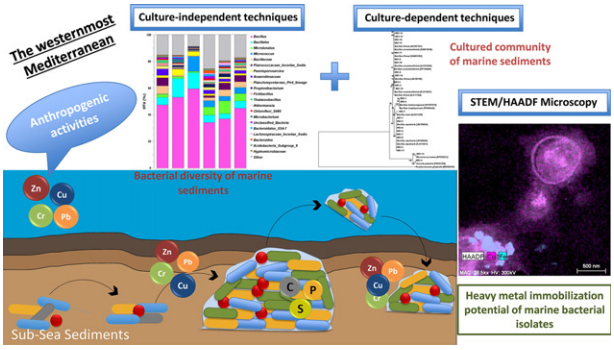
HIGHLIGHTS

- The westernmost Mediterranean is highly sensitive to anthropogenic pressure.
- NGS showed mostly *Bacillus* and *Micrococcus* as dominant in the deep-sea sediments.
- Culturable bacteria revealed mostly the presence of Firmicutes and Actinobacteria.
- Marine culturable bacteria bioaccumulate heavy metals within the cells and/or in EPS.
- Lead precipitates in the sediment bacterial cells as pyromorphite.

GRAPHICAL ABSTRACT



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ABSTRACT

The role of microbial processes in bioaccumulation of major and trace elements has been broadly demonstrated. However, microbial communities from marine sediments have been poorly investigated to this regard. In marine environments, particularly under high anthropogenic pressure, heavy metal accumulation increases constantly, which may lead to significant environmental issues. A better knowledge of bacterial diversity and its capability to bioaccumulate metals is essential to face environmental quality assessment. The oligotrophic westernmost Mediterranean, which is highly sensitive to environmental changes and subjected to increasing anthropogenic pressure, was selected for this study. A sediment core spanning the last two millennia was sampled at two intervals, with ages corresponding to 140 (S1) and 1400 (S2) yr BP. High-throughput sequencing showed an abundance of *Bacillus*, *Micrococcus*, unclassified members of *Planococcaceae*, *Anaerolineaceae*, *Planctomycetaceae*, *Microlunatus*, and *Microbacterium* in both intervals, with slight differences in their abundance, along with newly detected ones in S2, i.e., *Propionibacterium*, *Fictibacillus*, *Thalassobacillus*, and *Bacteroides*. Canonical correspondence analysis (CCA) and co-occurrence patterns confirmed strong correlations among the taxa and the environmental parameters, suggesting either shared and preferred environmental conditions, or the performance of functions similar to or complementary to each other. These results were further confirmed using culture-dependent methods. The diversity of the culturable bacterial community revealed a predominance of *Bacillus*, and *Micrococcus* or *Kocuria*. The interaction of these bacterial communities with selected heavy metals (Cu, Cr, Zn and Pb) was also investigated, and their capacity of bioaccumulating metals within the cells and/or in the extracellular polymeric substances (EPS) is demonstrated. Interestingly, biomineralization of Pb resulted in the precipitation of Pb phosphates (pyromorphite). Our study supports that remnants of marine bacterial communities can survive in

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deep-sea sediments over thousands of years. This is extremely important in terms of bioremediation, in particular when considering possible environmentally friendly strategies to bioremediate inorganic contaminants.

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1. Introduction

The significance of the deep biosphere in the Earth system's processes has been clearly demonstrated (Parkes et al., 2014). Yet most of the microbial world remains to be discovered. Relatively little is known about the patterns of microbial distribution in many marine habitats (Nemergut et al., 2011). This is particularly true for deep-sea sediments (>1000 m), despite the fact that they constitute the largest ecosystem, covering about 95% of the total oceanic bottom and 67% of the Earth's surface (Nemergut et al., 2011). In marine environments, sub-seafloor ecosystems are subjected to particular conditions that include high salinity and pressure, low temperatures, and variable oxygen concentrations. These conditions generate an evolutionary pressure on marine microorganisms, differentiating them from their terrestrial counterparts, with significant implications for their genetic and metabolic diversity (Lam, 2006).

The origin of these communities is now increasingly studied, and it has been confirmed that buried microbial communities represent a subset of taxa from the sediment at the time of burial, meaning they exist as relics of past surface communities that were buried over time (Walsh et al., 2016; Petro et al., 2017). Bacteria and archaea are closely linked with the physico-chemical processes following deposition (e.g., Parkes et al., 2014). They are key to all biochemical cycles and are crucial for the functioning of marine ecosystems. Microbial communities also affect the physicochemical conditions in sediments, including the degree of oxygenation, nutrient availability and pH (Fierer et al., 2007; Fierer and Jackson, 2006; Hansel et al., 2008; Lüdemann et al., 2000; Zhang et al., 2017b; Zhou et al., 2004). For example, marine microorganisms are responsible for the degradation of organic matter in the ocean and are thus key for maintaining the balance between produced and fixed carbon dioxide (Geetha et al., 2008; Huerta-Diaz and Reimer, 2010). Understanding and identifying sediment bacterial communities is therefore crucial if we are to anticipate the responses of marine ecosystems to future environmental changes.

Marine sediments in regions under high anthropogenic pressure constantly increase heavy metal concentration, which may lead to significant pollution. This is a highly relevant issue in the Mediterranean region, known to be highly sensitive to environmental changes. In fact, it has been identified as a "hotspot" for global change impacts (e.g., Durrieu de Madron et al., 2011; Malanotte-Rizzoli et al., 2014). The westernmost Mediterranean has also been subjected to an active water mass exchange since the Gibraltar Arc system was established, making this region of exceptional environmental interest. Intensive marine traffic and the risk of spills from ship carriers leave the westernmost Mediterranean particularly vulnerable to anthropogenic pollutants, including heavy metals that may interact with marine microbial populations and affect fate and behavior.

A better knowledge of the microbial diversity and of the capability of marine bacteria to bioaccumulate metals within the cells and/or in their extracellular polymeric substances (EPS) is furthermore essential to gain new insights into the impact of marine microbial populations in the concentration and enrichment of heavy metals in such aquatic environments. It would help when designing bioremediation strategies (e.g., Agnello et al., 2016), in particular when natural attenuation and biostimulation are considered. For instance, the characteristics of marine microbial communities and the specific role of different marine sediment microorganisms in heavy metal accumulation are still not clearly understood. Very few metagenomic studies have been performed on complex marine deep-sea sediment communities, and almost none investigate their possible relationship with metals. In marine environments, indigenous bacteria could be involved in the accumulation and concentration of contaminating metals through different processes, including reduction (Vandieken et al., 2012; Holden and Adams, 2003), biomineralization (Morcillo et al., 2014), and biosorption by EPS-forming biofilm populations (Iyer et al., 2005). Members of different bacterial taxa (*Arcobacter*, *Colwellia* and *Oceanospirillaceae*) identified in manganese oxide-rich marine sediments were described to couple acetate oxidation with manganese reduction (Vandieken et al., 2012). Morcillo et al. (2014) reported the role of *Idiomarina,* a marine bacterium widely distributed in marine and hypersaline habitats, in the precipitation of soluble uranyl ion into insoluble U phosphate minerals. In turn, González-Muñoz et al. (2008) described the ability of these marine bacteria to precipitate Mg-rich carbonates (Ca-Mg kutnahorite). The exopolysaccharide produced by the marine bacterium *Enterobacter cloacae* reportedly has excellent chelating properties with respect to cadmium, copper, and cobalt (Iyer et al., 2005). Martinez-Ruiz et al. (2018) demonstrated the role of many marine bacteria in the concentration of Ba, underlining the role of the EPS in different microbial processes.

Thus, studying the bacterial diversity of marine environments may eventually lead to a selection of novel marine bacteria with new abilities, e.g. the remediation of metal-contaminated environments. Culture-independent studies and the use of next generation sequencing approaches have revealed the high microbial diversity and complexity of bacterial assemblages inhabiting deep-sea sediments (Rath et al., 2011). Despite their wide application in the field of microbial ecology, however, molecular techniques do not suffice to recover the whole array of marine bacterial abilities. Culture-dependent techniques, in combination with culture-independent techniques, remain the most practical method to determine bacterial diversity and activity in marine environments.

Within this context, in the present work, both culture-independent and -dependent techniques were applied to study the bacterial communities in deep-sea sediments of the westernmost Mediterranean (Alboran Sea basin) and their interactions with heavy metals — in particular, chromium (Cr), copper (Cu), zinc (Zn), and lead (Pb). Due to its environmental sensitivity and vulnerability, as one of the most oligotrophic regions in the world, this area was chosen as a case study to further explore microbial communities preserved in deep-sea sediment. We first performed direct DNA extraction and amplification from the sediments to determine the composition and structure of marine bacterial populations by means of Next Generation Sequencing (NGS) (Illumina MiSeq). We then cultured the marine bacterial communities to study their interactions as a whole with different trace metals using state-of-the-art microscopic techniques combined with element mapping analysis. The metal remediation potential of the isolated marine communities is discussed and evaluated.

2. Material and methods

2.1. Sediment sampling

Samples of marine sediments were collected from a 40 cm-long core, collected at a multicore station, GP02 (Lat. 35°47.261 N, Long. 04° 32.089 W), during the Gasalb-Pelagia oceanographic cruise (R/V Pelagia) in the Alboran basin, the westernmost Mediterranean (Fig. 1). The selected core is located at 1305 m water depth. The sediments at this site consist of homogeneous green-brownish hemipelagic nannofossil-rich clays. The analyzed sediment core spans the last two millennia (yr. cal. B.P.). Two different depth intervals: 2–3 cm (S1)

Fig. 1. Localization map showing the sampled site at the westernmost Mediterranean Sea.

and 31–32 cm (S2) were sampled and stored at −20 °C for further laboratory testing; five samples were collected at each interval for representative analyses.

Samples were also prepared for analyses of sediment composition. The mayor element composition was measured in triplicate by X-ray Florescence spectrometry (XRF) using a –4 kW wavelength dispersive spectrometer (Bruker S4 PIONEER). Determination of the trace element composition was performed using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) with a Perkin Elmer Scienx-Elan 5000 spectrometer. Samples were acidified in triplicates to pH 2 with ultrapure HNO_3 + HF for cation/trace elements using Re and Rh as internal standards. The respective instrumental errors reported were ±2% and ±5% for elemental concentrations of 50 ppm and 5 ppm.

2.2. Metagenomic analyses of the marine sediments: culture-independent studies

2.2.1. DNA extraction from marine sediments

DNA was extracted directly from marine sediments (S1 and S2). Three replicates were performed for each sediment interval. First, 0.5 g of marine sediment was placed in a sterile screw-cap tube containing 0.3 g of glass beads (size: 0.1–0.3 mm) and one glass bead 3–5 mm in diameter. One milliliter of lysis buffer (100 mM Tris-HCl [pH 8.0]; 100 mM EDTA [pH 8.0]; 100 mM NaCl; 1% polyvinylpyrrolidone [PVP]; and 2% SDS), 24 μL freshly made lysozyme (10 mg/mL), and 2 μL proteinase K (20 mg/mL) were added to each tube. The tubes were vigorously shaken for 2–3 min in vortex, after which mechanical lysis of the cells was performed twice using a Fastprep (FastPrep™ FP120, Carlsbad, CA) at a speed of 5.5 m s^{-1} for 45 s with intermittent cooling on ice. The tubes were incubated at 37 °C for 30 min, and then at 60 °C for 30 min. Next, the tubes were centrifuged for 5 min at 14,000 ×*g* at room temperature and the supernatant was collected in new 15-mL Falcon tubes. The pellets were mixed with another 1 mL of lysis buffer and the FastPrep step was repeated twice, followed by centrifugation under the same conditions as before. The supernatants were transferred to the first 15-mL tubes mixed with the supernatants obtained from the first centrifugation. The supernatants were mixed with one volume phenol: chloroform:isoamyl alcohol (PCI-25:24:1, pH 8), by gently inverting. The aqueous and the organic phases were separated by centrifugation at 4 °C for 10 min at 1500 ×*g* and the upper (aqueous) phase was washed with one volume of phenol:chloroform (PC-1:1) and centrifuged under the same conditions. The DNA was then precipitated by adding one volume of isopropanol and 1/10 volume of 3 M sodium acetate (pH 5), incubated for 1 h at −80 °C and centrifuged at 4 °C for 30 min at 5000 ×*g*. Subsequently, the DNA was dissolved in 35 μL Tris (5 nM, pH 8.5)-TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA), previously heated to 65 °C. Extracted DNAs were quantified on Qubit 3.0 Fluorometer (Life Technology) and then stored at −20 °C until all extractions were completed and ready for transport to LGC Genomics (Berlin, Germany).

2.2.2. Amplification and sequencing

Extracted DNA from each sample was amplified using the bacterial 16S rRNA gene primers 341F (5′-TCC TAC GGG NGG CWG CAG-3′) and 785R (5′-GAC TAC HVG GGT ATC TAA KCC-3′) (Klindworth et al., 2013). These primers are suitable for detecting a wide range of bacterial taxa, amplifying the hypervariable V3-V4 region. The PCR amplification and Illumina libraries were constructed and sequenced at LGC Genomics (http://www.lgcgroup.com/) as described in Carlson et al. (2018). Purified amplicon, barcode and primer complexes were sequenced on Illumina MiSeq, producing 2 × 300-bp paired-end reads.

2.2.3. Bioinformatics and diversity analyses

The paired-end reads were first quality controlled, combined, and then clustered into OTUs (at 97% identity) through the Quantitative Insights into Microbial Ecology (QIIME v1.8) pipeline (Caporaso et al., 2010) prior to alignment. Classification of sequences was performed using the 16S Mothur-Silva SEED r119 database. Clustered and

annotated OTUs were finally analyzed in Explicet 2.10.5 (Robertson et al., 2013), generating pie charts and stacked bars showing the relative abundance of OTUs, and mean values by sediment sample/replicate.

Species richness was measured by means of alpha-diversity metrics (Chao1, Shannon diversity index, and observed species), indicating whether a change in the number of OTUs between the different marine sediment samples occurred. Beta-diversity — the similarity between the identities of taxa and their abundances by sediment sample — was assessed using Bray-Curtis distances. In the different marine sediments, the bacterial community structure was analyzed through the use of weighted UniFrac distance measured in QIIME, the output being visualized with principal coordinate analysis (PCoA). Data are available in the NCBI database under the accession number PRJNA556449.

2.2.4. Statistical analysis

Data were compiled and transformed in Microsoft Excel. Significant differences in phyla relative abundance ($p < 0.05$) between the studied sediment intervals were determined by one-way ANOVA testing after normalization of all counts using SPSS software. In addition, similarity percentages (SIMPER) were carried out to investigate significant genera in the marine sediments, with PAST3 software. In all cases, values of $p \leq 0.05$ were considered statistically significant.

2.2.5. Correlation and network analyses

In order to further study possible correlations between the bacterial diversity and sediment physico-chemical parameters, Canonical Correspondence Analysis (CCA) was performed using PAST3 software and a cut-off of >0.5% of the total bacterial population. In addition, network analyses based on Pearson correlations and a threshold of >0.5% of the bacterial community were conducted in R environment using MASS and RESHAPE2 libraries within the Vegan package. Network visualization and modularization were performed on the interactive platform of CYTOSCAPE (Shannon et al., 2003).

2.3. Culturable marine bacterial community: culture-dependent studies

2.3.1. Culture and isolation of marine bacteria

Sediment cultures were used to obtain the different marine isolates. Each sediment sample collected at the two depth intervals (S1 and S2) was resuspended in 0.5 mL of NaCl (3.5%) solution and homogenized briefly by vortex. One milliliter of the mixture was transferred to 100 mL of marine broth (MB) medium (DIFCO Laboratories, USA) and incubated for 48 h and for 7 days at 28 °C under shaking at 180 r.p.m. After each incubation time, 20 mL of the cultures were separated and prepared for their microscopic analysis.

In order to obtain bacterial isolates, the samples (after 7 days of incubation of the sediment) were serially diluted up to 10^{-6} in sterilized NaCl (3.5%) solution and 100 μL aliquots of the diluted sample suspensions were spread on solid MB culture medium (per triplicate) followed by incubation at 28 °C for 3–7 days. After incubation, colonies from the agar plates were picked on the basis of their morphology and subcultured in fresh agar medium until pure isolates were obtained.

2.3.2. Molecular identification of the marine bacterial isolates

All obtained isolates were identified based on sequence similarity and phylogenetic analysis of 16S rRNA gene sequences. Bacterial genomic DNA was extracted using the methodology described in Jroundi et al. (2010). The 16S rRNA gene was PCR-amplified with two universal primers, fD1; 5′-AGAGTTTGATCCTGGCTCAG-3′ and rD1; 5′-AAGGAG GTGATCCAGCC-3′. PCR was carried out using the method described in Jroundi et al. (2010). PCR products were purified using the MBL-PCR QuickClean purification kit (MBL, Molecular Bio Laboratory, Spain) and sequenced with the same primers used for amplification. Each sequence of the 16S rRNA gene was compared with those of the NCBI database to find closely related species and to choose reference sequences for the phylogenetic analyses. A phylogenetic tree was reconstructed by means of the neighbor-joining method (Saitou and Nei, 1987) based on the distance matrix generated according to Kimura's two-parameter model (Kimura, 1980) using MEGA6 software (Tamura et al., 2013). Bootstrap analysis using 1000 sequence replications was used to evaluate the confidence level of the tree topology. The sequences were deposited at NCBI GenBank under the accession numbers from MN238728 to MN238753.

2.4. Heavy metal interactions with the isolated marine bacterial community

To study the interactions of metals with the marine bacterial isolates, Pb, Cu, Cr, and Zn were selected. A stock solution of each metal was prepared at 0.1 M by dissolving the appropriate quantity of the metal salt in $NaClO_4$. The stock solutions were sterilized by filtration through 0.22 μm nitrocellulose filters and stored at 4 °C until used. Working solutions were prepared by diluting the stock solutions to two different concentrations of 0.1 and 0.5 mM in sulfate free synthetic seawater (SSW) (Martinez-Ruiz et al., 2018), amended with 0.1 g/L of peptone (SCHARLAU, Sharlab S.L., Spain). Culturable marine community isolates destined to interaction with the heavy metals from both sediment intervals (S1 and S2) were grown and maintained in liquid MB medium. Cells were grown to the late exponential phase (for 48 h) and aliquots of 20 mL of the bacterial cultures were centrifuged at 10,000 r.p.m. for 10 min at 4 °C. The collected cells were rinsed to remove residues from the medium three times with NaCl solution (3.5% w/v) and resuspended in sterile Erlenmeyer flasks with 20 mL of SSW amended with the corresponding metal at each concentration. Three replicates of each metal and concentration were prepared. The flasks were incubated for 48 h at 28 °C under shaking at 180 r.p.m. After the incubation time, 20 mL of sediment cultures used as controls (obtained as described above) and of the bacterial cells in contact with the different heavy metals were separated and prepared for their observation.

2.5. Electron microscopy analyses

Scanning electron microscopy of variable pressure (VPSEM) and high-resolution transmission electron microscopy (HRTEM) were used for analyses of the bacterial cells after each treatment. The bacterial cultures from each experiment (at 48 h and at 7 days) were centrifuged (at 10,000 ×*g*, for 10 min) and the pellets were washed 3 times with NaCl (3.5%) solution to remove the interfering ingredients of the medium, and to then be resuspended in either 1 mL of MB culture medium or in saline solution. Afterwards, 0.5 mL of each bacterial suspension was fixed in 0.5 mL of 2.5% glutaraldehyde solution (prepared in 0.2 M cacodylate buffer with 0.4 M sucrose and osmolarity of 1220 mOsM) for 4 h at 4 °C. The fixed cells were harvested and supported on a crystal/glass previously embedded in Poli-L-Lysine, then placed in a damp chamber for 24 h. The samples were then dehydrated in gradients of ethanol (at 50%–70%–90%–100% 3×; 10 min each) and exposed to critical point drying (LEICA EM CPD 300 Critical Point Dryer). The samples were coated with carbon before their observation under VPSEM (Zeiss SUPRA40VP model, coupled with energy-dispersive X-ray microanalysis [EDX]). For observation with HRTEM, thin sections of cells obtained from the different heavy metal treatments were prepared as described in Merroun et al. (2005) and were deposited on carbon-film-coated copper grids for all treatments except for copper-treated cells, which were deposited on gold grids to avoid interferences. Samples were examined using a high-angle annular dark-field scanning transmission electron microscope — (HAADF/STEM) FEI TITAN G2 60–300 microscope with a high brightness electron gun (X-FEG) — operated at 300 kV and equipped with a Cs image corrector (CEOS). The high resolution STEM is equipped with a HAADF detector and EDAX energy dispersive X-ray. Digital X-ray maps were collected on selected areas of the samples and mapped for all heavy metals. Selected area electron diffraction (SAED) patterns were also acquired for the identification of possible crystalline phases.

3. Results

3.1. Sediment composition

Data regarding the chemical composition of the different sediment samples involved in this study are given in Table SI-1 (Supplementary material). Contents of major elements Al, Ca, Fe, K and Mg from both studied sediment intervals are also included. The distribution of these elements (expressed as % oxides) was similar in the two intervals, though there were slight changes with depth. The concentrations of Na_2O, K_2O, MnO, and Fe_2O_3 were somewhat higher in interval S1 than in S2 sediment. In the latter, the concentration of CaO was higher, while distributions of MgO, Al_2O_3, SiO_2, P_2O_5, and TiO_2 were similar for both intervals. In terms of trace element composition, the concentration of elements such as Co, Cu, Zn, Zr, Pb, V, Be, Ba, Mo, La, among some others, was higher in interval S1 than in S2, whereas U, Ni, Sr, Li, Rb, and Cs were higher in the S2 sediment interval. Other trace elements were similarly distributed in the two intervals.

3.2. Metagenomic analyses of the marine bacterial communities

3.2.1. Richness and diversity of the bacterial communities

Alpha-diversity analysis revealed no significant differences in the bacterial richness regardless of the metrics used (Table 1). Chao1 and ACE richness estimators, as well as Taxa richness and Individual indexes, indicated high and comparable bacterial richness in the communities of the two sediment intervals. This was confirmed by the Shannon diversity index and Simpson diversity index (Table 1), and also by Good's coverage index (data not shown), all of which provide not only the simple species richness (i.e., the number of species present) but also the distribution of abundance of each species (the evenness of the species) among all the species in the community. The two sediment samples displayed high richness and had similar alpha-diversities. Yet beta-diversity revealed significant differences in the bacterial community structure and abundance of the two sediments. Rarefaction curves showed that all samples reached a plateau, meaning that the sequencing was deep enough to detect the most phylotypes (Supplementary_material S1 and Supplementary Fig. S1). Results of weighted UNIFRAC PCoA and of the Bray-Curtis index (Heatmap) showed significant clustering of the replicates by sediment sample (Fig. 2). Accordingly, samples S1 and S2 appear to have different bacterial community structures; this is held to mark a substantial difference in the distribution of the bacterial taxa within the communities, although similar genera were observed in the two sediments with different percentages of relative abundance.

3.2.2. Taxonomic complexity of the total marine bacterial communities

A total of 241,406 bacterial 16S rRNA gene sequences were recovered for both sediment samples in triplicate. The sequences consisted of 40,728 for S1R1, 39,057 for S1R2, 38,098 for S1R3, 40,257 for S2R1, 40,512 for S2R2, and 42,574 for S2R3, and all were used for community analyses by QIIME. OTUs were assigned by clustering sequences with over 97% sequence identity. A total of 2162 OTUs were distributed at phylum level into 42 different phyla, as shown in Fig. S2 (Supplementary material S2). At genus level, the detected OTUs comprised 516 different genera in the bacterial communities of the sediment samples (Fig. 3). The upper sediment sample (S1) was dominated by the genus *Bacillus*, accounting for 53.51% of the total community, followed by unclassified *Bacillales* (10.6%), *Micrococcus* (3.83%), *Paenisporosarcina* (3.34%), unclassified *Bacillaceae* (2.50%), *Planococcaceae* Incertae_Sedis (2.01%), unclassified *Anaerolineaceae* (1.90%), *Microlunatus* (1.58%), *Planctomycetaceae* Pir4_lineage (1.14%), and *Microbacterium* (1.03%), among others having a relative abundance of <1%. A few differences in the bacterial community composition and structure were observed in the lower sediment sample (S2), where more genera with an occurrence of >1% were represented. Here, the most abundant genus was also *Bacillus* (38.90%), followed instead by *Microlunatus* (7.13%), unclassified *Bacillales* (5.00%), *Micrococcus* (4.24%), unclassified *Bacillaceae* (3.90%), *Planococcaceae* Incertae_Sedis (3.74%), unclassified *Anaerolineaceae* (2.37%), *Planctomycetaceae* Pir4_lineage (1.85%), *Propionibacterium* (1.77%), *Paenisporosarcina* (1.30%), *Fictibacillus* (1.29%), *Thalassobacillus* (1.18%), *Lachnospiraceae* Incertae_Sedis (1.05%), and *Bacteroides* (1.00%), among others of low abundance (<1%).

3.2.3. Statistical analyses

Similarity of percentages analysis (SIMPER) was used to determine the relative contribution of each individual genus to the dissimilarity between the two sediment clusters. The average Bray-Curtis dissimilarity and the contribution of each genus to the total dissimilarity between communities in both sediment intervals were calculated; the top major genera responsible for the microbial community difference (>98% contribution to cumulative dissimilarity) is summarized in Table 2. Among them, *Bacillus* had the largest dissimilarity contribution (21.11%), followed by unclassified *Bacillales* (8.05%), *Microlunatus* (7.928%), *Micrococcus* (7.345%), *Planococcaceae* Incertae_Sedis (4.647%), unclassified *Bacillaceae* (4.223%), *Anaerolineaceae* (3.021%), *Paenisporosarcina* (2.932%), *Propionibacterium* (1.757%), and *Microbacterium* (1.6%), among others. According to the One-Way ANOVA ($p \leq 0.05$) test, the presence of some significant differences in community composition at the genus level between the two sediments ($p \leq 0.05$) was confirmed. Particularly, significant differences in the relative abundance ($p \leq 0.05$) were obtained for genera such as *Bacillus*, *Microlunatus*, *Jeotgalibacillus*, *Cloacibacillus* and *Bifidobacterium*, where the *p*-values were 0.039, 0.008, 0.034, 0.024, and 0.000, respectively (Supplementary Table 1).

3.2.4. Correlations and network analyses

To further analyze and visualize the correlation between the target major genera (>0.5% of relative abundance) representing the bacterial communities and the physical and chemical properties of the sediments (S1 and S2), a canonical correspondence analysis (CCA) was performed (Fig. 4A). Both the first and second axes were positively correlated with high concentrations of Co, Cu, Pb, Mo, Zn, and Sn, and negatively correlated with U, Sr, Be, Zr, and Ni, among other environmental parameters. Regarding the bacterial community, *Bacillus*, *Micrococcus*, *Paenisporosarcina*, *Microbacterium*, *Megasphaera* and *Enterobacteriaceae*

Table 1
Inference statistics at genus level of the sediment samples. The species richness (Taxa_richness, CHAO1, ACE), evenness (Pielou's evenness, Simpson_1-D), and diversity (Shannon_H, Fisher's alpha).

Samples	Individuals	Taxa_richness	CHAO1	ACE	Pielou's evenness	Simpson_1-D	Shannon_H	Fisher's alpha
S1R1	46,930	320	326	330.8	0.45	0.76	2.64	46.3
S1R2	48,430	214	217.6	217.3	0.43	0.69	2.31	28.8
S1R3	44,725	236	247.1	245.7	0.33	0.62	1.82	32.7
S2R1	49,351	287	291.4	293.9	0.59	0.87	3.36	40.4
S2R2	44,540	248	266.9	266.8	0.55	0.84	3.03	34.6
S2R3	48,373	315	321.8	325.2	0.49	0.78	2.81	45.2

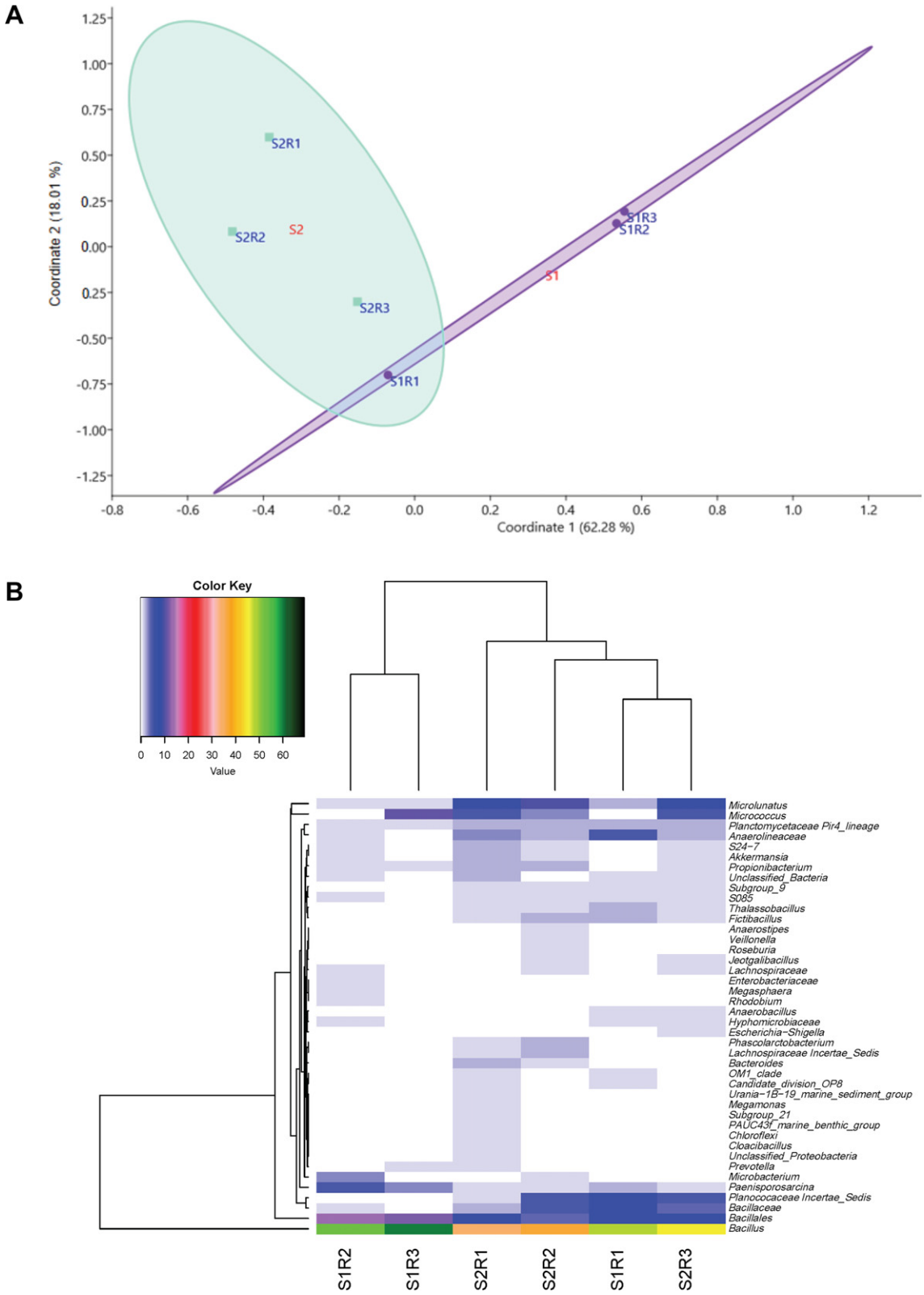

Fig. 2. PCoA plot showing the relationship between the bacterial community structures of the marine sediments S1 and S2 based on Bray Curtis index (A); and Heatmap based on the relative abundance of the genera with an average abundance of >0.5% in at least one sample (B).

displayed significant positive correlation with Co, Cu, Pb, Mo, Zn, and Sn, while *Microlunatus*, *Megamonas*, *Propionibacterium*, *Bacteroides*, *Akkermansia*, *Veillonella*, and *Urania_1B_19_Marine_sediment_group*, among some others, were positively correlated with U, Sr, Be, Zr, and Ni. Similarly, *Jeotgalibacillus*, *Anaerobacillus*, *Fictibacillus*, *Thalassobacillus*, Pir4_lineage, and *Hyphomicrobiaceae* were positively correlated with Cr, La, Ta, V, Li, Ce, among other trace elements of the marine sediments.

In addition, and based on strong correlations ($\rho > 0.8$ or $\rho < -0.8$), network analysis was performed to analyze the co-occurrence and co-

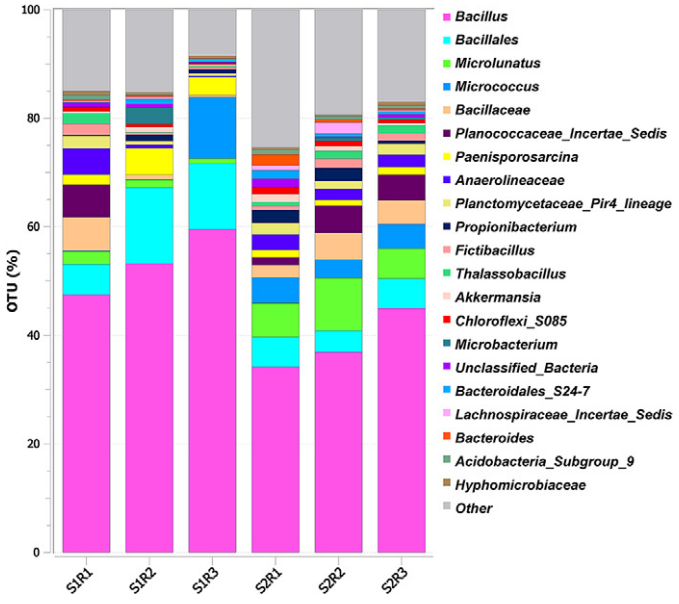

Fig. 3. Taxonomic distribution of marine sediment bacterial communities at genus level. S1R1, S1R2, and S1R3: replicates of the upper sediment interval (S1); S2R1, S2R2, and S2R3: replicates of the lower sediment interval (S2).

exclusion patterns among the bacterial communities and the physico-chemical parameters of both sediments. As shown in Fig. 4B, a total of 68 nodes and 442 edges were represented. Topological characterization commonly used in network analysis was calculated to describe the complex network of interrelationship among bacterial taxa and environmental parameters (Supplementary material S3). These correlations revealed putative associations among the major genera and particular environmental parameters, supporting the results obtained by the CCA analysis. For example, the positive correlations between Cu, Co, Zn, Pb, Mo, and *Bacillus*, *Bacillales*, *Paenisporosarcina*, *Microbacterium*, *Megasphaera* and *Enterobacteriaceae* were also observed by CCA analysis, likely indicating that changes in oxygenation (in turn related with the abundance of these metals) may promote the proliferation of these genera. Based on the modularity classification and compared to a random association, the network could be divided into 8 major modules, which are clusters of nodes interacting more among themselves than with the others. The three largest modules, I, VI, and VII, were respectively occupied by 12, 13, and 22 of the 68 total vertices. The co-occurring bacterial taxa and physiochemical parameters of each module are summarized in Table SI-2.

3.3. Characterization and phylogenetic analysis of sediment culturable-bacteria

The mean numbers of culturable bacteria in the marine sediment samples S1 and S2 were 2.56×10^9 Colony Forming Unit (CFU)/g and 2.6×10^9 CFU/g, respectively. A total of 26 culturable bacteria, exhibiting distinct colony characteristics in terms of size, pigmentation, opacity, texture, form, elevation and margin surface, were selected from both sediments for further molecular identification. Approximately 1500 bp of 16S rDNA region of the bacterial strains were amplified and sequenced for phylogenetic analysis. Based on their molecular characterization, similar results were obtained in intervals S1 and S2 (Table 3). From the S1 interval, 20 strains were affiliated to two major phyla

Table 2
SIMPER analysis of bacterial community dissimilarity (>98% of contribution to cumulative dissimilarity) of the two sediment (S1 and S2) intervals

Taxon	Avg. dissimilarity (%)	Contribution to dissimilarity (%)	Cumulative dissimilarity (%)	Mean abundance (%)	
				S1	S2
Bacillus	7.344	21.11	21.11	53.4	38.7
Bacillales	2.8	8.048	29.16	10.6	4.98
Microlunatus	2.758	7.928	37.09	1.58	7.1
Micrococcus	2.555	7.345	44.43	3.82	4.22
***Planococcaceae* Incertae_Sedis**	1.617	4.647	49.08	2	3.72
Bacillaceae	1.469	4.223	53.3	2.49	3.89
Anaerolineaceae	1.051	3.021	56.32	1.9	2.37
Paenisporosarcina	1.02	2.932	59.25	3.33	1.29
Propionibacterium	0.6111	1.757	61.01	0.731	1.76
Microbacterium	0.5635	1.62	62.63	1.03	0.342
Planctomycetaceae Pir4_lineage	0.5126	1.474	64.1	1.15	1.89
Thalassobacillus	0.4667	1.342	65.44	0.74	1.17
Fictibacillus	0.4412	1.268	66.71	0.88	1.28
***Lachnospiraceae* Incertae_Sedis**	0.4321	1.242	67.96	0.232	1.04
Bacteroides	0.3926	1.129	69.08	0.217	1
Phascolarctobacterium	0.3105	0.8926	69.98	0.229	0.699
Jeotgalibacillus	0.2741	0.7878	70.76	0.133	0.681
Unclassified_Bacteria	0.2732	0.7854	71.55	0.551	0.818
S24-7	0.2717	0.7809	72.33	0.455	0.835
Akkermansia	0.2551	0.7331	73.06	0.558	0.968
Subgroup_9	0.209	0.6007	73.66	0.361	0.639
Megamonas	0.2011	0.5779	74.24	0.00809	0.405
S085	0.2008	0.5773	74.82	0.545	0.915
Cloacibacillus	0.1898	0.5456	75.37	0.0139	0.394
Anaerobacillus	0.1791	0.5147	75.88	0.204	0.433
Megasphaera	0.1676	0.4818	76.36	0.305	0.211
Anaerostipes	0.155	0.4455	76.81	0.00973	0.32
Roseburia	0.148	0.4254	77.23	0.0339	0.308
Enterobacteriaceae	0.145	0.4167	77.65	0.302	0.192
Bifidobacterium	0.145	0.4167	78.07	0.0281	0.318
Urania-1B-19_marine_sediment_group	0.1398	0.4018	78.47	0.0812	0.343
OM1_clade	0.1396	0.4012	78.87	0.217	0.395
Prevotella	0.134	0.3851	79.25	0.306	0.435
Veillonella	0.1314	0.3776	79.63	0.0524	0.28
Escherichia-Shigella	0.1314	0.3776	80.01	0.125	0.387
Lachnospiraceae	0.1313	0.3773	80.39	0.274	0.498

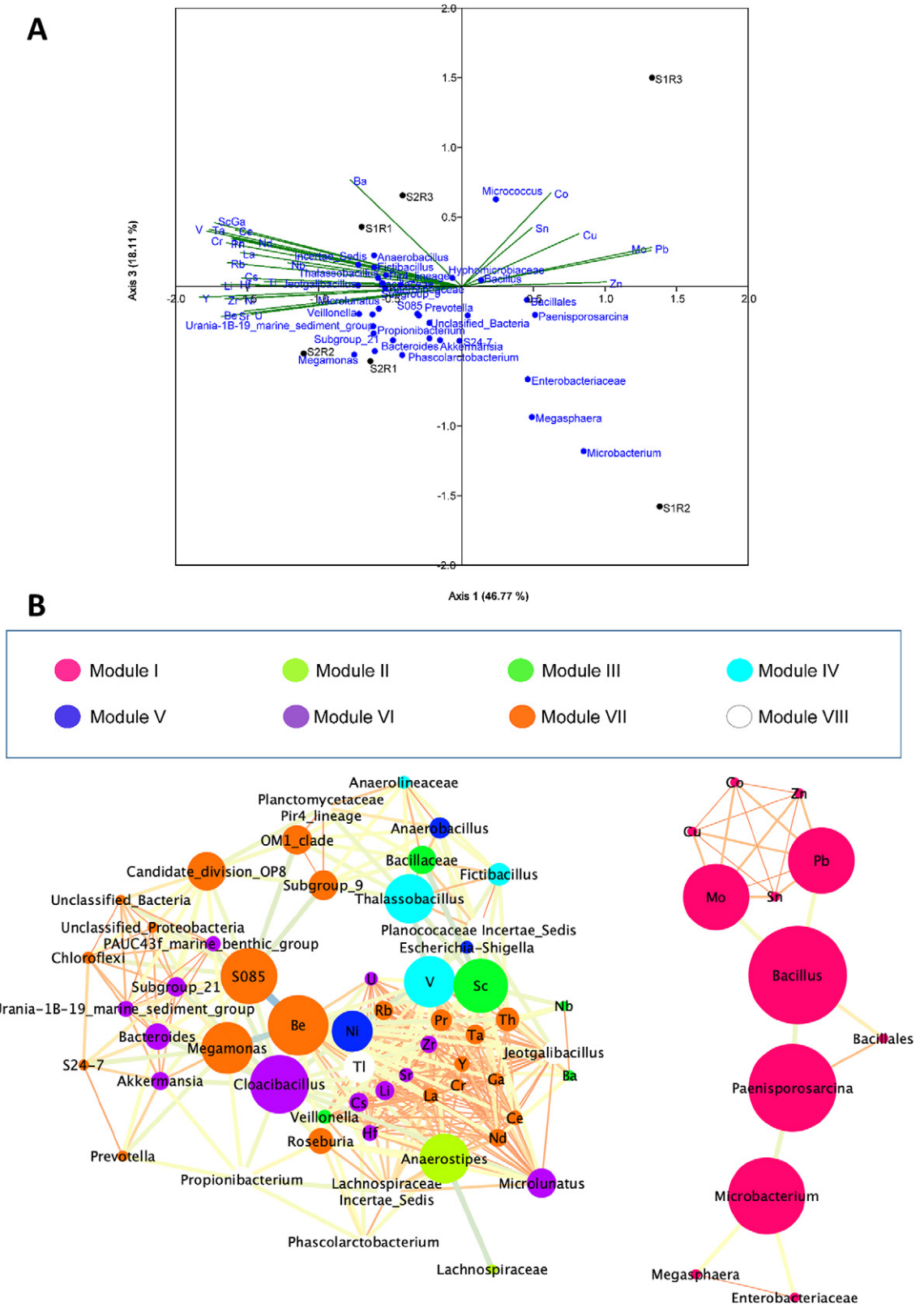

Fig. 4. Correlation analyses showing complex relationships between the bacterial community and the physico-chemical parameters of both sediment samples. A) Canonical correspondence analysis (CCA) plot revealing the relationships between the target bacterial genera and the geochemical parameters. B) Network analysis revealing the co-occurrence patterns among bacterial taxa and environmental parameters. The nodes are colored according to modularity class. A connection represents a strong positive correlation based on Pearson's correlation coefficient (ρ of >0.8). The size of each node is proportional to the number of connections, i.e., the degree.

(Firmicutes and Actinobacteria), whereas 8 strains isolated from the S2 interval were also affiliated to the two phyla Firmicutes and Actinobacteria. In both sediments, Firmicutes was the most dominant phylum: represented in S1 by 95% and in S2 by 87.5% of the total cultured bacterial community. Actinobacteria was represented by 5% in S1 and 12.5% in S2 of the total cultured population. The phylogenetic

Table 3
Molecular characterization and phylogenetic classification of the bacterial communities isolated from the marine sediments.

Isolate	Closest relative on basis of 16S rRNA gene	Phylum	Family	Similarity percentage
Sediment S1				
MS1-7	*Bacillus* sp. PK2	Firmicutes	*Bacillaceae*	100%
MS1-17	*Bacillus aquimaris* strain NIOT-Ba-31	Firmicutes	*Bacillaceae*	99.5%
MS1-4	*Bacillus aquimaris* strain K-W2	Firmicutes	*Bacillaceae*	100%
MS1-1	*Bacillus aquimaris* strain K-W2	Firmicutes	*Bacillaceae*	99.9%
MS1-11	*Bacillus oceanisediminis* strains 3–4	Firmicutes	*Bacillaceae*	99.6%
MS1-15	*Bacillus oceanisediminis* strain KSW29	Firmicutes	*Bacillaceae*	99.9%
MS1-13	*Bacillus oceanisediminis* isolate KSW 29	Firmicutes	*Bacillaceae*	99.8%
MS1-12	*Bacillus oceanisediminis* strain SR115-B5	Firmicutes	*Bacillaceae*	99.7%
MS1-2	*Bacillus* sp. 20140105	Firmicutes	*Bacillaceae*	99.9%
MS1-3	*Bacillus firmus*, isolate CV93b	Firmicutes	*Bacillaceae*	99.6%
MS1-6	*Bacillus firmus* strain D8	Firmicutes	*Bacillaceae*	100%
MS1-8	*Bacillus firmus* strain D8	Firmicutes	*Bacillaceae*	100%
MS1-9	*Bacillus firmus* strain niuC	Firmicutes	*Bacillaceae*	99.3%
MS1-10	*Bacillus firmus* strain XJSL1-4	Firmicutes	*Bacillaceae*	99.9%
MS1-18	*Bacillus firmus* strain KJ-W9	Firmicutes	*Bacillaceae*	99.7%
MS1-19	*Bacillus* sp. BAB-5146	Firmicutes	*Bacillaceae*	98.4%
MS1-16	*Bacillus oceanisediminis* strains 3–4	Firmicutes	*Bacillaceae*	99.9%
MS1-14	*Micrococcus luteus* strain OH4847	Actinobacteria	*Micrococcaceae*	100%
Sediment S2				
MS2-1	*Bacillus baekryungensis* strain CW126-A01	Firmicutes	*Bacillaceae*	99.5%
MS2-2	*Bacillus aquimaris* strain K-W2	Firmicutes	*Bacillaceae*	99.1%
MS2-3	*Bacillus aquimaris* strain NIOT-Ba-31	Firmicutes	*Bacillaceae*	99.5%
MS2-6	*Bacillus aquimaris* strain CCMM B685	Firmicutes	*Bacillaceae*	99.6%
MS2-8	*Bacillus aquimaris* strain NIOT-Ba-31	Firmicutes	*Bacillaceae*	99.5%
MS2-5	*Bacillus hwajinpoensis* strain CCMM B652	Firmicutes	*Bacillaceae*	99.3%
MS2-7	*Bacillus humi* strain LMG 22168	Firmicutes	*Bacillaceae*	97%
MS2-4	*Kocuria palustris* strain R-39201	Actinobacteria	*Micrococcaceae*	99.7%

placement of the isolates is shown in the Supplementary Fig. S3. Firmicute was found to be the overall predominant group in the bacterial community of both sediment intervals. Among this group, the low G + C content microorganism *Bacillus* was the most dominant genus, being related to four different species in the S1 sediment interval (*B. aquimaris*, *B. oceanisediminis*, *B. firmus*, and *Bacillus* sp.) and another four species in the S2 sediment interval (*B. baekryungensis*, *B. aquimaris*, *B. hwajinpoensis*, and *B. humi*). Surprisingly, four strains of *B. oceanisediminis* and eight strains of *B. firmus* isolated in the current study appeared to be close and clustered together in a clade separated from the rest of the *Bacillus* strains. Actinobacteria was the other group of bacteria found in both sediments S1 and S2. Among the bacterial community, the well-known high G + C bacterial strains *Micrococcus* (MS1-14) in sediment S1, and *Kocuria* (MS2-4) in sediment S2, each formed a distinctive clade from that of *Bacillus*.

3.4. Electron microscope analysis of marine bacterial cells

SEM micrographs of the cultured bacterial communities obtained from the sediments S1 and S2 are shown in Fig. 5. Different bacterial cell morphologies were observed among the communities, in accordance with the molecular identification studies. The cells were moreover seen to have a large amount of EPS — which serves to maintain the cells attached to each other and to the sediment particles — along with pilis and fimbriae at the cell surface, which could be used for the formation of biofilms as well as the concentration of metals (Fig. 5-B and 5-D). SEM–EDX spectra also reveal some elements from the clay and carbonate particles (Fig. 5-E and -F).

3.5. Heavy metals and marine bacterial community interactions

The enriched cultures of the bacterial communities isolated from the S1 and S2 sediments were tested for their ability to interact with different heavy metals such as Pb, Cu, Cr and Zn at two different concentrations (0.1 mM and 0.5 mM). These bacterial communities were chosen as representative of the aerobic heterotrophic community inhabiting these sediments that could be enriched in the presence of carbon and nitrogen sources. Bacterial communities from each sediment interval were grown in a liquid marine broth medium (MB), washed with NaCl solution, and suspended in SSW amended with the corresponding concentration (0.1 or 0.5 mM) of the metal (Pb, Cu, Cr, Zn).

To determine the ability of the bacterial communities isolated from each sediment interval to interact with the metals tested here, and to determine the cellular localization of the metal accumulated, a combination of VPSEM, STEM/HAADF and HRTEM analyses were performed. SEM observations demonstrated that Cr, Cu, Pb, and Zn had accumulated in the bacterial community of both sediment intervals (S1 and S2) after 48 h, regardless of the metal concentration (Supplementary material S4 and Figs. S4 and S5).

HRTEM analyses were further applied to determine the precise cellular localization of Cr, Cu, Pb, and Zn precipitates. In the case of Pb, Fig. 6 shows the presence of polyphosphate grains enriched in Pb within some cells in addition to electron-dense Pb precipitates on the cell walls and within the EPS in the extracellular space. EDX element-distribution maps and spectra (Fig. 7) show the elemental distribution of these precipitates, indicating they are mainly composed of Ca, P, and Pb. Furthermore, the Pb phosphate deposits accumulated at some cell surfaces were morphologically different from those precipitating inside cells of other bacterial strains, meaning the morphology of the Pb accumulates is bacterial-species specific. The Pb phosphate deposits range from amorphous to crystalline pyromorphite, as evidenced by SAED and diffraction pattern analyses. In particular, d-space measurements in diffraction rings confirmed the presence of pyromorphite (Fig. 6). Some Fe was observed in the precipitates located within the EPS in the extracellular space, which derived from the accumulation by the cells of this metal taken from the SSW. The Cu peak results from the copper grid used to support the specimen, and the U peak from the uranium used to contrast the cells. Zn precipitated mainly in the EPS in the extracellular space, and only sparingly on the walls of some cells (Figs. 8 and S6). The elemental composition of these precipitates also reflects the presence of Fe, Zn, and P. The sediment bacterial community accumulated Cu on cell walls and on the EPS in extracellular space (Fig. 9). Elemental composition of these accumulates furthermore showed the presence of P, some Fe and Ca; Pb and U derived from the cell contrasting. In view of

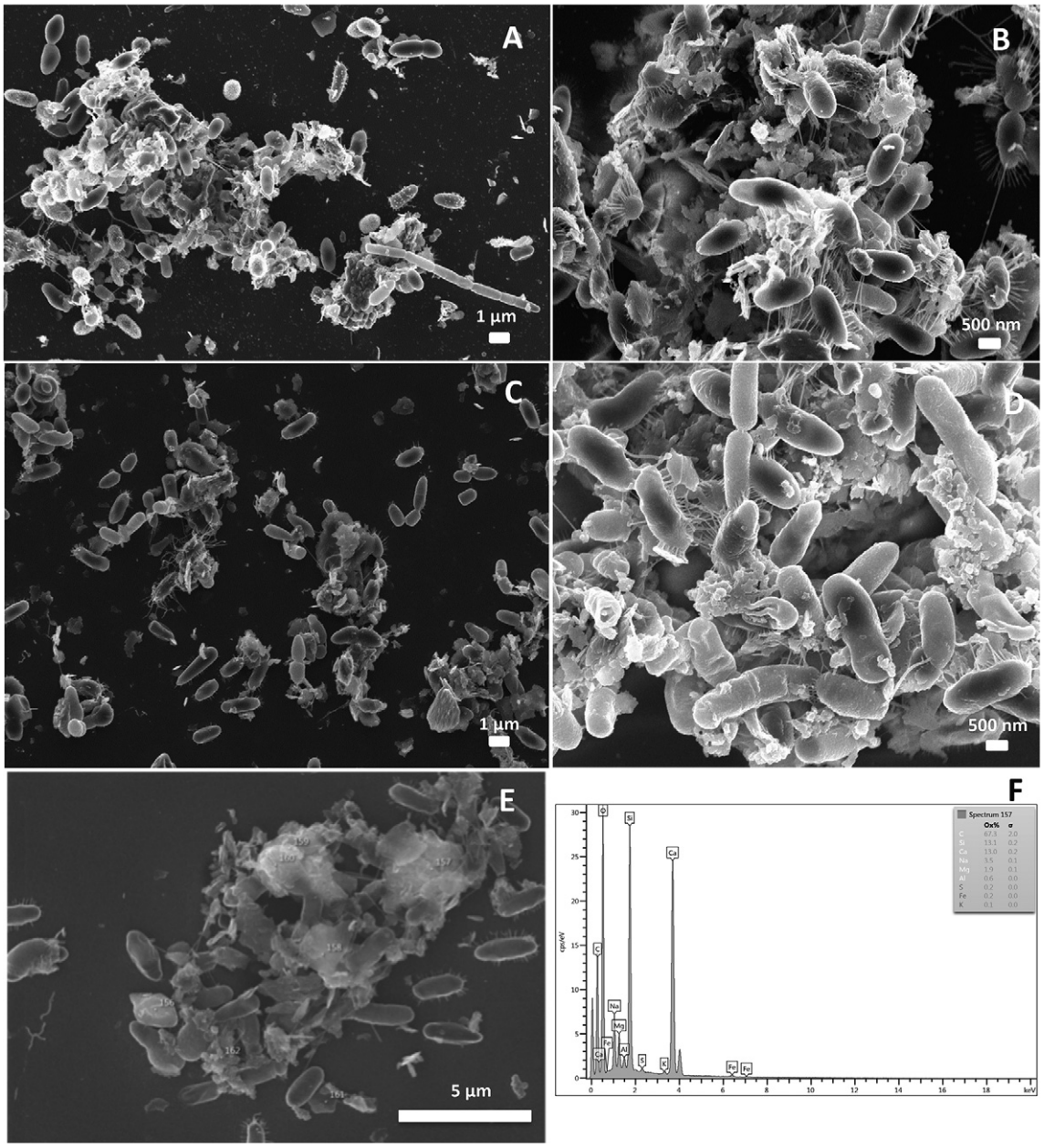

Fig. 5. SEM photographs of cells and EPS from the bacterial communities isolated from the marine sediment S1 (A and B) and sediment S2 (C and D). E) and F) SEM photograph of the bacterial community in S2 and the corresponding EDX spectrum.

these results, EDX maps were used to investigate the presence and concentration of carbon in the metal accumulates. Fig. 9-E depicts a high concentration of C where the metal (mainly Cu and Zn) precipitated. This could mean these accumulates were effectively bound to the EPS produced by the bacterial cells, and not merely a result of abiotic precipitation. Meanwhile, Cr accumulated mainly in the EPS in the extracellular space, along with P, Ca and Fe (Fig. 10).

4. Discussion

It is well known that benthic deep-sea environments are the largest ecosystems on Earth, and microbes inhabiting such environments therefore represent a significant proportion of the biosphere (e.g., Danovaro et al., 2017; Parkes et al., 2014). However, the abundance and structure of deep-sea ecosystems is still poorly known (Colman et al., 2017; Nemergut et al., 2011; Petro et al., 2017; Polymenakou et al., 2005; Singer et al., 2015). The ubiquity of life in sub-seafloor sediments has drawn the attention of more research in recent years (e.g., D'Hondt et al., 2004; Jørgensen, 2017; Kallmeyer et al., 2012; Meister, 2015; Parkes et al., 2005, 2014), as its comprehension is crucial for identifying ecologically compatible mechanisms influencing biogeochemical cycles in the deep-sea sediments. In addition to dispersal and deposition patterns (Zhang et al., 2017b), microbial abundance and diversity depend largely upon environmental conditions such as sediment nature and composition (D'Hondt et al., 2004; Gilbert et al., 2009; Parkes et al., 2014; Sogin et al., 2006). In turn, microorganisms may influence the geochemical conditions within sediments (Beck et al., 2011; Danovaro, 2018; Hoshino et al., 2011; Marshall et al., 2018; Vuillemin et al., 2018; Walsh et al., 2016). Remarkable, for instance, is the role that bacteria and their EPS could play in mineral precipitation and in concentrating diverse chemical elements. It is well known that microbes can mediate the precipitation of diverse minerals — some of the best studied examples are carbonates, in particular dolomite — and the

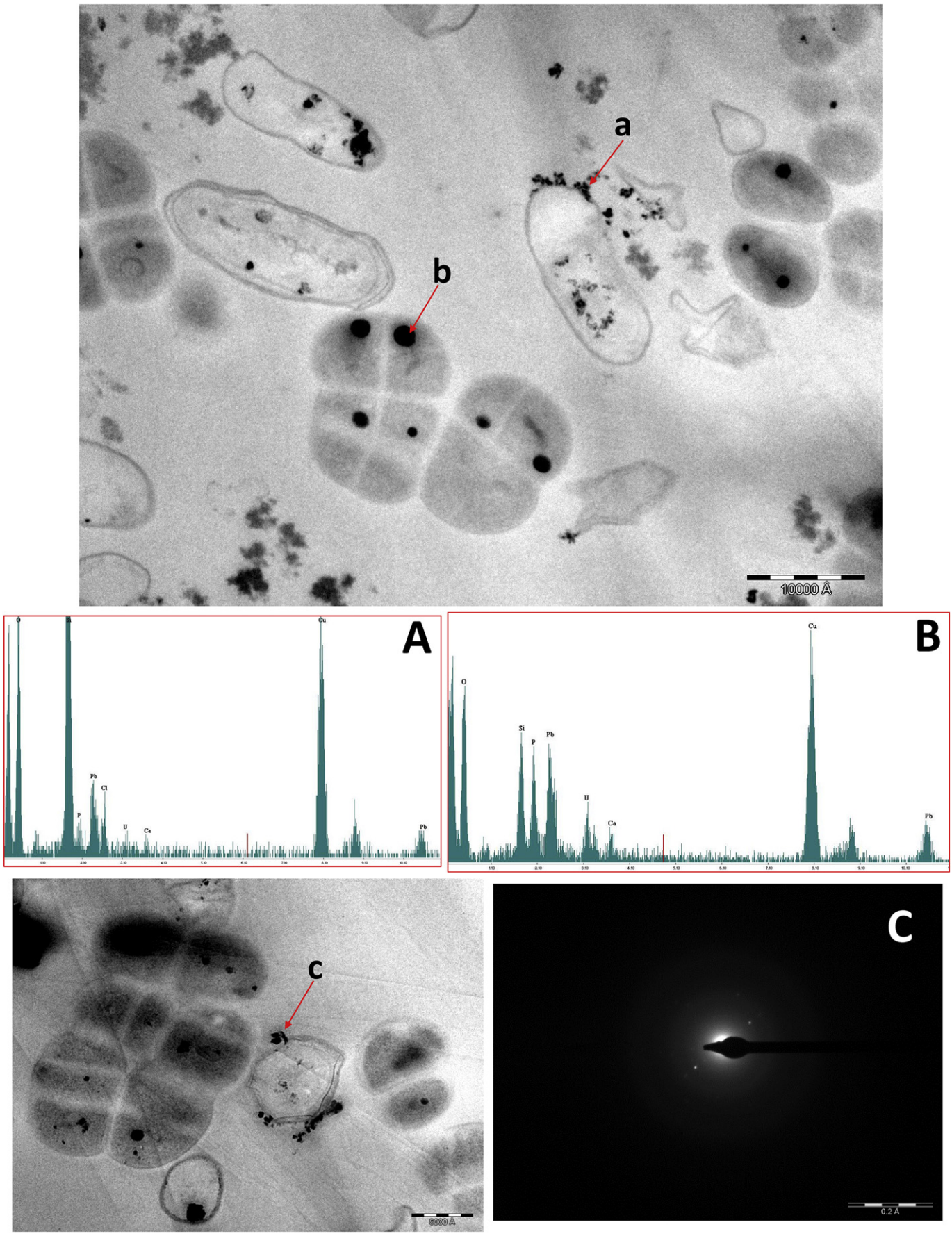

Fig. 6. HRTEM photograph of the bacterial cell-Pb interaction showing the extracellular and intracellular precipitates and their corresponding EDX spectra. A) and B) show EDX spectra of the precipitates a and b, respectively. C) SAED pattern of the Pb phosphate precipitate (c) confirming the presence of pyromorphite as the main mineral phase.

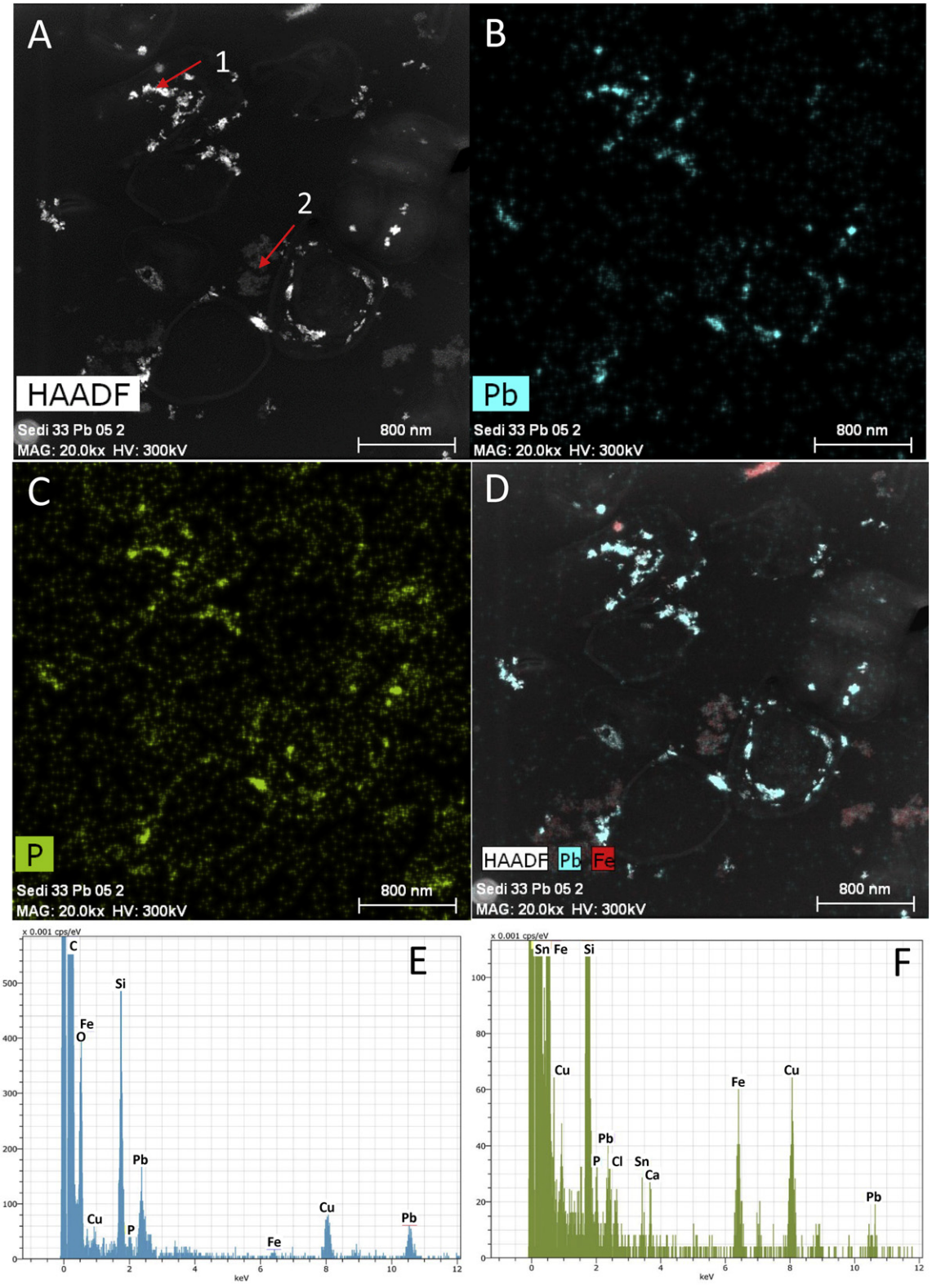

Fig. 7. HRTEM/EDX maps of Pb, P, Fe in the bacterial cells. A) High-angle annular dark field HAADF/STEM image and B), C), and D) corresponding EDX maps showing the distribution of Pb and P and a combined map of Pb and Fe, respectively in bacterial community of the marine sediment. E) and F) show EDX spectra of the precipitates 1 and 2 from image A.

precipitation of marine barite in the ocean water column has been related to bacterial activity (Gonzalez-Muñoz et al., 2012; Martinez-Ruiz et al., 2018, 2019). Nonetheless, the potential role of microbes inhabiting deep-sea sediments in mineral precipitation within sediments, e.g. during diagenesis, remains largely unexplored. Such processes can be particularly relevant for the sediment environment (Parkes et al., 2014).

The results obtained here demonstrate the capability of the analyzed bacterial strains to accumulate and concentrate Cr, Cu, Zn, and Pb. These elements bind to the bacterial cells as well as to the

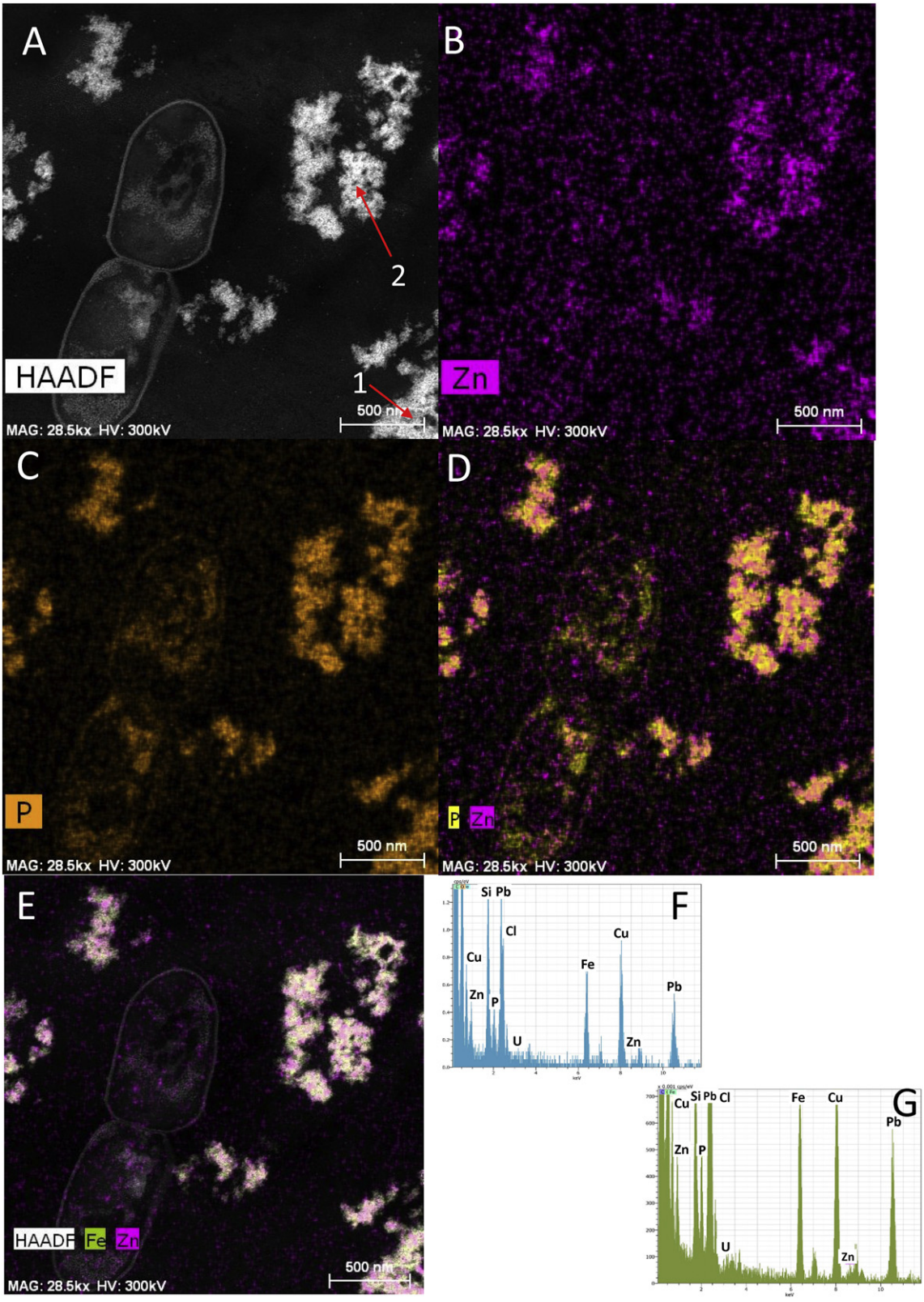

Fig. 8. HRTEM/EDX maps of Zn, P, Fe in the bacterial cells. A) High-angle annular dark field HAADF/STEM image; B), and C), corresponding EDX maps showing the distribution of Zn and P; D), and E) a combined map of Zn and P, and of Zn and Fe, respectively in bacterial community of the marine sediment. E) and F) show EDX spectra of the precipitates 1 and 2 from image A.

EPS in the extracellular space, and even accumulate in the cytoplasm in some cases. This finding has significant implications, since these are elements commonly used as redox proxies in sediments accumulated over geological time scales. It also sheds light on the factors controlling how they bind to sediment material. Because sediments are under increasing anthropogenic pressure, implying large amounts of pollutants, it is essential to determine the relationships surrounding bacterial diversity and pollutant metal bioaccumulation to devise bioremediation strategies (Fonti et al., 2015; Micheli et al., 2013). The quality of an ecosystem, such as that of marine sediments,

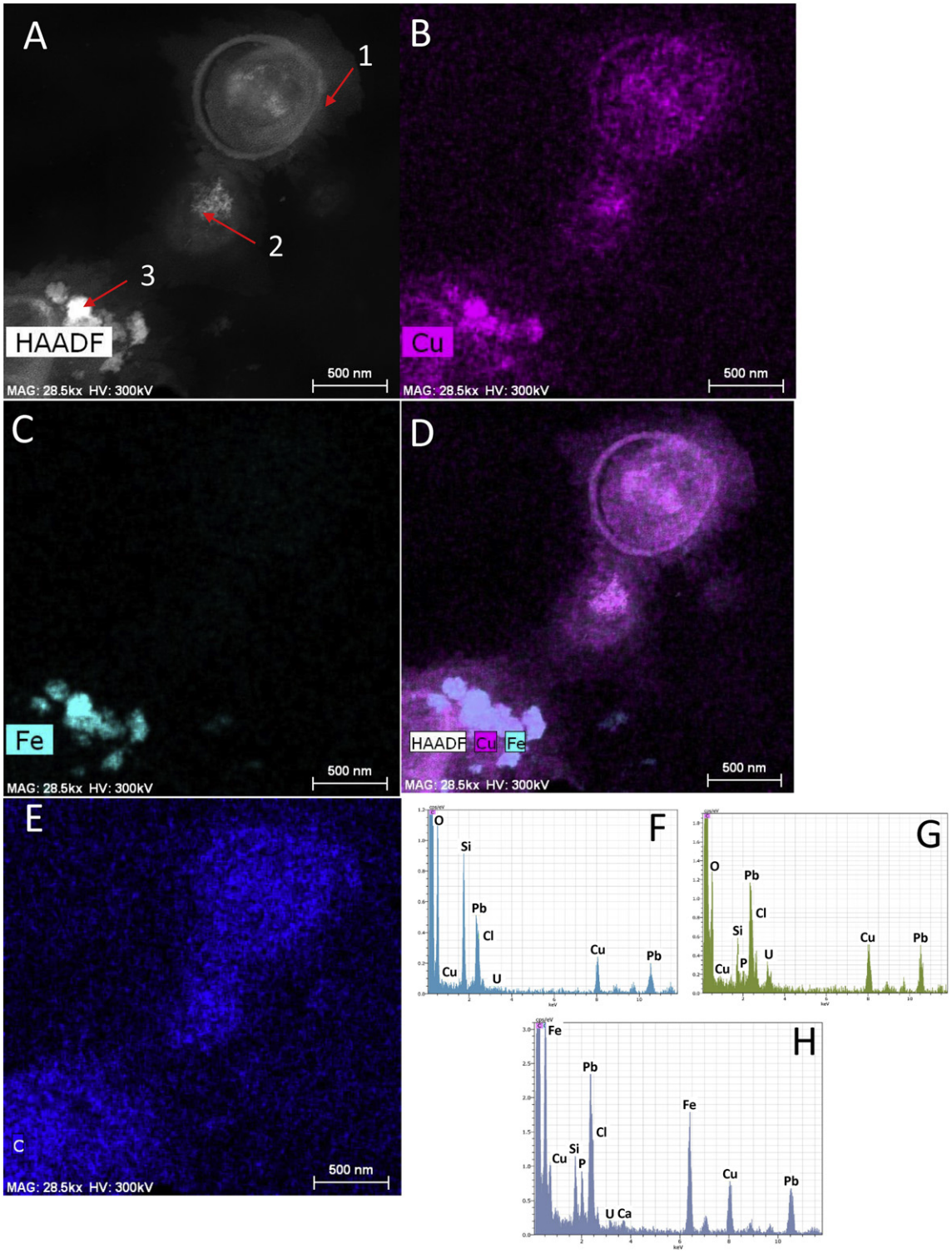

Fig. 9. HRTEM/EDX maps of Cu, Fe, C, in the bacterial cells of the marine sediment. A) High-angle annular dark field (HAADF) STEM image; B), C), and E) The corresponding EDX maps showing the distribution of Cu, Fe, and C, and D) a combined map of Cu and Fe in the bacterial community of the marine sediment. F, G and H show EDX spectra of the precipitates 1, 2, and 3, respectively, from image A.

is strongly tied to microbial processes, which in turn are influenced by the microbiome diversity (Glöckner et al., 2012). Owing to their short life cycle and genetic organization, microorganisms rapidly undergo a number of changes induced by contaminated environments, conferring on them the metabolic capacities to transform or even remove pollutants from their habitats (Fonti et al., 2015; Povedano-Priego et al., 2017).

According to our results, Firmicutes (77.04% and 62.73%), Actinobacteria (9.79% and 16.74%), Chloroflexi (3.08% and 4.41%), Proteobacteria (3.33% and 3.96%), Planctomycetes (2.20% and 3.30%), Bacteroidetes (1.38% and 3.14%), and Acidobacteria (0.59% and 1.32%) would be the most predominant phyla in the Mediterranean marine sediments at the studied intervals, S1 and S2. Most of these phylotypes have likewise been identified in other marine environments,

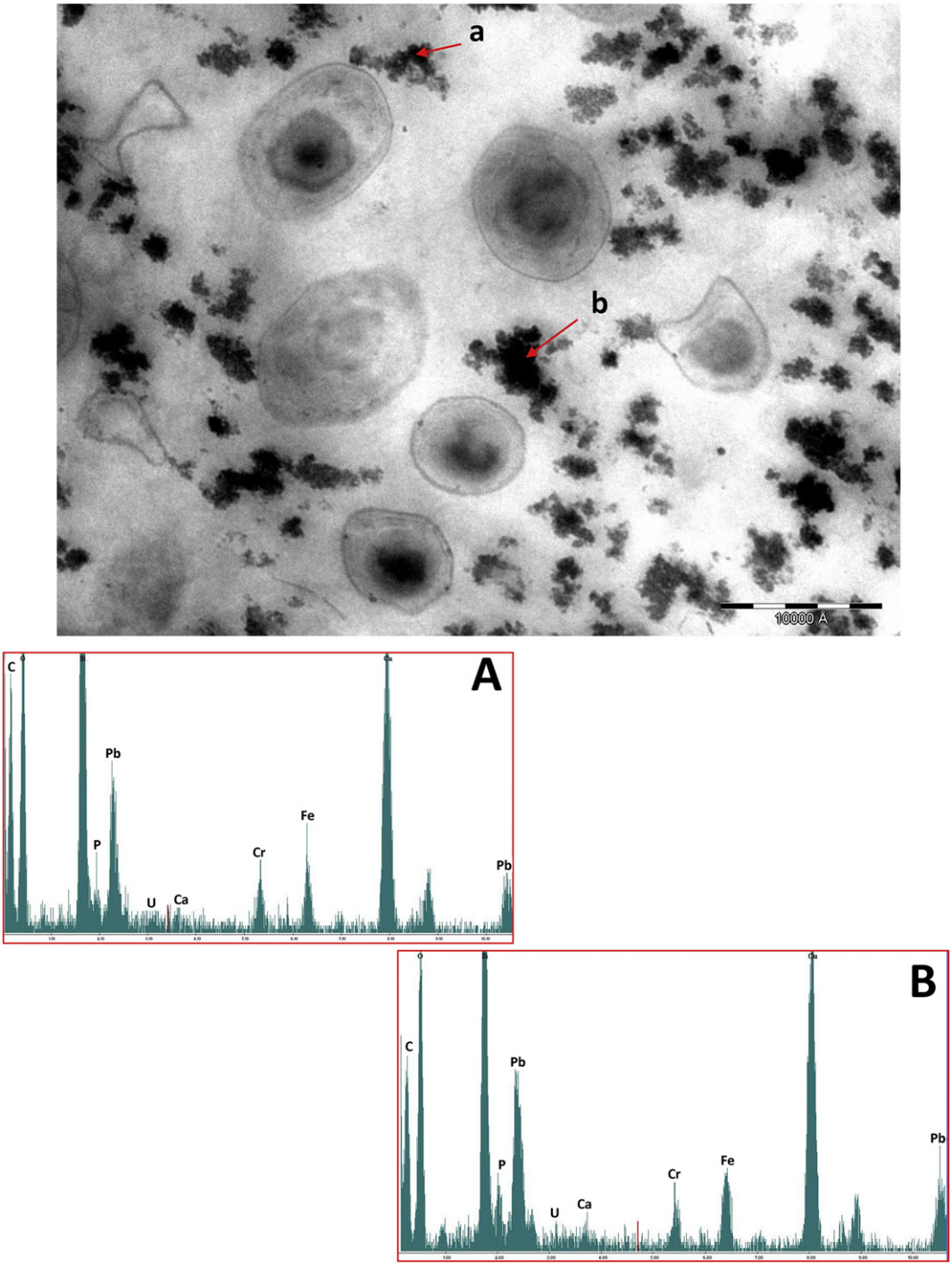

Fig. 10. HRTEM photograph of the bacterial cell-Cr interaction showing the localization of Cr precipitates in the EPS within the extracellular space of the bacterial community of the marine sediment and their corresponding EDX spectra. A) and B) EDX spectra of precipitate a and b, respectively.

particularly in the Mediterranean area (Catania et al., 2016; Fonti et al., 2015). For instance, Chloroflexi has been widely found as a main player in the transformation of organic compounds in freshwater and marine sediments (Matturro et al., 2016). Similarly, Planctomycetes, detected in contaminated sediments, is capable of oxidizing ammonia aerobically and anaerobically (Quero et al., 2015). The bacterial composition of such sediments appears to be dependent on diverse environmental factors (e.g., oxygen content, food availability, past environmental conditions) and interactions. Complex communities were observed in both sediment intervals studied here, meaning only a partial description of their functions can be put forth.

It has been proposed that a greater number of bacterial taxa may account for higher and more variable functions in the marine ecosystem (Bodelier, 2011; Krause et al., 2014). Bacteria like *Bacillus*, *Microlunatus*, *Micrococcus*, *Paenisporosarcina*, *Anaerolineaceae*, *Propionibacterium*, and *Fictibacillus*, among others, dominate in Mediterranean marine sediments. Many *Bacillus* species are known to be heavy metal-resistant bacteria. They have been isolated from mine tailing soils in China (Yu et al., 2014) and Germany (Merroun et al., 2005), from marine polluted sediments, and from the Aliaga ship dismantling zone in Turkey (Kacar and Kocyigit, 2013). Similarly, *Paenisporosarcina*, with a large occurrence in these sediments, are reportedly psychrophilic or

psychrotolerant bacteria, isolated from cold sediments (Reddy et al., 2013). Interestingly, *Microlunatus* species were detected in and dominated these marine sediments. Members of this genus, frequently isolated from different soils (Wang et al., 2008), exhibit many potential advantages for managing environmental pollution (Zhang et al., 2017a). The fact that they were recently detected for the first time in a marine environment in the Chukchi Shelf sediments (Yuan et al., 2014) comes to support the considerable diversity of these Actinobacteria, widely present within Mediterranean sediments. *Anaerolineaceae* plays a key role in the initial activation of *n*-alkane biodegradation in oil-contaminated environments (Liu et al., 2019). Liang et al. (2015, 2016) reported the frequent occurrence of members of this family in methanogenic *n*-alkane-degrading microbial communities derived from oily sludge and the production water of petroleum reservoirs.

Taking into consideration the potential production of organic acids (such as acetate) by *Anaerolineaceae* and its high abundance in surface sediments, we should underline their possibly essential role in the survival of our sediment bacterial community. In both sediments studied here, strong positive correlations moreover revealed putative associations between the dominant bacterial taxa and sediment composition. This finding would be supported by the consistent results of CCA and network analyses: positive correlations were observed between typical redox sensitive elements such as Cu, Co, Zn, Pb, Mo and *Bacillus*, *Micrococcus*, *Bacillales*, *Paenisporosarcina*, *Microbacterium*, *Megasphaera* and *Enterobacteriaceae*, suggesting that changes in oxygenation governing the concentrations of the metals may promote the proliferation of these genera. Consistent results were also commonly observed for the rest of the environmental factors-genus associations. The co-occurrence patterns among the bacterial genera were derived from microorganisms either sharing similar ecological niches or specific traits. In this study, therefore, strong ecological associations evolved as a cluster of robust co-occurrence correlations. Such observations lead us to surmise that some co-occurrence patterns reflect associations of bacteria performing functions that are similar or complementary to each other, while others may co-occur due to shared and preferred environmental conditions.

Interestingly, and supporting the bacterial diversity analyses, the marine cultured bacterial communities were enriched and dominated by *Bacillus* species and by either *Micrococcus* or *Kocuria* in S1 and S2 sediment intervals. The cultivation of aerobic or facultative anaerobic strains from deep-sea sediments has already been described in the literature (Fonti et al., 2015, and references therein). In this study, the isolation of bacterial strains under aerobic conditions may be explained by the fact that some strains living in marine sediments are facultative anaerobes (e.g. *Bacillus*); or that they are strictly aerobes that were inactive *in situ* ("in dormancy"). The biogeochemical interactions among abiotic and biotic processes influencing metal mobility are still largely unknown in marine environments, although their understanding is crucial to determine which bacterial strains are most closely involved in the changes occurring in contaminated marine sediments. Few studies have looked into metal/radionuclide interaction with marine bacteria such as *Idiomarina* sp., which proved able to biomineralize U as uranium phosphate (Morcillo et al., 2014). To date, however, the interaction of marine sediment bacteria with other metals such as Cr, Cu, Zn, and Pb has been largely overlooked. In the present study, the interaction of marine bacterial communities with such heavy metals comes to highlight their ability for metal sorption, biomineralization and bioaccumulation within the cells, on the cell walls, and/or on the EPS in the extracellular space.

In oxygenated seawater, Cr is mostly present as a chromate anion, CrO_4^{2-} and may also appear as Cr(III) in the aquahydroxyl ion, $Cr(H_2O)_4(OH)^{2+}$ (e.g., Calvert and Pedersen, 1993). Under normal oxygenated seawater, the chromate anion is soluble, but under anoxic conditions, Cr(VI) is reduced to Cr(III), forming aquahydroxyl cations and hydroxyl cations ($Cr(OH)^{2+}$, $Cr(OH)_3$, $(Cr, Fe)(OH)_3$), which can readily form complexes with humic/fulvic acids or be adsorbed to Fe- and Mn-oxyhydroxides (e.g., Algeo and Maynard, 2004). As Cr does not form insoluble sulfide, it is easily lost to the water column (Morse and Luther, 1999). Its accumulation in the enriched marine bacterial community studied here is in line with other reports of *Bacillus*-affiliated strains, capable of Cr(VI) reduction, detected in different Cr polluted environments (Chandhuru et al., 2012; Dhal et al., 2010; Rehman et al., 2008; Zeng et al., 2019). The *Bacillus* species, featuring high tolerance, uptake and reduction abilities, could prove useful in the remediation of Cr-contaminated environments. Likewise, Cr-reducing isolates belonging to the genus *Micrococcus* have exhibited great metal resistance in many Cr-contaminated environments (Bizani and Spagiari, 2016; Teles et al., 2018). However, particularly under suboxic environments, Cr is significantly enriched in certain marine sediments (Calvert and Pedersen, 2007). Thus, its potential bioaccumulation in the microbial biomass can account for the enrichment in sediments. We might speculate that most bacterial strains in the marine community isolated in this study possess the capacity to bioaccumulate Cr and further serve for remediation strategies.

Meanwhile, Cu is mostly present in the form of organometallic ligands, and to a lesser extent as $CuCl^+$ ions in solution in oxic marine environments (e.g., Algeo and Maynard, 2004; Calvert and Pedersen, 1993). Cu also behaves as a micronutrient, and its complexation with organic matter is well known, as is its adsorption onto particulate Fe–Mn-oxyhydroxides. Yet when organic matter degrades and Fe–Mn-oxyhydroxides dissolve, Cu may be released to seawater. Under the reduction conditions commonly encountered in marine sediments following deposition, Cu is reduced and subsequently incorporated in sulfides as pyrite, or it may even form its own sulfide phases, CuS and CuS_2 (e.g., Morse and Luther, 1999), since it can be fixed by clay minerals (Pedersen et al., 1986). Bioaccumulation in the bacterial biomass could be an additional factor accounting for the fixation of this metal in marine sediments. In fact, Cu biosorption to the bacterial cells, previously reported in many *Bacillus* species endowed with S-layers, support that structural elements of the envelopes are responsible for such activity (Allievi et al., 2011).

The behavior of Zn is rather complex. In oxic marine environments, Zn behaves as a micronutrient and may be present as soluble Zn^{2+} cations or $ZnCl^+$ ions, but it is usually present as complexes with humic/fulvic acids (Algeo and Maynard, 2004; Calvert and Pedersen, 1993). Like other trace metals, it can be adsorbed into particulate Fe–Mn-oxyhydroxides, and upon organic matter degradation, it may be released to pore waters (Calvert and Pedersen, 2007; Tribovillard et al., 2006). Like Cu, under reducing conditions it can be incorporated as ZnS, a solid solution phase in pyrite, or even form its own sulfides (Daskalakis and Helz, 1993; Huerta-Diaz and Morse, 1992; Morse and Luther, 1999). Nevertheless, the role played by bacterial communities in its absorption and fixation in the sediments is not well known. As with the previous metals, our results highlight that the marine bacterial community can satisfactorily bioaccumulate Zn in the EPS in extracellular space and, sparingly, on the walls of some cells. These results are well in line with previous reports on the capacity of many *Bacillus* species to bioaccumulate this metal (Allievi et al., 2011; Catania et al., 2016; da Costa and Duta, 2001; Fonti et al., 2015).

In turn, Pb is widely reported to show a strong affinity for Fe/Mn-oxyhydroxide phases in sediments (Chakraborty et al., 2016). As a pollutant, its bacterial bioaccumulation has been broadly demonstrated (e.g., Tiquia-Arashiro, 2018). Variations in oxygen concentrations would significantly impact Pb speciation as Fe/Mn-oxyhydroxide and organic matter, its major hosting phases in marine sediments; thus, its bioaccumulation in bacterial cells plays a major role in their concentration. Indeed, some of the bacterial species described in this study, e.g. *Bacillus*, were found to be Pb-resistant bacterial strains, demonstrating a biotechnological potential for the bioremediation of Pb from contaminated marine sediments (Pepi et al., 2016) or multiple metal-contaminated saline environments through biosorption (Mohapatra

et al., 2019). *Bacillus* is also known to thrive in the presence of Pb owing to surface proteins involved in metal association acting as biosorbants of this metal (Allievi et al., 2011). They can form spores as a strategy to survive unfavorable environmental conditions. Our results are also significant in that they point to the precipitation of pyromorphite leading to Pb immobilization by phosphate as an effective mechanism in bioremediation strategies and support its potential in Pb contaminated settings (e.g. Sharma et al., 2018).

Overall our findings suggest that the microbial communities inhabiting marine sediments entail effective mechanisms able to concentrate metals. This role deserves further exploration, as bioaccumulation holds great interest: firstly, to understand how trace metals concentrate in marine sediments, for the purpose of paleoenvironmental reconstructions; and secondly with regard to pollutants, and how these marine bacterial communities may serve to accumulate them in sediments.

5. Conclusions

Microbial communities have been shown to play an essential role in biogeochemical processes in marine sediments. Nonetheless, the capability of such communities to bioaccumulate trace metals is still largely unexplored. This potential is especially important in terms of paleoenvironmental reconstructions and redox cycling within sediments, yet also for bioremediation strategies, which could involve natural attenuation and biostimulation. Our study demonstrates that microbial communities inhabiting marine sediments constitute an effective mechanism for concentrating diverse metals — in particular the precipitation of Pb as pyromorphite — and clearly holds potential for bioremediation strategies given the severe Pb pollution affecting living organisms, including humans. According to our results, *Bacillus*, unclassified *Bacillales*, *Micrococcus*, unclassified members of *Planococcaceae*, and *Anaerolineaceae*, *Microlunatus*, *Planctomycetaceae*, and *Microbacterium* are the dominating genera, showing only slight differences in their percentages of abundance between the two studied intervals. In the older interval, *Propionibacterium*, *Fictibacillus*, *Thalassobacillus*, *Lachnospiraceae* Incertae_Sedis, and *Bacteroides* were also obtained. Strong correlations among the taxa and the sediment physicochemical parameters were confirmed by CCA and network analysis. The results further demonstrate that bacterial biomass in marine sediments may constitute a major sink for a wide range of pollutants, which is highly relevant for regions under substantial anthropogenic pressure and heavy metal accumulation. The Mediterranean is especially sensitive in this sense, and the westernmost Mediterranean is subjected to intensive maritime traffic, entailing pollution risks and other environmental issues. A better knowledge of its microbial diversity and of the capability of marine bacteria to bioaccumulate metals is therefore essential for environmental quality assessment, and should be further explored.

Author contributions

M.T.G.M. and F.M.R. conceived the concept, led this project and designed the experiments setup. F.J. performed the experiments and contributed to analyze the results. M.T.G.M. and F.M.R. contributed by performing the microscopic studies. F.J. and F.M.R. participated in the writing of the original Draft. F.J., F.M.R., M.L.M, and M.T.G.M. contributed to the review and editing of the paper.

Declaration of competing interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135660.

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