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Isolation of biocrust cyanobacteria and evaluation of Cu, Pb, and Zn immobilisation potential for soil restoration and sustainable agriculture

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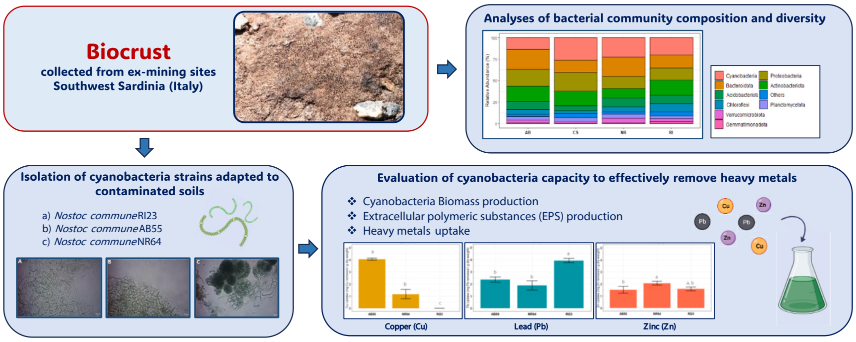
HIGHLIGHTS

- Cyanobacteria comprised 26 % of the bacterial community in metal-contaminated soils.
- *Nostoc commune* strains from ex-mining sites varied in growth rates and EPS production.
- *N. commune* strains were able to effectively immobilise copper, lead, and zinc.
- Cu immobilisation was strongly associated to cyanobacterial EPS production.
- Results suggest potential for soil bioremediation with indigenous cyanobacteria.

GRAPHICAL ABSTRACT



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ABSTRACT

Soil contamination by heavy metals represents an important environmental and public health problem of global concern. Biocrust-forming cyanobacteria offer promise for heavy metal immobilisation in contaminated soils due to their unique characteristics, including their ability to grow in contaminated soils and produce exopolysaccharides (EPS). However, limited research has analysed the representativeness of cyanobacteria in metal-contaminated soils. Additionally, there is a lack of studies examining how cyanobacteria adaptation to specific environments can impact their metal-binding capacity. To address this research gap, we conducted a study analysing the bacterial communities of cyanobacteria-dominated biocrusts in a contaminated area from South Sardinia (Italy). Additionally, by using two distinct approaches, we isolated three *Nostoc commune* strains from cyanobacteria-dominated biocrust and we also evaluated their potential to immobilise heavy metals. The first isolation method involved acclimatizing biocrust samples in liquid medium while, in the second method, biocrust samples were directly seeded onto agar plates. The microbial community analysis revealed Cyanobacteria,

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Bacteroidota, Proteobacteria, and Actinobacteria as the predominant groups, with cyanobacteria representing between 13.3 % and 26.0 % of the total community. Despite belonging to the same species, these strains exhibited different growth rates (1.1–2.2 g L^{-1} of biomass) and capacities for EPS production (400–1786 mg L^{-1}). The three strains demonstrated a notable ability for metal immobilisation, removing up to 88.9 % of Cu, 86.2 % of Pb, and 45.3 % of Zn from liquid medium. Cyanobacteria EPS production showed a strong correlation with the removal of Cu, indicating its role in facilitating metal immobilisation. Furthermore, differences in Pb immobilisation (40–86.2 %) suggest possible environmental adaptation mechanisms of the strains. This study highlights the promising application of *N. commune* strains for metal immobilisation in soils, offering a potential bioremediation tool to combat the adverse effects of soil contamination and promote environmental sustainability.

1. Introduction

Over the last decades soil contamination by heavy metals, primarily caused by industrial activities, agricultural practices, and natural sources, has become a prominent and concerning environmental and public health issue on a global scale (Ilyin et al., 2004; Lu et al., 2012; Zhang and Wang, 2020; Alengebawy et al., 2021; European Environment Agency, 2023). Different levels of soil pollution due to heavy metals have been detected all around the globe (Su, 2014; Hou et al., 2020; Liu et al., 2022). For instance, in China, about 10 % of agricultural soils have been found to be affected by heavy metal pollution (Kou et al., 2018). In other countries, including France, Sweden, Slovakia, Hungary, and Austria, over 200,000 sites have been categorized as heavy metal contaminated. Meanwhile, Greece and Poland have reported about contaminated 10,000 sites (Su, 2014). In Italy, despite the absence of comprehensive studies on heavy metal contamination of soils, this issue affects many localities in various regions of the country (Buccheri et al., 2018; Agrelli et al., 2020; Cicchella et al., 2020). In contrast to organic pollutants, metal(loids) are not easily degraded by microbes or chemicals (Raffa et al., 2021). Consequently, after their introduction into the soil, the total concentration of these metal(loids) can remain for an extended period depending on the type of soil and nature of the metal (Bolan et al., 2014). For example, in the Montevecchio-Ingurtosu mining district in Southern Sardinia (Italy), >30 years after mining activity ceased, we observe that the landfill materials still exhibit substantial, and at times remarkably high, concentrations of Pb (up to 77,681 ppm), Zn (up to 48,194 ppm), and to a lesser extent, Cu (up to 3154 ppm), far exceeding the permissible limit values set by European Directive 2008/50/CE (Fanfani et al., 2001).

It is widely recognized that the overuse of fertilizers and pesticides contributes to heavy metal contamination in the soil. Indeed, fertilizers may contain traces of heavy metals such as Cd, Cu, Zn, Pb, or As (Hou et al., 2020; Lu et al., 2012; Nziguheba and Smolders, 2008). These elements are frequently incorporated into micronutrient fertilizers or as components of balanced fertilizer formulations. For instance, Cu has several applications in agriculture due to its use as an essential micronutrient for plants and as an effective fungicide (Lamichhane et al., 2018; Moreira et al., 2022). Cu-based compounds are extensively applied as treatments to protect seeds and plants from fungal and bacterial pathogens, particularly for olive groves and fruit trees (Rehman et al., 2019). In these specific crops, it is common for Cu concentration levels to exceed established limits (Ballabio et al., 2018; Provenzano et al., 2010; Steinmetz et al., 2017; European Commission, 2018). Also, phosphate fertilizers can be associated with the presence of metals, including Ni, Cd, Zn, Pb, As, and Cr. This correlation stems from the natural presence of these metals in phosphate rock, a common phosphorus source for fertilizer production (Gupta et al., 2014; Nziguheba and Smolders, 2008). The introduction of Pb from fertilizers can result in soil contamination, hazarding crop health and food safety (Nacke et al., 2013; Khan et al., 2018). Once Pb is present in the soil, it tends to remain there for an extended period, potentially persisting for decades or even centuries, and may eventually continue to pollute the soil, putting at risk the ecosystem due to its high persistence (Weissengruber et al., 2018; Frank et al., 2019). Considering these data, the search for effective solutions to address heavy metal contamination in agricultural soil is emerging as an increasingly pressing concern.

The remediation of metal-contaminated sites has traditionally relied on physical remediation methods such as soil thermal desorption and soil replacement, as well as chemical remediation approaches including chemical leaching, chemical stabilization, electrokinetic remediation-permeable reactive barrier, and chemical oxidation/reduction (Bolan et al., 2014; Dhaliwal et al., 2020; Song et al., 2022). However, these techniques sometimes lack selectivity and often render metal ions more soluble and mobile, leading to groundwater pollution and negatively affecting soil quality. Chemical/physical methods are also laborious and expensive, and many times provide limited results for heavy metal contamination removal (Wu et al., 2010). Therefore, considering reagent toxicity, unselectivity, inefficacy and high cost of traditional remediation techniques, bioremediation techniques have emerged as more environmentally friendly and economically viable alternatives. These include phytoremediation and microbial remediation, which are respectful of both the environment and human health (Mohapatra et al., 2019; Priya et al., 2022; Rizvi et al., 2020; Shen et al., 2022). Among various microorganisms, the employment of cyanobacteria, a widely distributed phylum of photoautotrophic bacteria, has attracted scientific attention. Cyanobacteria offer potential for bioremediation of heavy metals both by biosorption and bioaccumulation processes (Al-Amin et al., 2021). The effectiveness of cyanobacteria in heavy metal immobilisation has primarily focused on water cyanobacteria strains for application in wastewater treatment (Ahmad, 2022). However, investigation of soil cyanobacteria for application in heavy metal immobilisation has been much more limited, despite their potential for treating contaminated soils in both natural and agricultural environments (Ciani and Adessi, 2023; Cui et al., 2020; Dutta et al., 2022; Potnis et al., 2021).

In soils, cyanobacteria usually form assemblages with other photoautotrophic organisms (algae, lichens, mosses) and heterotrophic organisms (fungi, bacteria) residing on top of the uppermost millimetres of soil, forming the community known as biological soils crust or biocrust (Weber et al., 2022). Biocrusts cover approximately 12 % of the Earth's terrestrial surface (Rodriguez-Caballero et al., 2018), including contaminated areas and regions with extreme temperatures and salt concentrations (Xiao et al., 2022). These communities exhibit remarkable resistance to harsh conditions and may survive in situations with high levels of heavy metal contamination (Fan et al., 2023). Additionally, biocrusts are important for soil health and ecosystem resilience, due to their recognized effects improving soil water availability, fertility, biodiversity and on erosion control (Chamizo et al., 2016; Antoninka et al., 2020; Román et al., 2021). Biocrust-forming cyanobacteria could be a promising solution for soil heavy metal immobilisation due to their unique characteristics, such as the ability to grow in contaminated soils and the production of extracellular polymeric substances (EPS). Cyanobacteria possess a cell wall characterized by a thicker peptidoglycan layer and with a higher degree of cross-linking among the polysaccharidic chains in comparison with other Gram-negative bacteria (Hoiczyk and Hansel, 2000). Due to the presence of these negatively charged EPS cell layers, cyanobacteria are capable of passively absorbing high amounts of solubilized metals (De Philippis et al., 2011; Ciani and Adessi, 2023). Indeed, the synthesis of EPS by cyanobacteria plays a crucial role in interfering with the mobility and bioavailability of heavy metal ions in the soil. Cyanobacteria EPS chelate and immobilise heavy metals, mitigating their harmful effects and facilitating their removal from the environment (De Philippis et al., 2011). To further improve the efficacy of cyanobacteria in heavy metal remediation, it is necessary to identify suitable species with key functional characteristics, such as high metal immobilisation capacity, easy and rapid ex-situ culture, and soil survival and colonisation. Native species collected and isolated from contaminated environments, already adapted to the site-specific conditions to be restored, potentially can show these traits. However, there is limited research focusing on how the adaptation of cyanobacteria to their specific environment can influence their metal-binding capacity.

To fill in this gap, in this study, the bacterial communities of cyanobacteria-dominated biocrusts inhabiting a contaminated area in South Sardinia (Italy) were analysed at the phylum level and different cyanobacteria strains were isolated and tested for their ability to immobilise heavy metals. Therefore, the objectives of this work were: i) to examine the bacterial community composition and diversity of biocrust communities in four heavy metal contaminated sites; ii) to isolate and identify cyanobacteria strains from contaminated soils, and thus potentially applicable for soil bioremediation; and iii) to evaluate the growth in liquid medium, the EPS production and the heavy metal removal of the isolated strains. Therefore, this research directly addresses the global issue of soil contamination by heavy metals, with a specific focus on exploring the use of native cyanobacteria in soil restoration and sustainable agriculture.

2. Material and methods

2.1. *Study sites and biocrust sampling*

The study sites consist of ex-mining areas, located in the Medio Campidano and Carbonia-Iglesias provinces in Southwest Sardinia, Italy (Fig. 1).

South Sardinia region is rich in mineral resources, and mining activities have significantly contributed to the local economy during the 20th century (Manca et al., 2019). The minerals extracted in this area include Pb, Zn, Fe, Mn, Cu, coal, salt, and other minerals (Fanfani et al., 2001; Bacchetta et al., 2015; Manca et al., 2019). Lead and zinc were particularly important due to their historical significance and economic value, with abundant ore deposits supporting a thriving mining industry (Vacca et al., 2012).

For this study, we selected four localities that have experienced intense mining activity (Fig. 1). The localities and GPS coordinates are listed in Table 1.

Mean annual precipitation ranges approximately from 600 to 800 mm, and average annual temperatures fluctuate between 15 and 17 °C. Climate is characterized by dry spring and summer and wet autumn and winter (Dore et al., 2020).

Soils show sandy-loam texture, with pH ranging from 5.4 to 6.7, soil organic carbon content is low and varies from 1 to 16 g kg^{-1}, and average total nitrogen content is ~2 g kg^{-1} (Vacca et al., 2012; Nicolas et al., 2022). These mining sites are significantly affected by heavy metal contamination, especially from Cd, Cr, Pb, and Zn (Satta et al., 2012; Boi et al., 2021). Additionally, mine wastes in the area exhibit significant heterogeneity in terms of metal concentrations. This heterogeneity is primarily attributed to the diverse extraction methods employed throughout the history of mining operations in the region (Bacchetta et al., 2017; Boi et al., 2021). Vegetation cover is low and the most frequent plant species in the area are *Rumex bucephalophorus*, *Pistacia lentiscus*, *Helichrysum microphyllum* subsp. *Tyrrhenicum*, *Euphorbia cupani pithyusa, Juncus acutus,* and *Carlina corymbose* (Angius et al., 2011; Sprocati et al., 2014; De Giudici et al., 2015; Medas et al., 2019; Fois et al., 2023). Biocrusts are present forming small patches in the inter-shrub spaces and mainly consist of well-developed cyanobacteria biocrusts.

The soil samples were collected in April 2022. At each site, three soil samples at 1 cm of depth were randomly taken from cyanobacteria-dominated biocrust communities with a sterile trowel and directly stored in 50 mL sterile Falcon® tubes and kept dry in the dark until their use. Part of the samples (~1 g) was weighed and stored at −20 °C for further metagenomic analysis. The resting soil was air-dried, sieved to 2 mm, and ground for the determination of Cu, Pb, and Zn concentrations, using ICP-MS (iCAP TQ, Thermo Scientific). These metals have been selected due to their significant impact on the phenomenon of agricultural soil contamination. Before the analysis, the samples underwent acid digestion using a microwave oven, specifically employing a single

Table 1
Sites and coordinates of biocrust samples.

Site	Coordinates	Biocrust sample
Mine waste Sanna	39°32′03.7″N 8°31′41.8″E	CS
Rio Irvi banks	39°32′47.9″N 8°28′40.6″E	RI
Naracauli	39°31′09.2″N 8°29′23.5″E	NR
Ex-mine S. Acqua Bona	39°28′36.3″N 8°30′07.1″E	AB

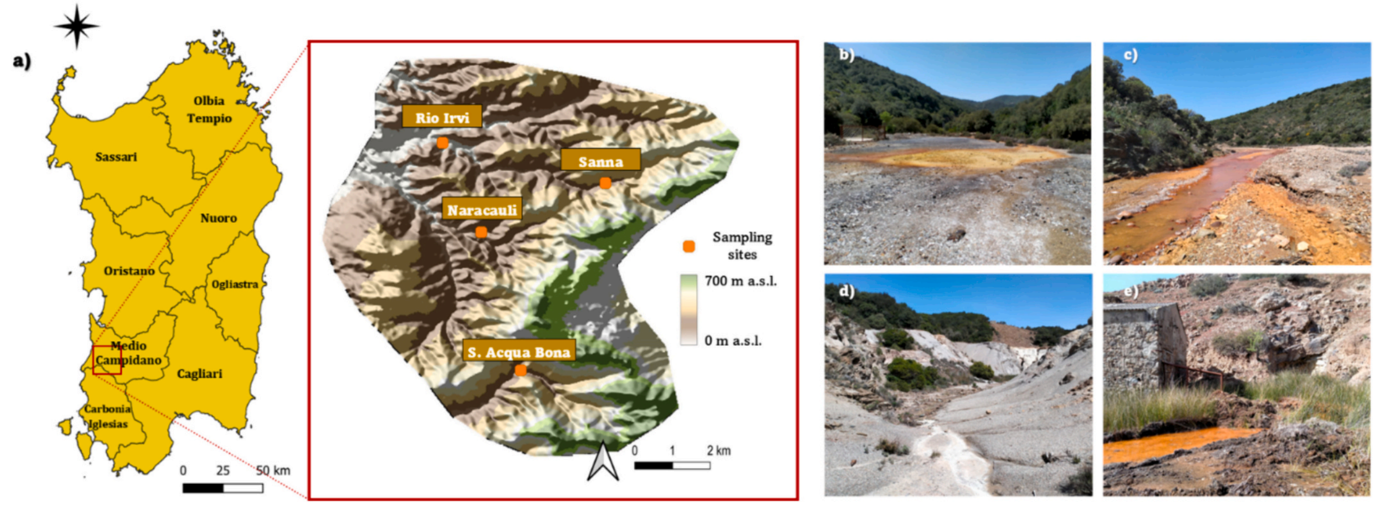

Fig. 1. General view of the study area (a). Location of sampling sites: Mine waste Sanna, CS (b), Rio Irvi banks, RI (c), Naracauli, NR (d), and Ex-mine S. Acqua Bona, AB (e).

reaction chamber digestion system (UltraWAVE "ERC", MILESTONE).

2.2. *Analyses of microbial community composition*

Biocrust samples were first pre-treated with three freeze–thaw cycles and ground in a mortar using a pestle (Loza et al., 2013). DNA extraction from biocrust was performed using the QIAGEN DNeasy PowerSoil kit (Qiagen, Germantown, MD, USA) following the manufacturer's protocol. The microbial community composition was analysed using Next Generation Sequencing (NGS). For library preparation, a "two-step PCR protocol" was employed, which involved DNA amplification, using primers modified with universal tags, followed by enzymatic purification using Thermostable Exonuclease I. Subsequently, a second PCR step was carried out where Illumina Nextera XT indexes were attached to the universal tags. The samples were then normalized, multiplexed, and sequenced using the MiSeq platform (Illumina, San Diego, CA, USA). The sequence data were processed with Qiime2 tools version 4.0. Initially, adapter trimming was performed utilizing Cutadapt. To achieve this, raw sequences from the Illumina MiSeq platform were prefiltered, which entailed removing primer sequences from both forward and reverse reads. This operation was executed with the default parameters of Cutadapt. After adapter sequence removal, read denoising was carried out utilizing the DADA2 toolset. This encompassed a series of steps including read quality filtering and trimming, estimation of error rates, dereplication, read merging, and the detection of chimeric sequences. The resulting outcome of these preprocessing steps yielded a feature table that served as the foundational dataset for subsequent analyses. In the following steps, each sample was analysed individually. To refine the dataset, a feature filtering approach was applied, leading to the exclusion of features representing <0.01 % of the total data. Additionally, singleton features were removed. Taxonomy was assigned using trained sequences (OTUs at 99 %) from Silva database version 138. The alpha biodiversity within the biocrust samples was quantified by the Shannon and the Simpson diversity index (1-D), and the Inverse Simpson diversity index (1/D) by using R Statistical Software (v4.2.1; R Core Team, 2021). These indices are commonly used to assess and compare the richness and evenness of species in ecological communities, providing valuable insights into the overall diversity of the biocrust samples.

2.3. *Cyanobacteria strains isolation and identification*

Two different isolation methods were employed to obtain cyanobacteria isolates from the biocrust samples. According to Rippka et al. (1979), small biocrust fragments (~ 0.5 g) were placed in Eppendorf tubes® with BG11$_0$ medium, shaken, and kept at 19 ± 1 °C and at 40 μmol photon m^{-2} s^{-1} for 4 h to allow cyanobacteria acclimatization. Then, 400 μL of medium was seeded onto agar-solidified Petri dishes with BG11 and BG11$_0$ media at 1.5 % (*w*/*v*), added with cycloheximide (0.1 mg mL^{-1}) to avoid fungal contamination. BG11 medium contains nitrogen, while BG11$_0$ medium, without nitrogen source, is specifically used to select nitrogen-fixing cyanobacteria (Stanier et al., 1971). Furthermore, biocrust samples of ~1 cm^2 were positioned directly in both BG11 and BG11$_0$ agar medium in the centre of the dish. The Petri dishes were kept at the temperature of 19 ± 1 °C, with a 12:12 photoperiod, and at 40 μmol photon m^{-2} s^{-1} of irradiance for 6 weeks. After this time, single colonies were re-streaked on fresh agar plates and this process was repeated until a single morphotype was obtained (Roncero-Ramos et al., 2019).

Identification of cyanobacteria strains was carried out using a multiphase approach that combined morphological characterization by microscope observation and molecular analyses (Roncero-Ramos et al., 2019). The isolated strains were observed under a light microscope at a magnification of 40 X and the morphological identification was based on literature (Komárek and Anagnostidis, 2005; Komárek, 2018; Singh et al., 2020) combined with the use of GenBank (https://submit.ncbi.nlm.nih.gov) of cyanobacteria genera (Hauer and Komárek, 2022). The results of the morphological identification were further confirmed by the genetic identification. Genomic DNA was extracted from pure cultures using the GeneMATRIX Plant & Fungi DNA Purification Kit. The 16S rRNA gene was amplified using the forward primer 359F (5′- GGG GAA TYT TCC GCA ATG GG -3′) and reverse primer 781Ra/781Rb (5′- GAC TAC TGG GGT ATC TAA TCC CAT T -3′/5′- GAC TAC AGG GGT ATC TAA TCC CTT T-3′) (Nübel et al., 1997). The products were purified using the Wizard® SV Gel and PCR Clean-Up System Purification Kit and sequenced in both directions using the Sanger method by AB 3500 DNA analyser (Life Technologies, Thermo Fisher Scientific Inc., US). For the 16S rRNA gene sequences, the closest matching taxa were searched using the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov), and nucleotide sequences were deposited under the accession numbers P256154, PP256155, and PP256156.

2.4. *Growth curves and EPS production*

Once isolated, cyanobacteria strains were first inoculated in multiwell plates and then relocated in flasks with BG11 liquid medium and incubated for 14–19 days at room temperature ($T = 25 \pm 1$ °C), irradiance of 200 μmol photons m^{-2} s^{-1} and aerated with air sterilized by filtering (0.22 μm, Millex EMD Millipore™). Cyanobacteria strains were grown in 100 mL Erlenmeyer flasks, in triplicate, starting from an initial concentration of ~0.5 g L^{-1}. During incubation, cyanobacteria growth was monitored through measurements of dry weight and chlorophyll *a*. In addition, the production of total and released exopolysaccharides (EPS) at the beginning and at the end of the experiment (after 14 days) were analysed.

The dry weight of culture was determined by filtering 5 mL of the culture onto pre-weighed filter papers with 0.45 μm pore size. The filter was then dried at a temperature of 80 °C for 24 h (Roncero-Ramos et al., 2022). The concentration of chlorophyll *a* was determined according to Singh et al. (2016). 5 mL of culture was centrifuged at 2500 ×*g* at 9 °C of temperature for 7 min. The supernatant was discarded, then 5 mL of methanol was added to the pellet, vortexed and heated at 70 °C of temperature for 5 min. The sample was then centrifuged again at 2500*g*, and the absorbance of the supernatant was measured using a spectrophotometer at 665 nm and 750 nm wavelengths. The chlorophyll *a* content was determined using the equation of Ritchie (2006):

$$\mathrm{Chl}\ a = 12.9447 \times (\mathrm{Abs665} - \mathrm{Abs750}) \times \frac{\mathrm{Vm}}{\mathrm{Vs}} \tag{1}$$

Chl *a* is Chlorophyll *a* (μg mL^{-1}), Abs665 and Abs750 are the values of absorbance at 665 nm and 750 nm wavelengths, respectively, Vm is the volume of methanol and Vs is the sample volume (mL).

The content of both total and released EPS was determined using the phenol-sulfuric acid method (Dubois et al., 1956). For the released EPS, 5 mL of culture was previously centrifuged at 4000*g* for 30 min and 1 mL of the supernatant was taken to quantify carbohydrate content (Chamizo et al., 2020). When EPS amount exceeded the detection limit, 1:5 or 1:10 dilutions were applied to the samples, after which the phenol-sulfuric assay was applied on 1 mL aliquots of the samples. This method is based on the principle that carbohydrates, when reacted with concentrated sulfuric acid, undergo dehydration and form furfural derivatives. The absorbance was measured at 488 nm, with glucose used as standard for comparison.

2.5. *Heavy metal removal experiments*

The isolated cyanobacteria strains were tested for their ability to immobilise Zn, Pb, and Cu, which were selected due to their presence at high concentrations at the selected study sites as well as their noteworthy relevance in the context of agricultural soil pollution resulting from fertilizer inputs. A preliminary treatment of the cultures was carried out using 0.1 M HCl to remove the metal ions bound to the negatively charged groups (Micheletti et al., 2008). After 30 min, the cultures were centrifuged at 2500 ×g for 10 min and resuspended 3 times in deionized water to remove residual HCl. The metal solutions of Cu, Pb, and Zn were respectively prepared using copper nitrate (VWR Chemicals), lead (II) nitrate (VWR Chemicals), and zinc sulfure (Scharlau). Each cyanobacterial strain was incubated at the concentration of 2 g L^{-1}. Individual metals were added to the culture with a concentration of 10 mg L^{-1}. For each treatment, 3 replicates were set up, each with a volume of 100 mL. The negative control was a solution of the selected metal without cyanobacteria. The cultures were incubated for 90 min at 100 rpm shaking at the temperature of 25 ± 1 °C, providing an optimal timeframe for the accumulation of heavy metals by cyanobacteria (Chojnacka et al., 2005; Micheletti et al., 2008). During the experiment, the cultures were maintained at pH 4. After 90 min, the cultures were filtered through a 0.45 μm membrane. The final metal concentration was detected with ICP-MS (iCAP TQ model, Thermo Scientific), and the metal removal (mg metal removed g^{-1} dry weight) was calculated with the following formula:

$$q = V\frac{Ci - Ct}{m} \tag{2}$$

Where V is the sample volume (mL), Ci and Ct are the initial and final heavy metal concentrations, respectively, and m is the dry weight value for sample (g) (Volesky and May-Phillips, 1995).

2.6. Statistical analysis

Repeated measures ANOVA was used to examine variations among cyanobacteria strains in dry weight and chlorophyll *a* values over time. One-way ANOVA was employed to analyse differences in released EPS production, total EPS production, and metal removal among cyanobacteria strains, and when statistically significant, the Tukey post-hoc test was applied for multiple comparisons. All variables were previously tested for normality and homogeneity of variance using the Shapiro–Wilk and Levene's test. Non-metric multidimensional scaling (NMDS) was used to visually detect relations between cyanobacteria's capacity to immobilise heavy metals and the presence of heavy metal in the place of isolation. All statistical analyses were performed using R Statistical Software (v4.2.1; R Core Team, 2021) and IBM SPSS Statistics (v29.0; IBM Corp). Results were considered significant when p value < 0.05.

3. Results

3.1. Biocrust bacterial community composition and diversity

By NGS sequencing we obtained 133,890 high-quality reads, classified into 1202 operational taxonomic units (OTUs). We found nine main phyla across all biocrust samples. Cyanobacteria, Bacteroidetes, Proteobacteria and Actinobacteria were the most abundant phyla, with different proportions depending on the biocrust sample (Fig. 2). Cyanobacteria were prevalent in CS (26.0 %), RI (19.9 %), and NR (22.5 %), while they exhibited a lower relative abundance (13.3 %) in AB. Bacteroidota and Proteobacteria respectively accounted for 14.4 % and 21.8 % of the relative abundance in CS, 15.5 % and 14.3 % in RI, 22.7 % and 14.1 % in NR, and 23.8 % and 19.3 % in AB. Actinobacteria exhibited a similar abundance in CS, RI, and AB (around 17 %), but showed a lower abundance in NR (11.2 %). Other phyla characterizing biocrust samples included Acidobacteriota, Chloroflexi, Planctomycetota, and Verrucomicrobiota.

Among the taxa with lower representation (<2 %), the phyla Gemmatimonadota, Armatimonadota, Myxococcota, Bdellovibrionota, Patescibacteria, Firmicutes, and Deinococcota were present in all biocrust samples, while Chlorophyta and Abditibacteriota were found only in CS, NR, and RI. Crenarchaeota, RCP2-54, WPS-2, Methylomirabilota, and Sumerlaeota were exclusively detected in RI, while Dependentiae was identified in AB, CS, and NR. Nitrospirota, SAR324_clade, and Fibrobacterota were uniquely found in AB, NR, and CS, respectively.

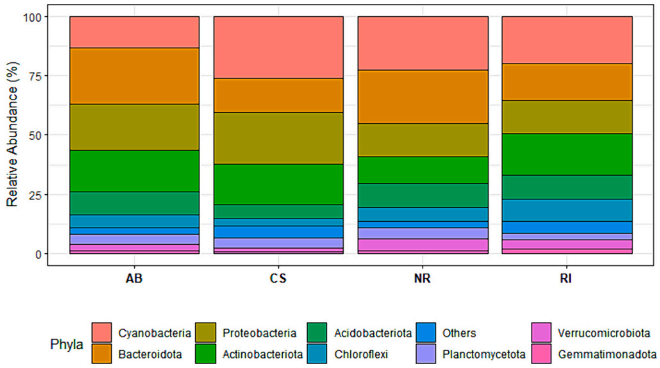

Fig. 2. Relative abundance of dominant bacteria phyla (>1.0 % of total reading) for the four study sites: Ex-mine S. Acqua Bona (AB), Mine waste Sanna (CS), Naracauli (NR), and Rio Irvi banks (RI).

According to Shannon, Simpson and Inverse Simpson index, the biocrust bacterial biodiversity was highest for AB and CS sites, followed by RI and finally by NR (Fig. 3). The Shannon index ranged between 4.4 and 5, the Simpson index between 0.95 and 0.99, and the Inverse Simpson between 20 and 70.

3.2. Identification of cyanobacterial strains

We isolated 56 cyanobacterial morphotypes from the study sites: 10 from CS, 14 from RI, 13 from NR, and 19 from AB. Of all the isolated strains, we focused on the Nostocaceae family, which proved to have strong bioremediation properties (Morales et al., 2017; Williams et al., 2019). Notably, members of the Nostocaceae family were found to be present in all sampled sites, emphasizing their widespread distribution. After this screening, three *Nostoc-like* cyanobacteria strains (RI23, AB55, and NR64), filamentous with interconnected cells, heterocysts, and gelatinous mucilage surrounding the filaments, were selected (Fig. 4). 16S rRNA molecular analysis confirmed the three isolated cyanobacterial strains as *Nostoc commune* with similarity values ranging between 99.2 and 99.75 % according to NCBI database.

3.3. Growth curves and EPS production

Growth curves of the three isolated cyanobacteria strains were assessed by measurement of dry weight and chlorophyll *a* overtime. Both time and strain had a significant effect ($p < 0.001$) on dry weight and chlorophyll *a* content (Fig. 5). *Nostoc commune* AB55 strain showed the highest biomass and chlorophyll *a* content, reaching its maximum growth between 7 and 9 days and resulting in a biomass of 2.2 g L^{-1} and chlorophyll *a* content of 12.4 g L^{-1}, whereas strains RI23 and NR64 showed significantly lower biomass and chlorophyll *a* content (Fig. 5). The maximum dry weight recorded for these strains was between 1.1 and 1.3 g L^{-1}, while the maximum chlorophyll *a* content was approximately 2.7 g L^{-1}. The growth peak was reached after 9 days for strain RI23, after 12 days for strain AB55, and after 14 days for strain NR64.

There were significant differences in released EPS ($p = 0.000329$ at day 0 and $p = 0.00675$ at day 14) and total EPS ($p = 0.000171$ at day 0 and $p = 0.000942$ at day 14) between strains. The strains AB55 and NR64 showed the highest released EPS concentrations, both at the beginning (day 0) of the experiment and after 14 days. The strain RI23 showed a significantly lower released EPS content (Fig. 6). There were no significant differences in the production of released EPS between AB55 and NR64. The strain AB55 also exhibited the highest total EPS content, with an average value of 1786 mg L^{-1} after 14 days. The strains

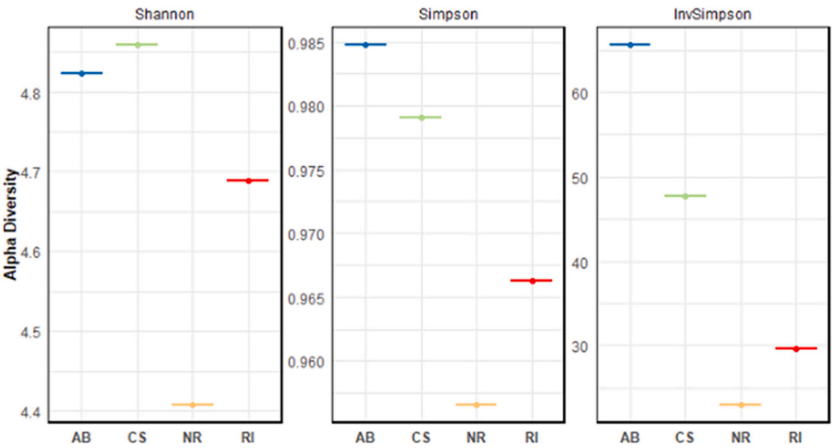

Fig. 3. α-diversity indices (Shannon, Simpson, Inverse Simpson) calculated for the four study sites: Ex-mine S. Acqua Bona (AB), Mine waste Sanna (CS), Naracauli (NR), and Rio Irvi banks (RI).

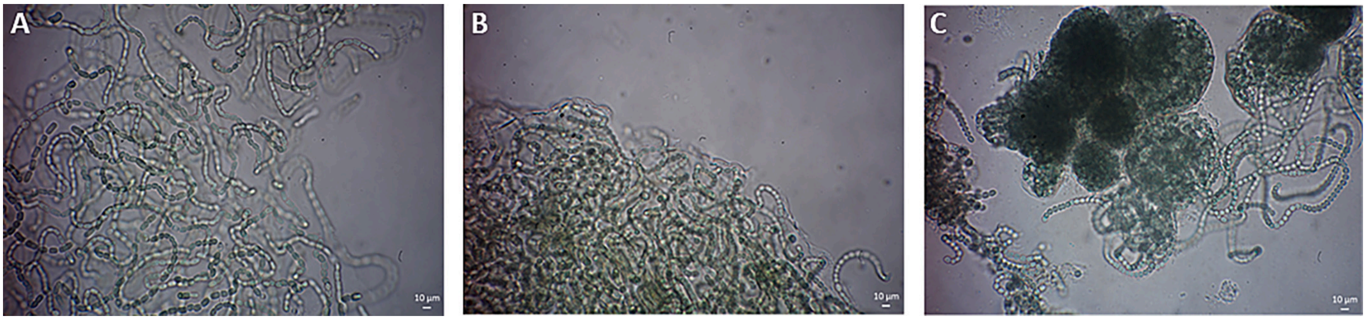

Fig. 4. Microphotographs of cyanobacterial strains isolated from biocrust: a) Nostoc commune RI23, b) Nostoc commune AB55, c) Nostoc commune NR64. Scale Bar = 10 µm.

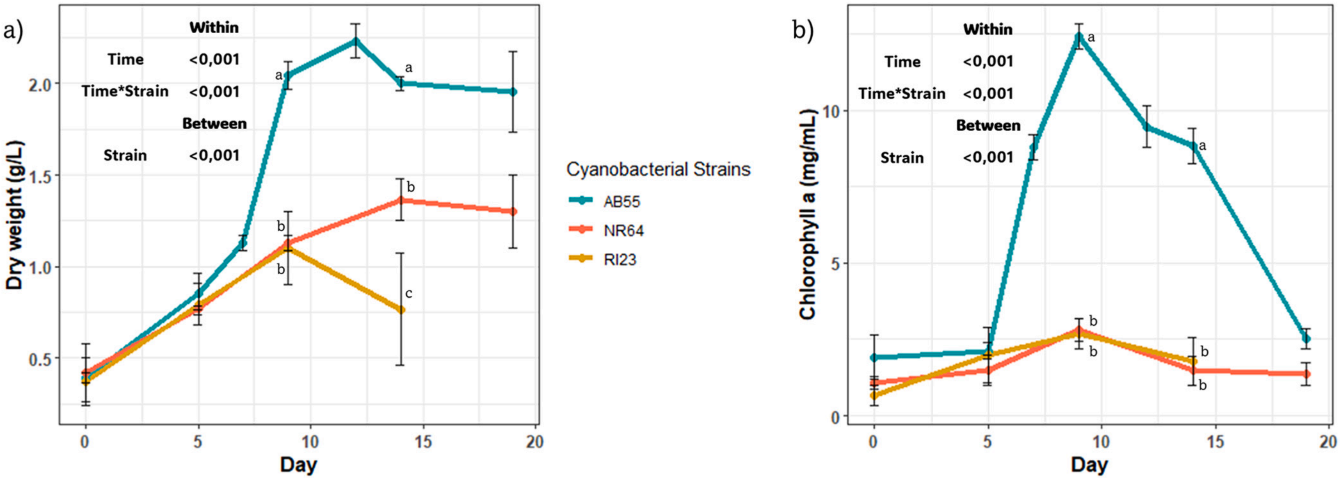

Fig. 5. Growth curves based on dry weight (a) and chlorophyll *a* (b) measurements. Means and standard deviations are presented along with *p*-values from repeated measures ANOVA for both “Between” and “Within” subject comparison. Different letters are included only in case of significant differences between strains at each day.

NR64 and RI23 showed a significantly lower total EPS content, with values consistently lower, both at the beginning and after 14 days of incubation.

3.4. Heavy metal immobilisation

The metal removal capacity differed according to the metal and the cyanobacteria strain (Fig. 7). The strains showed significant differences in Cu ($p = 0.000367$), Pb ($p = 0.000298$), and Zn ($p = 0.0355$) removal from the liquid medium. The AB55 and NR64 strains demonstrated the ability to immobilise all tested metals (Cu, Pb, Zn), whereas strain RI23 did not exhibit any Cu removal capacity. For Cu immobilisation, strain AB55 showed the highest removal capability, removing up to 88.9 % of the metal from the liquid medium, and strain NR64 immobilised up to

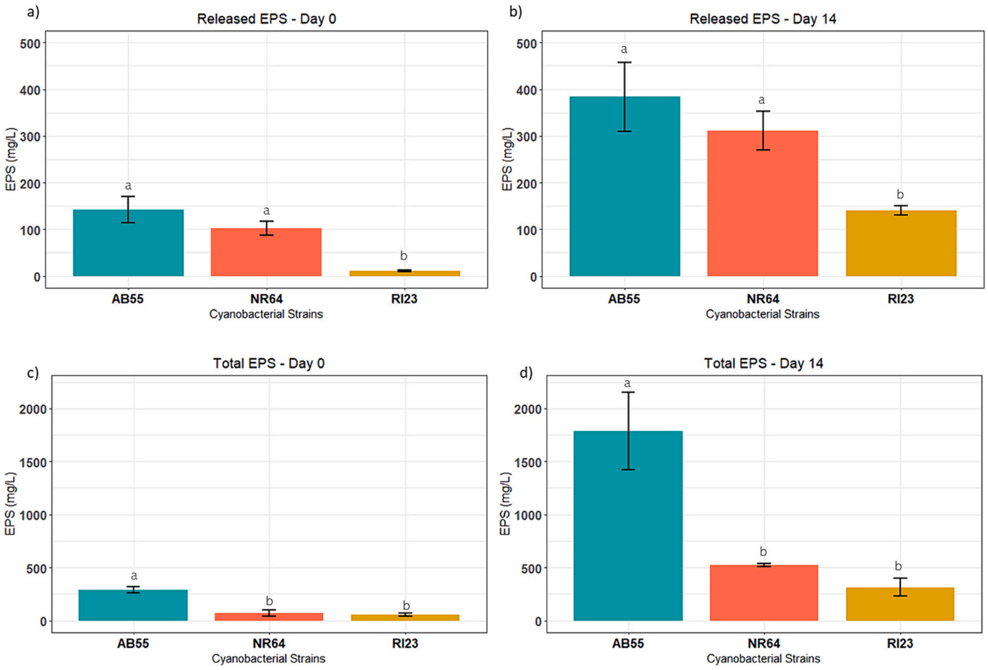

Fig. 6. Released (a and b) and total EPS (c and d) content in cyanobacterial cultures on day 0 and day 14. Letters mark significant differences ($p < 0.05$) in EPS production among different strains. All strains showed a significant increase in EPS production after 14 days.

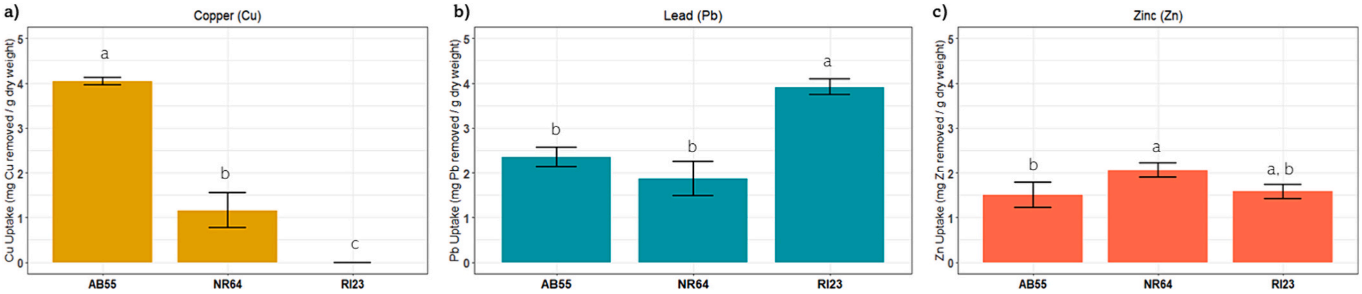

Fig. 7. Heavy metal immobilisation (q), expressed as mg of metal removed per g of biomass dry weight, by the three isolated cyanobacterial strains: *Nostoc commune* RI23, *Nostoc commune* AB55, and *Nostoc commune* NR64. Letters mark significant differences (p < 0.05) in q among different strains.

25.5 % of Cu. In the case of Pb, the strain RI23 showed the highest Pb removal, up to 86.2 % of Pb in solution. Strains AB55 and NR64 had a lower capacity for Pb immobilisation, at 40 % and 50 % respectively. All strains were less effective in Zn uptake, with percentages below 50 %. NR64 was the most efficient, removing 45.3 % of the Zn in solution, while AB55 was the least efficient, with only 33 % removed.

Fig. 8 illustrates the NMDS showing the different cyanobacteria strains, the content of both released and total EPS by the cyanobacterial strains, cyanobacteria capability to immobilise the selected heavy metals, and the content of these metals in the isolation environment. Average heavy metal concentrations on each study site are reported in Table 2. The NMDS ordination indicates an association between Pb content in the soil from which the cyanobacteria was isolated and the ability of cyanobacteria to immobilise this metal.

Such association between metal content in the cyanobacteria isolation sites and cyanobacteria removal capability was not observed for Cu and Zn. Meanwhile, Cu removal was associated with high contents of total and released EPS.

4. Discussion

4.1. Biocrust bacterial community composition

The microbial communities of biocrust samples from four heavy metal-contaminated sites in Sardinia were characterized by the presence of four predominant bacterial phyla: Cyanobacteria, Bacteroidota, Proteobacteria and Actinobacteria. These phyla were found in varying abundances across the samples, in agreement with previous studies on biocrust from arid soils (Moreira-Grez et al., 2019; Gabay et al., 2022; Vega-Cofre et al., 2023). Furthermore, while some less prevalent phyla were consistently present in all samples, others were exclusive to specific samples, such as Crenarchaeota, RCP2–54, WPS-2, Methylomirabilota, and Sumerlaeota, which were found solely in the RI sample. All these phyla collaborate to support the structure and functionality of biocrusts, contributing to nutrient cycling, soil conservation, and the maintenance of the ecosystem (Wang et al., 2023). Among these microorganisms, Cyanobacteria play a pivotal role as primary producers and pioneers in the establishment of biocrust communities (Román et al., 2018; Deng et al., 2020; Rubio and Lázaro, 2023). The relative abundances of cyanobacteria in the analysed samples, ranging from 13.3 % to 26 %, are relatively high compared to other phyla. This pattern is characteristic of biocrusts in their early stages of development,

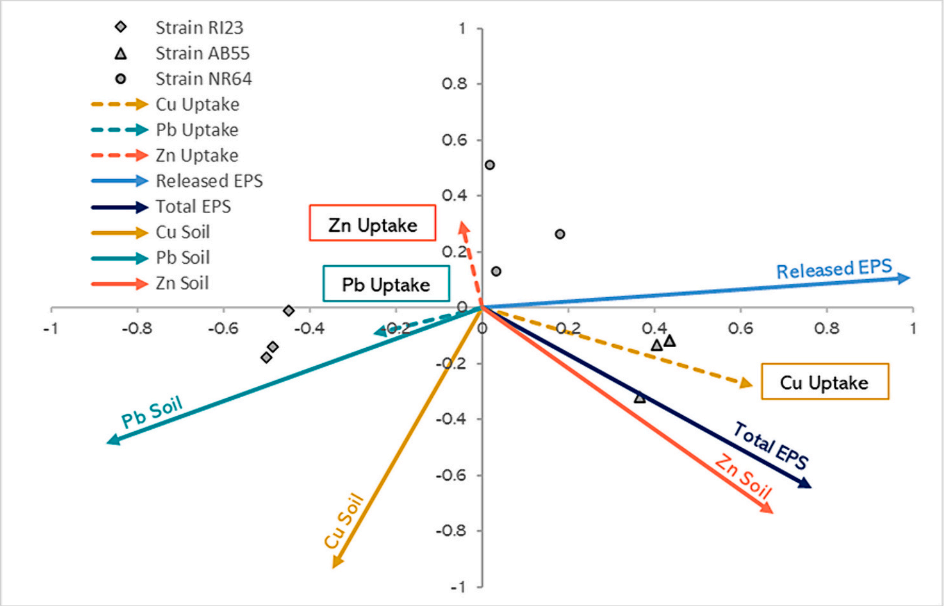

Fig. 8. Non-metric multidimensional scaling (NMDS) ordination of Biocrust cyanobacteria strains. Vectors indicate the magnitude and direction of correlations between the strains, EPS production, metal removal, and heavy metal content in the soil from which the strains were isolated.

Table 2
Heavy metal values for the four sampling sites.

Study sites	Al g kg^{-1}	Cr mg kg^{-1}	Mn g kg^{-1}	Fe g kg^{-1}	Ni mg kg^{-1}	Cu mg kg^{-1}	Zn g kg^{-1}	As mg kg^{-1}	Cd mg kg^{-1}	Pb g kg^{-1}
CS	15.02	34.97	2.33	42.51	23.92	89.89	8.54	44.07	33.06	1.78
RI	7.05	36.47	3.43	62.7	30.88	303.25	12.27	115.68	40.42	16.04
NR	4.39	27.21	3.99	69.25	31.97	178.59	24.77	129.41	82.85	2.32
AB	4.42	18.72	32.56	42.55	395	234.57	229.57	119.88	233.47	2.51

where cyanobacteria are the primary colonizers and dominant (Miralles et al., 2020; Lan et al., 2022). The decrease in the proportion of cyanobacterial abundances in AB and RI was accompanied by an increased proportion of other soil bacterial phyla, such as Bacteroidota, Actinobacteriota, Proteobacteria, and Acidobacteriota. All these phyla play a key role in nutrient cycling and are involved in the decomposition of soil organic matter. Actinobacteria break down complex organic matter, such as cellulose and lignin (Bao et al., 2021), Acidobacteria are adapted to acidic pH conditions and play a role in soil stabilization (Kim et al., 2016; Kalam et al., 2020). Proteobacteria have been recognized for their significant contribution to combating wind erosion and facilitating nitrogen fixation (Gundlapally and Garcia-Pichel, 2006). The presence of these phyla in contaminated sites suggests their potential tolerance to heavy metals. It is also hypothesized that some members of these phyla may be actively involved in either the bioaccumulation or the reduction of heavy metals (Jroundi et al., 2020; Fathollahi et al., 2021).

Regarding the biological diversity of the studied biocrusts, the higher Shannon index for CS and AB in comparison to RI and NR indicates a greater diversity within the community of microorganisms. This suggests that there are likely many different species present, and these species are distributed relatively evenly within the biocrust samples. Meanwhile, the lower Simpson values also found in NR and RI, indicate a reduced dominance of specific species within the biocrust samples. Compared to other studies assessing microbial community diversity in cyanobacteria-dominant biocrusts (Miralles et al., 2020; Wei et al., 2022; Barrón-Sandoval et al., 2023), biocrusts from our study sites exhibit lower α-diversity values. This lower α-diversity can be explained by the heavy metal contamination in the study sites. Metal toxicity harm sensitive species, and competitive interactions between species could benefit resistant ones leading to alterations in soil community relationships (Li et al., 2020; Shuaib et al., 2021).

Limited research has been conducted on the analysis of biocrust composition in contaminated sites. Previous studies examining the effect of various disturbance factors on biocrusts also demonstrate that disruptive agents such as heavy metal contamination, grazing disturbance, and other human activities, lead to a decrease in the number of species and functional traits within both plant and bacteria forming biocrust communities (Bao et al., 2019; Gabay et al., 2022; Sánchez et al., 2022). On the whole, analysis of microbial community composition and diversity in biocrusts from metal-contaminated sites contributes to a better understanding of how the presence and concentration of different metals may affect biocrust structure and composition as well as identify potential microorganisms able to grow under these conditions and potentially applicable as inoculum for soil bioremediation.

4.2. Growth and EPS production capabilities of isolated cyanobacterial strains

The isolation of the cyanobacterium *N. commune* from the three different study sites confirms its ability to adapt to terrestrial environments and its cosmopolitan distribution. Indeed, its presence extends across diverse climates, ranging from tropical zones to polar regions, as reported in other studies (Tamaru et al., 2005; Sand-Jensen and Jespersen, 2012; Roncero-Ramos et al., 2019).

Our results reveal distinct growth capabilities of isolated *N. commune* strains (AB55, RI23, and NR64) as shown by the substantial variability in biomass production and chlorophyll levels across the three strains. Indeed, strain AB55 stands out for its productivity, while strains RI23 and NR64 exhibit lower performance (Fig. 5). The results obtained match with recent studies on *N. commune* growth in the same medium and similar growth conditions (Diao and Yang, 2014), where different strains have demonstrated varying in growth ranges. Cyanobacterial biomass increases in parallel with an increase in EPS synthesis (Chamizo et al., 2020). The AB55 strain showed the highest growth and the highest total and released EPS amount. Nonetheless, strain NR64, which showed a limited growth in the liquid medium, synthesized a high amount of released EPS, comparable to that of strain AB55. This variability in EPS synthesis among strains is consistent with values previously reported for cyanobacteria where strains belonging to the same species exhibited differences in terms of polysaccharide production (Cruz et al., 2020; Alvarez et al., 2021). Variability in both biomass and EPS production among different strains of *N. commune* may be attributed to intraspecific genetic variations that affect their ability to adapt to specific culturing conditions. Genetic mutations or differences in genetic composition could lead to variations in biomass, chlorophyll, and EPS production (Wright et al., 2001; Jungblut et al., 2021). Another explanation could be that some strains might be adapted to grow and produce EPS in specific environmental conditions, showing increased growth and EPS production only in particular contexts. Indeed, *N. commune* strains have exhibited variations in growth and EPS production in response to changes in temperature and light conditions, as well as differences in composition and pH of the growth medium (Pereyra and Ferrari, 2016; Tripathi et al., 2019). In the examined sites, the soil's physico-chemical alterations resulting from contamination may stimulate EPS production, thereby potentially augmenting their capacity for heavy metal immobilisation.

This information can be valuable for selecting suitable strains for large-scale cyanobacterial biomass production for biotechnological applications, particularly applicable in agriculture and bioremediation.

4.3. Cyanobacteria potential for heavy metal immobilisation

Cyanobacteria have been regarded as a potential tool for heavy metal removal due to their efficient metal-binding capabilities and implementation of mechanisms such as biosorption, bioaccumulation, and biotransformation (Chakdar et al., 2022). Our experimental findings indicate that all three isolated strains exhibit the capability to immobilise the selected heavy metals, except for strain RI23, which appears ineffective in removing Cu. Notably, there are substantial variations in immobilisation capacity, particularly for Cu. Among the tested metals, Cu stands out with the highest absorption capacity, and strain AB55 demonstrates remarkable efficiency by removing up to 88.9 % of this metal from the liquid solution. These results are consistent with previous studies conducted on cyanobacteria from different environments, not associated with biocrusts, which described variations in heavy metal absorption across different cyanobacterial species and strains within the same species (Micheletti et al., 2008; Roy et al., 2015; Atoku et al., 2021). These significant differences in Cu immobilisation could be attributed to the differential production of EPS by *N.commune* strains. Indeed, the three analysed strains exhibit a correlation between both total and released EPS production and Cu removal, a relationship that is absent for the other analysed heavy metals (Fig. 8).

The presence of EPS produced by cyanobacteria enhances the biosorption process, which is considered the major mechanism for the removal of heavy metals, involving several mechanisms, such as ion exchange, adsorption, surface complexation, precipitation, and chelation (Bhatt et al., 2022; Ciani and Adessi, 2023). The EPS matrix contains abundant ionizable functional groups, such as carboxyl and hydroxyl groups. These groups can interact with cationic species in a solution, indicating the potential for serving as metal-binding sites (De Philippis and Micheletti, 2017; Priya et al., 2022). A recent investigation showed that EPS extracted from sewage sludge had higher efficacy in Cu removal compared to other heavy metals examined in the study (Pb, Ni, and Zn) (Pagliaccia et al., 2022). This finding aligns with the outcomes obtained in our research. For the other two tested metals (Pb and Zn), no clear correlation was found between EPS production and metal immobilisation (Fig. 8). Pb and Zn removal by *N.commune* strains may be attributed to different mechanisms or factors not directly related to EPS production, such as active bioaccumulation, intracellular complexation with ligands, and uptake through active processes (Al-Amin et al., 2021).

According to the NMDS analysis, which demonstrates an association between the presence of Pb in the site of cyanobacterial isolation and the removal capacity of this metal by the strain isolated from the site in question (RI23), the observed differences in Pb immobilisation could arise from environmental adaptation. Cyanobacterial adaptation to specific Pb-contaminated environments may enhance their abilities to immobilise Pb through a range of physiological and biochemical adaptations that enable them to survive and thrive under stressful conditions (Chakdar et al., 2022). These changes may include the expression of specific genes involved in Pb resistance and detoxification, as well as the activation of metabolic pathways that promote lead absorption and reduction within bacterial cells (Tripathi and Poluri, 2021; Priya et al., 2022).

Our findings are consistent with earlier studies that have identified the ability of *N. commune* strains to retain heavy metals. Specifically, the infrared analysis of cyanobacterial biomass revealed the presence of essential functional groups such as amino, carboxyl, hydroxyl, and carbonyl groups, demonstrating their pivotal role in the biosorption of Cd and Zn (Morsy et al., 2011). Additionally, in another research, due to its proficiency in accumulating heavy metals, *N. commune* was suggested as a model species for monitoring heavy metal pollution (Patova et al., 2000). All these studies have been conducted in aquatic environments and focused on their application for wastewater decontamination. However, they also open the possibility for their use in the immobilisation of metals from the soil, an aspect that has been barely explored to date.

The demonstrated capabilities of the analysed cyanobacteria strains for heavy metals removal suggest them as promising candidates for application in the bioremediation of both agricultural soils and water.

5. Conclusions

The present study offers insightful information about the possible application of native cyanobacteria for metal immobilisation as a bioremediation technique for sustainable agriculture and soil restoration. By comprehending the intricate relationship among microbial communities, strain diversity, and metal immobilisation processes, we facilitate the creation of novel bioremediation approaches customized to environmental settings. From the findings of this study, it is evident that the diversity of cyanobacteria in biocrusts from contaminated soils is lower than in uncontaminated soils, suggesting that only some adapted species can survive in these environments. Consequently, three strains of *Nostoc commune* have been isolated, showcasing remarkable metal-binding capabilities. These strains can absorb up to 88.9 % of copper (Cu), 86.2 % of lead (Pb), and 45.3 % of zinc (Zn) in liquid medium. The high metal removal capability of the isolated native cyanobacteria supports their potential use as soil inoculants to immobilise a wide range of heavy metals, thus representing an environmental-friendly tool for bioremediation of metal-contaminated sites to combat their harmful effects on agricultural productivity and ecosystem health. Future research on the bioremediation capability of native cyanobacteria, in conjunction with developments in biotechnological applications, holds significant promise for reducing the negative consequences of soil contamination and advancing environmental sustainability.

CRediT authorship contribution statement

Carlotta Pagli: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Sonia Chamizo:** Writing – review & editing, Supervision, Investigation, Formal analysis, Conceptualization. **Giada Migliore:** Writing – review & editing, Resources, Investigation. **Lorenza Rugnini:** Writing – review & editing, Supervision. **Giovanni De Giudici:** Writing – review & editing,

Resources. **Roberto Braglia:** Writing – review & editing, Supervision. **Antonella Canini:** Writing – review & editing, Supervision. **Yolanda Cantón:** Writing – review & editing, Visualization, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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