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Semi-continuous cultivation of EPS-producing marine cyanobacteria: A green biotechnology to remove dissolved metals obtaining metal-organic materials

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ABSTRACT

Given the necessity for bioprocesses scaling-up, the present study aims to explore the potential of three marine cyanobacteria and a consortium, cultivated in semi-continuous mode, as a green approach for *i*) continuous exopolysaccharide-rich biomass production and *ii*) removal of positively charged metals (Cu, Ni, Zn) from mono and multi-metallic solutions. To ensure the effectiveness of both cellular and released exopolysaccharides, weekly harvested whole cultures were confined in dialysis tubings. The results revealed that all the tested cyanobacteria have a stronger affinity towards Cu in mono and three-metal systems. Despite the amount of metals removed per gram of biomass decreased with higher biosorbent dosage, the more soluble carbohydrates were produced, the greater was the metal uptake, underscoring the pivotal role of released exopolysaccharides in metal biosorption. According to this, *Dactylococcopsis salina* 16Som2 showed the highest carbohydrate productivity (142 mg L^{-1} d^{-1}) and metal uptake (84 mg Cu g^{-1} biomass) representing a promising candidate for further studies. The semi-continuous cultivation of marine cyanobacteria here reported assures a schedulable production of exopolysaccharide-rich biosorbents with high metal removal and recovery potential, even from multi-metallic solutions, as a step forward in the industrial application of cyanobacteria.

Introduction

Toxic metals are one of the most severe concerns threatening human and environmental health due to their harmful and non-biodegradable nature. Metals pollution is mostly caused by anthropogenic activity; in particular mining, electroplating, and tanneries industries produce effluents with high metals content [1]. Several technologies have been proposed so far for the abatement of these pollutants to meet regulatory standards limits, such as ion exchange, chemical precipitation, solvent extraction, membrane filtration, and adsorption [2,3]. Nevertheless, there are some constraints to the feasibility of these methods, including the high energy and cost requirements, the low efficiency at low metals concentration and/or the poor selectivity as well as the generation of secondary wastes [2,4,5]. In addition, the necessity to reduce waste production by also implementing their recovery and reutilization has become pivotal during the last decades. Hence, developing eco-compatible technologies to meet circular economy standards is challenging nowadays.

In this context, biosorption is one of the most promising alternative strategies for metal removal, making possible the recovery and the reuse of the biosorbed pollutants from biological material, including cyanobacteria [6].

Cyanobacteria are cosmopolitan photoautotrophic microorganisms able to withstand various environmental conditions [7]. One of the strategies developed for cell protection against abiotic and biotic stresses, as well as for adhesion and motility, is the production of exocellular polymeric substances [8,9]. These complex polymers are mainly made by exopolysaccharides (EPS) and, to a lesser extent, proteins, nucleic acids, lipids, and vitamins [8]. Cyanobacterial EPS include a more condensed fraction (i.e., the capsule, a thick layer around the cell, or the sheath, a thin layer surrounding cells or cell groups) and/or a less condensed fraction, loosely associated with the cells and without a

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defined structure, named slime. Polysaccharides belonging to these fractions can be partly released and solubilized in the surrounding medium, taking the name of released polysaccharides (RPS) [8,10]. Cyanobacterial EPS are pretty often characterized by a strong anionic character due to the presence of one or two different uronic acids as well as sulfate, carboxylic, and phosphate groups, representing potential binding sites for positively charged metals, thus promoting biosorption process [3,6,11]. In addition, the physicochemical properties of EPS, such as their rheological, emulsifying, and sticky properties, make them attractive polymers in several applications [12]. Hence, the implementation of a circular approach coupling the exploitation of cyanobacteria for the bioremoval of metallic ions from wastewaters and the valorization of the metal-organic materials recovered may lead to several advantages from an environmental and economic point of view [7]. Since biosorption is mainly based on the rapid, reversible, and passive binding of ions from aqueous solutions onto functional groups on the surface of the biomass [13], the cells are not directly affected by metals concentration, which are toxic at high values; besides, they may undergo several sorbing/desorbing cycles, promoting a longer shelf life of these biosorbents, compared to the bioaccumulation process [6,14,15].

Many unicellular or filamentous cyanobacteria (e.g., belonging to *Anabaena*, *Nostoc*, *Tolypothrix*, *Cyanothece,* and *Synechococcus* genera) are able to produce huge quantities of EPS [9,10]. Within EPS-producing cyanobacteria, marine cyanobacteria represent promising candidates for biosorption process, due to the massive production of EPS [9,16–18], and their cultivation requirements, since the higher pH and salinity compared to freshwater cultures can reduce the contamination risk, while seawater can be used as water source to decrease the water footprint of the process [16,19,20].

The role of cyanobacteria in the biosorption process has already been demonstrated but the studies are often reduced to a few strains or metals, generally suspending cells in metal-containing solutions [21,22]. In contrast to employing free cells, the utilization of confined systems holds the potential to enhance metal removal by harnessing soluble components such as RPS. Additionally, these systems offer advantages in efficiently recovering spent biosorbents. This facilitates their reuse in successive adsorption-desorption cycles, recovery and return of the metals to the production cycle, or valorization of the resulting metallic-organic materials, for example as hybrid catalysts or nanotechnology sector [7,14,23]. This approach may support the scaling-up and the circularity of the process. Additionally, the works that have been presented so far exploit biomasses obtained through batch cultivation [11,22] without considering other strategies to produce constant biomass in terms of quantity and quality (e.g., semi-continuous cultivation). According to these considerations, this work aimed to explore the Cu(II), Ni(II), and Zn(II) biosorption potential by three marine unicellular cyanobacteria and a consortium cultivated in semi-continuous mode as a strategy to get a constant weekly production of EPS-rich biosorbents. Weekly-harvested whole cultures of cyanobacteria were confined in dialysis tubings, and the effect on Cu(II), Ni(II), and Zn(II) removal of several parameters, including soluble and cellular carbohydrate content, biosorbent concentration and contact time, was investigated while metal uptake data were fitted with adsorption isotherms. Since more than one metals are generally present in real wastewater [24], the effect of the simultaneous presence of the three metals in a multi-metal system was also studied.

Material and methods

Selection and identification of the cyanobacteria used as biosorbents

Four cyanobacteria cultures from the Cyanobacteria Cultures Collection of the Department of Agriculture, Food, Environment and Forestry (via Maragliano 77, University of Florence, IT) that were previously isolated from saline environments [25] were selected for their ability to remove HM [26]. The cultures were kept in 250-mL Erlenmeyer flasks containing sea-water medium enriched as follows (g L^{-1}): $NaNO_3$, 1.5; Na_2HPO_4, 0.04; $NaHCO_3$, 0.1; NaCl, 28; ferric ammonium citrate, 0.006; citric acid, 0.006; Na_2 EDTA, 0.001 and 0.5 ml L^{-1} of trace metal solution [25] inside a rotatory shaker (Innova 44B, New Brunswick, USA) at a constant temperature of 30 °C, light intensity of 15 μmol photons m^{-2} s^{-1} and stirring speed of 100 rpm.

To extract genomic DNA, the bacterial cultures were centrifuged and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0) at OD600 = 1, then 740 μL of the bacterial suspension was added with 20 μl 100 mg mL^{-1} lysozyme and incubated at 37 °C for 30 min. Subsequently, 40 μL 10 % (w/v) SDS and 8 μL 10 mg mL^{-1} proteinase K were added, and the bacterial suspension was incubated at 56 °C for 1 h. After centrifugation, the supernatant was 10-fold diluted and used as a template for 16 S rRNA gene amplification.

The 16S rRNA gene was amplified with cyanobacteria-specific primers CYA106F and an equimolar mixture of CYA781R(a) and CYA781R(b) [27]. The reactions were performed in a 20 μL volume containing 2 μL of DNA. The composition of the reaction mixture was the following: 1 × reaction Buffer, 2 % tween 20, 200 μM of dNTPs, 0.1 mg mL^{-1} of bovine serum albumin, 0.1 μM of each primer, and 0.05 U of Taq DNA polymerase (Thermo Scientific, Massachusetts, U.S.A.). PCR conditions consisted of an initial denaturation at 95 °C for 5 min, 30 cycles at 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 90 s, and a final extension at 72 °C for 8 min. The PCR amplicons were purified with the Genejet PCR purification kit (Thermo Scientific) and sequenced.

FASTA files of closely related sequences were retrieved from the Genbank database (NCBI) and used for phylogenetic analysis. The sequences were aligned in MEGA 11 software [28], and a phylogenetic tree was constructed using the neighbor-joining method and Jukes–Cantor distance with 1000 bootstraps. The 16S rRNA gene amplicon sequence data are available at the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) with the accession numbers OQ945753 - OQ945752 - OQ945751.

Growth condition

The cultures were grown adopting seawater-enriched growth medium [25], as previously described, in 1-L bubbled flat glass bottles with an illuminated surface-to-volume ratio of 25.5 m^{-1}. The bottles containing 0.8 L of culture, were bubbled with a sterile air/CO_2 mixture (99:1, v-v) at a flow rate of 0.2 L per L of culture per minute to support biomass and carbohydrate productivity. The amount of CO_2 was regulated to maintain pH 9 ± 0.2. The temperature of the room was kept at 25 °C ± 1. The cultures were continuously illuminated by a LED lamp with 200 μmol photons m^{-2} s^{-1} receiving 0.35 mol photons daily. A semi-continuous harvesting regimen was adopted after a week of growth, replacing 50 % of the culture volume with fresh medium once a week.

Experimental plan

The cyanobacteria were cultivated for three weeks, monitoring chlorophyll *a*, cellular, and soluble carbohydrate content. 0.4 L of each culture were weekly harvested to evaluate their capability to remove a specific metallic ion from aqueous solutions through biosorption assays (specifically described in “Biosorption experiment” section of material and methods). A total of 3 harvests per culture were used to check the removal of Cu^{2+}, Ni^{2+}, and Zn^{2+} (henceforth, Cu, Ni and Zn, respectively). Three stock solutions of 1 g L^{-1} Cu ($CuCl_2$), Ni ($NiCl_2$), and Zn ($ZnCl_2$) were prepared and used to obtain the starting solutions of the assays. Two different experiments, named biomass and metal assays, were set for each harvest, carried out to i) assess the effect of biomass and carbohydrates concentration on metal removal and ii) identify the adsorption isotherm that fits better with this process; respectively.In biomass assay, the specific uptake (expressed per gram of biomass) and

the absolute metal removal from an initial metal concentration of 10 mg L^{-1} were evaluated adopting four biomass dilutions: undiluted cultures as well as 1.5, 2, and 3-fold diluted cultures. In the metal assay, four different starting metal concentrations in the solutions were tested: 2.5, 5, 10, and 15 mg L^{-1} of Cu, Ni, and Zn. Each assay lasted 24 h and three replicates for each sample were set. In addition, three replicates of the blank, consisting of pre-treated cultivation medium, were added for each condition. The cultures with higher efficiency in metal removal were cultivated for one week as described above and adopted in three-metal systems to evaluate the effect on metal biosorption of the simultaneous presence of more than one metal in the solution. The schematic representation of the experimental workflow is shown in Fig. 1.

Chlorophyll a, biomass, and carbohydrate quantification

The quantitative analysis of chlorophyll *a* (Chl*a*) was performed to monitor the growth of cyanobacteria, the extraction was carried out adopting ethanol 96 %, following the protocol described by Ritchie [29]. Cellular and soluble carbohydrates were quantified in the harvested cultures every week, while total carbohydrate concentration was measured on pre-treated cultures (the pre-treatment is described in the following section "Biosorption experiments"). For cellular, soluble, and total carbohydrates (cCH_2O, sCH_2O, tCH_2O, respectively) quantification, the phenol-sulfuric acid method [30] was adopted for pelletized cells, the supernatant obtained after centrifugation (7000 *g*, 10 min), and for whole cultures obtained after the pretreatment, respectively. To evaluate biomass productivity during the semi-continuous cultivation and the biomass concentration of the biosorbents, biomass dry weight quantification was carried out after the pre-treatment since the measurements during the growth were not reliable due to the viscosity of the cultures. Thus, cell suspensions obtained after the pre-treatments were filtered on dry pre-weighted MCE membrane filters (0.45 μm pore size, 47 mm diameter) and weighted after drying the filters at 105 °C per 4 h. Weekly data on tCH_2O and DW concentration (*C*) of the biosorbents obtained after pre-treatment of the cultures in the three weeks of growth under a semi-continuous regimen were adopted to evaluate metal specific-uptake during biosorption experiments (see section below) and to calculate average carbohydrate and biomass volumetric productivity (VP, Eq. (1), expressed as g tCH_2O L^{-1} d^{-1} and g DW L^{-1} d^{-1}, respectively), carbohydrate productivity per gram of biomass (BP, Eq. (2), defined as g tCH_2O g^{-1}DW) and biomass productivity per illuminated surface (ISP, Eq. (3), expressed as g DW m^{-2} d^{-1}) and per amount of photons (PhP, Eq. (4), expressed as g DW mol $Ph.^{-1}$ d^{-1}), for each week, adopting the following formula:

$$VP = (C_n - C_{n\text{-}7})/7 \quad (1)$$

$$BP = (C_{CH2On} \times V)/(C_{DWn} \times V) \quad (2)$$

$$ISP = (VP \times V)/IS \quad (3)$$

$$PhP = (VP \times V)/Ph \quad (4)$$

where *V* is the volume of the culture (L), *n* is the day of the harvest of the culture; IS is the surface of the flat bottle illuminated by the LED light (m^2), *Ph* is the number of mol of photons received by the culture in a day (mol ph.).

Biosorption experiments

For bioremoval assays (Fig. 1), the cultures were confined in large nitrocellulose dialysis tubings (MW cut-off 12–14 kDa, S/V 1.9 cm^{-1}, Medicell Membranes Ltd, UK), dialyzed first against deionized water (16 h), then against HCl 0.1 M (30 min), and finally against deionized water overnight. According to the literature [4,6], the pre-treatment is necessary to remove the highly concentrated salts of the cultivation medium through an ion-exchange mechanism, making available the binding sites for HM. The same pre-treatment was carried out with dialysis tubings only containing the cultivation medium, which was taken as blank. To assess the biosorption ability of the strains, the pre-treated cultures or the blank confined in smaller dialysis tubings (MW cut-off 12–14 kDa, S/V 3.7 cm^{-1}) were dipped in Cu, Ni, or Zn solutions and maintained under controlled conditions (25 °C for 24 h, continuous agitation). The pH of the metal solutions was kept in the range of 5–5.5 [1] during the assays by adding NaOH or HCl 0.5 M to avoid metal precipitation and the effect of different pH due to varying types of biosorbents. In biomass assay, the cultures were diluted with distilled water after the pre-treatment. Residual Cu, Ni, and Zn concentrations in the HM solutions were determined after 24 h since preliminary trials revealed that 24 h were enough to reach the equilibrium (unpublished results). The HM quantification was carried out by adopting an adaptation of the bicinchoninate,

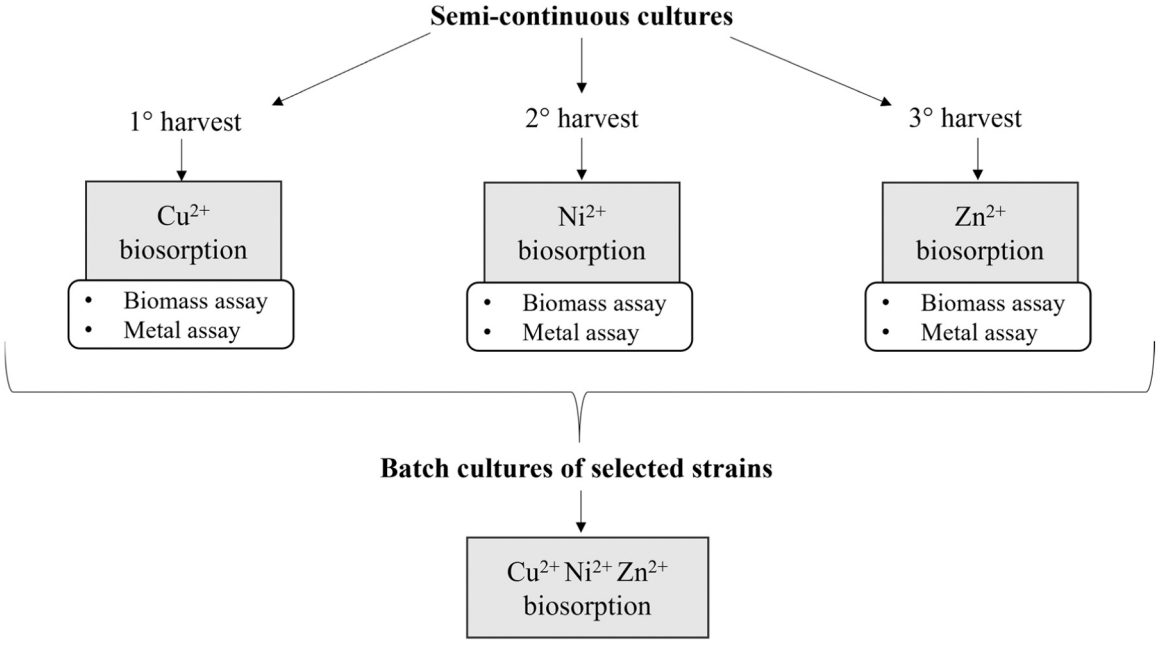

Fig. 1. Schematic representation of the experimental workflow. The aim and the set-up of each assay are specifically described in "Experimental plan" section.

1-(2-pyridylazo)-2naphthol and Zincon method (HI 93702, HI93740 and HI93731 reagents kit, respectively, Hanna Instruments Srl, Italy) following manufacturer's instructions. For the three-metal assay, a starting solution containing 10 mg L^{-1} of Cu, 10 mg L^{-1} of Ni, and 10 mg L^{-1} of Zn was prepared, and the trial was set as described above. After 24 h, the amount of metal was quantified by ICP-OES (iCAP 7400 ICP-OES Analyzer, Thermo Fisher Scientific, US) following the method APAT CNR IRSA 3020 Man 29 2003.

The μg of metal removed were calculated by subtracting the value of the metal solution treated with cyanobacterial culture from the value of the blanc and multiplying the result for the volume of the solution. Specific metal uptake q, expressed as mg of metal removed per gram of biomass or carbohydrate, was calculated as follows:

$$q = V(C_b\text{-}C_t)/m \quad (5)$$

where V is the volume of the metal-containing solution (L), C_b and C_t represent the metal concentration (mg L^{-1}) in the blanc and the treated samples, respectively, m is the amount of biomass or carbohydrate in the biosorbent (g) obtained multiplying the DW to the volume of the biosorbent. For the three-metal assay, specific uptake was divided for the atomic weight of the single metals, and the result was expressed as mmol of metal g^{-1} DW.

Adsorption isotherms

The data obtained from the metal bioremoval assay were plotted according to the two most widely used isotherms for biosorption (i.e., Langmuir and Freundlich) [31] for each biosorbent and metal as previously described [21,32].

Langmuir adsorption isotherm is expressed in its simplified form:

$$C_e(q_e)^{-1} = (bq_{max})^{-1} + C_e(q_{max})^{-1} \quad (6)$$

Where q represents the mg of metal adsorbed per g of adsorbent; q_{max} is the amount of metal adsorbed at saturation representing total biomass binding sites; b is a constant related to the energy and affinity of adsorption; and C_e is the equilibrium (final) concentration of the metal in the solution, expressed as mg L^{-1}.

Freundlich adsorption isotherm is expressed in its linearized form:

$$\ln q = \ln K_f + n^{-1} \ln C_e \quad (7)$$

where q represents the mg of metal adsorbed per g of adsorbent; K_f and n are constants related to the adsorbent capacity and the energy of sorption, respectively; and C_e is the equilibrium (final) concentration of the metal in the solution, expressed as mg L^{-1}. For each model, the coefficient of determination (R^2) as well as the model's parameters (q_{max} and b for Langmuir, K_f and n, for Freundlich), were estimated to check the fitting of the model and to compare different biosorbents.

Scanning electron microscopy (SEM) observations

Confined 16Som2 cultures were harvested after contact with Cu solution (16Som2 + Cu) and then centrifuged or freeze-dried. Centrifuged cells were fixed with 2.5 % glutaraldehyde in phosphate buffer (0.1 M, pH 7.0) at 4 °C overnight. The dehydration was performed using an ethanol series (25–100 %; v/v). Then, the samples were subjected to Emitech K850 Critical Point Drying (CPD) using liquid CO_2 as transitional fluid. The same treatment was performed on confined 16Som2 culture after the contact with water solution as negative control (16Som2 + H_2O). All the samples were gold-coated twice using Emitech K500x sputter at 35 mA for 3 min. Specimens were observed using Philips SEM 515 at 18 kV coupled with a Pentax K-5 camera to acquire micrographs.

Data analysis

Data are shown as absolute removal (μg of metal removed) or specific metal uptake, expressed as mg metal g^{-1} DW of biomass, to compare data in the literature, mmol of metal g^{-1} DW of biomass, to consider the stoichiometric effect in the multi-metal assay, or mg metal g^{-1} tCH_2O, to evaluate the effect of carbohydrate content. The effect of biomass concentration on Cu, Ni, and Zn biosorption was analyzed with one-way ANOVA and Fisher's LSD test at the 5 % significance level. As different experiments were set for each biosorbent, one-way ANOVA was performed on specific or absolute HM removal through a multiple comparison among different biomass dilutions of the same biosorbent. Pearson product-moment correlation was performed between biomass dry weight, cellular and soluble carbohydrate content, total carbohydrates content, and biomass dry weight with absolute metal removal for each biosorbent and each metal. The correlation coefficients are associated with a P-value that tests the estimated correlations' statistical significance at the 95 % confidence level. Statistical analyses were conducted using the STATGRAPHICS® Centurion XVI software (Statgraphics Technologies, Inc., The Plains, Virginia).

Results and discussion

Cyanobacteria identification

All the cultures of selected cyanobacteria presented cylindrical-oval cells, 6–9 μm long. The presence of EPS was assessed by staining the cells with Alcian blue 0.1 % (Fig. 2). Sequencing of the 16S rRNA gene from cyanobacterial cultures showed that ET5, CE4 and 16Som2 were single cyanobacterial strains. At the same time, VI 22M was not formed by a single cyanobacterial strain. Microscopic observation of VI 22M suggested it should be a consortium of *Cyanothece*-like strains.

The evolutionary relationships of ET5, CE4, 16Som2 and other cyanobacteria whose sequences are available in the GenBank database (NCBI) are represented in the phylogenetic tree (Fig. S1). According to the phylogenetic tree and the similarity percentage in the Genbank database, ET 5 resulted closely related to *Halothece* sp. WR8 (99.99 %), CE 4 to *Cyanothece* sp. 115 (99.99 %), and 16Som2 to *Dactylococcopsis salina* PMC1177.19 (99.99 %). These genera belong to the *Cyanothece* cluster 3 "*Halothece*", order of Chroococcales, which includes extreme halotolerant, moderate thermophiles, and diazotrophs [33,34]. Cyanobacteria from *Cyanothece* genus have been often used for different application, including EPS and phycocyanin production, metal removal and nanoparticles synthesis [11,34–37].

Biomass and carbohydrate productivity

Chlorophyll *a* content and biomass and carbohydrate concentration evaluated during cyanobacteria cultivation in semi-continuous mode are shown in the Supplementary material (Fig. S2, Table S1). The average biomass and carbohydrate productivities were summarized in Table 1. 16Som2 revealed the highest total carbohydrate productivity:143 mg tCH_2O per Liter of culture per day, resulting in 1.56 g of tCH_2O per gram of biomass. These values consider the cellular and the soluble components that remained in the dialysis tubings after the pre-treatment (MW > 12 kDa). Kushwaha et al. [38] found lower carbohydrate productivity value for cyanobacteria cultivated under different abiotic conditions. Even though, they were able to maximize biomass and carbohydrate production, adopting a two-stage growth, suggesting that nitrogen starvation can be used for carbohydrate accumulation. The differences in terms of biomass productivity between tested cyanobacteria were not as marked (Table 1), showing values in line with those established by many cyanobacteria cultivated under standard conditions [38–40]. Differentiating cellular and soluble carbohydrate production, quantified before the pre-treatment, the cultures of 16Som2 and VI 22M generally showed the highest carbohydrate concentration (Supplementary

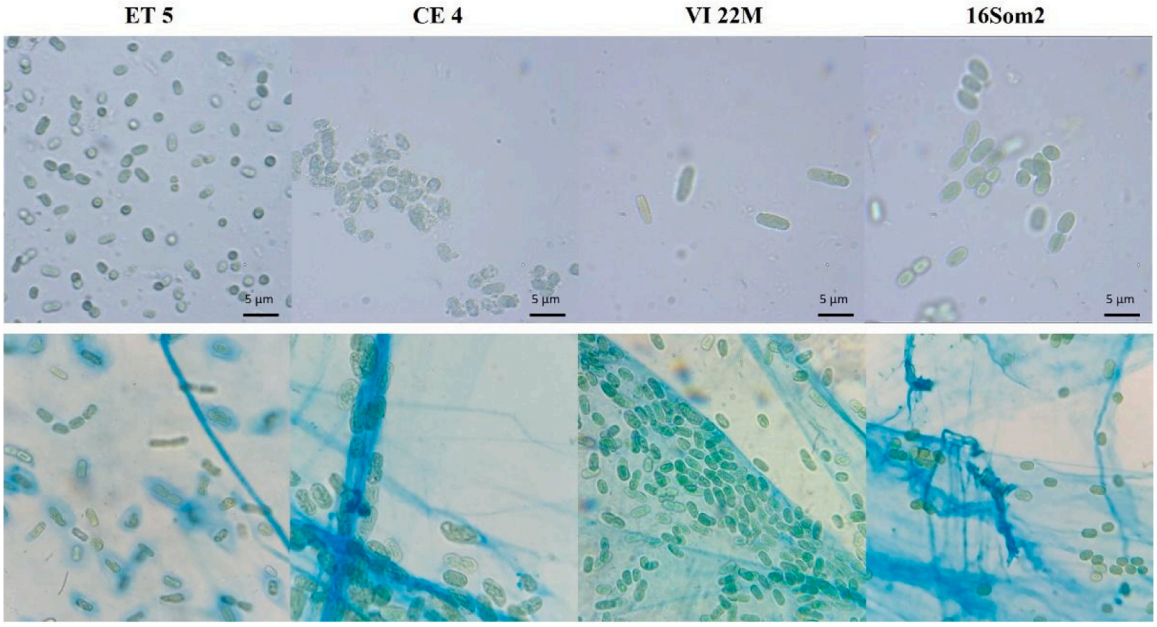

Fig. 2. Micrographs (magnitude 1000 ×) of selected cyanobacteria, without (above) and with (below) staining with Alcian blue. In the order from the left: ET 5, CE 4, VI 22M, 16Som2.

Table 1
Carbohydrate and biomass productivity of the cultures.

Culture	Carbohydrate productivity		Biomass productivity		
	cVP[a] mg tCH_2O L^{-1} d^{-1}	cBP[b] g tCH_2O g^{-1} DW	bVP[c] g DW L^{-1} d^{-1}	bISP[d] g DW m^{-2} d^{-1}	bPhP[e] g DW mol ph^{-1} d^{-1}
ET 5	70.00 ± 13.18	0.99 ± 0.32	0.06 ± 0.05	2.36 ± 2.01	5.40 ± 4.60
VI 22M	92.66 ± 59.15	1.34 ± 0.35	0.07 ± 0.01	2.74 ± 0.21	6.25 ± 0.49
CE 4	55.42 ± 30.24	0.53 ± 0.13	0.09 ± 0.04	3.64 ± 1.63	8.32 ± 3.73
16Som2	142.56 ± 19.23	1.56 ± 0.54	0.08 ± 0.07	3.27 ± 2.70	7.47 ± 6.16

Average ± standard error of the daily total carbohydrate and biomass productivity for each of the three weeks of growth expressed as:
[a] Carbohydrate volumetric productivity.
[b] Carbohydrate per gram of biomass productivity.
[c] Biomass volumetric productivity.
[d] Biomass per illuminated surface productivity.
[e] Biomass per amount of photons productivity.

material), resulting in an average cellular carbohydrate productivity of 129.9 and 104.4 mg cCH_2O per Liter of culture per day, respectively and an average soluble carbohydrate daily productivity of 54.3 and 87.1 mg sCH_2O per Liter of culture per day, respectively. Conversely, CE 4 and ET 5 showed the lowest values: 35.7 and 63.9 mg cCH_2O per Liter of culture per day, respectively, and 31.6 and 46.1 mg sCH_2O per Liter of culture per day, respectively. Semicontinuous cultivation allows the culture to be maintained in exponential growth, reaching greater amounts of biomass per unit volume of photobioreactor when compared with batch cultivation, but also of specific cellular components, including carbohydrates [41,42]. These results suggest the use of 16Som2 cultivated in semi-continuous mode for the production of EPS-rich biosorbents even at industrial-scale.

Biosorption experiments

To exploit an EPS-rich biosorbent, diluted or undiluted cultures were confined in dialysis tubings before being used for biosorption experiments. The results obtained through biomass assay revealed that specific HM uptake (Fig. 3) was generally enhanced by higher dilution factors, i.e. at lower biomass concentrations. Considering the biomass concentration of the pre-treated cultures (Supplementary Table S1), the dilution factor, and metal-specific uptake values (Fig. 3), the optimal biomass concentration ranged between 0.3–0.5 g DW L^{-1} for CE 4 and VI 22M. In contrast, for 16Som2 this range was wider, showing a metal-specific effect, with higher biomass concentration required to maximize Ni removal. Specifically, the maximum Cu specific uptake was reached by 16Som2, corresponding to an average of 83.9 mg Cu g^{-1} DW, whereas the maximum Ni and Zn specific uptake were 38.3 mg Ni g^{-1} DW and 40.3 mg Zn g^{-1} DW, shown by VI 22M and 16Som2, respectively. In terms of absolute removal (Supplementary Fig. S3), 16Som2 was the more performant biosorbent for all the three metals tested, removing an amount of Cu roughly twice the amount of Ni or Zn removed, probably because of the high carbohydrate concentration. ET 5 revealed an absolute metal removal (Supplementary Fig. S3) comparable to the other cyanobacteria only with the highest biomass concentration. According to the Pearson correlation coefficient (Supplementary Table S2), Cu and Zn removal were characterized by a statistically significant strong positive linear correlation with the total carbohydrate content (r 0.878 and 0.975, respectively). Pearson correlation performed between metals removed and dry weight or total carbohydrates content per each biosorbent revealed a strong positive linear correlation coefficient between metals removed by ET 5 and its carbohydrate content (r 0.719) and dry weight (r 0.878). On the contrary, the other biosorbents showed, when significant, a negative linear relationship between metals removed and their carbohydrate content and dry weight. Even though it is known that a high specific area and high number of active binding sites due to high biosorbent dosage are necessary to increase removal efficiency, this reduction in specific uptake may be attributed to reduced accessibility to the binding sites and metal ions diffusion due to increased viscosity for the high content of EPS [6,43]. Specific uptake expressed per total carbohydrate content (Fig. 4) by CE 4 resulted 7–3 times higher than Cu and Ni specific uptake of ET 5, respectively. Since CE 4 is characterized by an extremely high content of uronic acids (80 % of EPS DW), as previously described [25], metal-specific uptake may be positively affected even if the carbohydrate content and productivity per gram of biomass is lower than the

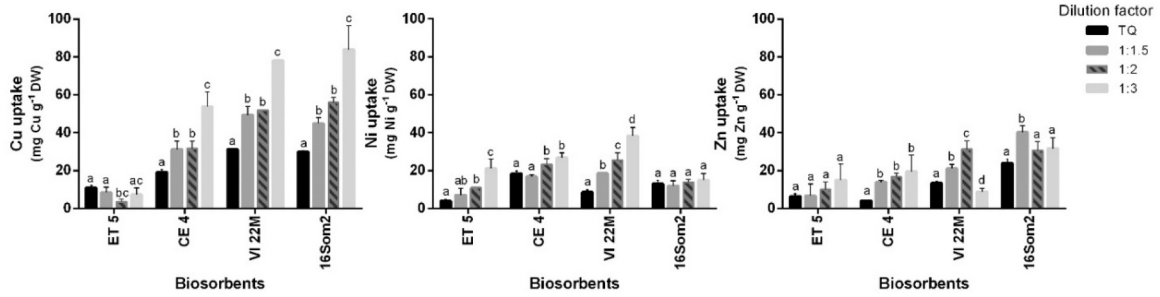

Fig. 3. Specific uptake expressed as mg of metal g^{-1} DW of undiluted cultures (TQ, black bar), or cultures diluted 1:1.5 (grey bar), 1:2 (grey bar with diagonal line), 1:3 (light grey bar) of ET 5, CE 4, VI 22M, 16Som2 (biosorbents). From the left, in the order: Cu, Ni, Zn specific uptake (mean ± sd, n = 3). Different letters between the same metal and biosorbent at different dilution factors mean a significant difference ($p < 0.05$).

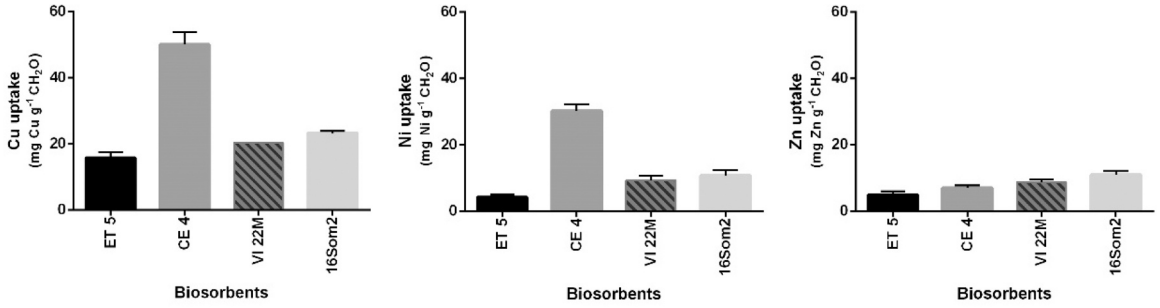

Fig. 4. Specific uptake expressed as mg of metal per gram of total carbohydrate (mg metal g^{-1} CH_2O) for each biosorbent represented by undiluted culture (TQ) of ET 5 (black bar), CE 4 (grey bar), VI 22M (grey bar with diagonal lines), 16Som2 (light grey bars). From the left, in the order: Cu, Ni, Zn specific uptake (mean ± sd, n = 3).

other strains. Similarly, despite the lower amount of EPS produced, Sharma et al. [44] found a more robust biosorption capacity of EPS from *Nostoc* sp. compared to those from *Gloeocapsa* sp. According to Pathirana et al. [45], acidic surface functional groups represent fundamental property for metal adsorption, and in particular, carboxylic groups are the most functional in providing binding sites for Cu^{2+}.

Specific HM uptake (q) at different concentrations at the equilibrium was plotted according to Langmuir and Freundlich adsorption isotherms as shown in Supplementary Fig. S4. The two models' linear transformation provided specific parameters reported in Table 2. According to the coefficients of determination (R^2), Freundlich isotherm largely explained Cu and Ni specific uptake by all the biosorbents, whereas Langmuir isotherm showed high R^2 values for CE 4 and 16Som2. On the other hand, the two models did not significantly explain Zn-specific uptake. Langmuir and Freundlich adsorption isotherms are often adopted to suggest the mechanisms of interaction between sorbate and biosorbent and to compare the obtained parameters between different biosorbents [3,21]. It has been assumed the presence of a monolayer surface without any interaction between adsorbed species and surface heterogeneity in Langmuir and Freundlich isotherms, respectively. Thus, multilayer surface, surface coverage degree, or porous solids are not considered [31]. The highest q_{max} values were shown by VI 22M with Ni and Cu. Nevertheless, the low R^2 values suggest a poor fitting of the Langmuir model with VI 22M data. Despite Cu removal by 16Som2 revealed the highest q_{max} (32.79) and high R^2 (0.997), the highest b value was observed for Cu removal by CE 4. Also, b values for Cu were always higher compared to Ni or Zn. From the Freundlich isotherm, the highest K_f values were attained by Cu removal performed by 16Som2 and CE 4. In addition, n values were always higher than 1, suggesting a favorable adsorption process. Namely, the highest n value was shown by Cu removal by CE 4, which also showed the highest b value, indicating a strong bond between CE 4 and Cu.

Freundlich model gives a better agreement of CE 4 equilibrium adsorption data, for the three metals, this may be caused by the precipitation of the cells of this cyanobacterium in the bottom of the dialysis tubings causing high biosorbent heterogeneity.

Febrianto et al. [31] summarized isotherms parameters obtained in a large number of published works. Generally, our results showed q_{max} and K_f values of a comparable order of magnitude towards the main biological materials listed in their work, including green and brown algae, lignocellulosic substrates, plants, bacteria, and fungi. Additionally, our q_{max} values were an order of magnitude higher than those obtained by vegetable shells, unmodified maize husk, baker's yeast, and some green algae but lower than those obtained by some chitosan-made

Table 2
Adsorption parameters and coefficient of determination (R^2) from Langmuir and Freundlich models.

		Langmuir			Freundlich		
Biosorbent	Metal	R^2	q_{max} [a]	b [b]	R^2	K_f [c]	n [d]
ET 5	Cu	0.237	15.53	0.08	0.819	1.45	1.33
	Ni	0.074	30.86	0.03	0.891	0.89	1.05
	Zn	0.025	-66.99	-0.01	0.456	1.50	2.19
CE 4	Cu	0.998	21.91	1.08	0.997	12.26	4.45
	Ni	0.971	23.17	0.30	0.973	5.91	2.14
	Zn	0.727	22.66	0.13	0.948	3.29	1.68
VI 22M	Cu	0.457	37.65	0.25	0.882	3.65	1.03
	Ni	0.577	63.21	0.02	0.994	1.49	1.06
	Zn	0.787	4.02	-0.87	0.107	4.57	4.69
16Som2	Cu	0.997	32.79	0.59	0.991	12.32	2.59
	Ni	0.996	15.93	0.40	0.891	5.27	2.48
	Zn	0.512	26.63	0.20	0.717	4.41	1.41

[a] q_{max} maximum adsorption capacity (mg/g).
[b] b Langmuir biosorption constant related to the affinity and free adsorption energy.
[c] K_f Freundlich isotherm constant (L/g) which indicates the adsorption capacity of the biosorbent.
[d] n reflects the adsorption intensity and suggests if the process is favorable ($n > 1$) or not ($n < 1$).

compounds, algae, crab shell particles, and EDTA-modified maize husk. On the other hand, only K_f values from Cu biosorption by CE 4 and 16Som2 resulted higher than a wide array of compounds, except non-living green alga *Cladophora fascicularis*.

To gain insights into the interaction between different metals and create a more realistic representation of biosorbent use, one-week-grown cultures of CE 4, VI 22M, and 16Som2 were adopted for multi-metal assay. The obtained data were compared with Cu, Ni, or Zn-specific uptake obtained during previous experiments with mono-metal solutions at different dilution factors, selecting the values obtained with the concentration of biomass more similar (Fig. 5). The total metal-specific uptake (expressed as mmol of metal removed per g DW) in multi-metal solutions resulted always higher (up to + 78 %) than in mono-metal solutions. Considering the metal removal of the single metals in multi- or single-metal solutions, Cu-specific uptake was positively affected by the multi-metal system for CE 4. In contrast, for VI 22M and 16Som2, the differences were not as marked. Ni and Zn specific uptake was negatively affected in the multi-metal system except for VI 22M, whose Ni uptake was slightly higher. According to different authors [26,46,47], metal ions characterized by highest affinity are rapidly sorbed to the biosorbents inducing a physicochemical modification of their surface or EPS conformation. This modification may reduce or increase the availability of the binding sites for the other metals, resulting in an antagonistic or synergistic effect in biosorption. These findings have important implications for real-world applications, since more than one metal generally co-exist in industrial wastewater [43]. Additionally, other factors like metal load, and interaction with other ions can influence metal uptake and selectivity in more complex systems [2].

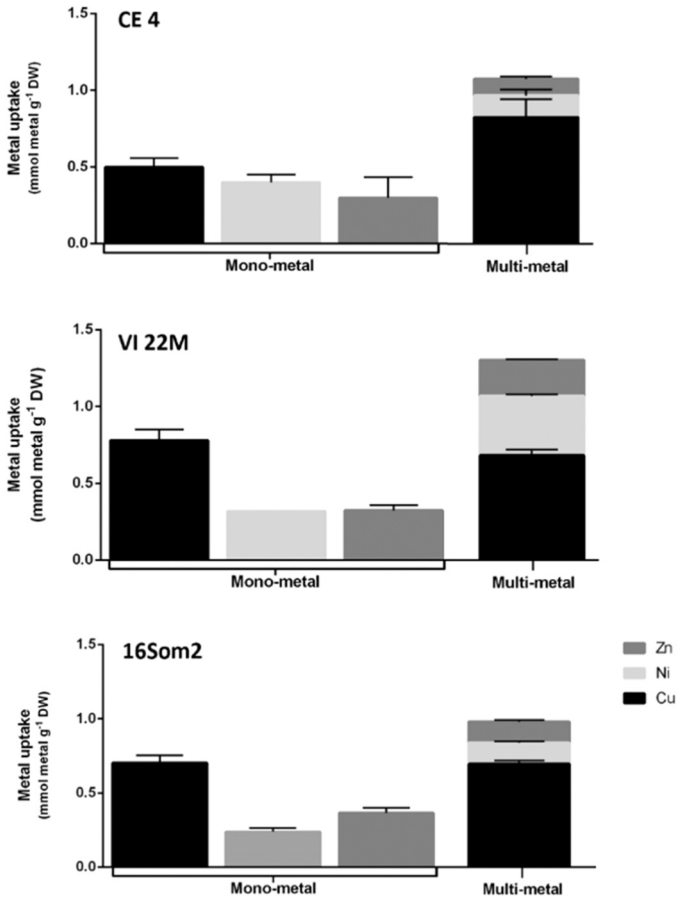

Fig. 5. Metal-specific uptake (mmol metal g^{-1} DW) in the order from left by CE 4, VI 22M, and 16Som2 (biosorbents) from mono-metal (on the left of each graph) and multi-metal solutions (on the right of each graph). Cu, Ni, and Zn specific uptake (mean ± sd, n = 3) are represented by black, light grey, and dark grey bars, respectively.

All the cyanobacteria have shown the maximum specific uptake for Cu both in mono and multi-metal systems, suggesting greatest affinity for this metal, as also confirmed by the higher *b* values obtained through the linearization of Langmuir isotherm. According to Pearson's hard soft acid base theory [48,49], the three metals are included in borderline ions showing a great affinity for hard and soft bases. Nevertheless, Zn is characterized by a lower electronegativity compared to Cu and Ni (1.65, 1.90, 1.91, respectively), negatively affecting its covalent index X^2r (where X is the electronegativity, r is the atomic radius), suggesting weaker affinity of Zn towards the binding sites. The lower-than-expected specific uptake of Ni may be attributed to the formation of highly stable aqueous complexes by Ni^{2+} in water, as suggested by Micheletti et al. [26]. In addition, Ni is usually present in minimal quantity in cyanobacteria, whereas Cu is an extremely important microelement as it works as a cofactor for several cuproenzymes, also involved in photosynthetic and respiratory electron transport processes [50,51] probably driving cyanobacteria to prioritize the uptake of copper from the external environment over other metals, likely contributing to a higher affinity to this metal also in biotechnological applications. However, despite these observations, further investigations are crucial to fully characterize the local environment of metals bound to cyanobacteria, including localization, oxidation state, and binding atoms. Such comprehensive analyses will elucidate binding mechanisms and metal selectivity, advancing our understanding of biosorption processes and enhancing biotechnological applications.

Microscopic observations of metal-enriched organic materials

The proposed process promotes the use of EPS-producing cyanobacteria for metals biosorption from polluted waters, obtaining metal-enriched organic materials. Given that the best performance in terms of specific and absolute Cu removal was obtained with 16Som2, we observed the cells of this cyanobacterium after the contact with Cu solution.

A higher surface roughness with exocellular polymeric substances and white dots are visible on the cells of 16Som2 + Cu sample compared to 16Som2 + H_2O sample (Fig. 6a–b). According to different authors [52,53], these features may suggest the presence of metallic salts on the surface of the cells. The freeze-dried whole culture of 16Som2 + Cu sample (Fig. 6c–d) appeared as a matrix of cells embedded within EPS filaments. The potential for the recovery and reuse of metals accumulated on cyanobacterial cells presents an avenue for sustainable resource management within biosorption processes. Techniques such as bioleaching, desorption, or combustion of the biomass in ashes followed by metal extraction and purification [5], may be employed to allow the reintroduction of metals into production systems. Additionally, according to many authors [5,23,54,55], these "hybrid" materials may be adopted as metal-based hybrid catalysts or antimicrobic materials. The valorization of the resulting metallic-organic materials can constitute further high-value compounds. The utilization of contaminated biomass is crucial for enhancing safe disposal methods and minimizing the risk of secondary environmental contamination while offering a cost-effective and sustainable approach to the synthesis of functional materials [5]. The polysaccharidic fraction released by cyanobacteria is not only rich in possible chelating sites for positively-charged pollutants [11,56], but is also characterized by intriguing physical and chemical properties. Incorporating this fraction into metallic-organic materials, their attractiveness for valorization purposes is enhanced, potentially leading to the synthesis of novel materials with diverse functionalities and applications. Moreover, the conversion of remaining cyanobacterial cells into value-added products represents an additional strategy for comprehensive resource utilization. These cells can be exploited for the production of pigments, antioxidants, bioactive compounds, or bioenergy through a biorefinery approach [57]. By exploiting all fractions of cyanobacteria

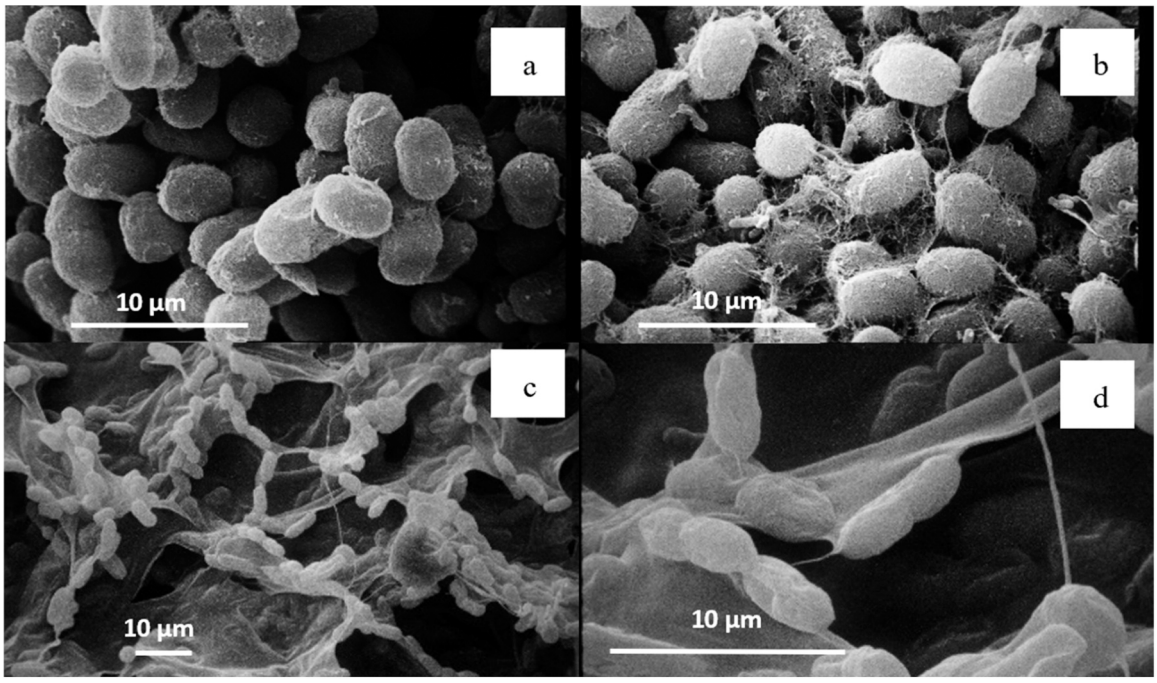

Fig. 6. 16Som2 cells obtained after the contact with water (a, 4000 magnitude), or with Cu (b, 4000 magnitude), above. 16Som2 freeze-dried whole culture after the contact with Cu at 1250 (c) and 5000 (d) magnitude, below. White bars indicate 10 µm.

with minimal to zero waste generation, the biorefinery concept maximizes resource efficiency and sustainability, aligning with principles of circular economy and waste minimization [57].

Conclusions

Cu, Ni, and Zn biosorption by marine cyanobacteria cultivated in semi-continuous mode here proposed, represents an eco-friendly and scalable approach due to *i*) schedulable and constant biosorbent production, *ii*) high carbohydrate content, positively correlated with metal-specific uptake, and *iii*) easy recovery of the metal-organic materials, that may be reused or valorized. *D. salina* 16Som2 resulted a promising candidate for the scaling-up of the process due to strongest carbohydrate productivity and metal uptake. Within the purpose of obtaining metal-rich biomasses to be valorized as added-value products, biosorbent dosage is an important parameter that needs to be considered to maximize specific uptake. Nevertheless, further studies are needed to chemically and structurally characterize these compounds and ensure their valorization.

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CRediT authorship contribution statement

Claudio Ratti: Writing – review & editing, Methodology, Formal analysis, Data curation. **Roberto De Philippis:** Writing – review & editing, Supervision, Conceptualization. **Matilde Ciani:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Francesca Decorosi:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Alessandra Adessi:** Writing – review & editing, Supervision, 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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2024.04.004.

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