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Mechanisms of copper and zinc bioremoval by microalgae and bacteria grown in nutrient rich wastewaters

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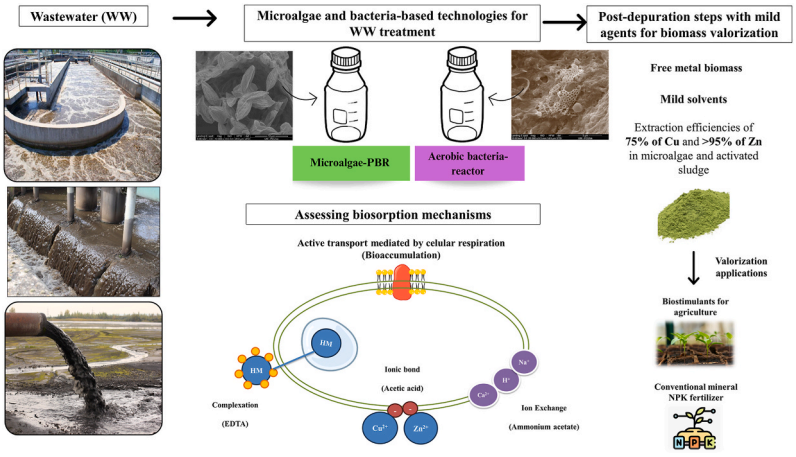
HIGHLIGHTS

- Sewage sludge more effective than microalga *S. almeriensis* to remove Cu and Zn from wastewater.
- Cu is more strongly biosorbed than Zn by both sewage sludge and microalgae.
- Metal biosorption on protonable electron donor groups is the main bioremoval mechanism.
- Cu and Zn are biosorbed on groups –COOH, –OH, –NH contained in proteins and carbohydrates of the cell membrane.
- Most metal accumulated by the biomasses is acid soluble/exchangeable and readily available.

GRAPHICAL ABSTRACT



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ABSTRACT

Swine farming produces large quantities of nutrient-rich wastewater, which often contains metals such as Cu and Zn, used as feed additives for pigs. These metals must be removed from the wastewater before discharge but their retention in the biomass can limit its subsequent utilization. Photobioreactors are a very promising alternative for swine wastewater treatment, as the consortium of microalgae and bacteria growing symbiotically in these reactors allows high nutrient and metal removal efficiency at moderate costs. This work studies the mechanisms of removal of Cu(II) and Zn(II) by the two types of microorganisms growing in these photobioreactors. A microalga commonly used in wastewater treatment (*Scenedesmus almeriensis*) and an activated sludge were kept in contact with synthetic wastewater containing 100 mg/L of Cu and Zn. After 72 h, *Scenedesmus almeriensis* removed 43% of Cu and 45% of Zn, while activated sludge removed 78% of Cu and 96% of Zn. Single and sequential extractions of the biomasses using different extracting reagents revealed that biosorption on

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protonable groups is the dominant removal mechanisms. Mild reagents solubilized 69% of Cu and 94% of Zn from the microalgae and 76% of Cu and 93% of Zn from the activated sludge. Low metal concentrations in the oxidizable and residual fractions evidenced minimal bioaccumulation inside the cells. FTIR and ESEM-EDX analysis confirmed biosorption by ion exchange and complexation as the main metal remediation mechanisms. The weak bonds of the biosorbed Cu and Zn ions are beneficial for the valorization of biomass and the obtaining of safe bioproducts.

1. Introduction

The intensification of livestock activities in recent decades has raised serious concerns about its environmental and health consequences, particularly in terms of greenhouse gas emissions and generation of large quantities of wastewater. Among the farms, the high use of water in the swine industry results in the generation of significant volumes of wastewater, rich in organic carbon, nutrients, and heavy metals like copper and zinc (López-Pacheco et al., 2021). These heavy metals are introduced to pig feed as growth-promoting additives, incompletely absorbed by the animals, and excreted and end up in swine wastewater (Vardhan et al., 2019). Swine manure varies widely in copper and zinc content, reaching up to 108 and 234 mg/L in swine wastewater respectively (ASAE, 2003; Zhang et al., 2011). Copper and zinc are also present in other types of wastewater, being often present in sewage sludge and related compost products (Wu et al., 2017).

The treatment of swine wastewater is usually carried out in biological reactors. Among biological methods, photobioreactors using consortia of green microalgae and aerobic bacteria are emerging as a promising technology to treat wastewaters containing high load of nitrogen, organic carbon and heavy metals as swine wastewater (García et al., 2019; Collao et al., 2022). This methodology is cost efficient as the main energy source used is sunlight, consumes carbon dioxide, do not require aeration and can be implemented on site. The biomass generated in biological wastewater treatment plants can be valorized to produce fertilizers, industrial peptides, biostimulants, biopesticides, animal feed, biofuels or other biocompounds (Aziz et al., 2019; Rojo et al., 2023), applying the biorefinery and circular economy concepts. There are many published works about the uptake of heavy metals by the microorganisms present in wastewater treatment bioreactors (Saavedra et al., 2018; Yin et al., 2019), but scarce information is available on the mechanisms of this removal and the methods for recovery of these metals. Bioremoval of heavy metal ions can occur by adsorption, a passive process in which the contaminants remain attached to the microorganism cell wall through weak interactions, thus resulting on reversible processes (Spain et al., 2021), or by bioaccumulation inside the cells. Due to the hydrophilic nature of the metals and the lipophilic nature of the cell membrane, diffusion processes into the cell involve irreversible equilibria (Monteiro et al., 2011).

Knowledge of metal uptake mechanisms is necessary to assess the stability and availability of the retained metal and to guarantee the safety of the bioproducts generated from biomass grown in wastewater treatment plants. Metal solubility tests and sequential extraction procedures provide information on microorganism-driven bioremoval mechanisms and the subsequent mobility of retained heavy metals during biomass valorization. These procedures involve the use of increasingly reactive extractants, such as strong electrolytes, weak acids for proton exchange, chelating agents, and reductants/oxidants. Sequential extraction is crucial for interpreting metal speciation (Pardo et al., 2013), but scarce research has been done applying sequential metal fractionation to metal accumulating algae and aerobic bacteria (Du et al., 2022; Oliveira et al., 2023). Fourier Transform Infrared Spectrometry (FTIR) is another useful tool that provides information about the functional groups related to the biosorption of metal ions (Tiquia-Arashiro et al., 2023). Scanning Electron Microscopy - Energy Dispersive X Ray spectroscopy (SEM-EDX) (Pytlik et al., 2018) evidences cell morphological changes caused by the biosorption of heavy metal ions and can confirm the presence of metal on the cell wall.

This study focuses on a comparative analysis of copper and zinc uptake mechanisms by the two types of microorganisms which grow symbiotically in wastewater treatment photobioreactors. *S. almeriensis* was selected as a representative of the microalgae because this specie is commonly used as inoculum in swine manure treatment photobioreactors with excellent results (Ciardi et al., 2022). Activated sludge from the aerobic reactor of an urban wastewater treatment plant was used as aerobic bacteria. The uptake capacity of Cu and Zn ions by each of these microorganisms was independently determined, working with nutrient rich solutions mimicking swine wastewater doped with the heavy metals. Single and sequential metal extractions, FTIR and ESEM-EDX analysis were carried out for each metal loaded biomass to determine the mechanism of uptake. The results from this research will provide the knowledge about removal of heavy metals in biological wastewater treatment plants necessary to design effective strategies to valorize the biomass grown in these systems. This information will ensure the production of safe bioproducts that meet regulatory requirements and contribute to sustainability goals.

2. Materials and methods

2.1. Microalgae and activated sludge

Monocultures of *S. almeriensis* microalgae in a synthetic culture medium were provided by the University of Almeria (Spain) and maintained in home-made Bristol freshwater medium at 21–23 °C under continuous agitation, applying an LED irradiance of 1200 μmol $m^{-2}{\cdot}s^{-1}$ in a 12-h photoperiod (Zambrano et al., 2023). The activated sludge was collected from the aerobic bioreactor of the municipal urban wastewater treatment plant located at Valladolid (Spain) and kept in the dark under aeration at 4 °C until its use. Fresh activated sludge was observed under the microscope (Leica DM 4000 B) and no microalgae or cyanobacteria were detected. Fresh samples of both inoculums were freeze-dried for subsequent FTIR and ESEM-EDX analysis.

2.2. Metal bioremoval experiments

Synthetic wastewater was used in these experiments, to maintain the cultures of the two types of microorganisms compared in this work. Real swine wastewater contains different microorganisms which would made impossible to study independently the heavy metals uptake by pure microalgae and by aerobic bacteria. The composition of the synthetic wastewater, per liter of final solution, was (Alcántara et al., 2015): 30 mg urea, 32.5 mg KNO_3, 4 mg $CaCl_2{\cdot}2H_2O$, 7 mg NaCl, 2 mg $MgSO_4{\cdot}7H_2O$, 110 mg peptone and 160 mg meat extract, resulting in 120 mg/L of total organic carbon (TOC), 45 mg/L of inorganic carbon (IC) and 60 mg/L of total nitrogen (TN). Cu and Zn were added together, as usually appear in the swine wastewater. Saavedra et al. (2018) shown that the uptake capacity of Cu and Zn by different microalgae species, including *S. almeriensis,* was higher working with multimetallic solutions than with monometallic solutions. Thus, appropriate volumes of 5000 mg/L standard solutions of Cu(II) and Zn(II) prepared from $CuCl_2{\cdot}2H_2O$ and $ZnCl_2$ were added to synthetic wastewater to achieve a concentration of 100 mg/L of each element. All the reagents described in the different experiments were of analytical grade and the solutions prepared using deionized (ultrapure) water.

Bioremoval experiments were carried out batch-wise in borosilicate glass bottles of 1 L capacity containing 500 mL of Cu and Zn loaded synthetic wastewater. Microalgae or bacteria inoculum was added to achieve biomass concentration of 4 g/L, expressed on a dry matter basis. The pH of the resulting suspension was adjusted to 7.5 with 0.1 M NaOH to simulate the environmental conditions existing in photobioreactors (Posadas et al., 2015) and aerobic bioreactors (Gola et al., 2020) treating nutrient rich wastewaters. Samples of both, microalgae and bacteria in Cu and Zn loaded synthetic wastewater, were kept at 25 °C under continuous stirring at 250 rpm in a multi-point magnetic stirrer, irradiated with 1200 μmol $m^{-2}{\cdot}s^{-1}$ using LED lamps arranged on top, working in 12-h photoperiod for 72 h (Acién et al., 2012; Gola et al., 2020). At the end of the experiment, for each sample, a 75 mL aliquot of suspension was collected to determine the final biomass concentration. The rest of the suspension was centrifuged at 7800 rpm for 10 min, the solid fraction was rinsed twice with 15 mL portions of deionized water, centrifuged after each washing. A portion of this solid was stored at 4 °C in the dark for further analysis of dry biomass, copper and zinc concentration, and single and sequential extractions. The rest of biomass was freeze-dried and stored in the dark for FTIR and SEM-EDX analysis. The percentage of metal (Cu or Zn) bioremoval (RE_m, %) for each sample was calculated from the metal uptake by the biomass according to:

$$\%\ \boldsymbol{RE_m} = \frac{\boldsymbol{q_m} \bullet \boldsymbol{C_{b-72}}}{\boldsymbol{C_{m-0}}} \times \mathbf{100} \quad (1)$$

where q_m is the metal bioremoval (uptake) capacity (mg Cu or Zn·g^{-1} dry biomass), $C_{m\text{-}0}$ (mg·L^{-1}) is the initial concentration of metal (Cu or Zn) in the solution and $C_{b\text{-}72}$ (g·L^{-1}) is the concentration of dry biomass in the photobioreactor after 72 h.

2.3. Selective solubilization of bioaccumulated metals

Selective redissolution of Cu(II) and Zn(II) was performed by single and sequential extraction procedures, using extractants with different chemical reactivity for the selective solubilization (chemical fractionation) of metal ions in order to elucidate the nature and strength of the interactions stablished between the metals and the biomasses, and hence the availability or solubility of the accumulated metals in subsequent biomass valorization processes.

The amount of metal (Cu or Zn) extracted from the biomass by each solvent with respect to the total metal accumulated by the biomass in the bioremoval experiments, is determined by the following expression.

$$\textit{Metal extraction}\ (\%) = \frac{C_{m,e}{\cdot}V_e}{q_m{\cdot}m_b} \times 100 \quad (2)$$

Where V_e is the volume of extractant (L), $C_{m,e}$ is the corresponding concentration of metal (mg·L^{-1} of Cu or Zn) in this extractant, q_m is the metal bioremoval (uptake) capacity (mg Cu or Zn·g^{-1} dry biomass) and m_b is the accurate weigh (g) of dry biomass taken.

2.3.1. Single extractions

Single extractions of Cu and Zn bound to the biomasses collected from the bioremoval experiments were performed in triplicate using ammonium acetate to desorb metal ions bound to the biomass surface by weak electrostatic interactions, acetic acid to extract metal cations bound by metal-proton exchange and EDTA to release metals strongly retained on the cell surface by complexing competition. An accurately weighed amount of 0.5 g of centrifuged wet biomass and 20 mL of extractant were shaken in an Intelli-Mixer RM-2M multimode automatic shaker at 55 rpm for 30 min (experimental conditions are detailed in Table S1 of Supplementary Material). The suspensions were then centrifuged at 7800 rpm for 10 min and the supernatants filtered through 0.45 μm Nylon filters. The filtrates were stored at 4 °C for subsequent quantification of Cu and Zn ions.

2.3.2. Sequential extraction procedure

A four-step metal fractionation method, adapted from the Tessier and BCR sequential extraction procedures (Tessier et al., 1979; Ure et al., 1993), was applied in duplicate to sequentially extract metal fractions retained by the biomass through different mechanisms. A portion of 0.5 g of the centrifuged biomasses from bioremoval experiments was placed in a 50 mL Falcon tube and Cu and Zn were sequentially extracted with 30 mL of four different reagents (0.1 M magnesium chloride (F1), 0.5 M acetate buffer (F2), 0.1 M hydroxylamine hydrochloride (F3) and hydrogen peroxide 30% w/v (F4)) under 55 rpm stirring. Further experimental conditions can be consulted in Supplementary Material (Table S2). After each extraction, the liquid phase was separated by centrifugation at 7800 rpm for 10 min, filtered through 0.45 μm Nylon filters and stored at 4 °C for Cu and Zn quantification, whereas the solid fraction was washed twice with deionized water and centrifuged to remove the remaining reagent before adding the next extractant. The residual metal fraction was determined in the final solid residue.

2.4. Analytical methods

The dry biomass of the pristine and metal loaded biomasses was determined gravimetrically by desiccation of a portion of the biomass in the oven at 105 ± 1 °C for at least 24 h until constant weight. The biomass concentration after 72 h of bioremoval experiments was obtained gravimetrically, determining the amount of dry biomass in a 75 mL aliquot of the suspension taken at the end of the contact period. Biomass growth ratio at 72 h was calculated as:

$$\text{Biomass growth ratio} = \frac{C_{b-72}}{C_{b-0}} \quad (3)$$

where C_{b-72} (g·L^{-1}) is the concentration of dry biomass in the photobioreactor after 72 h of contact time and C_{b-0} (g·L^{-1}) is the concentration of dry biomass in the photobioreactor at the start of the experiment.

Solid samples from biosorption experiments and the residual biomass obtained after step F4 of the sequential extraction procedure were dissolved for Cu and Zn analysis by microwave-assisted acid digestion. The sample portion was accurately weighed and digested with 10 mL of 69% nitric acid (Panreac, Spain) in a Milestone Ethos Plus microwave oven controlled with EasyWave 3 software (Milestone Srl, Italy). Digestion was carried out with a temperature ramp up to 180 °C for 20 min followed by 10 min at 180 °C. After cooling, the resulting solution was diluted to 30 g with deionized water.

The concentration of Cu and Zn in the diluted samples and in the filtered extracts obtained by single and sequential extractions was determined by inductively coupled plasma optical emission spectrometry, ICP-OES, with a Varian 725-ES instrument (Agilent, USA), applying validated internal procedures of the Instrumental Techniques Laboratory of the University of Valladolid (LTI – UVa). The emission lines at 324.754 nm and 213.857 nm were used for Cu and Zn measurement, respectively. For quality assurance of the analytical results, blanks, standards, spiked samples and the certified reference material TMDA 64.2 (Environment Canada, Canada), were included in the measurement sequence as quality control samples, considering a range within 10% of the true value for valid acceptance.

The FTIR spectra of freeze-dried portions of the microalgae and bacteria biomasses before and after Cu and Zn biosorption were registered to observe differences in the vibration bands. The FTIR spectra were recorded between 4000 and 500 cm^{-1} with a Bruker Tensor 27 FTIR spectrometer using attenuated total reflection sampling method (ATR-FTIR) and a highly sensitive DLATGS (deuterated L-alanine doped triglycine sulfate) detector with a resolution of 1 cm^{-1}. The morphological modifications of the biomass cells after metal biosorption and the elemental composition of the cell wall were analyzed using an environmental scanning electron microscope coupled to an Energy

Dispersive X Ray spectroscopy detector, ESEM-EDX. The ESEM-EDX analysis of the freeze-dried samples was carried out using an ESEM FEI-Quanta200FEG.

2.5. Statistical analysis

A two-way ANOVA was performed on the results of the individual metal extractions to determine whether there are significant differences between the percentages of Cu and Zn solubilized with three different reagents from the two compared biomasses. The post hoc Tukey's honestly significant difference (HSD) test was applied to assess whether the mean results for each factor level differed significantly. Statistical hypothesis testing was performed at 5% significance level using Statgraphics Centurion 19.2.01.

3. Results and discussion

3.1. Copper and zinc uptake by microorganisms from nutrient-rich wastewater

The biomass growth ratio calculated at the end of the experiments was 1.12 for microalgae and 1.03 for activated sludge. These low values could be related to the inhibitory effect of heavy metals, but also to the batch operation. Cu and Zn are essential to living organisms at low concentrations, but at high concentrations they become toxic. Cu(II) disrupts many microalgae metabolic pathways, such as photosynthesis, respiration, ATP (adenosine triphosphate) production, and pigment synthesis, as well as inhibits cell division. Zn(II) is a cofactor for enzymes participating in CO_2 fixation (carbonic anhydrase), DNA transcription and phosphorus acquisition (Expósito et al., 2021). Nevertheless, Cu and, in less extent, Zn, form very stable complexes with strong chelating ligands present in the solution (Ringbom, 1963). Therefore, the availability, and then the toxicity, of Cu and Zn ions are reduced in the presence of chelating agents. Some organic compounds added to the synthetic wastewater to mimic swine wastewater can exert such complexing effect, especially on Cu(II), thus reducing its removal efficiency. Saavedra et al. (2019), working with *Scenedesmus almeriensis* in synthetic wastewater, found a remarkable protective effect versus heavy metals toxicity by the presence of organic material in the culture media. Collao et al. (2022), working in a continuous photobioreactor fed with real swine manure doped with 100 mg/L of Cu and 100 mg/L of Zn, found statistically different values of total suspended solids (TSS) compared to the non-doped photobioreactor. TSS were lower in the Zn doped photobioreactor than in the non-doped control, but the Cu doping resulted in higher TSS than in the control. In any case, the doped photobioreactors continued to have metabolic activity, achieving steady states with removals of 83% and 81% of TOC and 46% and 58% of TN, for Cu and Zn, respectively, compared with 93% of TOC and 58% of TN in the control photobioreactor. Cu and Zn did not affect NH_4^+ removal efficiencies and the total phosphorus concentration in the effluents remained below the quantification limit (<1 mg/L) throughout the entire operating period in all the photobioreactors. In agreement with these results, the morphology of cellular structures observed by ESEM-EDX analysis does not show cell lysis or rupture of the cell walls of any of the biomasses at the end of the 72 h culture with heavy metals.

Bioremoval capacity, q_m, and bioremoval efficiency, RE_m, of Cu and Zn were calculated for a photobioreactor treating synthetic wastewater of composition mimicking swine wastewater with microalgae and bacteria biomasses grown during 72 h with high contents of Cu and Zn (100 mg/L). These concentrations were selected to operate under the most unfavorable conditions within the range of values found in the literature for these metals in PWW (Gao et al., 2018; Zeng et al., 2021). Additionally, these concentrations were previously used in other studies of the research group, allowing the comparison of results (Collao et al., 2022). Moreover, the use of high concentrations of heavy metals makes this application extensible to other wastewaters with a typically higher content of heavy metals such as those from mining or the textile industry (Aguilar et al., 2020; Saavedra et al., 2018).

The results, depicted in Table 1, are the average of the performed duplicates; the given uncertainties have been estimated as their standard deviation.

Microalgae *S. almeriensis* achieved acceptable removal efficiencies of both metals. Despite the different microalgae strains, metal concentrations, pH or contact times, our results seem to be comparable with previously published data obtained with *Neochloris oleoabundans* (Gu and Lan, 2021), *Desmodesmus* sp. (Liu et al., 2021), *Scenedesmus* sp. (Oliveira et al., 2023) and *Chlorella beijerinck* (Heidarpour et al., 2019). Metal removal values achieved with aerobic bacteria contained in activated sludge were much higher, yielding efficiencies of 78% Cu and 96% Zn. The higher generation of extracellular polymeric substances, EPS, on the surface of the bacteria and the higher ease of binding of metal cations due to the abundance of negatively charged functional groups in the bacteria, favors and enables the better metal uptake performance of the bacteria than of the *S. almeriensis*. (Gao et al., 2018; Zeng et al., 2021; Zhao et al., 2023).

3.2. Elucidation of metal bioremoval mechanisms by selective solubilization procedures

The percentages of metal extracted from dry microalgae biomass using 0.1 M ammonium acetate, 0.1 M acetic acid and 0.1 M EDTA in non-sequential (single) extractions were, respectively, 12.5, 76.3 and 66.4 % of Cu and 39.3, 96.8 and 77.4 of Zn, while from activated sludge (aerobic bacteria) the percentages were 5.8, 55.4 and 67.2% of Cu and 14.3, 100 and 81.4% of Zn (Fig. 1).

Two-way ANOVA (analysis of variance) was carried out on the results of extracted metal (%) to check if there are significant differences among the assayed levels of the factors biomass (microalgae, bacteria) and extractant (NH_4AcO, HAcO, EDTA) for each metal. Both factors and their interaction had a significant effect on the extraction of Cu and Zn (p-values <0.05). However, the effect of the extractant used was stronger than that of the biomass type as demonstrated the smaller p-value (larger F-ratio) of the former. The Tukey's HSD post-hoc test for comparison of means detected significant differences, at the 5% significance level, among most factor levels assayed. The only exception was the amount (%) of Cu extracted with EDTA and acetic acid, that did not differ significantly.

Ammonium acetate dissolved in water is a strong electrolyte with negligible acid, alkaline or complexing properties, that does not significantly alter the pH of the biomass grown in the photobioreactor but increases the medium ionic strength, decreasing the electrostatic attraction between the metal ion and the cell wall surface and thus causing desorption of metal ions retained by this mechanism. Acetic acid is a weak acid; at 0.1 M concentration it provides a pH 3, thus desorbing Cu and Zn linked to protonable functional groups present in the cell membrane, such as –COOH, –OH, or –NH groups, by cationic exchange. EDTA is a complexing agent that forms very stable complexes with Cu

Table 1
Uptake capacity (q_m, mg·g^{-1}) and bioremoval efficiency (RE_m, %) of 100 mg/L of Cu and Zn in synthetic wastewater by microalga *S. almeriensis and* aerobic bacteria in activated sludge after 72 h.

Biomass	Uptake capacity, q_m (mg·g^{-1})		Bioremoval efficiency, RE_m (%)	
	Cu(II)	Zn(II)	Cu(II)	Zn(II)
S. almeriensis	9.85 ± 0.27	10.38 ± 0.26	44.16 ± 0.82	45.23 ± 0.77
Activated sludge	18.61 ± 0.26	23.37 ± 0.30	78.35 ± 0.55	95.59 ± 0.54

Results and uncertainties calculated as the mean and standard deviation of two replicates, respectively.

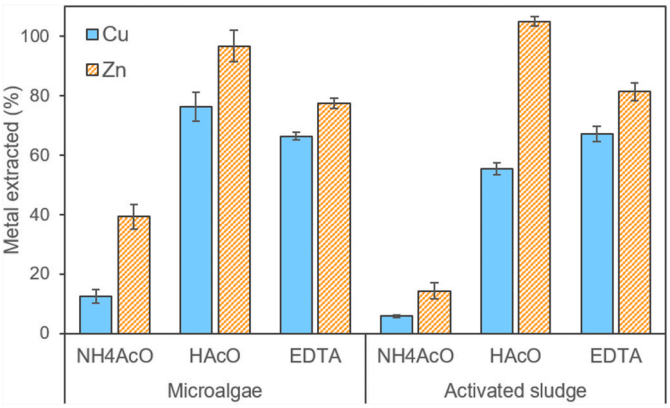

Fig. 1. Percentage of Cu(II) and Zn(II) extracted from metal loaded biomasses of microalgae *S. almeriensis* and activated sludge (aerobic bacteria) using reagents with different chemical properties at 0.1 M concentration. The vertical error bars are the standard deviations of three replicates.

and Zn cations, with higher affinity for Cu than for Zn at pH 7; thus, 0.1 M EDTA at pH 7 is able to compete for Cu and Zn ions strongly linked to electron donor functional groups in the cell surface, including protonable groups (Franklin et al., 2002).

The use of ammonium acetate for metal re-solubilization from biomass provided in general low yields, suggesting that the physical biosorption based on electrostatic attraction is of little relevancy for microorganisms. On the other hand, acetic acid showed a metal solubilization capacity comparable to that of EDTA for Cu(II) ions, while it was much more effective than EDTA for Zn(II) solubilization, releasing quantitatively the metal bioaccumulated by both microalgae and activated sludge (97% and 100% Zn, respectively). The different stability of EDTA complexes with Cu(II) and Zn(II) is related to the electronegativity and ionic radius of the cations, but also to the competing reactions of Cu (II) and Zn(II) with other electron donors and of EDTA with other electron acceptors in the reaction medium (Ringbom, 1963). Although in pure water Cu(II)-EDTA complex is more stable than Zn(II)-EDTA, the likely higher stability of the bonds of Cu(II) with electron donor groups on the cell membrane is the responsible of the lower Cu recoveries using EDTA as extractant (66 % from microalgae, 67% from activated sludge) in comparison with Zn solubilization (77% from microalgae, 81% from activated sludge).

Similar patterns were observed for metal single extractions from both microorganisms. However, desorption caused by ammonium acetate, which provides pH 7 and only contributes ionic strength, was less relevant for activated sludge, indicating stronger metal-microorganism bonds in this biomass. In addition, Cu was more efficiently released from activated sludge using EDTA, suggesting the presence of other non protonable electron donor groups in this biomass.

From these results it can be concluded that: (i) the metal ions incorporated to the microorganisms from wastewater are extracellularly adsorbed, i.e. bound to electron donor functional groups of the molecules forming the cell membrane (-OH, –COOH, –NH …); (ii) metal adsorption occurs likely on sugars and proteins present in the microalgae cell membrane, rich in protonable electron donor groups; (iii) Cu is more strongly biosorbed than Zn, as demonstrates the fact that Zn is more easily desorbed by the competing effect of all the extractants assayed.

In terms of biomass clean-up for application purposes, the best results for extraction of both heavy metals were achieved by 0.1 M acetic acid, for which >95% of Zn is released from both microalgae and activated sludge, while >75% of Cu is extracted from *S. almeriensis*. So, for instance, the washed biomass would meet the strictest requirements in the use of fertilizers for different applications. The washing of biomass with these mild solvents would not significantly affect the final content of biomolecules such as proteins or lipids. Chen et al. (2007) observed a solubilization of only 5% of the total protein content with an acid extraction at pH 4 for 4 days.

Some examples of similar extractants used for this purpose have been found in the literature. 8-hydroxyquinoline-5-sulphonate, a complexing agent, removed 25% of the total metal adsorbed by the microalga *T. weissflogi* (Price and Morel, 1990). Hassler et al. (2004) observed that green microalgae *Chlorella kesslerii* washed with EDTA released extracellular copper (80%) and zinc (90%). Levy et al. (2008) released Cu from marine microalgae grown in 50 mg/L Cu solutions: *Phaeodactylum* tricornutum (62%), *Tetraselmis* sp. (36%) and *Dunaliella tertiolecta* (91%). Franklin et al. (2002) used EDTA with *Chlorella* sp. and concluded that Cu and Zn exhibit the same adsorption mechanisms to the cell wall, presenting similar active sites on the cell surface, and that, as the concentration of the metal in solution increases, the proportion of extracellular metal also increases. These findings are, in general, in good agreement with the results obtained in the present study.

In order to further assess the bioaccumulation mechanisms of Cu and Zn by *S. almeriensis* and activated sludge, a sequential extraction scheme was applied to the biomass samples grown in synthetic wastewater simulating swine wastewater under the operational conditions described in section 2.3. Cu and Zn extracted from the biomass after each extraction step (F1 to F4 and residual fractions) are displayed in Table 2, in mg metal per g of dry biomass, and in Fig. 2, in percent fraction. The extraction scheme was applied in duplicate and results shown are the mean values.

In order to assess the accuracy of the procedure, the sum of the five metal fractions was compared with the total metal concentration determined in a sample portion of the biomass dissolved by microwave-assisted digestion with nitric acid (see Table 2). The accuracy of the sequential extraction procedure was evaluated as the percentage of relative error, comparing the sum of fractions with the total concentration measured directly on the biomass and considering the last as the true value. Relative errors below 5% were obtained for the sum of Cu and Zn fractions in microalgae and bacteria biomasses, thus validating the metal fractionation results despite the experimental complexity of the procedure, in which accumulation of errors may happen.

Fig. 2 shows the most prevailing metal fractions solubilized using this procedure and uncovers the involved metal biosorption mechanisms. In microalgae, most Zn is released with fraction F1, metal weakly bond through electrostatic attraction, whereas Cu is equally leached in fractions F1 and F2, metal bound to protonable groups exchangeable with protons, indicating a higher affinity of copper to bind to electron donor groups. The percentages of Cu and Zn extracted in step F1 with magnesium chloride were significantly higher than the ones recovered with ammonium acetate, being both reagents used as neutral salts providing ionic strength. This difference could be attributed to the fact than Mg^{2+} forms complexes with strong electron donor functional

Table 2
Cu(II) and Zn(II) extracted in each step of the sequential extraction procedure, in mg/g as dry weight, total metal concentration in the initial biomass determined in a digested portion of sample, in mg/g, and the relative accumulated error of the sequential extraction scheme, in %. Results are the average of two duplicates.

Sequential extraction step	Metal concentration (mg/g)			
	S. almeriensis		Activated sludge	
	Cu(II)	Zn(II)	Cu(II)	Zn(II)
F1: 0.1 M $MgCl_2$	2.89	7.50	1.39	5.17
F2: 0.5 M acetate buffer	3.83	2.63	13.11	15.69
F3: 0.1 M $NH_2OH{\cdot}HCl$	0.21	0.30	0.92	1.22
F4: 30% H_2O_2	1.25	0.21	3.45	0.30
Residual fraction: 69% HNO_3	1.61	0.09	0.27	0.13
Sum of fractions (F1+F2+F3+F3+residual)	9.79	10.73	19.14	22.51
Total metal in digested biomass	9.85	10.38	18.61	23.37
Difference (procedure relative error, %)	−0.61	3.37	2.85	−3.68

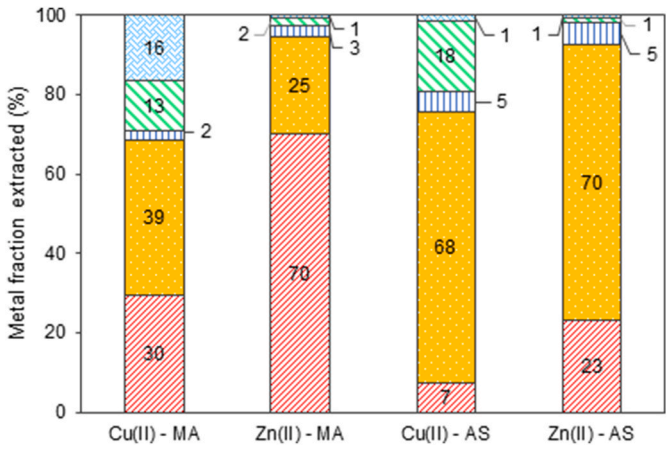

Fig. 2. Metal fractionation results, in percentage fraction, of the sequential extraction of bioaccumulated metals Cu and Zn by microalga *S. almeriensis*, MA, and by activated sludge rich in aerobic bacteria, AS. F1: $MgCl_2$, F2: acetate buffer; F3: $NH_2OH{\cdot}HCl$; F4: H_2O_2; Residual fraction.

groups on the cell membrane, thus competing with Cu and Zn ions retained by those chemical structures and causing a larger desorption of heavy metals, whereas ammonium has not that competing capacity. For activated sludge, 68% of Cu and 70% of Zn are released in fraction F2, indicating a higher stability of the biosorbed metals. The easily exchangeable fractions F1 and F2 contain the most available Cu and Zn ions which, if the biomass is disposed in the environment (as fertilizer), could be released to the soil and groundwater as a result of slight changes in the pH or salinity of rain or irrigation water in contact with the biomass. Therefore, washing the biomass grown in metal containing wastewater with a weak acid before its reutilization would be advisable.

The reducible fraction F3 (solubilized with diluted hydroxylamine hydrochloride at room temperature) was negligible. The fraction F4 was solubilized in strong oxidizing conditions (concentrated hydrogen peroxide at 85 °C) that could cause major destruction of the biomass, thus releasing metals strongly biosorbed and bioaccumulated inside the cells. The oxidizable and residual fractions can be considered non-available in normal environmental conditions, but could pass to the valorization products if aggressive treatments are used to release the desired biomolecules (Rojo et al., 2023). For Cu, a non-available 30% for microalga *S. almeriensis* and 20% for the activated sludge have been obtained, while for Zn the percentage drops to 3% and 2%, respectively. These low values can be considered negligible as they are below the estimated error of the sequential extraction procedure (up to 4%, Table 2).

Both single and sequential extractions confirmed that adsorption is the dominant bioaccumulation mechanism of heavy metals Cu and Zn for both microalgae and bacteria, being activated sludge better biosorbent than the microalga *S. almeriensis*. Cu showed a higher binding capacity through metal-organic complexes than Zn. The bioavailability of Cu and Zn, estimated as the sum of the easily solubilizable F1 and F2 fractions, is close to 75% for Cu and to 95% for Zn, with small differences between the microorganisms. The biosorbed Cu and Zn ions can be easily desorbed with mild reagents (weak acids, strong electrolytes), thus it is advisable to remove the metals from the biomass prior to its valorization to avoid toxic metals entering the biorefinery process and the recovered byproducts. For example, in the case of a high concentration of heavy metals in the photobioreactor feed, such as that studied in this work, washing with a weak solvent such as acetate buffer would allow the commercialization of high concentration wet biomass as fertilizer, complying with the most demanding legal quality requirements. Extraction with a weak solvent can be expected to have no effect on the composition of the biomass. Lee et al. (2017) recovered only 5% of the proteins from *Chlorella vulgaris* using Na-phosphate buffer (similar to acetate buffer proposed in this work) combined with an ultrasound treatment. The treatments proposed in this work do not include ultrasound treatment and *S. almeriensis* is reported to be a robust and resistant microalgae strain due to its cell wall thickness, so that negligible solubilization of biomolecules is expected (Dunker and Wilhelm, 2018; Spain et al., 2021).

Using a sequential extraction procedure not detailed, Oliveira et al. (2023) studied the metal fractionation of Zn in microalgae *Scenedesmus* sp. grown in swine wastewater containing 10–70 mg/L Zn using contact times of 1 and 40 days. The extracted fractions were interpreted as soluble (weakly bound), bound to carbonates (acid soluble), Fe and Mn oxides (reducible), organic compounds (oxidizable), and the residual fraction. This interpretation, also used by other authors, seems more adequate for soils and rocks than for biological matrices were the presence of carbonates and metal oxides is minor. They found that Zn is incorporated into the cell with time, becoming less available after long treatments. However, the time required for the major incorporation of Zn inside the cell is much longer than standard hydraulic residence times. Fuentes et al. (2008) addressed the sequential metal fractionation of sludges and observed that Cu and Zn were mostly recovered in the oxidizable fraction, while in our study bioaccumulated Cu and Zn are mostly associated with the exchangeable/acid soluble fractions (F1+F2). The prevalence of available metal fractions in our experiment favors the reutilization of the harvested biomass since metal redissolution can be performed easily with mild washing reagents. Yuan et al. (2011) applied the BCR sequential extraction procedure to sewage sludge and found that Cu was mainly bound to the oxidizable and residual fractions, whereas Zn was found in the fractions acid soluble/exchangeable, reducible and oxidizable. Li et al. (2010) investigated the bioacummulation of Cu and Zn in *Eisenia fetida* fed on swine manure and estimated their bioavailability using the Tessier sequential extraction procedure (Tessier et al. 1979), considering the fractions exchangeable, acid soluble and reducible as bioavailable, and concluded that Cu and Zn accumulated by the bacteria are available. The variety of experimental conditions makes data comparison difficult.

On a whole, once mechanisms of heavy metal removal have been studied, future research is needed to evaluate the effect of operating conditions such as composition of the feed, the contact time and the initial biomass concentration on the mechanisms of heavy metal removal of photobioreactors and aerobic reactors, in order to optimize the complete biomass valorization process. The presence of heavy metals can also influence the biochemical composition of the biomass cultivated in photobioreactors. As an example, the protein content in *Scenedesmus* sp rose from 49% in a control situation in the absence of heavy metals to 53.5% in presence of Zn (Oliveira et al., 2023). Also, the presence of heavy metals can influence the hydrolysis yields, especially if enzymatic hydrolysis is used.

3.3. *Effect of copper and zinc on microorganisms cell wall*

Infrared spectroscopy plays a fundamental role in surface characterization research as it provides valuable information about the molecular structures present on the surface of interest. Infrared spectra were obtained before and after Cu and Zn biosorption from freeze dried aliquots of the biomasses using the ATR sampling method. The FTIR spectra recorded for microalgae and activated sludge are displayed in Fig. 3A and B, respectively. The biomasses show similar absorption patterns, indicating that functional groups and molecular composition of microalgae and bacteria cell walls are alike.

A clear decrease in the intensity of the bands is observed once the metal has been biosorbed. This can be explained by the fact that when metals, which have a high mass in comparison with the usual atoms in biomolecules (C, O, H, N, S …), are incorporated on the membrane, they cause a decrease in the vibrational frequency of the substituted groups which results in an important decrease of the intensity signal with respect to the unsubstituted groups. From the analysis of the

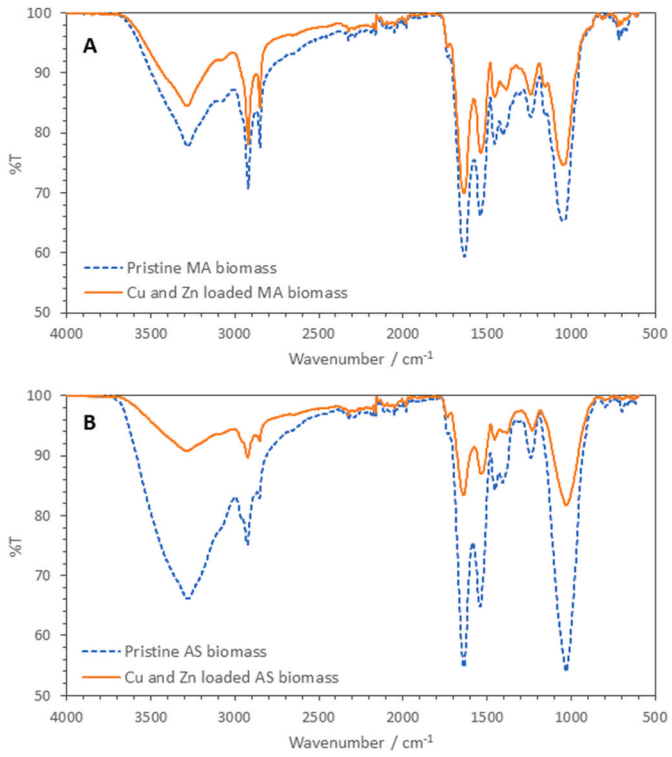

Fig. 3. FTIR spectra of pristine and metal loaded biomass for (A) green microalga *S. almeriensis* and (B) activated sludge. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

characteristic FTIR bands, the composition of the cell wall surface of the microorganisms and the membrane functional groups involved in metal binding may be elucidated. The characteristic absorption bands of microalgae and bacteria and their correspondence with the vibration frequency of functional groups are summarized in Tables S3 and S4 of the Supplementary Material. For both biomasses, a broad absorption band is observed between 3280 and 3284 cm^{-1}, which can be assigned to stretching vibration of the O–H bond, indicating the presence of strong hydrogen bonding. Narrower bands registered at frequencies 2919-2925 cm^{-1} correspond to vibrations of aliphatic C–H bonds. The intense absorption bands between 1633 and 1637 cm^{-1} and 1536-1540 cm^{-1} can be assigned to asymmetric stretching of carboxylic C═O bonds and their esters and to amide structures (Gu and Lan, 2021). On the other hand, the bands between 1454 and 1455 cm^{-1} are due to a weaker, symmetric type of vibration of C–O bonds of carboxyl groups. In the 1230-1238 cm^{-1} range, bands can be attributed to C–N amide bonds, deformations in the vibration of carboxylic groups or O–C═O bonds. Finally, the intense bands between 1028 and 1047 cm^{-1} can be assigned to C–O bonds of alcohols and C–N and P–O bonds.

As stated above and can be seen in Fig. 3A and B, biosorption of Cu and Zn causes a noticeable decrease of characteristic FTIR bands, this attenuation being more significant in active sludge than in the microalga. This agrees with the observations made in biosorption and solubilization experiments showing that the bacteria are capable to uptake larger amounts of heavy metals per gram of dry matter. These results suggest the formation of new bonds during the metal biosorption process that would displace lighter ions or molecules responsible for the vibrations observed in the pristine biomass. Similarly, the formation of complexes between Cu and Zn ions and hydroxyl and carboxyl groups could be affirmed, since some bands show a displaced wavenumber. As a result, the presence of O–H, C═O groups coming from carbonyl groups, N–H, C–O–C groups coming from esters, amino acids and phosphate groups can be highlighted for being responsible for the formation of new bonds with the metal ions. These results confirm the presence of lipids, amino acids (proteins) and polysaccharides on the cell membrane. Due to the high intensity of the O–H, C═O and N–H signals in both spectra, it is confirmed that the cell membrane composition of the microorganisms is rich in lipids and proteins (Vargas, 2019), likely as lipoproteins. Therefore, although the biological matrices used in this work are very complex and many other mechanisms such as bioaccumulation may occur, it can be concluded that metal biosorption is the dominant process and that the two main biosorption mechanisms are based on ion exchange and complex formation reactions, mainly with protonable groups.

ESEM images were registered at different magnifications and are shown in Fig. 4 (microalga) and Fig. 5 (activated sludge). According to ESEM imaging, pristine microalgae presents a structure of small, almond-shaped striated globules (Fig. 4A1 and 4A2). After biosorption, these domains are observed to be in closer contact, forming a more compact group of globules (Fig. 4B1 and 4B2). Fig. 5 depicts ESEM images taken from freeze dried activated sludge before (Fig. 5A1 and 5A2) and after Cu and Zn biosorption (Fig. 5B1 and 5B2). The observed circular and filamentous structures could correspond with different bacteria strains present in the material. It is difficult to appreciate a change in the structure of the surface after biosorption as the images show a mixture of bacteria, sludge and other components present in this complex material.

The spectra registered using energy dispersive X-ray spectroscopy coupled to ESEM (ESEM-EDX) on gold-coated biomass samples allowed the identification of chemical elements present in the biomass surface, before (Fig. 4A3 and 5A3 for microalgae and activated sludge, respectively), and after the contact with metal containing wastewater (Fig. 4B3 and 5B3). The EDX spectra evidenced the occurrence of Cu and Zn biosorption on the biomass surface after the 72 h treatment of wastewater containing 100 mg/L of Cu and Zn. The intensity of the Cu and Zn peaks, proportional to the element concentration, confirms again the lower retention of Cu and Zn by the microalgae in contrast with activated sludge (Fig. 4B3 and 5B3). It should be noted that the presence of aluminum in the activated sludge samples is due to the use of aluminum flocculants in urban wastewater treatment processes. Other elements identified in activated sludge (Fig. 5A3 and 5B3) were silicon or magnesium, likely due to the presence of small amounts of sand and clay in the sludge, coming from the treated urban wastewater.

4. Conclusions

The green microalga *S. almeriensis* and aerobic bacteria from activated sludge resulted effective in removing Cu(II) and Zn(II) from nutrient-rich wastewater, such as swine wastewater. Activated sludge showed approximately twice uptake capacity than *S. almeriensis* for both heavy metals, attributed to the higher production of extracellular polymeric substances, EPS, and abundance of negatively charged functional groups in bacteria. Acetic acid 0.1 M extracted the highest percentages of metal in a single extraction from the loaded biomasses (76.3% Cu and 96.8% Zn from *S. almeriensis* and 55.4% Cu and 100% Zn from activated sludge). Sequential extractions showed high bioavailability and mobility of Cu and Zn from both biomasses, with easily solubilizable fractions of about 75% for Cu and 95% for Zn. These results show that metal ions are mainly bond to biomass through biosorption mediated by complex formation with protonable electron donors and the bioaccumulation inside the cell is low. FTIR and ESEM-EDX confirmed Cu and Zn presence on the biomass cell surface. Mild solvents as acetic acid or acetate buffer demonstrated the feasibility of biomass clean-up to produce safe bioproducts. Further investigation is needed to assess how operational conditions influence the mechanisms underlying heavy metal removal to optimize the complete biomass valorization processes.

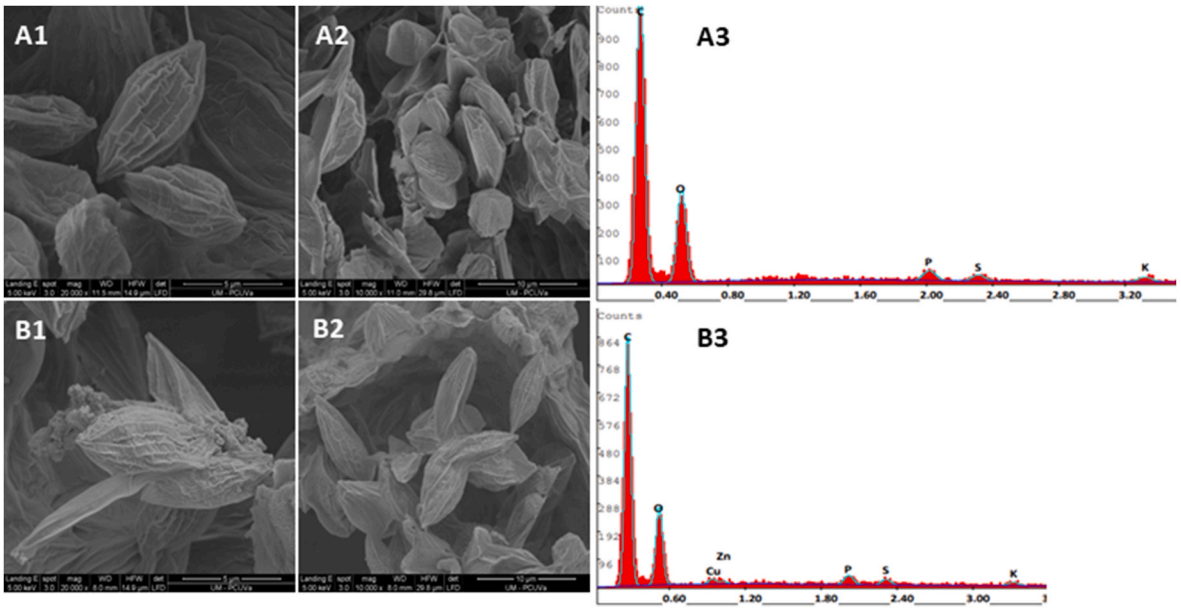

Fig. 4. ESEM images of pristine (A1, A2) and metal exposed (B1, B2) biomass of microalga *S. almeriensis* recorded at 20,000 × (A1, B1) and 10,000 × (A2, B2) magnification. ESEM-EDX spectra confirm the biosorption of Cu and Zn on the biomass cell membrane after wastewater treatment (A3, B3).

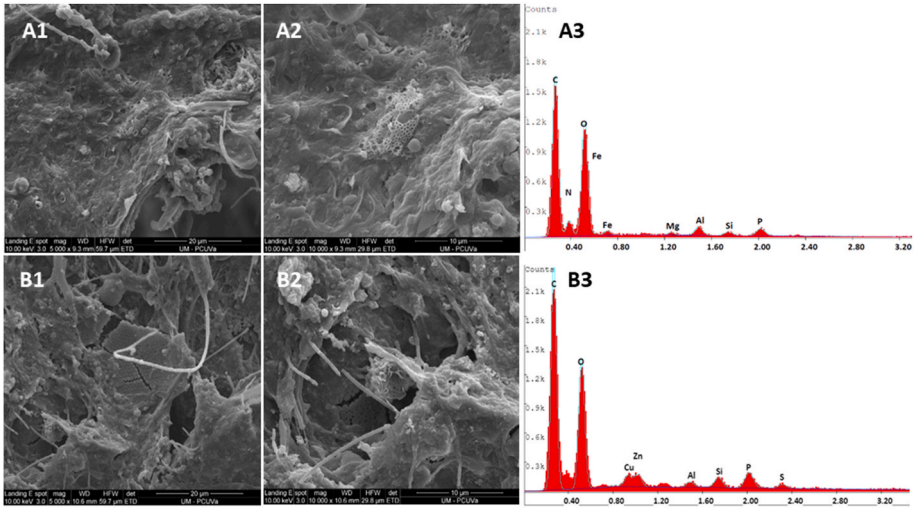

Fig. 5. ESEM images of pristine (A1, A2) and metal exposed (B1, B2) biomass of activated sludge (aerobic bacteria) recorded at 5000 × (A1, B1) and 10,000 × (A2, B2) magnification. ESEM-EDX spectra confirm the biosorption of Cu and Zn on the biomass cell membrane after wastewater treatment (B3).

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CRediT authorship contribution statement

Beatriz Antolín: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Alba Torres:** Investigation, Data curation. **Pedro A. García:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Silvia Bolado:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Marisol Vega:** Writing – review & editing, Visualization, Supervision, Resources, Funding acquisition, Formal analysis, 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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.141803.

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