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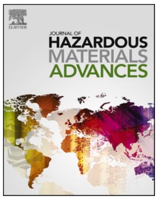

Evaluation of a microalgal–bacterial consortium for Cu and Zn removal using synthetic livestock waste solutions

Camino García Balboa *, Carlos Castelló Pinel, Eduardo Costas Costas, Victoria López-Rodas, Javier Pineda Pampliega

Complutense University of Madrid, Spain

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A B S T R A C T

The essential livestock production has increased in recent years, leading to a rise in its waste generation. One strategy to repurpose this waste is its use as fertilizer. However, the presence of heavy metals such as copper (Cu) and zinc (Zn) poses a contamination risk, making their removal essential before application. This study explores the use of a natural microalgal-bacterial consortium (Nacelle), in which microalgae and bacteria interact to remove Cu and Zn from livestock waste–simulating solutions. Two strategies were evaluated: biosorption with dried biomass and bioaccumulation with living cells. Biosorption was faster, but bioaccumulation achieved higher efficiency, reaching 93.7% metal retention. Exposure to metals also induced changes in microbial composition, with enhanced bacterial growth and reduced microalgal abundance. This study constitutes an essential first step, highlighting the promising potential of the 'Nacelle' consortium for the safe reuse of livestock waste as fertilizer.

1. Introduction

Veterinary medicine has achieved significant advancements in animal nutrition and animal health, enhancing conditions in livestock farms. All these advancements have contributed to an overall improvement in the well-being of individuals, leading to greater productivity. However, this increase in productivity comes with an added problem: a rise in livestock waste production, being its proper management one of the main challenges in livestock farms. The One Health Joint Plan of Action (2022–2026) (Yagüe-Muñoz et al., 2010), endorsed by the FAO, WHO, and UNEP in 2022, marked a significant step toward highlighting the importance of collaboration in environmental, animal and human health sectors. Within this context, the relevance of environmental preservation was strongly underscored. The EPA highlights the benefits of utilizing livestock waste as manure (US EPA, 2016). This practice is consistent with recycling strategies and circular economy frameworks due to the valuable components of the livestock waste for the soil. (Moral et al., 2005) analyzed the composition of slurries from various livestock species (e.g., cattle, horse, pig, poultry, sheep, goat, rabbit, and ostrich) in relation to their use as manure. Organic matter contents ranged from 39 % to 69 %, carbon contents varied between approximately 22 % and 41 %, and organic nitrogen ranged from about 1.5 % to 2.9 %, clearly demonstrating their potential use as manure properties. However, this potential is not without limitations, since insufficient characterization of their composition may lead to significant risk.

Animal feedstock includes supplementary additives, which are the source of heavy metals found in the livestock waste at significant concentrations (Brugger and Windisch, 2015). Heavy metals are persistent, bioaccumulative, and potentially toxic, and can accumulate in agricultural soils following repeated applications. Their presence may reduce soil microbial diversity and impair essential soil functions. Moreover, heavy metals can be absorbed by crops, not only affecting their growth negatively, also including them into the food chain, ultimately posing long-term risks to human health.

Due to the low intestinal absorption of metals approximately 95 % of the administered dose is excreted (Chen et al., 2022), contributing to metal accumulation in livestock waste. Slurry, the liquid from the mixture of animal excreta and urine, is characterized by low organic matter content, a low C/N ratio, and high concentrations of macro and micronutrients, being the part of the livestock waste where metals tend to accumulate. The metals and their concentration vary depending on the type of feeding and other characteristics of the exploitation. Copper (Cu) and zinc (Zn) are the predominant, with concentrations between

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* Corresponding author.
E-mail address: camino@ucm.es (C.G. Balboa).

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4.8–5.1 and 39.0–59.7 mg/L respectively, as they are used as growth promoters (Antezana et al., 2016). Other metals such as Al, B, Cd, Co, Fe, Li, Mn, Mo, Ni and Pb may also be present in slurries (Nicholson et al., 1999). The removal of these metals is an essential step to enable the safe use of slurry as manure. And, considering that the aqueous phase constitutes 95 % of the slurry, we believe it is feasible to employ a biotechnological approach based on aquatic microorganisms to remove the metals present in this aqueous fraction.

Biotechnological is an alternative increasingly used to remove heavy metals from polluted water environments (Fashola et al., 2024). It offers benefits such as the feasibility to be implemented in bioreactors located in situ, the low cost, and the effectiveness because biomass, the key active factor, is renovated itself. Biotechnology applied to metal uptake has been explored with plants (Ali et al., 2013), fungi (Kumar et al., 2019), yeasts (Morata and Loira, 2017), bacteria (Roy et al., 2024), and microalgae (Leong and Chang, 2020). Regarding the use of unicellular organisms, two different strategies have been tested: the selection of isolated strains (Vishwakarma et al., 2024) (Ma, 2024) (Zhang et al., 2024), or the use of microbial consortium. In this last case, two possible approaches have also been tested: artificial consortium, composed of selected pure strains that are forced to grow together in an artificial and pre-designed proportions (Che et al., 2024); or autochthonous consortium, obtained from samples collected in polluted environment (Abd Elrazak et al., 2016). One keyadvantage of using a naturally occurring consortium is that the initial adaptation phase to the pollutant is avoided. In contrast, artificial consortium requires multiple stages, including design, testing, adaptation, and selection of resistant cells prior to achieving effective degradation or biocapture, a process that is considerably more time-consuming even when successful.. Considering these advantages, we propose employing a native microbial consortium, specifically amixed consortium where microalgae and bacteria coexist naturally. This a novel approach aimed at taking advantage of the synergic activity of both types of microorganisms, enhancing the removal of contaminants through complementary mechanisms. Microalgae supply oxygen that supports bacterial metabolism, while bacteria release CO_2 and nutrients that promote algal growth, establishing a stable and self-sustaining system. The coexistence of these two types of organisms further enhances the consortium’s robustness against environmental fluctuations (pH, nutrients, metal concentrations). Complementary mechanisms are also involved in metal removal, with microalgae mainly contributing through cell wall biosorption and intracellular bioaccumulation, while bacteria enhance removal via metal biotransformation, mineral precipitation, and the production of metal-binding exopolymers..

Several studies have shown that this type of mixed microalgae-bacteria consortium could be used to treat metal-polluted wastes from different industrial activities, such as mining, chemical processing, leather manufacturing, textile industry and electroplating. In an artificial monometallic system with Cd, a *Chlorella salina–Bacillus subtilis* consortium was tested with success to uptake Cd, encountering a maximum Cd removal of 51.66 % (Yu et al., 2022). Dead biomass from a *Chlorella sorokiniana- Ralstonia basilensis* artificial consortium was tested for Cu(II), Ni(II), Cd(II), and Zn(II) uptake, obtaining a maximum Cu(II) adsorption of 8.5 ± 0.4 mg/g at an initial Cu(II) concentration of 20 mg/L. The yields for other metals were lower because a living culture did not tolerate Cu, which proved toxic (Muñoz et al., 2006). A complex waste composed of phenols, oil spills, and Cu, Ni, Mn and Fe as metals was treated with a synthetic microalgal-bacterial consortium (*Phormidium-Stichococcus)* obtaining a 62, 62, 90, 70 and 64 % of each metals removal respectively (Safonova et al., 2004). In Mubashar et al. mixed culture of *Chlorella vulgaris* and *Enterobacter* sp. was tested for the remediation of water from the textile industry. Chromium (Cr), cadmium (Cd), copper (Cu), and lead (Pb) concentrations decreased by 79 %, 93 %, 72 % and 79 %, respectively.

To date, biotechnological approaches, particularly artificial microalgae–bacteria consortia, have been applied for removing heavy metals from various industrial wastewater sources. However, their application to animal slurry or livestock waste remains limited. To our knowledge, only a few studies have addressed this context. For instance, Wang et al. explored metal removal using bacterial systems, while Blanco-Vieites et al. (2024) investigated nitrate removal with microalgae. These examples highlight the overall lack of research on metal remediation in animal-derived waste, and the even greater scarcity of studies employing combined microalgal–bacterial consortia.The production of livestock waste is increasing, and its use as manure represents a promising strategy for recycling it. However, its safe application requires the removal of potentially toxic metals. Biotechnological approaches appear to be particularly suitable for this purpose. However, to date, very few studies have investigated the use of microorganisms for metal removal from livestock waste. In this context, we propose the use of a natural microalgal–bacterial consortium. The use of consortia is a novel approach that offers advantages in removal efficiency, and employing a natural consortium reduces the adaptation time of the organisms. This study represents a preliminary study at lab conditions, using synthetic solutions containing the two main metals found in livestock waste: copper (Cu) and zinc (Zn). In this study, we first evaluated the consortium’s resistance and adaptability to metal exposure. Upon confirming its survival, we assessed its biocapture efficiency for Cu and Zn using two complementary strategies: either living cells or dried biomass. Simultaneously, we investigated the genotypic and phenotypic responses of the consortium under metal-induced selective pressure, providing a comprehensive understanding of its behavior and potential for sustainable metal remediation.The promising results obtained represent a significant step forward toward the safe and sustainable management of livestock waste, which is often applied to land without adequate sanitary or environmental safeguards.

2. Methods

2.1. Microalgae-bacteria consortium: sample site and composition

The microalgae-bacterial consortium “Nacelle” was isolated from a biological sample collected from a wind turbine blade of the Chiripa Eolic plant in Guanacaste Costa Rica. The plant is located 10 km from Arenal lake, 30 km from Arenal volcano, and 50 km from the coast. Preliminary observations using optical microscopy equipped with fluorescence evidenced the presence of prokaryotes and fluorescent eukaryote cells. The genome of the microbial sample was sequenced, resulting in a microbial consortium constituted of around 56 % of microalgae and 44 % of bacteria (in terms of the number of lectures in DNA identification). Only one microalgal species was identified as *Diplosphaera chodatii*, a eukaryotic green microalgal of the phylum *Chlorophyta*, class *Trebouxiophyceae* and order *Prasodiales*. This species is known for its ability to adapt to extreme environments, due to its resistance to high desiccation conditions (Medwed et al., 2024) and commonly found in symbiotic relationships with various fungi in lichenized forms (Gueidan et al., 2023) (Gueidan et al., 2023). The remaining biological material (44 % of the consortium) corresponded to bacteria, with 93 % from the phylum *Proteobacteria* and 7 % from *Bacteroidetes*. Of the prokaryotic component of the consortium, 72 % was composed of three bacterial species within the phylum *Proteobacteria* and class *Alphaproteobacteria*. Two orders were represented: *Sphingomonadales* and *Rhizobiales* at a ratio of 50:50 %. Regarding the species, the three more abundant were identified as *Porphyrobacter* sp.HT-58–2, *Ochrobactrum anthropi* and *Blastomonas fulva*, with relative abundances of 30, 23 and 19 % respectively. The genus *Porphyrobacter* belongs to the family *Erythrobacteraceae* and was first proposed by Fuerst et al. in 1993 (Fuerst et al., 1993). It is an aerobic anoxygenic phototrophic bacteria (Liu et al., 2017), whereas most species within this family are photosynthetic (HANADA et al., 1997) (Hiraishi et al., 2002). *Ochrobactrum* anthropic is a gram-negative bacterium, strictly aerobic, motile, oxidase-positive and indole-negative bacilli. The different species of the genus *Ochrobactum* are found in a wide variety of environmental habitats, including soil, water and air (Yagüe-Muñoz et al., 2010). *Blastomonas fulva* is an aerobic photosynthetic bacterium, next to *Microcystis* in metabYeolic and energetic requirements and evolved in the degradation of microcystin. Full details of the species are provided as supplementary material.

2.2. *Microalgae-bacteria consortium cultures*

Once the biological sample ("Nacelle" sample) was disaggregated in sterile distilled water, then was inoculated and grown in sterile aerated 50 mL cell culture flasks (Cellstar® Cell Culture Flasks, Greiner Bio-One). To determine the best growth conditions, two different culture options were tested: 1) BG-11 standard broth (Sigma-Aldrich®) to promote the growth of microalgae and 2) BG-11 standard broth, just as described, but supplemented with Luria-Bertani (LB) medium at 2.5 % to facilitate the growth of microalgae and aerobic heterotrophic bacteria possibly present in the sample.

Triplicates of both conditions were prepared as follows: 10 mL (10^5cells/mL) of the mixed consortium culture, was inoculated into: 1) 90 mL of BG-11 medium and 2) 89.5 mL of BG-11 + 0.5 mL of LB. Cultures were then grown under constant conditions of light ($\lambda = 390$ - 700 nm) and temperature (22 ± 2 °C) in sterile aerated 50 mL cell culture flasks. At day 6, Cultures were transferred axenically every 20 days to keep the mid-log exponential growth. the number of microalgal cells was 4.16×10^6 cells/mL and 6.56×10^6 cells/mL in BG-11 and BG-11+LB medium, respectively. After 7 days of growth, cultures were checked under an optical microscope (ZEISS®) observing a rapid growth of green microalgae cells and bacteria. To determine to what extent the proliferation of bacteria in the culture affected microalgae growth, microalgae counting was carried out using a Fast-Read®102 counting chamber (BioSigma®). This result proved that the presence of bacteria favours the growth of microalgae and therefore, we decided to use BG-11+LB to grow the Nacelle consortium for the biouptake tests.

The growth rate of microalga and bacteria at the consortia was estimated as $\mu = \text{Ln}\ (X_i\text{-}X_o)/\Delta t$, where X_i and X_0 are the cellular concentration at 30 days and the starting moment, just prior to inoculate the culture media, respectively. μ was 0.63 and 0.52 cells/ml.day for microalgal and bacteria respectively.

2.3. *Microbial sample identification and characterization*

"Nacelle" sample was subjected to an in-depth phylogenetic analysis. Preliminary observations under optical and fluorescence microscope showed the presence of prokaryotic and fluorescence eukaryotic cells.

Procaryotic cells in the sample were identified by rRNA 16S high-throughput gene sequencing via Oxford Nanopore technology. For this, DNA was extracted by enzymatic digestion and column purification. The extracted DNA was then subjected to PCR amplification of the complete rRNA16S gene using primers 27F - 5′AGAGTTTGATCMTGGCTCAG 3′ and 1492R - 5′ CGGTTACCTTGTTACGACTT. A tag was added to the purified amplicon via ligation, using SQK-NBD114.96 kit, and was then sequenced via Oxford Nanopore technology, MinION FLO-MIN114 R10.4.1. Readings were then reanalysed under high-quality parameters, filtered by size (1300–1800 nt) and quality ($q > 15$). The taxonomic assignment of the readings was done using EMU software. Species with fewer than 10 reads were discharged.

To identify eukaryotic species, the 18S rRNA region was amplified from the extracted DNA and sequenced by both strands using automatic sequencing on an ABI 3730 device. The sequence obtained was compared via Blast with the NCBI nr/nt database.

2.4. *Bioadsorption assay*

The potential use of the "Nacelle" microalgae-bacteria consortium as a biosorbent for the treatment of animal livestock wastewater was also explored. For this, bimetallic aqueous solutions of Cu and Zn were prepared from stock solutions at different concentrations. Two stock solutions of Cu ($CuSO_4 \cdot 5H_2O$, Sigma-Aldrich®) and Zn ($ZnSO_4 \cdot 7H_2O$, Sigma-Aldrich®) at 10 g/L (divalent cation) each one was prepared and sterilized by filtration (Millipore Steritop ®, Sigma-Aldrich). These were then used to prepare bimetallic solutions at two working concentrations: a) 5 mg/L of Cu and 50 mg/L of Zn; b) 50 mg/L of Cu and 500 mg/L of Zn.

Microalgae-bacteria consortium "Nacelle" cultures were grown for 20 days in 1 L cell culture flasks (Cellstar® Cell Culture Flasks, Greiner Bio-One) under previously described culture conditions to get abundant biomass for experiments. Once cultures reached the saturation stage, biomass was harvested by centrifugation at 4000 rpm for 15 min. The pellets were dried in an oven at 50 °C for 48 h and ground to a fine powder.

Biouptake tests were prepared as follows: 100 mg of dried biomass was suspended in 20 ml volume in a 100 mL Erlenmeyer flask under two experimental solutions: a) 5 mg/L of Cu and 50 mg/L of Zn; b) 50 mg/L of Cu and 500 mg/L of Zn, and kept on a rotary shaker at 150 rpm, 22 °C. Two different contact times were selected: 15 and 60 min. Triplicates of each concentration and exposure time were prepared.

Afterward, biomass was harvested by centrifugation of the whole culture at 4000 rpm for 15 min, and supernatants and pellets were separated and stored until chemical analysis.

2.5. *Metals biouptake (bioadsorption plus bioaccumulation)*

Two kinetic aspects were explored in cultures (living cells) of the "Nacelle" consortium: growth of bacteria and microalgae, in the presence and absence of metals, and the biocapture of Cu and Zn as cells grow.

Three replicates of the microalgae-bacteria consortium were prepared for each of the two conditions (metal presence and absence). 20 ml of the cultures were carried out in sterile aerated 50 mL cell culture flasks (Cellstar® Cell Culture Flasks, Greiner Bio-One) and kept under the previously established growth conditions (microalgae-bacteria consortium cultures) and in the presence and absence of metals (controls). . The living cell cultures were exposed to metal concentrations of 1mg/L Cu and 10 mg/L Zn. The concentration of Cu and Zn was smaller than in the case of bioadsorption trials because previous tests with increasing concentration of both metals exhibited a diminution in kinetic. So, we decided to select an intermedial conditions.

Five ml of each culture was sampled at 0, 72, 168, 240, and 720 h. The selection of these sampling times is based on previous work by our group, in which changes in metal uptake capacity were observed at these specific time points. This samples were selected to monitor cell growth (cytometry analysis), cell morphology (fluorescence microscopy) and chemical analysis (ICP-MS). Biomass and supernatants were harvested by centrifugation (4000 rpm for 15 min) for spectroscopic analysis. Cell numbers for both populations (microalgal and bacterial) were quantitatively measured by flow cytometry in a Cytek™ Aurora spectral cytometer. For this purpose, 50 μlaliquots were collected from each replicate over the five sampling days and diluted in PBS. Samples were incubated in the dark with SYTO™ Green Fluorescent Nucleic Acid Stains (InvitroGen®) and Propidium Iodide (ThermFisher®)to distinguish between live and dead cells.

Prior to the analysis, discrimination criteria between microalgae or bacteria criteria based on their size and complexity were established via Forward versus Side Scatter gating. After this, the populations of living and dead bacteria and microalgae were determined by differences in fluorescence derived from the fluorochromes, considering that the autofluorescence of the algae did not mask the results. SYTO™ fluorochrome penetrates both viable and non-viable cells, and propidium iodide fluorochrome binds to DNA, so it penetrates only damaged cells. In this way, the comparison of fluorescence profiles allows the discrimination between viable and non-viable cells. Cytometric results were processed using Microsoft™ Excel software.

Any morphological variations present in the culture over time were observed by confocal fluorescence microscopy using an Olympus IX83 confocal microscope with a STED super-resolution system (Abberior™). The 50 microliter aliquots taken at each sampling time were stained with SYTO™, Propidium Iodide and Calcofluor White (Sigma-Aldrich). SYTO9™ and propidium iodide allow differentiation between viable and non-viable cells, while Calcofluor White binds to the walls of the microalgae, allowing for a better observation of its structure. Blue cellular walls and read chloroplasts which auto-fluoresce inside the cells let to define cellular integrity. Images obtained were then processed using Fiji Image J software.

2.6. *Chemical analysis*

The pellets from biosorption and bioaccumulation assays were first subjected to acid digestion with 5 mL of a HNO_3-H_2O_2 mixture (4:1 v/v) in Teflon beakers and gentle heating on a hotplate until a transparent solution was achieved. Sample solutions were then evaporated until almost dried, and the residues were dissolved in 3 M HNO_3. One milliliter of each of these solutions was brought to a final volume of 10 mL with Milli-Q water, and Cu and Zn concentrations were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using a quadrupole instrument BRUKER Aurora Elite equipped with a collision cell (iCAP Q, Thermo FisherScientific), applying external calibration quantification method and internal standardization.

The total concentration of Cu and Zn in supernatants was directly quantified in the liquid samples after being acidified and diluted, when necessary, by inductively coupled plasma optical emission spectroscopy, ICP-OES (SPECTRO Arcos, SPECTRO Arcos III). Analyses were performed in the Centre for Research Assistance (CRA), CAI of geological techniques of the Complutense University of Madrid.

2.7. *Statistical analysis*

To evaluate if there were differences in the microalgae growth based on the presence of metals (living cells), we used a generalised mixed model. We include “cell number” as the dependent variable (with a lognormal distribution); “time” (0, 72, 168, 240, 720 h), and “treatment” (control and metals) as factors, including also their interaction, and “flask” as a random factor. A similar approach was used for measuring the effect of the presence of metals in bacterial growth but using a general mixed model due to the normality of the residuals of the model.

To test the dependence of Cu and Zn biocapture (living cells) with the time of exposure, one general mixed model was designed for each metal. In both, we used the metal concentration as the dependent variable; time (0, 72, 168, 240, 720 h) as a factor, and flask as a random factor.

Regarding the dried biomass, we wanted to compare if the amounts of Cu and Zn bioadsorbed was affected by the time of exposure. For this, we designed two general mixed models (one for each metal), using “metal concentration” as the dependent variable, “time” (15 and 60 h) as a factor, and “flask” as a random factor. Finally, to test if the bioadsorption capacity was related to the initial metal concentration on the medium, we designed two linear models using “metal concentration in cells” as the dependent variable and “concentration” (high or low) as a factor.

In some models the random factor “flask” was included to avoid pseudo-replication since samples taken at different time points come from the same flask.

All models were validated by visual inspection of the residual graphs to verify the assumptions of normality of the residuals and homogeneity of the variances. All analyses were performed in R 4.4.2. using the R packages “emmeans” (1.10.5) (Length and Piaskowski, 2017), “glmmTMB” (1.1.10) (Brooks et al., 2017) and “nlme” (3.1–166) (Pinheiro and Bates, 2002). The package ggplot2 (3.5.1) (Wickham, 2016) was used for graph design.

3. Results and discussion

3.1. *Growth kinetic of the consortium in a media with Cu and Zn*

The growth kinetic of microbial consortia to 1 mg/L of Cu and 20 mg/L of Zn differed between the microalgal and bacterial components. The microalgal component was significantly affected by metals at 240 and 720 h of growth (Chi-square4 = 456, $p <$ 0001) (Fig. 4). The growth rate in the microalgal component of the consortia diminished from 0.634 to 0.592 cells/mL when it was grown in the presence of metals. Cu is an essential element for microalgal growth, as it plays a key role in photosynthesis, respiration, and defence against Reactive Oxygen Species (ROS) via superoxide dismutase (SOD) enzyme, being growth-limiting factor (Cavalletti et al., 2022). However, these beneficial effects are concentration-dependent and vary in function of the species presented and environmental conditions, such as the presence of other metals. Beyond specific thresholds, Cu negatively impacts microalgae, leading to reduction in growth rates, photosynthesis inhibition, respiratory impairments, and morphological alterations, including changes in cell and organelles size (Cavalletti et al., 2022). Regarding Zn, low concentrations aided the growth of certain algae, while high concentrations slowed growth and reduced cell division (El-Agawany and Kaamoush, 2023). When Cu and Zn are present together, the cellular response to their combined toxicity involves the extracellular adsorption and the binding of significant amounts of both to EPS (extracellular polymeric substances) and cell wall functional groups (Ye et al., 2023).

Regarding the bacterial component of the consortium, it was significantly affected by the presence of metals at 2fatro40 and 720 h (Chi-square4 = 483, $p <$ 0001) (Fig. 4). In contrast to the microalgal response, where growth decreased under metal exposure, the bacterial component exhibited an increase in cell numbers under the same conditions. Bacteria increased their growth rate from 0.518 to 0.550 when are in the presence of Cu and Zn. This result has been yet described in bacteria, having found a worrying increase in bacterial resistance to both metals because of their increasing presence in soils and water (Poole, 2017). These results demonstrate that our Nacelle consortium is capable of surviving in media rich in Cu and Zn. Our next step is to assess its efficiency in capturing these metals.

> These differences in the effects of metals on microalgal and bacterial growth were expected. Although metals can negatively affect bacterial growth, it is well established that many bacterial species are naturally capable of tolerating high metal concentrations, even use them as electron donors, which is why bacteria have been widely used in bioremediation processes (Escamilla-Rodríguez et al., 2021). In contrast, microalgae are generally more sensitive to metal exposure, although they can be highly effective in metal removal when concentrations are not excessively high (Yang et al., 2025). Therefore, the use of a mixed consortium may enhance overall system performance by enabling synergistic interactions between the complementary characteristics of both microbial groups.

3.2. *Cu and Zn biouptake by the consortium*

The “Nacelle” microalgal-bacterial consortium was tested to evaluate its potential use biouptake of Cu and Zn in a synthetic solution, as essential prior step for its use in remediating slurry from animal livestock. Several organisms, cells, and even materials such as shells (Chenet et al., 2024) or tree barks (Şen et al., 2015) have been tested as biological alternatives to purify metal-polluted waters. We discarded non-renewable materials from an environmental conservation point of view. The selection of self-renewable alternatives, such as the living cells of consortium, has the advantage of the self-regeneration of the sorbent

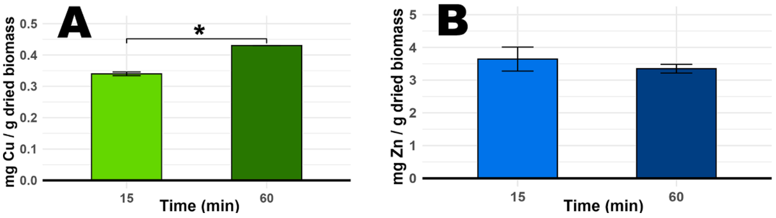

Fig. 1. Bioadsorption of Cu (A) and Zn (B) as a function of contact time. Keys and * indicate significant differences between groups.

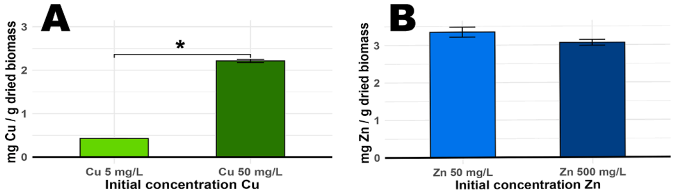

Fig. 2. Effect of the initial metal concentration in the bioadsorption of Cu (A) and Zn (B). Keys and * indicate significant differences between groups.

material. Moreover, regarding the microalgal component, there is the added benefit of the consumption of atmospheric CO_2 and the liberation of oxygen. Additionally, the discarded material may be reused and even metals could be recycled (Suresh Kumar et al., 2015).

To gain a comprehensive understanding of this consortium's potential to remove Cu and Zn from livestock-derived slurry, we use two approaches: the living consortium and its dried biomass. For the dried biomass, only bioadsorption was evaluated. In the case of living cells, we estimate the kinetic of the combined bioadsorption and bioaccumulation processes and assess the effects of metals on the phenotypic and genotypic behaviour of the indigenous consortia.

3.2.1. Dried biomass: bioadsorption

Dried biomass from the microalgal-bacterial consortia “Nacelle” was tested for its ability to uptake Cu and Zn. A solution containing both metals at the habitual concentration in waste livestock (Oliveira et al., 2021) (Collao et al., 2022) was mixed with 100 mg of the dried biomass, maintained at constant stirring of 150 rpm and 22 °C. We evaluate two different factors regarding the bioadsorption of Cu and Zn: the influence of contact time and the effect of the initial metal concentration.

To determine the influence of contact time, two periods of 15 and 60 min were tested. After the first 15 min, 34 ± 1 and 364 ± 63 mg/g of Cu and Zn, respectively, were recovered in pellets of dried biomass. These values increlased up to 43 ± 0 and 335 ± 23 mg/g after 60 min (Fig. 1). A statistical difference was observed in the Cu concentration between the two contact periods tested ($F_{1,2} = 243$; $p < 0.01$) but no difference was found in relation to Zn ($F_{1,2} = 0.651$; $p = 0.504$). The yield is consistent with reported values, with mean values for bacteria between 70–125 mg/g (Fathollahi et al., 2021).

The initial metal concentration had also influenced the yield of Cu bioadsorption ($F_1 = 2253$; $p < 0.001$) (Fig. 2). With an initial concentration of 5 and 50 mg/L of Cu and Zn, respectively, 43 ± 1 mg /g of Cu and 335 ± 23 mg/g of Zn were recovered after 60 min. When the initial concentration was increased up to 50 and 500 mg/L of Cu and Zn, respectively, 221.3 ± 6.5 mg/g of Cu and 306.3 ± 13.5 mg/g were retained in the dried biomass.

These results suggest that Cu exhibits a greater affinity for biomass sites in comparison to Zn. When the concentration of Cu and Zn was increased by 10 units, an increase of 178.3 mg/g was achieved in the case of Cu. In contrast, 28.66 mg/g of Zn was displaced from the binding sites. We have selected divalent salts of Cu and Zn ($CuSO_4 \cdot 5H_2O$; $ZnSO_4 \cdot 7H_2O$), then there are no differences in terms of charge between the divalent cations of both metals. Another factor such as ionic exchange or surface complexation may also affect the results (Mahlangu et al., 2024). These results are consistent with those previously reported, where it was observed that, due to its chemical properties, Cu binds much more strongly to organic matter than Zn, whether in living microalgae (Antolín et al., 2024), sewage sludge Antolín et al., 2024(), or even in organic compounds such as carboxylated biosorbents (Pan et al., 2022).

In the conditions tested in our research, the saturation limit for Zn was around 300 mg/g of dried biomass, whereas the saturation limit was not reached for Cu. In a similar study, a microalgal-bacterial consortium composed of *Ralstonia basilensis* strain in symbiosis with the microalga *Chlorella sorokiniana* was tested for its potential to uptake Cd(II), Ni(II), Cu(II), and Zn(II). The authors also reported a preference for Cu biouptake from the medium (Muñoz et al., 2006) assuming that there was no competition for adsorption sites and suggesting that each metal binds to different locations of the surface cells. A similar behaviour was observed in our study: when the initial concentrations of 5 and 50 mg/L for Cu and Zn were tested, Cu bound to new available sites, and Zn remains almost constant. (Muñoz et al., 2006) reported similar yields, with a maximum Cu(II) adsorption of 8.5 ± 0.4 mg/g achieved with an initial Cu(II) concentration of 20 mg/L. These results reveal a certain specificity that could be exploited for selective Cu biouptake and potential reuse of the metal. The selective biorecovery of scarce metals or high-value elements is very relevant under the principles of circular economy, thus avoiding the environmental impact of mineral exploitation and metallurgical operations.

Although there is limited literature related to the application of mixed bacteria-microalgal consortia for metal biouptake from polluted solutions, it has been proved that microalgal and bacteria establish a complex relation, normally symbiotic (Zhao et al., 2023), in which the extracellular substances excreted by both species play a key role in the interaction, survival conditions and also in the biocapture of metals (Muñoz et al. 2006).

The differential contribution of the bacterial and microalgal components of the “Nacelle” consortia to the adsorption process was not estimated in the present study. However, both, bacterium and microalgal have successfully been tested for the remediation of metal-polluted waters previously. Regarding the role of microalgae on the biorecovery of metals, several species have been exploited under different conditions. One of the latest reviews in the field (Mahlangu et al. 2024) presented examples of yields that may vary from 0.46 μg/Kg of Cd with *Spirirulina*; 0.00722, 0.043 and 0.096 mg/Kg for Hg, Cd and Pb using *Chlamydomonas reinharditii*; 140 mg/g of Pb with *Spirogira* and 96.20 mg/g of Cd(II) with *Parachlorella* sp. The stability of the metal-microalgal bind is often provided through the interaction between positively charged metal and the anionic groups (e.g., polysaccharides, carbohydrates, hydroxyl groups) exposed on the surface of the microalgal cells walls, as well as other groups in the extracellular polymeric substances (EPS) (Mahlangu et al., 2024). *Diplosphaera chodatii*, the microalgal component of the consortia tested in this study, belongs to *Chlorophyceae*. Other microalgal from the same genus, such as *Chlamydomonas reinharditii*, and from other orders, such as *Spirogyra, Spirulina* or *Parachlorella*, have also shown very good results in the biosorption of metals such as Hg (II), Cd (II), Pb (II) and Cu (II) (Mahlangu et al., 2024).

Regarding bacteria, there have been numerous attempts to exploit bacteria for metal capture, particularly heavy metals such as Cd (II), Cr

Table 1
Percentage of metals biorecovered.

Starting conditions	% Cu recovered		% Zn recovered		% Cu+Zn recovered	
DRIED BIOMASS						
	15 min	60 min	15 min	60 min	15 min	60 min
100 mg dried biomass/ 20 mL 5 mg/L Cu; 50 mg/l Zn	34	43	36,4	33,50	70	79,4
100 mg dried biomass/ 20mL 50 mg/L Cu; 500 mg/ l Zn		22,13		30,63		52,76
LIVING CELLS						
1 mg/L Cu, 5 mg/L Zn						
0 h, 1856,743 cells/mL	6,5		6,5		13	
72hours 5964,573 cells/mL	14,8		59,7		74,5	
168 h 11,634,456 cells/mL	18,2		61,8		80	
240 h 20,782,140	17,5		59,8		77,3	
720 h 69,273,800 cells/mL	25,7		68,0		93,7	

(II), Pb(II), Zn(II), Cu(II), Ni(II) and Mn(II). The Firmicute phyla (not encountered in the consortia) showed the highest overall biosorption efficiency (both living and dead biomass). The living biomass of Proteobacteria, which is very abundant in our consortia, has shown the best biosorption performance (Fathollahi et al. 2021).

These results suggest that, even when using only dried biomass, our consortium proves to be capable of capturing both metals, although its capacity to capture Cu is notably higher.

3.2.2. *Living cells: biouptake (bioadsorption plus bioaccumulation)*

The consortium “Nacelle” has demonstrated its ability to uptake both Cu and Zn. However, the dynamics of the process differ significantly. There is an effect of time of exposure regarding the amount of Cu uptake ($F_{4,8} = 35.7$, $p < 0.001$) (Fig. 5). The value at 72 h is higher than the initial value, however, this amount continues increasing, with the maximum amount at 720 h. Regarding Zn uptake, there is also a time effect ($F_{4,8} = 95.95$, $p < 0.001$) (Fig. 5). However, there are no differences regarding the amount uptake at 72 h with the rest of the study points (168, 240 and 720 h). These findings indicate differential kinetic patterns in metal uptake by the consortium, with Cu exhibiting a continuous accumulation over time. In contrast, Zn uptake reaches a saturation point at earlier time intervals. These results are consistent with those reported by Maznah et al., 2012, who observed that Chlorella sp. displayed a higher biosorption capacity for Cu than Zn. They attributed this difference to the greater affinity of Cu ions for functional groups such as carboxyl and phosphate present on the algal cell surface, as well as differences in the chemical properties and complexation behaviour of Cu Zn in aqueous environments, being Cu is more reactive, having, higher tendency to bind with biomass.

3.2.3. *Bioadsorption vs biouptake*

Bioadsorption and bioaccumulation are processes involved in the biouptake of metals, but their mechanistic aspects differ significantly. Bioadsorption refers to the binding of elements on the cells surface through different mechanisms such as chelation, ionic interaction, ion-exchange or coordination with functional groups on the cell surface. Thus, it does not require living material; in fact, at its most extreme, only surfaces with active groups are needed. In contrast, bioabsorption (or bioaccumulation) needs the activity of living cells, involving enzymes, transport mediators, and intracellular detoxification mechanism once metals have entered the cell (Chojnacka, 2010).

A significant amount of scientific literature has been published comparing bioabsorption and bioadsorption (Kaduková and Virčíková, 2005) and it is often suggested that bioadsorption is preferred in terms of effectiveness. However, our results challenge this assumption. As shown in Table 1, bioadsorption is beneficial in kinetic terms: it is a quicker process. However, if referring to the yield of the process, the maximum, 93.7 % of Cu+Zn retention was only achieved with living cells. Dead biomass only reached a yield 80 % after 60 min that was almost achieved with living cells (74.5 %) within 72 h. It should be noted that these values reflect the outcomes under the specific experimental conditions tested for each biomass type, rather than a direct one-to-one comparison between living and dead biomass. Biotechnological approaches based on dried biomass are preferred because of the ease of their management. However, biomass must first be dried, which is a high-energy-consuming process. In contrast, living microalgal-bacterial consortia, such as the one used in this study, are less demanding in terms of material and energy inputs, self-sustaining and gets a high yield.

3.3. *Genotypic and phenotypic changes*

3.3.1. *Genotypic evolution of the consortium*

The potential for bioremediating heavy metal-polluted environments of different origins, such as mining, electronic industries, urban wastes, smelting sites, metal-manufacturing plants, and tanneries has been studied from two perspectives: the use of dried biomass or the exploitation of the metabolic activity of living cells (Singh, 2020). Both options offer benefits and drawbacks (Ayele et al., 2021), regardless of the microbial species selected.

In addition to the experiments previously described with dead biomass, we also decided to examine the behaviour of living cells to understand how this complex population evolves under the selective pressure of Cu (1 mg/L) and Zn (10 mg/L). Our focus was not only on comparing both options in terms of effectiveness but also on obtaining insights into the genome evolution of the consortia. This information could be useful in the future for designing a consortium *ad hoc*, by selecting species that are more resistant to metals, while avoiding sensible species. Furthermore, it may be possible to select determinant genes relevant to the decontamination process (Arunraja et al., 2023).

The presence of Cu and Zn did not dramatically change the genotypic composition of the microbial consortia, indicating that the relative abundance of species remained stable. However, *Diplosphaera chodatii*, the only microalgal species present in the consortia, which represents 56 % of the total microbial biomass, was reduced to 42 % in terms of biomass when the consortia when exposed to Cu and Zn, suggesting a greater impact on algal growth. Up to certain concentrations, metals such as Cu and Zn are considered micronutrients for microalgae (Chugh et al., 2022), but higher concentrations trigger mechanisms of detoxification such as biosorption. Cu affects photosynthetic activity (Rocha et al., 2021) and induces chloroplast dysfunction, generation of reactive oxygen species (ROS), and a reduction in growth rate (Cavalletti et al., 2022). The response to increased concentrations of Cu and Zn has been studied in *Pavlova viridis* (*Prymnesiophyceae*), revealing oxidative damage in the microalgal cells, which led to an increase in lipid peroxidation and activation of some antioxidant enzymes such as superoxide dismutase (SOD) (Li et al., 2006). Relating the effect of Zn on microalgae, a EC50 of 15 mg/L was estimated in *Dunaliella tertiolecta*, lower concentrations of Zn accelerated the growth, while higher concentrations produce an inhibitor effect (El-Agawany and Kaamoush, 2023).

The evolution of the composition of the bacterial component of the consortia under the selection pressure of Cu and Zn was assessed by measuring species richness and calculating the Shannon (H) and Simpson (D) (Simpson, 1949) indexes. Richness decreased in 5 species (from 45 to 40), Shannon value (H) varied from 2.17 to 1.73 and inverse Simpson (1/D) from 5.33 to 3.52 after the metal addition. Before metal exposure, 92 % of bacterial biomass in the consortia was represented by

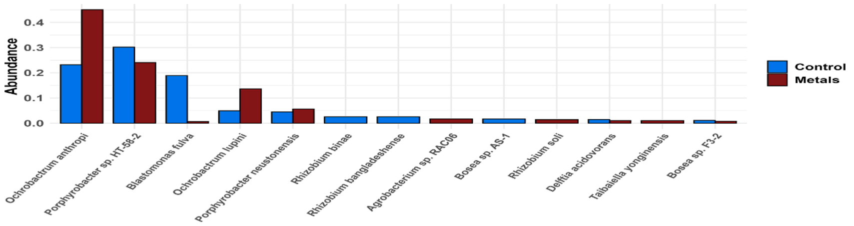

Fig. 3. Abundance of the ten most abundant species within each treatment.

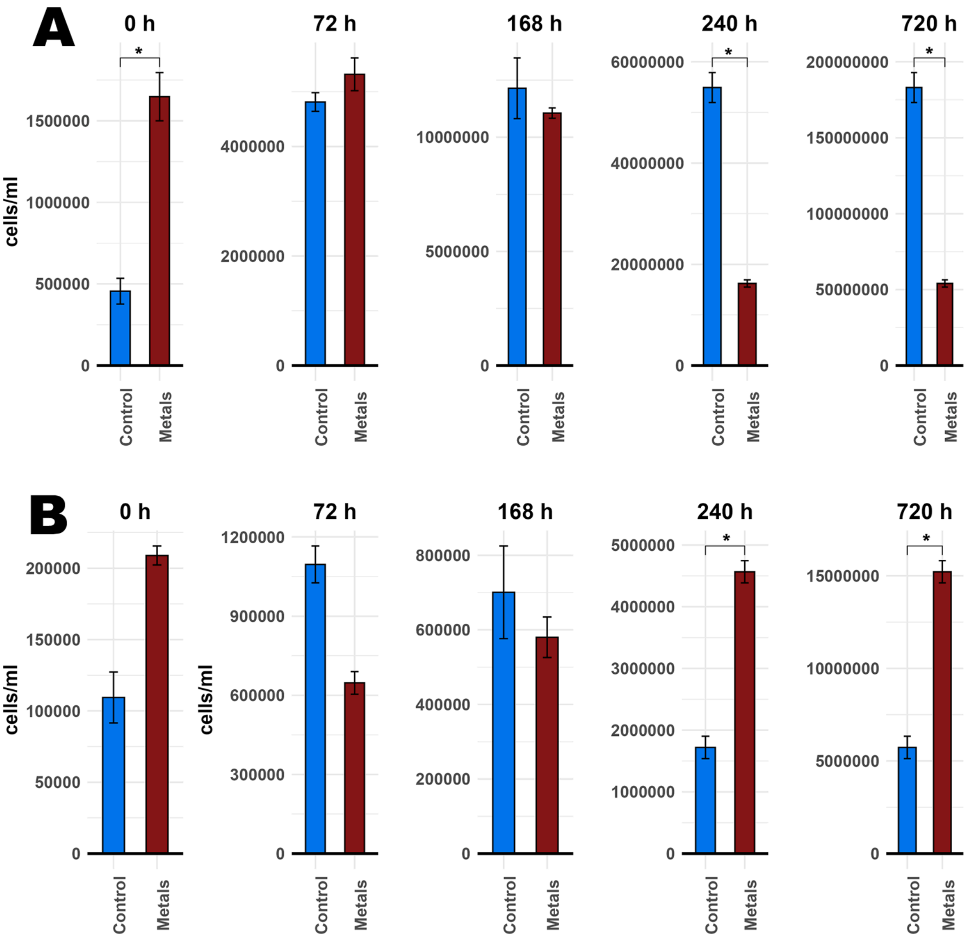

Fig. 4. Growth kinetic of the microalgae (A) and bacterial (B) components of the Nacelle consortium. Keys and * indicate significant differences between groups.

11 different species; after metal exposure, 93 % of the bacterial biomass was represented by only 8 species, representing a reduction in overall diversity. (Fig. 3). From a species-level perspective, 72 % of the total bacterial biomass in the indigenous consortia is represented only by three species: *Porphyrobacter* sp.*HT-58–2* (30 %), *Ochrobactrum anthropic* (20 %) and *Blastomonas fulva* (20 %). *Porphyrobacter* sp.*HT-58–2* decreased to 24 % after exposure to metals. *Blastomonas fulva*, which initially represented 18 % of the natural consortia, was affected by metals and diminished up to 0.63 %. However, *Ochrobactrum lupini* which only represented around 23 % in the natural consortia, enhanced up to reach 45 % in the presence of metals. Although no studies have directly demonstrated the ability of this bacterium to tolerate heavy metals, related species within the *Ochrobactrum* genus have been reported to possess metal resistance and bioaccumulation capabilities. *Ochrobactrum intermedium BPS-20* and *Ochrobactrum cicero BPS-26* have demonstrated not only the ability to survive Pb and Ni above 2000 mg/L and 800 mg/L, respectively, but also not suffer an inhibitory effect on the growth rate (Sharma and Shukla, 2021) . Another example from this genus is *Ochrobactrum MT180101*, which resisted Cu toxicity through multiple mechanisms, including binding metal cations to the cell wall, producing chelating and stabilizing compounds, transporting metals from the inner to the outer membrane, and enzyme-mediated biotransformation (Peng et al., 2019). Regarding Zn, *Ochrobactrum EEELCW01* has been shown to tolerate its presence without any impact on its survival (Huang et al., 2025) . All these examples of species from the same genus as the one identified in our study demonstrate that this genus possesses specific traits and mechanisms that allow its members to survive and be well adapted to metal-rich environments. These findings suggest that this species could be a promising candidate for future studies on metal removal from the environment.Thus, *Porphyrobacter* sp. *HT-58–2, Ochrobactrum antropi* and *Ochrobactrum lupini* were the most abundant and metal-resistant species to Cu and Zn under the concentrations studied.

From the original 45 bacterial species, after exposure of the consortia to Cu and Zn, 12 disappeared, 13 increased their abundance and 7 species not previously detected were identified under the selective

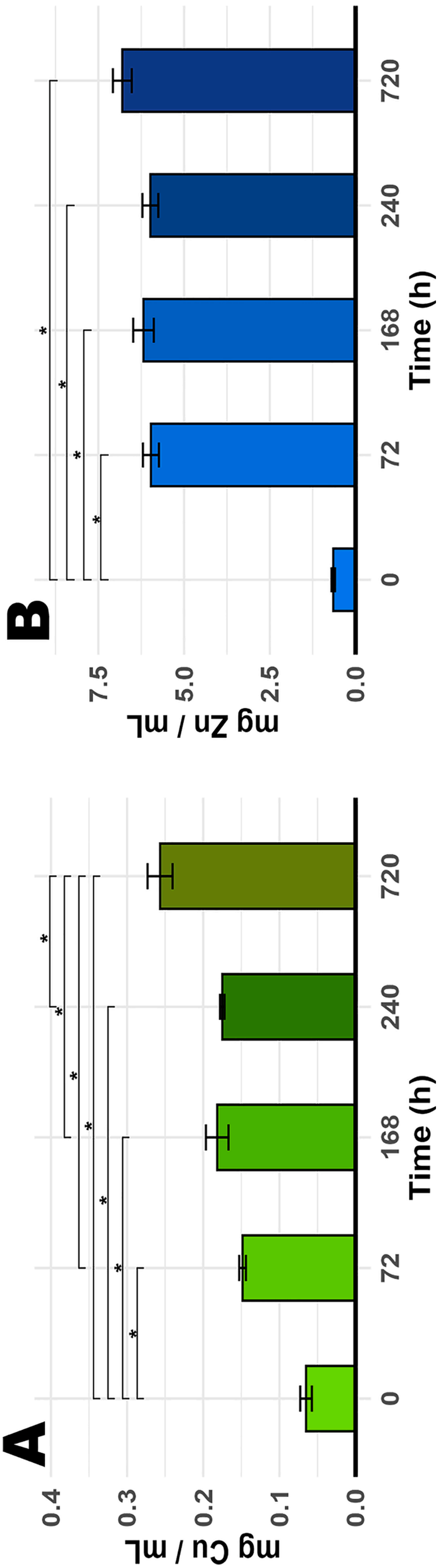

Fig. 5. Changes in the uptake of Cu (A) or Zn (B) with time. Keys and * indicate significant differences between groups.

pressure of the metals. Cu and Zn caused the disappearance of species that were present in low abundance (below 6 %) in the natural consortia and diminished the abundance of other more dominant such as *Porphyrobacter* sp. *HT-58–2* or *Blastomonas fulva*, which abundance was affected in a percentage of 6 and 18 % respectively.

The disappearance of bacterial species could be explained by the toxic effect of Cu and Zn (Song et al., 2018), which have been shown to alter and inhibit microbial functioning (Velázquez-Fernández et al., 2024), (Cavalletti et al., 2022). Cu and Zn are among the most toxic metals (as Hg, Pb, Cd, Cr, or As). However, unlike these metals, Cu and Zn may act as microelements with functional roles. This difference explains the differential responses of living organisms to their presence. In this way, the impact of Cu and Zn on a bacterial community may, among other effects, reduce biodiversity, but it may also promote the growth of resistant bacteria that adapt and increase in abundance (Sazykin et al., 2023).

The species that increased its abundance (with a non-significant increase) might have been favoured because Cu or Zn also may act as an enzymatic cofactor. Cu, Zn and nickel (Ni) are trace metals essential for some bacterial enzymes (Velázquez-Fernández et al., 2024). Finally, six not previously detected species, representing 0.3 % of the total, were detected in the consortia after exposure to the metals. From a quantitative perspective, these new species represent an insignificant contribution to the overall composition of the consortium. It is possible that these species were present initially but were not detected, or that existed in a resistant form (such as spores) that proliferated when other species disappeared, thus occupying their niches.

3.3.2. *Phenotypic response of the consortium*

3.3.2.1. *Cells morphology.* Among the expected phenotypic changes in our consortium is the absorption of Cu and Zn. However, since this change has already been discussed in Sections 3.2.2 and 3.2.3, this section will focus on another important phenotypic change: cell morphology. Samples from consortia cultures grown in the presence and absence of Cu and Zn were co-stained with SYTO9™ Green Fluorescent Nucleic Acid Stains (InvitroGen®) and Propidium Iodide (ThermFisher®, PI) to observe differences in bacterial and microalgal populations in terms of structure and quantity. As well known, during co-staining SYTO9 can enter all cells regardless of their membrane integrity, bind to DNA and RNA, and emit green fluorescence, while PI can only enter cells with compromised membranes, bind to DNA and RNA, and emit a red fluorescent signal (Rosenberg et al., 2019). So, this staining method gives us information on the extent to which Cu and Zn affect the viability of bacterial and microalgal cells.

Firstly, we selected the optimal channel to distinguish microalgal and bacterial populations (Fig. 6). Microalgal and heterotrophic bacteria could be differentiated based on their differences in fluorescence: microalgal (autofluorescent) and heterotrophic bacteria (cells stained with fluorescent chromophores) (Peniuk et al., 2016).

We also dye cells with Calcofluor White (Sigma-Aldrich), which binds to cellulose-like compounds. The blue fluorescence of calcofluor-dyed microalgal cell walls allowed us to determine if metals affect microalgal morphology and viability.

Then, the treatment of consortia samples taken at different moments of cultivation with Syto9, calcofluor, and propidium iodide let to observe morphological differences between cells in contact with metals and controls and to determine the degree of affection.

Cu and Zn differentially affect bacteria and microalgal cells. Microalgal cell walls were damaged in the presence of metals (Fig. 7, A), and calcofluor only linked to healthy cells. Microalgal autofluorescence was also evident in controls and cultures growth in presence of metals.

The composition of the consortia drastically changed as it grew in the presence of Cu and Zu. At the starting moment, microalgal relatively predominates over bacterial. But after 720 h, a clear succession in

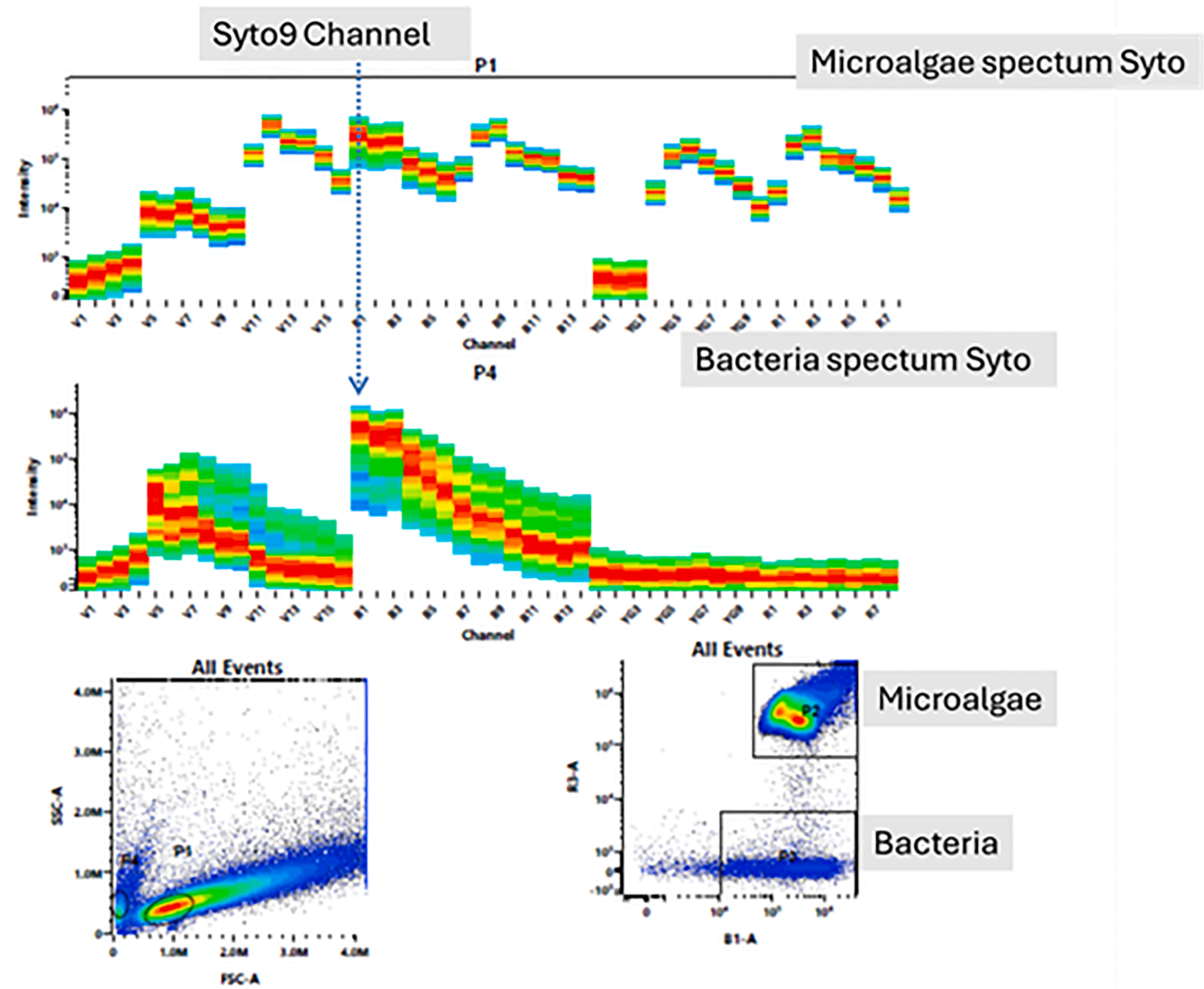

Fig. 6. Flow cytograms to differentiate microalgae and bacteria cells.

populations was observed, resulting in bacteria overgrowth microalgae (Fig. 7, B).

Finally, when comparing images of control and culture exposure to Cu and Zn after 720 h (Fig. 7, C), the ratio between the number of bacterial and microalgal cells increases significantly as the contact time between metals and consortium cells increases, and the number of dead bacterial cells also increases.

4. Conclusions

This study demonstrates the potential of the natural microalgal-bacterial consortium “Nacelle” for the biouptake of Cu and Zn from synthetic aqueous solutions, representing a preliminary step toward its application in livestock waste management. The consortium remained viable under metal exposure, and living cells achieved the highest metal recovery (93.7 % within 720 h), while dead biomass offered faster kinetics (79.4 % in 60 min), highlighting complementary operational strategies. However, while living cells showed high removal potential at low concentrations, a definitive comparative efficiency analysis requires experiments at equivalent concentrations.

Exposure to metals induced shifts in microbial composition, with Diplosphaera chodatii decreasing and Ochrobactrum lupini increasing, reflecting phenotypic and genotypic adaptation.

These findings indicate that “Nacelle” could be an effective tool to reduce Cu and Zn contamination when livestock waste is applied to land, linking the study’s advances to practical applications. Key limitations include the use of synthetic solutions rather than real slurries and the need to further elucidate the underlying metal uptake mechanisms. Future research should focus on direct testing in livestock waste, optimizing process conditions, and investigating the molecular and physiological strategies employed by the consortium for metal capture.

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CRediT authorship contribution statement

Camino García Balboa: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization. **Carlos Castelló Pinel:** Investigation. **Eduardo Costas Costas:** Resources, Project administration. **Victoria López-Rodas:** Resources, Project administration. **Javier Pineda Pampliega:** Writing – original draft, Formal analysis, Data curation.

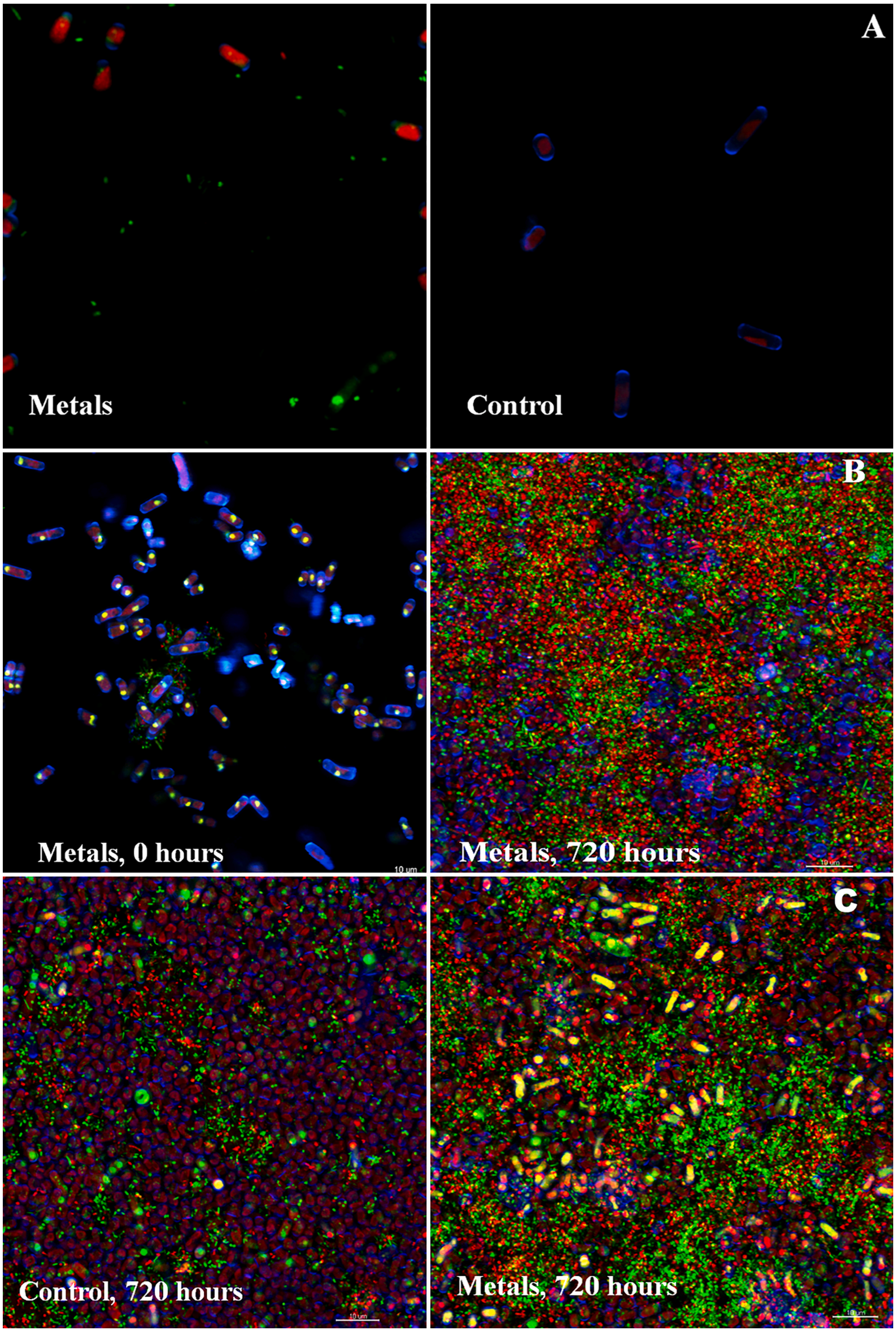

Fig. 7. .A) Changes in microalgal cell morphology, experimental (left) and control (right); B) consortia growth in the presence of metals: from the starting point (left) and to the final (720 hours); C) experimental culture (left) and control (right) after 720 hours.

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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