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Elucidation of heavy metal removal, transformation and distribution mechanisms in piggery wastewater treatment systems with microalgae and activated sludge

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List of abbreviations

Nomenclature	
ANOVA	Analysis of Variance
BR	Bioreactor
DMA	Dimethylarsonic acid
DoE	Design of Experiments
HM	Heavy metal
HRT	Hydraulic retention time
IC	Inorganic Carbon
IC-RE	Removal efficiency of Inorganic Carbon
ICP-OES	Induced Coupled Plasma-Optical
ICP-MS	Induced Coupled Plasma- Mass spectrometry
LW	Livestock waste
MMA	Monomethylarsonic acid
NH_4^+ -RE	Removal efficiency of NH_4^+
PBR	Photobioreactor
PWW	Piggery wastewater
RE	Removal efficiency
S/N	Signal to Noise
sP	Soluble phosphorus
TN	Total nitrogen
TN-RE	Removal efficiency of Total nitrogen
TOC	Total organic carbon
TOC-RE	Removal efficiency of Total organic carbon
TTE	Toxic Trace Elements

Resumen

La gestión de los purines de cerdo se ha convertido en uno de los mayores desafíos medioambientales de los últimos años debido al aumento de la producción de carne de cerdo destinada al consumo humano. Los purines son una mezcla líquida de excrementos de cerdo, agua, y en algunos casos, productos químicos utilizados en la limpieza de las instalaciones. Este subproducto de la producción porcina puede ser una fuente de contaminación si no se gestiona adecuadamente. Uno de los mayores problemas asociados a estos residuos es su alto contenido en nutrientes, debido a las altas cargas de N, P y materia orgánica. Es necesario por lo tanto una adecuada gestión, ya que una vez que se procede a su descarga en el medio ambiente, pueden generar problemas de contaminación y eutrofización de las aguas, afectando negativamente a los ecosistemas acuáticos. Por otro lado, el uso de aditivos minerales en la alimentación del ganado, y otros suplementos utilizados como promotores del crecimiento, hace que el 90% de los metales pesados que contienen, pasen a los purines, siendo solamente el 10% asimilado por el animal. Además, el agua de bebida es otro factor clave, y fuente de entrada de metales en la dieta del ganado. El cobre (Cu) es un oligoelemento esencial y las dietas ricas en Cu aceleran el crecimiento de los cerdos, aunque un exceso del mismo también puede perjudicar al ganado y a los seres humanos. El zinc (Zn) es otro oligoelemento esencial que se utiliza para prevenir infecciones y otras enfermedades que afectan al ganado. El arsénico (As) es un metaloide considerado por la Organización Mundial de la Salud (OMS), como una de las 10 sustancias más preocupantes para la salud pública. Este metaloide se encuentra en aguas subterráneas y acuíferos de la comarca de Tierra de Pinares en Castilla y León y, aunque este contaminante suele tener un origen antropogénico, la gran mayoría de los acuíferos y aguas subterráneas es de origen geogénico, resultado de las interacciones entre el agua y las rocas, dando lugar a la lixiviación de minerales cuando se dan las condiciones adecuadas. El cadmio (Cd) se utiliza ampliamente en la industria ganadera debido a su presencia en suplementos minerales inorgánicos junto con sales de zinc que contienen impurezas de cadmio, junto con plomo (Pb) y mercurio (Hg). El uranio (U), al igual que el arsénico, es un elemento de origen geogénico presente de forma natural en las aguas superficiales y subterráneas de Castilla y León y puede incorporarse a la dieta de los animales a través del agua de bebida.

Los procesos biológicos con microalgas y bacterias han ganado importancia en los últimos años debido a su alta efectividad y bajo coste. Además, es una tecnología sostenible con el medioambiente, robusta, que elimina simultáneamente materia orgánica, nutrientes y contaminantes (metales pesados) y que no genera residuos tóxicos. La biomasa generada es un consorcio de microalgas y bacterias, que puede valorizarse posteriormente como pienso o fertilizante, siempre que los metales bioacumulados no estén presentes en los subproductos recuperados o lo estén dentro de los límites permitidos según su uso. El agua tratada puede

reutilizarse como agua de riego. Por ello, en este trabajo se estudió el empleo de microalgas y bacterias vivas, así como sus consorcios con el objetivo de estudiar la bioeliminación de elementos tóxicos presentes en la fracción líquida de purín de cerdo. Concretamente se evaluó la eficacia de la especie de microalga *Scenedesmus almeriensis* y fango activo frente a la bioeliminación de Cu, Zn y As entre otros que se encuentran en menor concentración como Cd, Pb, Hg y U.

El presente trabajo se ha dividido en diferentes capítulos. El primer capítulo se ha centrado en el estado del arte en torno al empleo de microalgas y bacterias para el tratamiento de aguas residuales contaminadas con metales pesados. El segundo capítulo trata de establecer los objetivos generales y específicos del trabajo y su relación con cada uno de los capítulos. Por otro lado, en el Capítulo 3 se describen los Materiales y Métodos generales que se han estado empleando durante todo el desarrollo del presente trabajo.

En cuanto a la parte de resultados de este trabajo, en el primer capítulo de resultados, se ha estudiado el efecto del tiempo de contacto, la exposición a la luz, la concentración inicial de metal, y la presencia de materia orgánica y CO_2 en la biosorción de Cu y Zn en *S. almeriensis* pura, su consorcio de bacterias y fango activo (Capítulo 4). Se obtuvieron capacidades máximas de biosorción (expresado en mg de metal por gramo de biomasa seca) para Cu; 104.52 mg/g, 81.50 mg/g, y 67.71 mg/g, Para Zn(II) fueron de 121.65 mg/g, 96.71 mg/g, y 73.52 mg/g, a una concentración inicial de metal de 100 mg/L y un pH inicial de 7.5. Estos resultados permiten considerar el tratamiento de aguas residuales con carga de materia orgánica y altas concentraciones de metales en fotobiorreactores.

A partir de estas condiciones óptimas de biosorción se propuso un estudio para elucidar los principales mecanismos implicados en la eliminación de Cu y Zn por parte de *S. almeriensis* y de fangos activos formado por bacterias aerobias (Capítulo 5). Para ello, se propusieron solubilizaciones selectivas con diferentes disolventes suaves como ácido acético en concentración 0.1M, acetato de amonio o EDTA. mostrando que los iones Cu(II) y Zn(II) son biosorbidos principalmente por reacciones de intercambio iónico, complejación e interacción electrostática. Además, gran parte del metal retenido se encuentra en la biomasa en forma biodisponible, un 69% para el caso del Cu(II) y 94% para el Zn(II) para la microalga y 76% de Cu(II) y 93% de Zn(II) para los fangos aerobios. Este capítulo se corresponde con el contenido de la aportación científica de la tesis en forma de artículo científico, publicado en la revista Q1 Chemosphere: https://doi.org/10.1016/j.chemosphere.2024.141803

En el Capítulo 6 se estudió la efectividad de microalgas y fangos activos aerobios en la bioeliminación de especies inorgánicas As(III) y As(V) así como de la especie dimetilada DMA, ácido dimetilarsénico. Las microalgas eliminaron el 80% de DMA en 10 días, abriendo la posibilidad de utilizar microalgas en el tratamiento de aguas residuales. Por su parte, los fangos activos, fueron capaces de bioeliminar hasta un 80% de las especies inorgánicas As(III) y As(V) en un tiempo de contacto de 24 horas.

En el Capítulo 7 se evaluó la eficiencia de eliminación de cuatro elementos traza tóxicos (Cd, Pb, Hg, U) por la microalga *S. almeriensis* y fangos activos. *S. almeriensis* mostró altos porcentajes de eliminación, especialmente para Cd, Pb, y Hg, mientras que los fangos activos demostraron eficacia en la eliminación de todos los elementos traza tóxicos estudiados. La eliminación de carbono orgánico total (TOC) fue significativa tanto por *S. almeriensis* como por los fangos activos, alcanzando valores máximos del 92% y 88% respectivamente en presencia de Pb y U.

En el Capítulo 8 se implementó un Diseño de Experimentos de Taguchi para optimizar los parámetros de plantas de tratamiento de aguas residuales, utilizando fotobiorreactores de consorcios algas-bacterias y biorreactores con bacterias aerobias. Se logró la eliminación efectiva de Cu(II), Zn(II), As(V) y Cd(II) así como nutrientes presentes en la suspensión. Las eficiencias de eliminación de Cu(II), Zn(II), As(V) y Cd(II) oscilaron entre el 81-98%, 96-97%, 98-72% y 93-99% respectivamente, y los porcentajes de eliminación de carbono orgánico total (TOC), nitrógeno total (TN) y NH_4^+ oscilaron entre el 83-88%, 56-63% y 63-89% para biorreactores basados en microalgas y bacterias respectivamente.

Finalmente, en el Capítulo 9 se evaluó el rendimiento de un reactor en cascada de capa fina operando en régimen semi-contínuo con un HRT de 5 días con un consorcio de *S. almeriensis*-bacteria para la eliminación de Cu, Zn, As y nutrientes de agua residual de purín de cerdo real. Se lograron eficiencias de eliminación significativas tanto para los metales como para los nutrientes, demostrando la viabilidad de este enfoque para la depuración de aguas residuales. Los metales tuvieron un efecto negativo sobre TN-RE, NH_4^+-RE y la viabilidad de la biomasa. Se alcanzaron eficiencias de eliminación máximas de 77.1, 78.1 y 77.4 % para Cu, Zn y As respectivamente. Por otro lado, se alcanzaron eficiencias de eliminación máximas de 58, 94, 98 y 95% para TOC, TN, NH_4^+ y PO_4^{3-} respectivamente antes del dopaje con mezcla metálica, mientras que, en presencia de las especies metálicas, se alcanzaron eliminaciones de 48, 72, 76 y 85% de TOC, TN, NH_4^+ y PO_4^{3-} respectivamente.

Esta tesis constituye una primera aproximación al tratamiento de aguas residuales combinando la eliminación de nutrientes y metales pesados con microalgas y fangos activados, así como el efecto de estos contaminantes sobre la viabilidad de la biomasa crecida.

Abstract

Pig manure management has become one of the biggest environmental challenges in recent years due to the increasing production of pork for human consumption. Manure is a liquid mixture of pig excrement, water, and in some cases, chemicals used in cleaning facilities. This by-product of pig production can be a source of contamination if not properly managed. One of the major problems associated with this waste is its high nutrient content, due to high loads of N, P and organic matter. Proper management is therefore necessary, as once they are discharged into the environment, they can generate problems of water pollution and eutrophication, negatively affecting aquatic ecosystems. On the other hand, the use of mineral additives in livestock feed, and other supplements used as growth promoters, means that 90% of the heavy metals they contain pass into the manure, with only 10% being assimilated by the animal. In addition, drinking water is another key factor and source of metals entering the livestock diet.

Copper (Cu) is an essential trace element and Cu-rich diets accelerate growth in pigs, but too much copper can also harm livestock and humans. Zinc (Zn) is another essential trace element used to prevent infections and other diseases affecting livestock. Arsenic (As) is a metalloid considered by the World Health Organization (WHO) as one of the top 10 substances of public health concern. This metalloid is found in groundwater and aquifers in the region of Tierra de Pinares in Castilla y León and, although this contaminant is usually of anthropogenic origin, the vast majority of aquifers and groundwater is of geogenic origin, resulting from interactions between water and rocks, leading to the leaching of minerals when the right conditions are met. Cadmium (Cd) is widely used in the livestock industry due to its presence in inorganic mineral supplements together with zinc salts containing cadmium impurities, along with lead (Pb) and mercury (Hg). Uranium (U), like arsenic, is an element of geogenic origin naturally present in surface and groundwater in Castilla y León and can be incorporated into the diet of animals through drinking water.

Biological processes with microalgae and bacteria have gained importance in recent years due to their high effectiveness and low cost. Moreover, it is an environmentally sustainable, robust technology that simultaneously removes organic matter, nutrients and pollutants (heavy metals) and does not generate toxic waste. The biomass generated is a consortium of microalgae and bacteria, which can be subsequently valorized as feed or fertilizer, provided that the bioaccumulated metals are not present in the recovered by-products or are present within the limits permitted according to their use. The treated water can be reused as irrigation water. Therefore, in this work, the use of live microalgae and bacteria and their consortia was studied in order to study the bioelimination of toxic elements present in the liquid fraction of pig manure. Specifically, the efficacy of the microalgae species *Scenedesmus almeriensis* in the bioelimination

of Cu, Zn and As, among others found in lower concentrations such as Cd, Pb, Hg and U, was evaluated.

This work has been divided into different chapters. The first chapter focuses on the state of the art in the use of microalgae and bacteria for the treatment of wastewater contaminated with heavy metals. The second chapter tries to establish the general and specific objectives of the work and their relationship with each of the chapters. On the other hand, Chapter 3 describes the general Materials and Methods that have been used throughout the development of this work.

As for the results part of this work, in the first chapter of results, the effect of contact time, exposure to light, initial metal concentration, and the presence of organic matter and CO_2 on the biosorption of Cu and Zn in pure *S. almeriensis*, its consortium of bacteria and an activated sludge has been studied (Chapter 4). Maximum biosorption capacities (expressed in mg of metal per gram of dry biomass) were obtained for Cu; 104.52 mg/g, 81.50 mg/g, and 67.71 mg/g, for Zn(II) they were 121.65 mg/g, 96.71 mg/g, and 73.52 mg/g, at an initial metal concentration of 100 mg/L and an initial pH of 7.5. These results allow considering the treatment of wastewater with organic matter load and high metal concentrations in photobioreactors.

Based on these optimal biosorption conditions, a study was proposed to elucidate the main mechanisms involved in the removal of Cu and Zn by *S. almeriensis* and activated sludge formed by aerobic bacteria (Chapter 5). For this purpose, selective solubilisations with different mild solvents such as acetic acid at 0.1M concentration, ammonium acetate or EDTA were proposed, showing that Cu(II) and Zn(II) ions are biosorbed mainly by ion exchange reactions, complexation and electrostatic interaction. Furthermore, much of the retained metal is found in the biomass in bioavailable form, 69% for Cu(II) and 94% for Zn(II) for the microalgae and 76% Cu(II) and 93% Zn(II) for the aerobic sludge. This chapter corresponds to the content of the scientific contribution of the thesis in the form of a scientific article. Published in Chemosphere (Q1): https://doi.org/10.1016/j.chemosphere.2024.141803

In Chapter 6, the effectiveness of microalgae and aerobic activated sludge in the bioelimination of inorganic As(III) and As(V) species as well as the dimethylated species DMA, dimethyl arsenic acid, were studied. The microalgae removed 80% of DMA in 10 days, opening up the possibility of using microalgae in wastewater treatment. The activated sludge, on the other hand, was able to bioeliminate up to 80% of the inorganic species As(III) and As(V) in a contact time of 24 hours.

In Chapter 7, the removal efficiency of four toxic trace elements (Cd, Pb, Hg, U) by the microalgae *S. almeriensis* and activated sludge was evaluated. *S. almeriensis* showed high removal rates, especially for Cd, Pb, and Hg, while activated sludge showed efficiency in the removal of all toxic trace elements studied. Total organic carbon (TOC) removal was significant for both *S. almeriensis* and activated sludge, reaching maximum values of 92% and 88% for Pb and U, respectively.

In Chapter 8, a Taguchi Design of Experiments was implemented to optimise wastewater treatment plant parameters, using algae-bacteria consortia photobioreactors and bioreactors with aerobic bacteria. Effective removal of Cu(II), Zn(II), As(V) and Cd(II) as well as nutrients present in the suspension was achieved. The removal efficiencies of Cu(II), Zn(II), As(V) and Cd(II) ranged from 81-98%, 96-97%, 98-72% and 93-99% respectively, and the removal rates of total organic carbon (TOC), total nitrogen (TN) and NH_4^+ ranged from 83-88%, 56-63% and 63-89% for microalgae- and bacteria-based bioreactors respectively.

Finally, in Chapter 9, the performance of a cascade thin-layer reactor operating in a semi-continuous regime with a 5-day HRT with a *S. almeriensis*-bacteria consortium was evaluated for the removal of Cu, Zn, As and nutrients from real pig manure wastewater. Significant removal efficiencies were achieved for both metals and nutrients, demonstrating the feasibility of this approach for wastewater treatment. Metals had a negative effect on TN-RE, NH_4^+-RE and biomass viability. Maximum removal efficiencies of 77.1, 78.1 and 77.4 % were achieved for Cu, Zn and As respectively. On the other hand, maximum removal efficiencies of 58, 94, 98 and 95 % were achieved for TOC, TN, NH_4^+ and PO_4^{3-} respectively before doping with metal mixture, whereas, in the presence of the metal species, removals of 48, 72, 76 and 85 % were achieved for TOC, TN, NH_4^+ and PO_4^{3-} respectively.

This thesis constitutes a first approach to wastewater treatment combining the removal of nutrients and heavy metals with microalgae and activated sludge, as well as the effect of these pollutants on the viability of the grown biomass.

Chapter 1:

Introduction

1. Introduction

Intensive livestock farming worldwide to feed a growing population has resulted in serious environmental problems such as soil degradation and pollution of surface and groundwater. Piggery wastewater (PWW) contains high concentrations of organic matter, nitrogen (N) and phosphorus (P). (López-Sánchez et al., 2022). Therefore, humanity faces great challenges to treat livestock waste properly and prevent overexploitation of natural resources. This can be achieved through a circular bioeconomy approach, which aims at sustainable production using biological resources such as PWW as raw materials. A circular bioeconomy uses innovative processes to produce biomaterials and bioenergy while reducing the consumption of new resources (López-Pacheco et al., 2021). In terms of manure, Europe's manure production is approximately 1.27 billion tons (2018) (Köninger et al., 2021). Some EU countries face significant problems complying with EU Directive 91/676/EEC. Pig farms produce manure with contents up to 7000 mg N/L and 600 mg P/L, which is not degradable in conventional systems and requires energy-intensive systems for processing. Contamination by metals and other pollutants today is an issue that affects wastewater very closely, and exposure to microorganisms, plants and humans. In addition, the harmful effects of heavy metals on human health are widely known. Heavy metals contamination is one of the main environmental issues in industrialized areas. The misuse and mismanagement of waste resulting from these human activities, both industrial and domestic, leads to the contamination of large bodies of water and threatens the viability and survival of aquatic ecosystems with consequences for the environment and even for humans (**Figure 1.1**).

Figure 1.1. Principal sources of water bodies contamination and its pollutants

It is for this reason that the use of microalgae for the retention of metal ions present in wastewater represents many advantages due to its widespread use in aquaculture or biofuel production, and its application for this type of process is quite feasible and inexpensive, and it can be used to obtain high value products. They adapt to a wide variety of environmental conditions, which makes microalgae very versatile microorganisms. Microalgae have high growth rates; low nutrient content is needed compared to other biomass organisms. And, finally, it is a type of biomass that does not generate toxic substances unlike others such as fungi and bacteria. Furthermore, the fact that metal uptake processes by microalgae are characterized by the fact that there is not a very high tolerance to the existence of some toxic heavy metals or to high concentrations of others that are essential, causes bacteria to play a very important role because, through their detoxification mechanisms, they can better withstand the load of these metalloids and non-essential heavy metals that are toxic. Bacteria have been widely used ign wastewater treatment due to their accessibility, low cost and abundance in the environment. They take up the O_2 produced by the microalgae, giving rise to interesting interactions to study, such as cooperative interactions, such as association, symbiosis or mutualism (Ramanan et al., 2016), or competitive or antagonistic interactions such as parasitism or commensalism (Liu et al., 2017). In order to explore research niches in the treatment of pig wastewater contaminated with metals in the last 10 years, a Scopus search was carried out. Keywords: Swine wastewater and heavy metals were used. A total of 267 papers were used to perform bibliometric analysis by VOSviewers 1.6.16. In **Figure 1.2A**, the different colours represent different research topics in this field. The size of font and circles represent the appearance frequency. **Figure 1.2B** shows the publication timing of these topics.

A

B

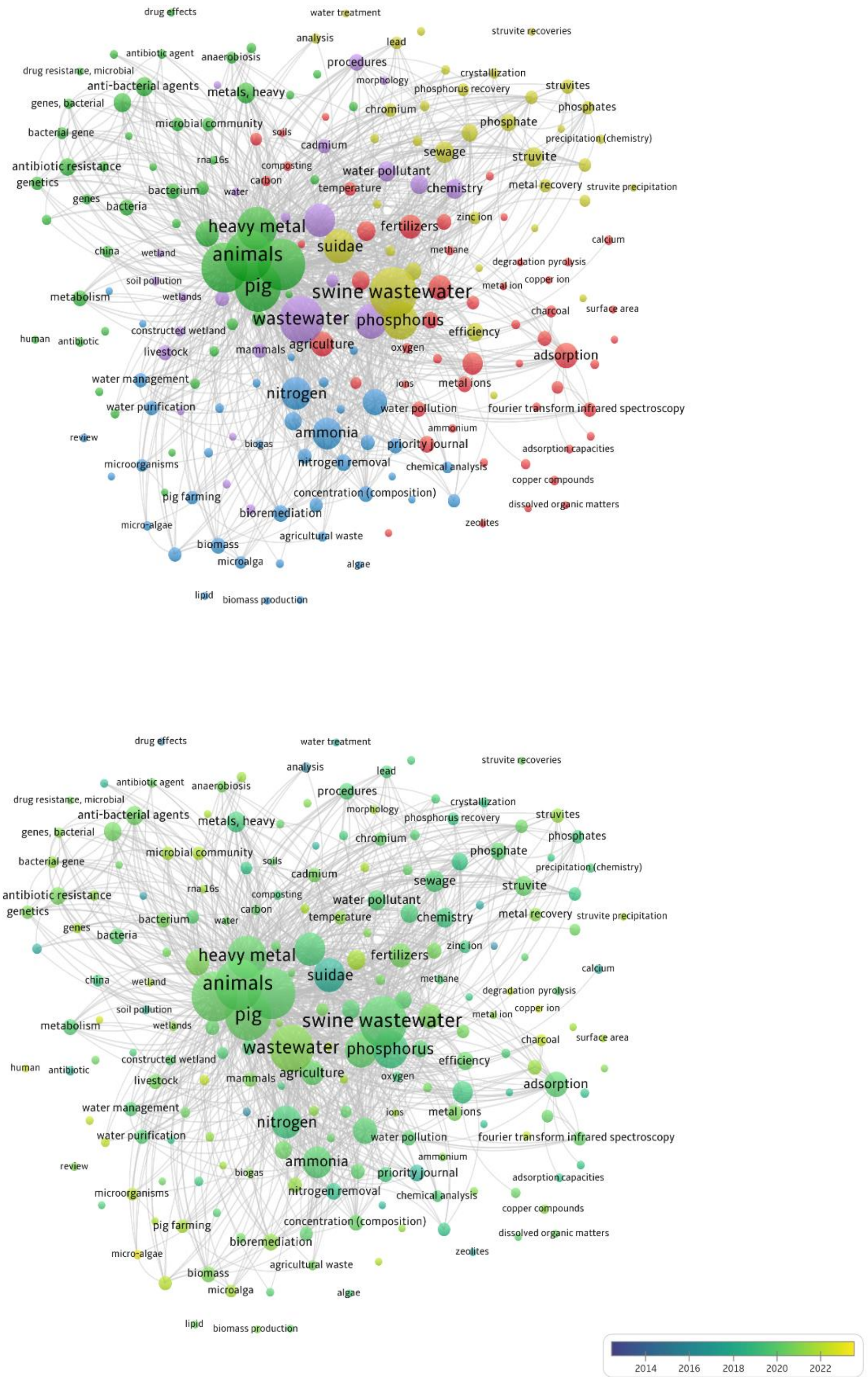

Figure 1.2. Bibliometric analysis of swine wastewater treatment technology based on recent years (2013–2024) of literature sorted by (A) technology category, same color represents similar research topics. (B) chronological order, timeline represents the chronological order of these research topics.

1.1 Heavy metals. Definition, classification and general aspects

Heavy metals are a group of chemical elements that mainly includes transition metals, semi-gmetals, lanthanides and actinides. Among the many classifications proposed, either by atomic weight or density, heavy metals are the chemical elements which have a density five times that of water. (Suresh Kumar et al., 2015a). For its part, IUPAC states that its definition has a "misnomer" due to lack of scientific consistency and due to the alternative use of the term toxic metal, around which there is also some controversy due to the lack of consensus on its accurate definition. Heavy metals are considered to be one of the main environmental pollutants. Today is an issue that affects especially wastewater and their exposure to microorganisms, plants and humans. This can be harmful, and toxicity can be expressed by different pathways: displacing essential metal ions from biomolecules and other biologically functional groups, blocking essential functional groups of biomolecules, including enzymes, modifying the active conformation of biomolecules, such as enzymes and polynucleotides and disrupting the integrity of biomolecules and modifying some other biologically active agents. In recent years, methodologies based on microorganisms have tried to displace the old conventional techniques most used as filters, membranes, chemical precipitation, etc., much more expensive and effective only for high concentrations of pollutants, which does not make them suitable for treating water containing metalloids and heavy metals in concentrations at trace level.

Organic compounds may suffer from degradation processes in the environment and form fewer toxic species. In contrast, metals and semimetals are not biodegradable, so they remain in the ecosystem, leading to bioaccumulation in the food chain, causing serious environmental and human health problems. With metal accumulation, they can cause DNA damage, kidney abnormalities, organ failure, allergies, decreased fertility, and more. Therefore, it is important to reduce the concentration of these toxic trace elements (TTEs) in wastewater (Ayangbenro and Babalola, 2017; Laskar et al., 2017; Leong and Chang, 2020). On the other hand, many of the metals present in nature occur naturally in the earth's crust as part of minerals and as a result of these activities previously mentioned, soil, surface water, and groundwater can quickly become contaminated by heavy metals. Many of them act as nutrients and trace elements such as Cu, Zn, Mo, Mn, Ni, Fe, Co and V, as they fulfil metabolic functions that are crucial for various biochemical physiological processes, and many of them are part of enzymes or proteins. However, high doses can be harmful to the organisms. Other elements such as Cd, Hg, Pb, Ag or As can have huge effects on the organisms in trace amounts, causing acute and chronic poisoning in humans (Azeh Engwa et al., 2019). The sources and potential hazards to humans from exposure to these toxic elements are shown in **Table 1.1.**

Table 1.1. Source, potential health effects in humans and on microorganisms of different metal species Adapted from (Ayangbenro and Babalola, 2017)

Metal	Source	Potential health effects from long term exposure in humans	Effects on microorganisms
Copper	Copper refineries, paint, textile industries, etc.	Gastrointestinal distress. Long-term exposure: Liver or kidney damage.	cellular function disruption and inhibits enzyme activities
Zinc	Manufacturing, mining, petroleum refining, plumbing	Ataxia, depression, gastrointestinal irritation, kidney and liver failure, prostate cancer, vomiting.	Death, decrease in biomass, inhibits growth
Arsenic	Mining activity, pesticides, volcanic rocks, sediments, smelters, etc.	Brain damage, cardiovascular and respiratory disorder, dermatitis, skin cancer	Deactivation of enzymes
Cadmium	Fertilizers, mining, pesticides, plastic industry, refining, welding	Kidney damage, bone disease, lung and prostate cancer, lymphocytosis	Acid nucleic damage, protein denaturing, cell division and transcription inhibition and inhibits carbon and nitrogen mineralization
Lead	Coal combustion, electroplating, battery manufacturing, mining, pigments, paints, etc.	Infants and children: Delays in physical or mental development; children could show slight deficits in attention span and learning abilities; Adults: Kidney problems; high blood pressure	Acid nucleic and protein denaturing
Mercury	Earth's crust, volcanic activity, fossil fuels, thermoelectric power plants, mining	Kidney damage	Population size decreased. Protein denaturing, disrupt cell membrane and inhibit enzyme activities
Uranium	Soil, sediments.	Increased risk of cancer, kidney toxicity	May interfere with vital cellular functions, such as cellular respiration and DNA replication

Because of the potential of this trace metal to enter the food chain, monitoring of food, water and soils is currently being closely monitored to prevent any type of toxin from entering the food chains and indirectly affecting humans. (Alam and McPhedran, 2019). For this reason, different national and international organizations impose limits for discharge water, inland water and water for human consumption.(U.S. EPA, 2009) as ahown in **Table 1.2.**

Table 1.2. Maximum contaminant level for heavy metals according to Ministerio de Agricultura Alimentación y Medio ambiente (2015) and USEPA, (2009)

Metal	Continental waters limit (μg/L)	Limit in other surface waters (μg/L)	Human consumption limit DMA (mg/L) (Ministerio de Agricultura Alimentación y Medio ambiente, 2015)	EPA (mg/L) (U.S. EPA, 2009)
Copper	120	25	2	1.3
Zinc	500	60	not legislated	5
Arsenic	50	25	0.01	0.01
Cadmium	not legislated	not legislated	0.005	0.005
Lead	not legislated	not legislated	0.005	0.015
Mercury	not legislated	not legislated	0.001	0.002
Uranium	not legislated	not legislated	0.030	not legislated

1.2 PWW composition and typical heavy metals used in pig farming

In recent years, the number of livestock for human consumption has risen sharply from $4.9 \cdot 10^9$ in 2013 to $5.1 \cdot 10^9$ in 2017 according to (FAOSTAT, 2024). Within this data, Europe account for around 8% of the total head of cattle ($1.2 \cdot 10^8$), 2% of that of goats ($1.9 \cdot 10^7$),11% of sheep ($1.3 \cdot 10^8$), and 19% of pigs ($1.9 \cdot 10^8$), this being the highest percentage in 2017. In Spain, swine livestock means $2.9 \cdot 10^7$of pigs, which represents 16.1% of this type of livestock of Europe. Swine farms generates waste called manure, with a strong ammonia odor and which is obtained by the mixture of animal defecation, washing water, and remains of feed. The composition of this manure can be very varied depending on the type of animals, breed, age, type of farm, feed, as well as the drinking water, which plays a fundamental role in the composition of this manure. Swine manure has a high-water content, being around 90%, an organic matter content around 60-70% in dry matter, 2-4 g/kg of nitrogen, 0.7-1 g/kg of phosphorus, 0.2-0.3 g/kg of magnesium, 0.9-1.4 g/kg of potassium as well as other metals such as iron, copper and zinc among others which will be discussed later. In addition, these residues contain volatile compounds such as hydrogen sulfide, volatile fatty acids and phenolic compounds among others (Ministry of Agriculture Fisheries and Food (2019)). The composition of these wastes gives them very interesting properties as fertilizers, since they have the essential nutrients that plants and other crops need to grow. However, this has inherent environmental problems such as exceeding the N or P concentration of the soil, which can lead to eutrophication problems if leaching and lixiviation process occurs and affect other ecosystems such as ground water. The maximum amount of N that can be added per hectare to be cultivated is limited to 170 kg of N according to European regulation *Directiva 91/676/CEE*. On the other hand, the liquid fraction of these swine manure, which is the object of study in this work, requires proper management due to the high load of N, P and K, organic matter, toxic compounds and metals. Biological treatment for the treatment and management of these wastes has been booming due to the high potential of microorganisms capable of organic matter removal from these wastewaters, as well as eliminating metals, heavy metals and other metalloids present in wastewater of this type, making it suitable for use as irrigation water, among others. In addition, another benefit of these technologies is the use of the biomass grown under these conditions, with a possible subsequent use as a biofertilizer or biostimulant, among others, due to its high protein content. Regarding typical metal used in pig farming it should be noted that Cu is an essential trace element that plays an important role in the metabolism of animals and plants, activating oxidative enzymes. Besides, Cu-rich diets accelerate pig growth. The mechanics of this promotion may be relevant for antibacterial activity, increasing feed intake, increased enzyme activity and induction of neuropeptide release. At correct Cu concentrations, this can promote the growth of microorganisms and their ability to convert and utilize the different carbon sources and transform it in form of polymer, thus promoting the decomposition of organic matter in the waste from agricultural activity (Yang et al., 2012). However, a high Cu concentration can stop all these processes exposed, due to the relationship between the concentration of this metal and the deactivation of the enzymatic activity. Excess copper can also harm livestock and humans. Zn is another essential trace metal that is part of more than 100 metalloenzymes and transcription factors for protein synthesis, it improves the performance of younger piglets by increasing their daily intake and growth rate, prevents enteric infection and diarrhea as is used as an antibiotic growth promoter (Zhang and Guo, 2009). However, Zn abuse increases both the diversity of *E. coli* in cattle and the proportion of multi-resistant *E. coli* strains. As is a metalloid that can be present in different inorganic and organic forms. A trace amount of As is essential for animal growth, as an As deficiency will cause abnormal reproduction (poor fertility and increased perinatal mortality) and depressed growth, as well as altering mineral concentrations in various organs (Wang et al., 2015). As, on the other hand, is used as a feed additive in animal diets all over the world, with consequent contamination of soil and water through the animal waste generated. The trivalent and pentavalent oxidation states species of As have toxic effects: neurotoxicity, nephrotoxicity, hepatotoxicity and reproductive toxicity (Liu et al., 2015). Cd is widely used in the livestock industry because of its presence in inorganic mineral supplements together with Zn salts (sulphates and phosphates) containing Cd impurities. Cd affects the growth of livestock, as it decreases the average daily feed intake and increases the feed/gain ratio (Sheng et al., 2004). Moreover, since not all the Cd content of these supplements is assimilated by the pig organism, most of it is excreted in the form of faeces, becoming part of the fertilisers and consequently contaminating soil and water, and is directly transfer to crops and feed, being an important route of human exposure to Cd. Pb has a high accumulation rate in the kidney of animals and humans and induces deformity and cardiovascular toxicity. Early Pb contamination of cattle comes from the use of Zn oxide used in the early stages of cattle weaning for the introduction of the fattening diet (Feng et al., 2018). Hg is a potent neurotoxic agent that accumulates in the liver, kidney and muscles of the animals. It is added to the slurry of these animals by disinfectants used in animal husbandry, and by the use of certain fish meals. Uranium is a geogenic trace element naturally present in surface and groundwater in Castilla y León and can be incorporated into the diet of animals through drinking water.

Among the most common concentrations of heavy metals found in this type of wastewater from livestock industry varies for Cu in values between 0.28 and 4.7 mg/L, although high values were found up to 148 mg/L (Collao et al., 2022; Zhang et al., 2011). For Zn, values from 0.98 and 12 were reported, while outliers as 234 mg/L were observed (Zeng et al., 2021), and for arsenic, values of up to 670 μg/L were found (Gao et al., 2018)(Gao et al., 2018). In the case of Cd(II), values between 0.022 mg/L (Zhang et al., 2011) and 0.35 mg/L (Moral et al., 2008), with moderate values of 0.16 mg/L (Creamer et al., 2010). Besides, although a wide range of concentrations have been found in the literature, the concentration of 100 mg/L was chosen to start the study in Chapter 4 for initial metal concentration of Cu and Zn. This was a consensus intermediate-high concentration among several results found in the literature for real piggery wastewater, which are shown in the **Table 1.3:**

Table 1.3. Typical copper and zinc concentrations in swine wastewater

Cu (mg/L)	Zn (mg/L)	Reference
108	450	(ASAE, 2003)
47	65	(Nicholson et al., 1999)
42	172	(Moral et al., 2008)
13.7	133	(Creamer et al., 2010)
59.4	234	(Zhang et al., 2011)

Similarly, a literature search was carried out on the typical composition of various liquid fractions of pig manures (**Table 1.4**).

Table 1.4. Principal swine wastewater composition from different sources

	(Hernández et al., 2013)	(García et al., 2017)	(Collao et al., 2022)	(Collao et al., 2022)	(Guo et al., 2020)	This work	This work
Origin	Cuéllar, (Spain)	Cantalejo, (Spain)	Segovia, (Spain)	Segovia, (Spain)	China	Almería, (Spain)	Narros, (Spain)
pH	7.5		-	-	6.74	8.5	7.9
TS (mg/kg)	3319.0		-	-	1014 (TSS)	14.4	37.3
VS (mg/kg)	1031.0		-	-		10.4	23.9
sCOD	465.0		-	-		-	-
sTOC (mg/L)		497	713	652.0	1054	707.2	34559.1
IC (mg/L)	-	82	-	-	277	890.4	1168.3
TC(mg/L)	-		-	-		1597.5	35729.3
TN (mg/L)	-	170	256	247.0	129	1243.9	8289.7
sP (mg/L)	47.5	2.5	3.8	3.6	13 (TP)	30.4	43.4
$\mathbf{NH_4^+}$ (mg/L)	12.3	-	141	164.0		516.0	2190.0
Cu (mg/L)	-	-	0.28	0.3	<0.6	0.3	3.2
Zn (mg/L)	-	0.37	0.98	0.8	0.63	2.7	7.2
As (mg/L)	-	-	2.04	1.9	<0.6	4.3	29.0
Cd (mg/L)	-	-	-	-	-	2.3	4.8

1.3 Physico-chemical methods for polluted water clean-up

There are different metal removal methodologies, based on the nature of their mechanisms. Within the conventional methodologies, they can be physical or chemical methods. In the physical methods, the removal of heavy metals occurs through adsorption mechanisms (Goswami et al., 2021). This can lead, at the same time, to easy desorption of these metals back into the environment, causing secondary contamination. Chemical methods include chemisorption mechanisms, in which metal binding occurs through chemical bonds with functional groups present on the adsorbent surface (Yan et al., 2022).

Physicochemical methods, includes chemical precipitation, (hydroxide precipitation, carbonate precipitation and sulfide precipitation), chemical oxidation or reduction using electrochemical techniques, including electrodeposition and electrodialysis, ion exchange (using resins, starch xanthates), reverse osmosis, evaporative recovery or even technologies based on adsorption using inorganic adsorbents (natural minerals, ores, clays and industrial waste) or organic adsorbents (e.g. plant or animal waste). Physico-chemical techniques are often inefficient (especially when metal concentrations are in the range of 1–100 mg/L), require high operating costs, and often produce polluting by-products. Most physico-chemical water treatment technologies such as chemical precipitation, evaporation, electrodeposition, ion exchange and membrane separation are inefficient and expensive for treating wastewater at low heavy metal concentrations. In addition, most conventional methodologies do not offer a high percentage of metal removal from the medium, require in some cases the regeneration of the adsorbent material, have a high initial investment, generate toxic residues and have moderate or low selectivity towards the target metal to be removed. On the other hand, the amount of energy and reagents required is very high. **Table 1.5** shows most of the advantages and disadvantages of physicochemical methods in heavy metal removal.

Table 1.5. Principal advantages and drawbacks of physical and chemical methods for metal removal from wastewaters. Adapted from Monteiro et al. (2012) and Cheng et al. (2019).

Methods	Advantages	Disadvantages
Physical methods		
Adsorption	High applicability to a wide variety of contaminants Depending on the biosorbent some selectivity is possible. Fast kinetics	Limiting process with respect to the nature of the adsorbent Chemical derivatization is required to improve sorption capacity
Ion exchange	High effectiveness High degree of regeneration and possibility of metal recovery	High pressure requirements High maintenance costs Expensive technique Sensitive to the presence of other particles in suspension
Membrane filtration	Low solid waste generation Small space requirement	Requires large initial capital investment. Low yield due to low flow rates
Reverse osmosis	Pure effluent generation and removes most of the heavy metals	Energy-intensive process Expensive
Chemical methods		
Chemical precipitation	Simple No metal selectivity	Large amount of metal-containing sludge generated High maintenance and sludge disposal costs Ineffective at high metal concentrations
Coagulation and flocculation	Bacterial inactivation capacity	High solvent consumption High sludge disposal requirement High maintenance costs
Flotation	Metal selectivity Removal of small particle Fast process	Requires large initial capital investment High maintenance and operation costs
Electrochemical treatment	Choice of conditions is a critical factor Moderate metal selectivity No chemical requirement Tolerance to suspended solids	Expensive for high concentration treatment
Evaporation	Pure effluent generation	Energy-intensive process Expensive

For this reason, effort has been made in recent decades to develop innovative, sustainable, cost-efficient and environmentally friendly technologies for the treatment and removal of pollutants in surface and inland water bodies and wastewater. In this context, the adoption of microalgae-based technologies has emerged as a promising alternative. These photosynthetic microorganisms have the ability to accumulate heavy metals efficiently through biosorption and bioaccumulation processes. These biological technologies comprise methodologies with low to moderate initial investment capital, eco-friendly with the environment and with the possibility of subsequent recovery of the generated biomass with mild solvent treatment, avoiding the generation of toxic by-products (Aziz et al., 2019).

These microorganisms possess functional groups, such as alcohol, amine, amide, carbonyl, carboxyl groups, on their cell surface, which interact with metal ions, and promote the adsorption of these HM to the cell wall. Precipitation, ion exchange, complexation, redox reactions, etc, are the main biosorption mechanisms. On the other hand, bioaccumulation processes also occur for the case of living biomass, an active process in which energy expenditure is required through cellular respiration of the microorganism. Moreover, this removal capacity is highly influenced by different factors, such as the initial metal concentration, the contact time or environmental factors.

1.4 Bioremediation

Wastewater bioremediation regarding metalloids and heavy metals has taken on great importance in recent years due to the demands that exist regarding the care of the environment. It can be defined as the process of utilizing living or dead organisms for the elimination of contaminants of interest. The use of biological microorganisms entails such a very effective, low cost and environmentally friendly technologies. It is therefore necessary to expand and explore new approaches to their application. As a result, microalgae and bacteria have been demonstrated to be highly effective in removing heavy metals and other pollutants such as Cu, Zn, As and other toxic elements from wastewater of many types. Among the types of biological material that can be used as biomass in the bioelimination of this type of pollutants, inert biomass, algal or microalgal biomass, vegetable biomass or from higher plants, microbial biomass, such as bacteria, fungi or yeasts have been used. The use of microalgae and other microorganisms for the retention of metal ions present in wastewater represents many advantages due to its widespread use in aquaculture, biofuel production, and the application for this type of process is quite feasible and at low cost, besides being also used to obtain high value products. They adapt to a wide variety of environmental conditions, which makes microalgae very versatile microorganisms. They have high growth rates; low nutrient content is needed compared to other biomass organisms. And, finally, it is a type of biomass that does not generate toxic substances unlike others such as fungi and bacteria. Moreover, the fact that uptake metal processes by microalgae are characterized by a low tolerance to the existence of some toxic heavy metals or high concentrations of others that are essential, means that bacteria play a very important role since, through their detoxification mechanisms, they can better withstand the load of these metalloids and non-essential heavy metals that are toxic. Five main processes are considered as bioremediation techniques (Suresh Kumar et al., 2015a; Abdel Maksoud et al., 2020); Phytoremediation described as the use of natural organisms (macroalgae and plants) to clean up pollutants *in situ*. They have a high absorption capacity. Biotransformation, as the conversion of toxic compounds into less toxic species, such as biotransformation of more toxic species of As to less toxic species, using biological systems (Papry et al., 2022). Biomineralization, different organisms have the ability to transform toxic compounds into their less toxic mineral forms (Cheng et al., 2023). Bioaccumulation, as an active metabolic process, it requires respiration to remove pollutants, which pass through the cell wall. Finally, biosorption, a metabolically independent process in which pollutants are retained on the cell surface, will be described in the following sections. This work has been focused on the study of biosorption and bioaccumulation mechanisms by living microorganisms, such as microalgae and activated sludge made up of a wide aerobic bacteria population.

1.5 Microalgae and bacteria. General aspects

Microalgae are considered eukaryote microscopic unicellular, photoautotrophic organisms that coexist in fresh water as well as in marine ecosystems (Cooper and Smith, 2015). The presence of amine, hydroxyl, carboxyl, sulphate, and phosphate groups in their cell surface, make possible the presence of potential metal binding sites for heavy metal bioremediation. Metal ion biosorption differs with the type and structure of the algal biomass, charge and chemical constitution of the heavy metal ion. Different microalgae, in live or dead forms, have been used, in batch or continuous systems, for in-situ bioremediation of different type of wastewater.

Microalgae play a crucial role in environmental remediation by consuming certain heavy metals as essential trace elements for enzymatic processes and cell metabolism. While metals like boron, cobalt, copper, iron, molybdenum, manganese, and zinc are beneficial, others such as arsenic, cadmium, chromium, lead, and mercury are toxic to microalgae. Interestingly, low concentrations of toxic metals can stimulate microalgae growth through hormesis. Some cyanobacterial species exhibit natural tolerance to heavy metal stress and can thrive in contaminated water. Microalgae also possess reactive groups that can form complexes with pollutants in wastewater, leading to flocculation and a reduction in total dissolved and suspended solids. The self-protection mechanisms of microalgae against heavy metal toxicity include immobilization, gene regulation, exclusion, chelation, and the production of antioxidants and reducing enzymes.

Microalgae and bacteria can perform biotransformation of inorganic species into their organic forms by microalgae is of interest. For instances, microalgae are able to reduce the toxicity of inorganic arsenic species by cell wall functional group-mediated oxidation of As(III) , complex formation, biotransformation to methylated species, biosorption through the cell membrane or excretion to the external environment via microalgal cell detoxification mechanisms (Papry et al., 2022). They can also form complexes with heavy metals without compromising their own activity, and these organometallic complexes are sequestered inside vacuoles to regulate metal ion concentrations in the cytoplasm, mitigating toxic effects. Additionally, heavy metals stimulate the biosynthesis of phytochelatins, thiol-rich peptides and proteins that interact with heavy metals, minimizing stress (Leong and Chang, 2020). Microalgae are generally photoautotrophic (inorganic carbon as a carbon source), although they can be heterotrophic (use organic carbon as a carbon source) or mixotrophic (they can use both types of carbon). Algae belong to the eukaryotic group of organisms. They are photosynthetic organisms (can convert CO_2 into biomass) and live in submarine surroundings. Attending to their size, they can be divided into two groups (Tebbani et al., 2014):

- Macroalgae. They are around 1 cm in size (multicellular organisms).
- Microalgae. Size in the micrometers scale, unicellular organisms.

Microalgae are the first directors of O_2. Likewise, microalgae still play a substantial part in the food chain as a principal supply of biomass. They acclimatize themselves to several surroundings and mediums and can be set up in both natural media and saline waters. They either live alone or via symbiotic relations with other microorganisms. The difference between plants and algae is that the latterly is more like “primitive” plantlike organisms that contain chlorophyll a, and perform photosynthesis, but they aren't as complicated as traditional plants (embryophytes) (Borowitzka, 2018; Tebbani et al., 2014). Microalgae are a veritably different group (the number of species ranges from 22000 to 26000). The six further essential groups are (Hemaiswarya et al., 2013): *Chlorophyceae* (green algae), *Phaeophyceae* (brown algae), *Pyrrophyceae* (dinoflagellates), *Chrysophyceae* (golden- brown algae), *Bacillariophyceae* (diatoms) and *Rhodophyceae* (red algae). Another photosynthetic microorganism capable of fixing carbon, nitrogen, phosphorus and heavy metals, among others, using the energy of the sun, are the purple bacteria or purple phototrophic bacteria (PPB). These bacteria are characterized with respect to other photosynthetic microorganisms by a superior metabolism that allows them to have higher growth factors, tolerance in a wider range of temperatures and to assimilate and fix nutrients and grow in a wider spectrum of wastewater composition. In addition, these bacteria can grow under different chemotrophic, phototrophic and mixotropic conditions, although if they coexist in an aerated wastewater treatment reactor, there may compete with other aerobic chemoheterotrophic microorganisms. Under aerobic chemotrophic conditions, these bacteria biodegrade pollutants through oxidative phosphorylation. Although PPB can grow under aerobic or anaerobic conditions, with chemotrophic or phototrophic metabolisms, respectively, anaerobic conditions probably favoured PPB growth more than that of any other chemotrophic bacteria (Sepúlveda-Muñoz et al., 2022)**.** In addition, there is a large amount of cellulose, hemicellulose and protein, which are used to form glycoproteins. (Romera et al., 2007). These components contain many functional groups with reactive and adsorption properties capable of forming coordination complexes with MPs ions (Anastopoulos and Kyzas, 2015).

Bacteria have been widely used in wastewater treatment, due to their great accessibility, low cost and abundance in the environment. They are found in water, organic matter, plants, animals, sediments, etc. They play a fundamental role in the degradation of chemical species and nutrients such as nitrogen, sulfur, phosphorus, etc. Among the microscopic organisms, bacteria, through aerobic and anaerobic treatment, are the most important organisms in wastewater treatment, since they use the nutrients existing in the matrix itself and can grow on it, without generating any toxic waste. (Gerardi, 2015; Gerardi and Zimmerman, 2005). Bacteria do not have a defined nucleus; their genetic material is dispersed inside the cell. In addition, their organelles do not have a well-defined envelope. Most bacteria have a size of 0.3-3 µm, depending on the shape of the cell body and flagellum, which will determine their ability to move and compete with nutrients and growth. According to their shape, the following types of bacteria can be described as Bacillus (Rod), Coccus (sphere), Spirilium (spiral) and vibrio (comma).

In terms of cell growth, bacteria use asexual reproduction, highly dependent on temperature. The genetic material is divided into two equal parts through a binary fission giving rise to two new cells identical to the original. However, genetic recombination occurs when there is recombination of two or more bacterial strains. It is a rapid process and results in the formation of so-called bacterial colonies.

To enter the bacterial cell, heavy metals must pass through an energy-demanding process through the different walls and membranes of the bacteria, among which the following can be distinguished:

1. **Bacterial capsule**: it is the most superficial layer and the one that confers mechanical resistance to disturbances in the environment. It is composed of glycoproteins and polysaccharides. It is the first barrier for heavy metals in the bioaccumulation process. On its surface there are various types of functional groups such as alcohol, carbonyl and carboxyl

groups, amines, amides and others, through which metal ions are bound to it by a rapid adsorption process.

2. **Bacterial wall**: Composed of peptidoglycans, it protects the cell against hypo- and hypertonic media. Comparable to that of plant cells.
3. **Plasma membrane**: similar to that of eukaryotic cells, they have a lipid bilayer and protein channels through which there is an exchange and transport of various substances, including metal ions. Based on the composition of the cell wall, two different types of bacteria can be established: gram-negative and gram-positive.

Cell composition is decisive because it determines which species are better adsorbed and bioaccumulated in case of working with live biomass, as the process highly depends on the surface involved. Bacteria, according to their sources of energy, can be of two types:

- **Autotrophs**. Those that generate their own energy, using carbon dioxide (CO_2) either through photosynthesis processes (phototrophs) or by using nitrogen or sulphur (chemotrophs).
- **Heterotrophs**. They must be nourished by consuming organic matter from other living beings, such as bacteria that decompose the flesh of dead animals. Bacteria living in wastewater media tend to be heterotrophs (they degrade organic carbon compounds to obtain energy). Most heterotrophs can tolerate a wide range of pH values (6.5–9.0) and temperature values (4–35°C). (Gerardi, 2015).

On the other hand, regarding the oxygen use, bacteria are divided into aerobes: They can only use free molecular oxygen to survive (the minority), facultative aerobes: They usually use oxygen, but in oxygen absence, they can also use nitrate or organic molecules to obtain energy, and anaerobes: They cannot use oxygen. In this work, aerobic bacteria from activated sludge will be used.

1.6 Bioremediation mechanisms

Removal of heavy metals by living microorganisms consists of a two-step mechanism. The first step is the rapid extracellular passive adsorption (biosorption), The second step is the slow intracellular active diffusion and accumulation of heavy metal and pollutants (bioaccumulation). Biosorption, is a physical-chemical process in which both organic and inorganic compounds present in an aqueous solution can be adsorbed through the surface of the microorganisms. This uptake occurs through interactions between metal ions present in the solution and functional groups on the surface of the microorganisms. Microalgae and bacteria possess functional groups on their surface such as phosphate, amine, sulfate, hydroxyl, and carboxyl groups, the latter being the most relevant (Gu and Lan, 2021). Therefore, the biosorption mechanism is a very complex process that mainly depends on the chemical composition of the cell surface of these microorganisms. Biosorption can be chemical, where the biomass-metal ion interaction occurs through a chemical process, either by ion exchange, complex formation, adsorption, chelation or microprecipitation; or physical biosorption, involving intramolecular forces such as Van der Walls or electrostatic forces, can also occur. Ion exchange is the predominant mechanism in the biosorption process by microalgae, since their cell surface contains ions such as K, Na, Ca, Mg, etc., which are usually linked to acidic functional groups through which exchange occurs. A release to the medium of these ions therefore occurs in this process. Another less common mechanism, but one that is also present in the biosorption process, is complexation, that is, the formation of complexes on the cell surface between the metal ion and the functional groups present in the microalgae (Cheng et al., 2023). One of the proposed mechanisms for Cu (II) biosorption by *Chlorella vulgaris* is complexation through its coordination with amine and carboxyl groups present in the polysaccharides that form part of the cell surface of the microalgae (Expósito et al., 2021). It should be noted that the interactions that occur in this type of mechanism are much stronger and give rise to much more stable complexes than the bonds that occur in ion exchange processes, so to regenerate the biosorbent, much stronger complexing agents, such as EDTA, are needed in this case. Microprecipitation can occur when either the pH drops dramatically during the biosorption process, or when the concentration of metal ion in the solution increases considerably and its supersaturation occurs (Bwapwa et al., 2017). This can lead to errors in calculating the biosorption capacity of the microalgae, since this phenomenon is completely independent of the nature of the surface of the microalgae. The main mechanisms of heavy metal retention by microalgae are summarised in **Figure 1.3.**

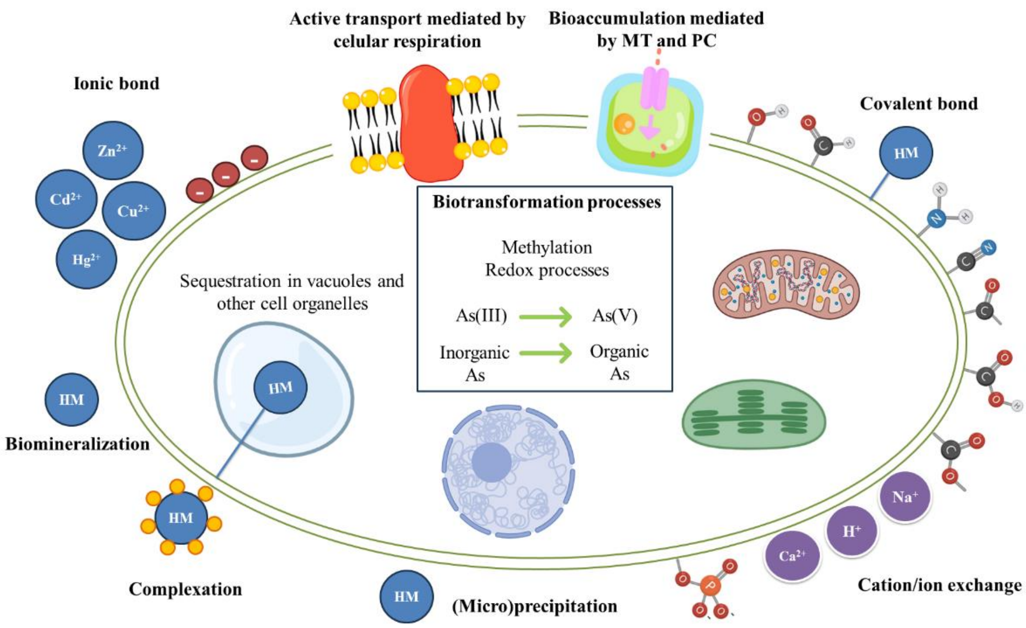

Figure 1.3. Mechanisms of heavy metal removal by microalgae cell

Among the main mechanisms involved in the removal of heavy metals by bacteria, can be found: Biosorption: Bacteria can adsorb heavy metals on their cell surface, and this is a process by which metal ions bind to functional groups on the surface of bacterial cells. Biotransformation: Bacteria can degrade or transform contaminant compounds, converting them into less toxic forms. For example, some bacteria have the ability to convert heavy metal ions into less soluble forms, facilitating their precipitation and removal from water. Precipitate formation: Some bacteria produce chemicals that can precipitate heavy metals into solid forms. This process reduces the mobility and toxicity of metals. Microbial reduction: Some bacteria can reduce metal ions, converting them into less toxic or insoluble forms. For example, microbial reduction of hexavalent chromium (Cr(VI)) ions to trivalent chromium (Cr(III)) or the reduction of As(V) to As(III). Ligand production: Bacteria can secrete organic compounds that act as ligands, forming complexes with heavy metals and reducing their toxicity. Thus, the ability of bacteria to remove heavy metals varies according to the bacterial species and the type of metal.

On the other hand, unlike the biosorption process, bioaccumulation is an active process in which metabolic mechanisms typical of living cells are involved, in which, ultimately, the metals are removed from the environment in which they are found and accumulate inside the cells. Bioaccumulation occurs when the rate of uptake of pollutants is greater than the rate of loss. Thus, the contaminants remain in the organism. Organisms can usually tolerate concentrations of chemicals up to a certain concentration above which these chemicals become toxic and harmful to the organism. Candidate organisms should therefore have broad tolerance to one or more pollutants and exhibit special properties in converting toxic elements into nontoxic forms, allowing them to retain the pollutants internally, thus reducing toxicity outside. Bioaccumulation occurs in two stages. Fast phase (molecules/elements are adsorbed). This stage corresponds to the biosorption process, which will be explained below. The first stage of this process consists of a physical-chemical interaction between the metal ion and the functional groups on the cell surface, i.e. a process similar to that of biosorption. The second stage involves the active transport of the metal ion into the cell through the cell membrane. Being an active diffusion process, it requires energy to be produced from the respiration developed by the cell metabolism, it is a slow and irreversible process in most cases. Furthermore, due to the hydrophilic nature of metals and the lipophilic nature of the cell membrane, this diffusion into the cell is mediated by proteins, creating communication channels between the extracellular and intracellular spaces (Monteiro et al., 2011).Once inside the cell, it has different defense mechanisms against the possible toxicity of these metals. These are mainly associated with proteins to give rise to metallothioneins (MT) and phytochelatins (PC) (Suresh Kumar et al., 2015). These organometallic complexes facilitate their introduction into vacuoles for better management of the concentration of these metals in the cytoplasm for excretion or their compartmentalization. Another metabolic protection mechanism possessed by microalgae is that of enzymatic methylation, which modifies protein structures to prevent potentially toxic metals from interacting with -SH groups (Suresh Kumar et al., 2015a). In **Table 1.6** the main differences between the two processes are shown.

Table 1.6. Characteristics of biosorption and bioaccumulation as main mechanisms for heavy metal removal by living microorganisms. (Adapted from Pandey and Keshavkant, 2021)

Biosorption	Bioaccumulation
Passive process, not controlled by metabolism	Passive process, not controlled by metabolism
reversible process	Partially reversible process
No cell growth	There is cell growth
Metals bind to the cell surface	Metals bind to both the cell surface and the interior of the cell surface.
Occurs at a one stage	Two stages process
Fast, metabolism-independent process, does not require the presence of nutrients	Slow metabolism-dependent process requiring the presence of nutrients
Unaffected by the presence of toxics or contaminants	Toxics affect process performance

1.7 Factors affecting heavy metal removal by living microorganisms.

Biosorption of heavy metal by living microorganisms is influenced by many factors, such as pH, initial metal concentration, contact time and other environmental factors. Regarding pH, acidic pH causes an increase of H^+ in the medium leading to increased competition of these protons with the metals for the binding sites on the cell surface of the microalgae (Samal et al. 2020). Lestari et al., (2020) evaluated Cd removal efficiency through the treatment with *Tetraselmis sp*., *Chaetoceros sp*., and *Nitzschia sp*. At low pH (pH=4) RE (removal efficiency) of 81, 53 and 68% respectively were obtained. On the other hand, for pH 7, the best %RE of 83, 77 and 84% were obtained. Under acidic conditions, the positive charge of the cell surface increases, thus causing an active competition between protons and metallic cations to bind to the binding sites of the cell membrane. In contrast, at higher pH, the cell membrane becomes negatively charged, promoting better performance in metal uptake by the microorganism. Regarding initial metal concentration, studies with different metals concentrations can be found in literature. In the study by Collao et al., a consortium of microalgae *Chlorella vulgaris*-bacteria was used to treat a swine wastewater with an initial concentration of 100 mg/L for Cu(II) and Zn(II) and 500 µg/L of As with removal rates of 83, 81 and 19% respectively . (Collao et al., 2022). The low removal efficiency of species that will be found in the medium as anionic species (arsenite, arsenate) may be largely due to the fact that the cell surface is negatively charged, so more effective electrostatic interactions will occur with positively charged or cationic species. In fact, metal binding to anionic functional groups on the cell surface was reported to be a general mechanism of metal resistance and a protection mechanism (Álvarez et al., 2012). On the other hand, few studies have been found in which the removal of Cd from the medium during the wastewater purification process has been studied. This is the case of the study by *Yang et al*., in which the removal of Cu, Zn and Cd by *Chlorella minutissima* UTEX2341 in a synthetic wastewater matrix was studied (Yang et al., 2015). The concentrations tested were for Cu, 12.7, 25.4 and 63.5 mg/L, obtaining bioelimination percentages of 83.60, 82.38 and 30.37% respectively for an initial biomass concentration between 7 and 8 g/L. For Zn, metal concentrations of 130.8, 261.6 and 392.4 mg/L were tested, obtaining bioelimination percentages of 62.05, 45.87 and 37.95% for initial biomass concentrations between 6 and 7 g/L, and finally for Cd, with metal concentrations of 22.48, 44.96 and 67.45 mg/L, bioelimination percentages of 74.34, 54.86 and 38.76% were obtained for initial biomass concentrations between 7 and 8 g/L. Both biosorption and bioaccumulation mechanisms were reported and biomass growth was not inhibited by the presence of high metal concentrations in the medium, except for high concentrations of Zn for a contact time of 7 days (Yang et al., 2015). On the other hand, in the study by Oliveira et al. (2023), the average removal of Cu and Zn from PWWs was 75.4% and 88.6% respectively. In this case, the photobioreactor was doped with concentrations between 0.5-3 mg/L Cu and 5-25 mg/L Zn in all possible combinations. (Oliveira et al., 2021).

Besides, effect of initial metal concentration can be evaluated through adsorption isotherm studies. Adsorption isotherms play an essential role in the study of removing heavy metals from wastewater. They elucidate the correlation between a metal's concentration in solution and the quantity adsorbed onto a material, typically a solid adsorbent employed in wastewater treatment. In the context of heavy metal removal, these isotherms elucidate how metals distribute between liquid and solid phases contingent upon variables like initial metal concentration, adsorbent properties, and environmental factors like pH and temperature. Analyzing these isotherms yields vital insights for designing cost-effective treatment systems, optimizing processes, and assessing adsorbents metal retention capacities, thereby tailoring removal strategies to meet specific pollutant removal targets. They are also widely applied (Babu Rao et al., 2016; Liu et al., 2008; Saravanan et al., 2010)

In terms of contact time, García et al., (2019) observed a decrease in Zn elimination as HRT decreased from 10 to 6 days, from 98 to 91% in terms of RE. Regarding organic matter (OM), Saavedra et al., (2019) evaluated the effects of OM and CO_2 on the bioremediation potential of *Chlorella vulgaris* and *S. almeriensis* by comparative test. The results showed that the presence of organic matter reduced the biosorption capacity of microalgae, and the adsorption capacity of arsenic by C*hlorella vulgaris* and *S. almeriensis* decreased from 2.2 and 2.3 mg/g to 0.0 and 1.7 mg/g, respectively. The addition of CO_2 not only decreased the adsorption performance of *Chlorella vulgaris* and *S. almeriensis*, but also inhibited the growth of *Chlorella*.

1.8 Heavy metal biosorption by living microorganisms

Cases of bioremediation with living microalgae and bacteria have been described, obtaining a biosorption capacity for Cu (II) of 124.4 mg/g with *Scenedesmus Obliquus* and a contact time of 1 h and at pH of 7 (Kumar et al., 2014), 16.16 mg/g with *Chlorella minutissima UTEX2341* with a contact time of 3 h and at pH of 4 (Yang et al., 2015) or 14.48 mg/g with *Chlorella vulgaris* with a contact time of 30 min and a pH of 3.5 (Mehta and Gaur, 2001a). In the case of Zn (II), biosorption capacities of 21.1 mg/g were achieved for *Spirogyra insignis* with a contact time of 2 h and at pH of 6 (Romera et al., 2007), 123.46 mg/g for *Chlorella minutissima UTEX2341* with a contact time of 3 hours and at pH of 6 (Yang et al., 2015) and 1.14 mg/g for *Sargassum sp*. with a contact time of 1 h and at pH of 5 (Sheng et al. 2004). Removals of 88% and 91.9% were achieved for Cu (II) and Zn (II) with *Chlorophyceae spp*. at pH of 7 and 5.5, 10 min and 3h respectively (Saavedra et al., 2018a). Numerous types of microalgae have been used in the bioelimination of different As species, such as *Sargassum glaucescens* in the simultaneous adsorption of As(III) and As(V) with maximum removal rates of 62 and 72% respectively (Tabaraki and Heidarizadi, 2018), *S. almeriensis*, *Chlamydomonas reinhardtii* and *Chlorella vulgaris* were tested for the removal of As(V) with maximum biosorption capacities of 5.0, 4.63 and 3.89 mg/g respectively (Saavedra et al., 2018a). And finally *Scenedesmus sp. IITRIND2* reaching biosorption capacities of 359.95 mg/g and 366.75 mg/g (Arora et al., 2017). For its part, Cd(II), Yang et al., (2015) studied the removal of Cu, Zn and Cd by *Chlorella minutissima* UTEX2341 in a synthetic wastewater matrix . The concentrations tested were 12.7, 25.4 and 63.5 mg/L for Cu, obtaining bioelimination percentages of 83.60, 82.38 and 30.37% respectively for an initial biomass concentration between 7 and 8 g/L. For Zn, metal concentrations of 130.8, 261.6 and 392.4 mg/L were tested, obtaining bioelimination percentages of 62.05, 45.87 and 37.95% for initial biomass concentrations between 6 and 7 g/L, and finally for Cd, with metal concentrations of 22.48, 44.96 and 67.45 mg/L, the bioelimination percentages obtained were 74.34, 54.86 and 38.76% respectively for initial biomass concentrations between 7 and 8 g/L. Both biosorption and bioaccumulation mechanisms were reported, and biomass growth was not inhibited by the presence of high metal concentrations in the medium, except for high Zn with a concentration of 100 mg/L over a contact time of 7 days. *Chlorella* and *Scenedesmus* show in general a very good performance and robustness against numerous heavy metals. *Chlorella pyrenoidosa* and *Scenedesmus acutus* showed a removal of 45.45% and 57.14% of Cd(II) in 8 days (P.S et al., 2021). For U(VI) the MCL (Maximum Contaminant Level) in drinking water, according to USEPA standards, is 30 µg/L. This limit is set to ensure that drinking water is safe for human consumption and that exposure to uranium through water is kept to levels considered acceptable for public health. Vogel et al. (2010) studied uranium (VI) biosorption by the green microalgae *Chlorella vulgaris* by varying uranium concentrations from 5 µM to 1 mM, and in the environmentally relevant pH range of 4.4 to 7.0. The uptake by live cells in 0.1 mM uranium solution at pH 4.4 was 14.3 mg U/g dry biomass at a contact time of 5 min. Dead cells reached an uptake of 28.3 mg U/g dry biomass. This corresponds to 45% and 90% of total uranium in solution, respectively. Zheng et al. (2018) studied the feasibility of U(VI) bioelimination by *Saccharomyces cerevisiae* at a biomass dose of 1g/L, 250 rpm, 30ºC and initial concentration of U(VI) of 10 mg/L. At pH of 5.5 a maximum biosorption of 66% was achieved. Above this pH, biosorption capacity decreases sharply. In the work of Cheng et al. (2023) *Ankistrodsemus* sp. has been used to treat uranium-contaminated water, and more than 98% of the uranium in solution can be removed in 96 h by the alga, when the initial uranium concentration ranges from 10 to 80 mg/L. Uranium removal remains constant throughout the 4 days of contact, but from this day onwards, a decrease in the percentage of removal of this metal is observed, due to an inhibition of the biomass growth with the nutrients already consumed. A heavy metal biosorption performance for different microalgae and bacteria is shown in **Table 1.7 and 1.8** respectively.

Table 1.7. Metal biosorption performance for different microalgae species and strain

Metal specie	Microalgae species	pH	Initial metal concentration	Biomass conc.	T	Contact time	Uptake (RE)	References
			(mg L^{-1})	(g L^{-1})	(C°)	(hour)	(mg g^{-1}/%)	
Cu (II)	*Chlorella vulgaris**	3.5	-	0.005	25	0.5	89.19	(Mehta and Gaur, 2001a)
	C. vulgaris	3.5	-	0.1	25	0.5	14.48	(Mehta and Gaur, 2001a)
	Scenedesmus obliquus	5-7	0.5-4	0.03	rt	1	124.4	(Kumar et al., 2014)
	Spirogyra insignis	4	50	-		2	19.3	(Romera et al., 2007)
	S. neglecta	4.5	100	0.1	25	0.16	115.3	(Singh et al., 2007)
	S. neglecta	4.5	50	-	25		30.17	(Singh et al., 2012)
	Spirogyra sp	5	100	-	25	1	38.2	(Lee and Chang, 2011)
	Ulothrix zonata	4.5		0.1	20	2	176.2	(Nuhoglu et al., 2002)
	Oscillatoria princeps	4	10	10	25	4	0,10 (90)	(Sulaymon et al., 2013a)
	Chlorella pyrenoidosa	2.00	-	-	-	96	(78)	(Moreira et al., 2019a)
	Tetraselmis marina AC16-MESO	-	5	-	20	72	(92)	(Cameron et al., 2018a)
	Chlorella pyrenoidosa	6,3	5	1,28	28	12	3,25 (83)	(Moreira et al., 2019b)
	Sargassum sp	5	-	-	-	1	0,81 (90)	(Sheng et al., 2004)
	Chlorella minutissima UTEX2341	4	-	4	28	3	16 (98)	(Yang et al., 2015)
Zn (II)	*Scenedesmus obliquus (ACOI598) **	6-7	75	0.02	25	24	429.6	(Monteiro et al., 2011)
	*S. obliqus(L)**	6-7	75	0.02	25	24	836.5	(Monteiro et al., 2011)
	S. obliqus(L)	6-7	50	0.02	25	1.5	209.6	(Monteiro et al., 2011)
	Spirogyra insignis	6	50	1		2	21.1	(Romera et al., 2007)
	Chlorella vulgaris	6	30-300	0,4	-	5	105,29	(El-Sheekh et al., 2016)
	Desmodesmus sp. MAS1	3,5	20	-	23	-	(68)	(Abinandan et al., 2019)
	Sargassum sp	5	-	-	-	1	1,14 (90)	(Sheng et al., 2004)
	Chlorella minutissima	6	-	4	28	3	123 (96)	(Yang et al., 2015)
As(III)	*Ulothrix cylindricum*	6	10	-	20	1	67.2	(Tuzen et al., 2009)
	Maugeotia genuflexa	6	10	4	20	1	2.4	(Sari et al., 2011)
	Mixture of green (Chlorophyta) algae and blue-green (Cyanobacteria) algae	4	50	10	20	3	3.5	(Sulaymon et al., 2013b) (Sulaymon et al., 2013a)
	Ulothrix cylindricum	6	10	4	20	1	2.45	(Tuzen et al., 2009)
	Chlamydomonas reinhardtii	9.5	12	1	-	3	4.63	(Saavedra et al., 2018b)
	Chlorella vulgaris	5.5	12	1	-	3	3.89	(Saavedra et al., 2018b)
	Scenedesmus almeriensis	9.5	12	-	-	3	5.0	(Saavedra et al., 2018b)
As(V)	*Spirulina sp.*	6	727	12	35	4	365	(Doshi et al., 2009)
Cd(II)	*Chlorella vulgaris*	4	200	0.75	20	2	85	(Aksu, 2001)
	C. vulgaris	4	150	1	25		87	(Aksu and Dönmez, 2006)
	*Chlamydomonas reinhardtii**	6			25	1	43	(Bayramoğlu et al., 2006)
	C. reinhardtii	7	989.21		23		145	(Adhiya et al., 2002)
	C. reinhardtii	5.0-6.0	100	0.20	25	1	67	(Bayramoğlu et al., 2006)

Tabla 1.7. Continued

	Codium vermilara	6	50	0.5		2	21.8	(Quílez et al., 2006)
	Scenedesmus quadricauda	5	10-50	0.20	rt	1	135.1	(Mirghaffari et al., 2014)
	Spirogyra insignis	6	50	1		2	22.9	(Romera et al., 2007)
Cr(III)	*Chlorella sorokiniana*	4		1	25		58.8	(AKHTAR et al., 2008)
	Rhizoclonium	4				2	11.81	(Onyancha et al., 2008)
	Spirogyra condensate	5				2	14.82	(Onyancha et al., 2008)
	Spirogyra sp.	5	50		25	3	28.81	(Bishnoi et al., 2007)
	Spirogyra sp.	5	50		25	3	29.15	(Bishnoi et al., 2007)
	Spirogyra sp.	5	50		25	3	30.21	(Bishnoi et al., 2007)
Cr(VI)	*Chlorella vulgaris*	1.5	250	1	25		140	(Gokhale et al., 2008)
	*Chlamydomonas reinhardtii**	2		0.6	25	2	18.2	(Arica et al., 2005)
	C. reinhardtii	2		0.6	25	2	25.6	(Arica et al., 2005)
	C. reinhardtii	2		0.6	25	2	21.2	(Arica et al., 2005)
	*Dunaliella sp.1**	2	100	1	25	72	58.3	(Dönmez and Aksu, 2002)
	*Dunaliella sp.2**	2	100	1	25	72	45.5	(Dönmez and Aksu, 2002)
	*Scenedesmus incrassatulus**	8.9			25	24	4.4	(Jácome-Pilco et al., 2009)
	Spirogyra sp.	2	5		18	2	14.7	(Gupta and Rastogi, 2009)
	Spirogyra sp.	4	-	1	30	2	265	(Yaqub et al., 2012)
Hg(II)	*Chlamydomonas reinhardtii**	6	100	0.20	25	1	72.2	(Tüzün et al., 2005)
	*C. reinhardtii**	5.0-6.0			25	1	89.5	(Bayramoğlu et al., 2006)
Ni(II)	*Chlorella miniata**	7.4	200	1	-	24	1.4	(Wong et al., 2000)
	C. sorokiniana	5			25	0.33	48	(Akhtar et al., 2004)
	*C. vulgaris**	7.4	100	2.5	-	24	0.641	(Wong et al., 2000)
	*C. vulgaris**	5		0.005	25	2	15.4	(Abu Al-Rub et al., 2004)
	*C. vulgaris**	5.5	100	2.5	25	0.5	23.47	(Mehta and Gaur, 2001a)
	C. vulgaris	5		0.1	25	2	15.6	(Abu Al-Rub et al., 2004)
	C. vulgaris	5.5	5		25	0.5	20.23	(Mehta and Gaur, 2001a)
	C. vulgaris	4.5	29.34	0.1	-	1	59.29	(Mehta and Gaur, 2001b)
	C. vulgaris	5.5	29.34		25	3	264.7	(Mehta et al., 2002b)
	C. vulgaris	5.5	200	0.8	25	3	437.84	(Mehta et al., 2002b)
	Odeonogonium hatei	5	50-100	0.8	25	80 min	40.9	(Gupta et al., 2010)g
	Odeonogonium hatei	2	-	1	45	110 min	14.6-28.2	(Gupta and Rastogi, 2009)
	Sphaeroplea sp	6	-	1	33	1.16	199.55	(Srinivasa Rao et al., 2005)
	Sphaeroplea sp	6	50	1	33	1	244.85	(Srinivasa Rao et al., 2005)
	Spirogyra insignis	6	80			2	17.5	(Romera et al., 2007)
Pb(II)	*C. fracta**	5			25	192	61.400	(Lamai et al., 2005)
	*Chlamydomonas reinhardtii**	5	100	0.20	25	1	96.3	(Tüzün et al., 2005)
	*Chlamydomonas reinhardtii**	5.0-6.0	10-50	0.20	25	1	253.6	(Bayramoğlu et al., 2006)
	Scenedesmus quadricauda	5	50	0.5	rt	1	333.3	(Mirghaffari et al., 2014)
	Spirogyra insignis	5	100	0.1		2	51.5	(Romera et al., 2007)
	S. neglecta	5	100		25	0.33	116.1	(Singh et al., 2007)
	Spirogyra sp	5	200	0.5	25	1	87.2	(Lee and Chang, 2011)
	Spirogyra sp	5	10		25	1.66	140	(Gupta and Rastogi, 2008)
	Ulva lactuca	5	23.8	0.76	20	1	34.7	(Sari and Tuzen, 2008)
U(VI)	*Chlorella vulgaris**	4.4	23.8	0.76	-	0.08	14.3	(Vogel et al., 2010)
	*C. vulgaris**	4.4	23.8	0.76	-	96	26.6	(Vogel et al., 2010)
	C. vulgaris	4.4			-	96	27	(Vogel et al., 2010)

Table 1.8. Metal biosorption performance for different bacteria

Metal specie	Bacteria	pH	Initial metal conc.	Biomass conc.	T	Contact time	Uptake (RE)	Reference
			(mg/L)	(g/L)	(^{0}C)	(h)	(mg/g / %)	
Cu (II)	*Pseudomonas Putida*	5	63.5	-	-	-	28.6	(Shen et al., 2017)
	Bacilus sp. (ATS-1)	-	200	-	-	-	16.25	(Dhanwal et al., 2018)
	Rhodobacter sphaeroides HY01	-	10	-	35	48	(96)	(Yang et al., 2016)
	Eichhornia spp and SRB	5-5.50	100	0.8-2	30	24	33.4 (85)	(Dave et al., 2010)
	Bacillus firmus MS-102	4	1000	0.85	25	0.17	860 (74.9)	(Salehizadeh and Shojaosadati, 2003)
	SRB, Desulfovibrio	-	25	-	-	-	(98.9)	(Kiran et al., 2017)
	Pseudomonas aeruginosa AT 18	6.25	50	0.5-1	-	2	86.95 (95)	(Pérez Silva et al., 2009)
Zn (II)	*Pseudomonas Putida*	4.5-5	65.3	-	-	-	26.1	(Shen et al., 2017)
	Bacilus firmus	-	0-500	-	-	-	418	(Salehizadeh and Shojaosadati, 2003)
	Streptomyces rimosus	7.5	100	3	20	4	30-80	(Mameri et al., 1999)
	Bacillus firmus MS-102	6	1000	0.85	25	0.17	722 (61.8)	(Salehizadeh and Shojaosadati, 2003)
	SRB, Desulfovibrio	-	5	-	-	-	(94.6)	(Kiran et al., 2017)
	Pseudomonas aeruginosa AT 18	7	80	0.5-1	-	72	77.5 (87.7)	(Pérez Silva et al., 2009)
As (III)	*Pseudomonas stutzeri WS9 + Micrococcus yunnanensis WS11 + Bacillus thuringiensis WS3*	7	7.5	0.6	37	8	11.92 (95)	(Aguilar et al., 2020)
	Bacillus arsenicus MTCC 4380	7	100	2	30	1.5	978.3 (93)	
	Bacillus cereus P1C1Ib	7	432.54	-	28	72	(72)	
As (V)	*Pseudomonas stutzeri WS9 + Micrococcus yunnanensis WS11 + Bacillus thuringiensis WS3*	7	9.00	0.6	37	6	15 (98)	(Aguilar et al., 2020)
	Bacillus arsenicus MTCC 4380	7	100.002	2	30	1.5	894 (96)	
	Bacillus cereus P1C1Ib	7	432.54	-	28	72	(84)	
Cd(II)	*Pseudomonas fluorescens 4F39*	5	95	0.4	30	1	28	(López et al., 2000)
Pb(II)	*Pseudomonas fluorescens 4F39*	5	176	0.4	30	1	24	
Hg(II)	*Pseudomonas fluorescens 4F39*	5	171	0.4	30	1	177	
U(VI)	*Pseudomonas fluorescens 4F39*	5	202	0.4	30	1	169	

1.9 Microalgae-bacteria consortium and real piggery WW applications

The relationship between microalgae and bacteria has been demonstrated over time with the main existing interaction being the exchange of matter and nutrients, in which the microalgae provide organic carbon from proteins and carbohydrates and O_2 which is used by bacteria in their respiration releasing CO_2, inorganic P, nitrogen compounds, vitamins and minerals, thus relieving the stress due to the existence of photosynthetic oxygen and the regulation of CO_2 concentration, leading to algal growth (Gonçalves et al., 2017). Mutualism is widely known as a biological interaction between two or more different species, whereby there is an exchange of nutrients, among other compounds, resulting in the growth of the consortium. However, these are some of the most important interactions between algae and bacteria. Non-cooperative interactions are also present in consortia of this type, as the release of certain compounds by both microalgae and bacteria can be detrimental to the other species. This is the case, for example, of the release of antibiotic substances by bacteria or metabolites from the photosynthesis of microalgae, which are harmful to bacteria. (Gonçalves et al., 2017). Competition for carbon fixation can also be established between microalgae and bacteria under low carbon conditions. In addition, it is reported in the literature that some bacteria that release streptomycin into the environment, affect the electron transport stage in the photosynthesis process of microalgae. (Perales-Vela et al., 2016). Unlike to cooperative interactions, in this type of antagonistic relationship only one of the species benefits, so that there is no clear relationship between the two species, unlike in parasitism, in which one species benefits at the expense of another, exerting negative consequences on it. Many types of bacteria have been reported as causing negative effects on microalgae through the breakdown of their cell wall by the action of glucosidases and cellulases among other enzymes. Once that cell wall is broken, the bacteria use their intracellular components as nutrients, resulting in reduced growth of the algal biomass or even death. Knowledge of all these interactions provides a better understanding of their modes of action and development, which allows for optimal conditions for the growth of this type of consortium or allows greater growth of the algal biomass at a lower cost. A relevant issue related to the development of intensive and factory farming such as pig farming is the generation of wastes with a high load of organic carbon, nitrogen (ammonia) and phosphorus (López-Pacheco et al., 2021). These nutrients cause serious problems with the discharge of untreated swine manure into the environment and can lead to eutrophication problems. The use of photobioreactors (PBRs) in wastewater treatment plants is an environmentally sustainable solution for the management of livestock waste such as piggery wastewater. Microalgae-bacteria photobioreactors (PBRs) have been developed for wastewater treatment and have been extensively studied in recent years, as they represent the lowest energy cost system for the removal of organic matter, inorganic compounds and pollutants. PBRs are based on the beneficial relationship between microalgae and bacteria. Microalgae use sunlight, nutrients and carbon dioxide produced in the heterotrophic decomposition of organic matter. (Figure 1.4).

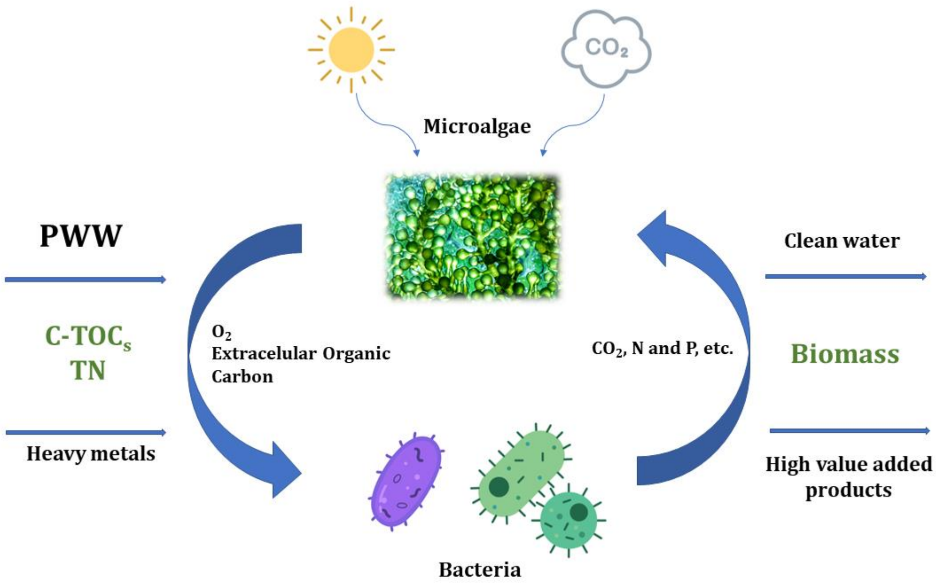

Figure 1.*4*. Nutrients and heavy metal assimilation scheme for a microalgae-bacteria consortia with a piggery wastewater (PWW) at photobioreactor inlet

It is a robust system for cleaning up this highly polluted waste, simultaneously removing organic matter, nutrients and toxic trace elements. The resulting biomass is a consortium of microalgae and bacteria, with different biosorption performance towards metalloid species. The biomass can be further valorized as animal feed or fertilizer, provided that bioaccumulated metals are not present in the recovered by-products. The treated water can be reused as irrigation water.

To develop large scale practical applications, microalgae can be cultivated in two different types of systems (Abinandan et al., 2019); closed photobioreactor (PBR) and open pool (open pounds) or open photobioreactor (**Figure 1.5**). Closed photobioreactors are a type of closed cultivation system that make up for the shortcomings described in the previous section on open pools. On the one hand, they have many advantages, such as the control of the operating conditions for optimal cultivation and the highest possible productivity, which ensures that these methodologies are capable of cultivating pure species. These systems also allow better protection against external environmental contamination and are less sensitive to external environmental conditions such as temperature and seasonal changes, from which pounds are affected. However, the high initial cost, as well as the energy management of the process still needs to be optimized for large-scale commercial application. (Bădescu et al., 2018).

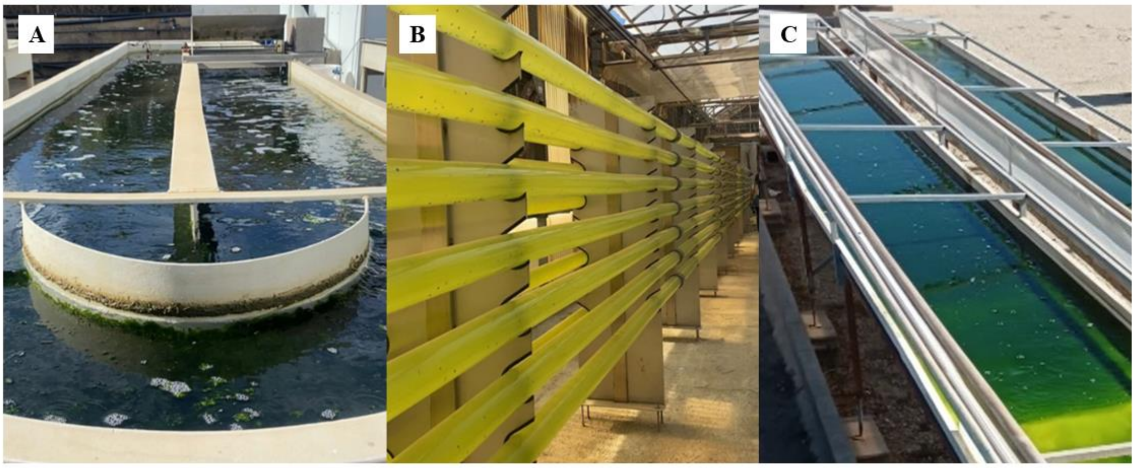

Figure 1.5. Open photobioreactor (A), closed bioreactor (B) and thin-layer photobioreactor (C) from University of Almería facilities

Due to all the after mentioned drawbacks, the use of open photobioreactors for the removal of nutrients and pollutants from wastewater using microalgae-bacteria consortia, has become increasingly widespread. The study of the effect of operational factors in photobioreactors for the treatment of wastewater from slurry is still under development. A compilation of the existing literature on the treatment of wastewater from piggery wastewater, together with the removal of heavy metals and other metalloids is presented in **Table 1.9**. However, there is still insufficient information on how metals influence the nutrient removal capacity of these microalgae and bacterial consortia, and the effect on biomass growth. Moreover, the composition of microalgae is rich in proteins, lipids and carbohydrates, so it is possible, after the use of the biomass for HM removal, to valorize this biomass to produce fertilizers, industrial peptides, lipids, biostimulants, biopesticides, animal feed or biofuels (Sahoo and Seckbach, 2015; Rojo *et al.*, 2023), applying the concepts of biorefinery and circular economy.

Table 1.9. Microalgae-bacteria based photobiroeactors performance combining heavy metals and nutrients removal from piggery wastewater (PWW)

PWW	Inoculum	HRT	Initial COD/TOC/TN/ NH_4^+/TP	Removal capacity COD/TOC/TN/NH_4^+/TP	Cu-RE	Zn-RE	As-RE	Cd-RE	Reference
		days	mg/L	RE%	%	%	%	%	
PWW from a farm located in Segovia, Spain	*Chlorella vulgaris)-bacteria consortia*	22	-/713/256/141/3.8	-/77/53/24/-	83	69	19	-	(Collao et al., 2022) **a**
PWW from a farm located in Segovia, Spain	*Chlorella vulgaris)-bacteria consortia*	22	-/652/247/164/3.6	-/92/61/53/-	81	81	19	-	
Swine wastewater from a farm in Wuhan of Hubei province, China	*Chlorella pyrenoidosa*	4	601/-/-/402/36.3	74.8/-/-/75.9	68.4	75.9	-	-	(Cheng et al., 2017) **b**
Piggery wastewater was obtained from plant of Jiaxing pig farm, Zhejiang, China	*Scenedesmus obliquus*	7	3200/-/-/120/129	65/-/63/-/71	-	-	-	-	(Lee et al., 2021)
		7	1600/-/60/-/64.5	75/-/74/-/81	-	-	-	-	
		7	800/-/30/-/32.2	63/-/58/-/85	-	-	-	-	
Piggery effluent was collected from commercial treatment facilities	*Scenedesmus quadricauda*	6	21600/-/4380/2945/142* dilution with 99.5% water	50/-/50/35/-	-	-	-	-	(Kim et al., 2016)
PWW obtained from livestock WW treatment plant of Zhejiang, China	*Chlorella. vulgaris*	10	2012/744/138/-/88	74/-/60-80/-/60-80	42	45	35	-	(Gao et al., 2018) **c**
PWW obtained from livestock WW treatment plant of Zhejiang, China	*Scenedesmus. obliquus*	10	2012/744/138/-/88	73/-/60-80/-/60-80	46	50	38	-	(Gao et al., 2018)
PWW obtained from livestock WW treatment plant of Zhejiang, China	*Chlorella. vulgaris with activated sludge*	10	2012/744/138/-/88	79/-/80/-/89	59	55	48	-	(Gao et al., 2018)
PWW obtained from livestock WW treatment plant of Zhejiang, China	*Scenedesmus obliquus with activated sludge*	10	2012/744/138/-/88	77/-/60-80/-/60-80	54	52	46	-	(Gao et al., 2018)
PWW	*Anaerobic granular sludge (AnGS)*	5	4334/-/-/-/-/	64/-/-/-/	99	50	-	-	(Zeng et al., 2021) **d**

a at initial concentrations of 100 mg/L of Cu 100 mg/L of Zn and 500 µg/L of As in separately experiments
b at initial concentrations of 2.2 mg/L of Cu 2.8 mg/L of Zn.
c at initial concentrations of 0.78 mg/L of Cu 0.92 mg/L of Zn and 690 µg/L of As
d at initial concentrations of 20.5 mg/L of Cu 35.4 mg/L of Zn.

The work of *Collao et al. (2022)*, indicate that nutrient removal in the photobioreactors was affected by the concentrations of zinc, copper and arsenic in swine wastewater. The authors measured the removal of total nitrogen, total phosphorus, nitrate, ammonium and phosphorus in the photobioreactors with different doses of metals. The results showed that the removal of total nitrogen and total phosphorus decreased significantly with increasing metal concentrations. Nitrate and ammonium removal also decreased, but to a lesser extent. Phosphorus removal remained relatively constant, except in the case of the photobioreactor with the highest arsenic dose, which showed a negative removal. The authors explained that these results were due to changes in the microbial communities caused by the metals, which affected the photosynthetic activity and metabolism of microalgae and bacteria. Metals could also compete with nutrients for binding sites on cell surfaces, making uptake difficult. In conclusion, the results demonstrate that current concentrations of zinc, copper and arsenic in swine wastewater compromise nutrient removal in photobioreactors due to changes in microbial communities (Collao et al., 2022). In terms of nutrient and metal removal, in the work of *Collao et al., (2022)*, for a *Chlorella vulgaris*-bacteria consortia PBR with an HRT of 22 days and an initial nutrient concentration of 713, 256 and 141 of $TOC,\ TN\ and\ N-NH_4^+$ respectively, achieves nutrient removals of 77, 53 and 24% respectively. As for metals, with an initial concentration of Cu, Zn and As of 100, 100 and 0.5 mg/L, eliminations of 83, 69, and 19%, respectively, were obtained (Collao et al., 2022).

On the other hand, in the work of *Gao et al., (2018)*, for the PBR treatment of wastewater from pig slurry with an initial concentration of nutrients of 744, 138 and 88 mg/L of TOC, TN and TP, respectively, nutrient eliminations of 60 to 80% are achieved. For metals, at initial concentrations of 0.78 mg/L of Cu 0.92 mg/L of Zn and 690 μg/L of As, bioeliminations of 42, 45 and 35% respectively are achieved for a consortium of *Chlorella vulgaris* with bacteria from the slurry. On the other hand, when the consortium used is that of *Scenedesmus obliquus* with bacteria, the results of nutrient removal are maintained, while those of metal bioelimination increase to removal percentages of 46, 50 and 38% for Cu, Zn and As, respectively (Gao et al., 2018). Moreover, *Ruiz-Martínez et al. (2012)*, evaluated the potential of microalgae for nutrient removal from effluents generated by an anaerobic membrane bioreactor (AnMBR) treating pig manure wastewater. The authors use a horizontal tubular photobioreactor to grow a mixture of indigenous microalgae and compare its performance with that of a conventional system based on nitrification-denitrification. The results indicate that the photobioreactor is able to remove 99% of the nitrogen and 95% of the phosphorus from the AnMBR effluent, with a biomass productivity of 9 g/m^2/d. The conventional system has a lower nutrient removal efficiency and a larger environmental footprint. The authors suggest that microalgae cultivation can be an integrated solution for resource recovery and energy production from wastewater from the AnMBR (Anaerobic

Membrane Bioreactor) (Ruiz-Martinez et al., 2012). The authors Cui el al. (2021), studied how *Myriophyllum aquaticum*, a plant that can be used for animal feed, can treat PWW that contains high concentrations of nitrogen, phosphorus, and heavy metals. They set up 12 ponds with 150 L of simulated SW and added different amounts of *M. aquaticum* (0.5, 1.0, or 1.5 kg per pond) or none (control) and monitored them for 75 days in the summer of 2019 in Nanjing, China. They found that *M. aquaticum* was very effective in removing N, P, and especially heavy metals from SW. The ponds with *M. aquaticum* had much lower levels of total N, NH_4^+-N, NO_3–N, NO2–N, dissolved organic N, Cu, Zn, and Cd than the control pond. The authors suggested that the optimal way to use *M. aquaticum* for SW treatment was to start with 1.0 kg of plant per pond and harvest it after 45 days in the summer. They also warned that the high levels of Cu and Zn in *M. aquaticum* could pose a risk for animal feed production and recommended further studies on their toxicity and regulation. Additionally, they observed that NH_4^+seemed to play a key role in the removal of heavy metals, especially Cd, by *M. aquaticum*, but the underlying mechanisms were unclear and needed more research. The average removal efficiencies of total N, $N-NH_4^+$, $N-NO_3^-$, $N-NO_2^-$, and dissolved organic N by *M. aquaticum* were 30.1%, 100%, 100%, 97.6%, 20.2%, and 39.8%, respectively, compared with the control pond. The average removal efficiencies of Cu, Zn, and Cd by *M. aquaticum* were 50.4%, 36.4%, and 47.9%, respectively, compared with the control pond. The concentrations of Cu and Cd in *M. aquaticum* at day 75 were in the ranges of 1.92-2.82 and 0.64-1.47 g kg(-1) DW, respectively, exceeding the corresponding limits of 1000 mg kg(-1) DW for Cu and 100 mg kg(-1) DW for Cd for a heavy-metal hyperaccumulator (Cui et al., 2021). One of the critical factors when implementing this type of PBR for wastewater treatment is the initial concentration of organic matter. *Guo et al. (2017)* established 300 mg/L as the optimal concentration of raw PWW to remove nutrient and heavy metals from the medium (Guo et al., 2017). In another study, COD level, 1200 mg/L, was proved to be the optimal concentration of PWW to remove nutrient and heavy metals lrom the medium (Hülsen et al., 2018). In other studies, the effect of the initial concentration of the metal species present in the wastewater has been studied. This is the case of the study by *Yang et al*., in which the removal of Cu, Zn and Cd by *Chlorella minutissima* in a synthetic wastewater matrix was studied (Yang et al., 2015). The concentrations tested were for Cu, 12.7, 25.4 and 63.5 mg/L, obtaining bioelimination percentages of 83.60, 82.38 and 30.37% for an initial biomass concentration of between 7 and 8 g/L. For Zn, initial metal concentrations of 130.8, 261.6 and 392.4 mg/L were tested, obtaining bioelimination percentages of 62.05, 45.87 and 37.95% for initial biomass concentrations between 6 and 7 g/L, and finally for Cd, initial metal concentrations of 22. 48, 44.96 and 67.45 mg/L, obtaining bioelimination percentages of 74.34, 54.86 and 38.76% for initial biomass concentrations of between 7 and 8 g/L.

García et al. (2018) studied the dynamics of microalgae population during PWW treatment in four open photobioreactors operated at 27 days of hydraulic retention time, and inoculated with *Chlorella sp*. (R1), *Acutodesmus obliquus* (R2), *Oscillatoria sp.* (R3) and in the absence of inoculum (R4). Efficient PWW treatment and Zn biosorption occurred regardless of the microalgae. *Chlorella sp.* exhibited a high tolerance to the pollutants present in PWW and the acclimation of native microalgae resulted in the highest biomass concentration. Initial concentrations of TOC were 459, 452 and 482 mg/L respectively. 285, 242, 227 and 294 mg/L for IC , 174, 166 and 165 mg/L for TN and 2.4, 2.1, 1.9 and 1.8 mg/L for TP. Removal efficiencies accounted for 86, 63, 82 and 90 % for R1, 87, 69, 83 and 91 for R2, 86, 71, 83 and 92% for R3 and 86, 62, 85 and 92% for control pond (R4) for TOC, IC, TN and TP. Regarding heavy metal removal, Zn-REs in R1, R2, R3 and R4 (control pond) accounted for 49 ± 6, 37 ± 6, 26 ± 5 and 49 ± 5%, respectively for an initial concentration of 0.9, 1.1, 1.3 and 0.9 mg/L of Zn, which resulted in average effluent Zn concentrations of 0.9 ± 0.2, 1.1 ± 0.1, 1.3 ± 0.3 and 0.9 ± 0.3 mg/L, respectively, at the end of the operational period. The determination of copper and arsenic removal efficiencies was not possible based on the low concentrations of these metals in the PWW. On the other hand, the study of *Zhou et al. (2019)*, present $s-COD$, $N-NH_4^+$ and $P-PO_4^{3-}$ removals of 55, 46 and 75%. In addition, the removal of Cu, Zn, As and Pb, reaching RE% of 93, 70, 11 and 72% respectively (Zhou et al., 2019). Hernández et al. (2013), treated the liquid fraction from pig manure in 5L photobioreactors with the microalgae *Chlorella sorokiniana* and activated sludge with an HRT of 10 days. Removal values of 58.1, 82.7 and 58% of sCOD, NH_4^+ and sP (soluble phosphorus) were obtained. The high percentage of ammonium elimination in this study is noteworthy, due to the low proportion of ammonium found with respect to other slurries. The initial concentration of ammonium in this PWW was 12 mg/L (Hernández et al., 2013). García et al. (2018) studied the dynamics of microalgae population during PWW treatment in four open photobioreactors operated at 27 days of HRT, and inoculated with *Chlorella sp*. (R1), *Acutodesmus obliquus* (R2), *Oscillatoria sp.* (R3) and in the absence of inoculum (R4). Efficient PWW treatment and Zn biosorption occurred regardless of the microalgae. *Chlorella sp*. exhibited a high tolerance to the pollutants present in PWW and the acclimation of native microalgae resulted in the highest biomass concentration. Initial concentrations were 459, 452 and 482 for TOC (mg/L) respectively. 285, 242, 227 and 294 mg/L for IC , 174, 166 and 165 mg/L for TN and 2.4, 2.1, 1.9 and 1.8 mg/L for TP. Removal efficiencies accounted for 86, 63, 82 and 90 % for R1, 87, 69, 83 and 91 for R2, 86, 71, 83 and 92% for R3 and 86, 62, 85 and 92% for control pond (R4) for TOC, IC, TN and TP. Regarding heavy metal removal, Zn-REs in R1, R2, R3 and R4 (control pond) accounted for 49 ± 6, 37 ± 6, 26 ± 5 and 49 ± 5%, respectively for an initial concentration of 0.9, 1.1, 1.3 and 0.9 mg/L of Zn, which resulted in average effluent Zn concentrations of 0.9 ± 0.2, 1.1 ± 0.1, 1.3 ± 0.3 and 0.9 ± 0.3 mg/L, respectively, at the end of the operational period. The determination of copper and arsenic removal efficiencies was not possible based on the low concentrations of these metals in the PWW. García et al. (2017) evaluated the performance of four open algal-bacterial photobioreactors operated at 26 days of hydraulic retention time during the treatment of 10 (×10) and 20 (×20) times diluted piggery wastewater under indoor (I) and outdoor (O) conditions for four months. The removal efficiencies of organic matter, nutrients and zinc from PWW, along with the dynamics of biomass concentration and structure of algal bacterial population were assessed. The highest TOC-RE, TP-RE and Zn-RE were 94, 100 and 83 respectively in indoors PBR in the x10 diluted PWW, while the highest TN-RE (72 %) was recorded outdoors in ×10 diliuted PWW. Guo et al. (2020) evaluated bioremediation performance of microalgae-based treatment technologies for nutrients and heavy metal removal in piggery wastewater. *Chlorella vulgaris*, *Scenedesmus obliquus* and *Neochloris oleoabundans* were selected for mono-cultivation or co-cultivation with fungi or activated sludge. The highest removal efficiency of total organic carbon, total nitrogen and total phosphorus in piggery wastewater were 87.29%, 87.26% and 90.17% by co-cultivation of *S. obliquus* with activated sludge.

1.10 Heavy metal bioavailability and recovery from microorganisms

Metal recovery studies using metal loaded biomass are crucial as a final step in the bioremediation process. It should be considered an important part of the overall bioremediation process. The presence of toxic metals in the harvested biomass could hinder the downstream valorization process as they could be transferred to the final recovered products. Therefore, in addition to the knowledge of the bioremediation capacity of these microorganisms, it is necessary to understand the metal-cell binding mechanisms and to evaluate the availability of the metal ions in the biomass in order to design appropriate valorization process to ensure safe by-products.

Weakly retained metals can be easily desorbed with mild washing reagents prior to biomass valorization. In contrast, strongly retained metals, inside the cell or forming strong bonds on the surface, could pass through the biorefinery processes to the recovered byproducts. Therefore, if the biomass is properly subjected to a cleaning process, it may subsequently be disposed of in the environment as fertilizer or could be released into the soil and groundwater. Hence, it is necessary in some cases to perform a biomass clean up after metal uptake from wastewater, with a weak acid or mild solvent before valorization. Thus, the bioavailability of metals after adsorption should be studied. For this purpose, leaching tests with different mild solvents, such as, NaCl(aq), diluted NaOH, $CaCl_2$, $MgCl_2$, etc. have been performed. Salts are employed to desorb metal ions bound to the biomass surface by weak electrostatic interactions, weak or diluted acids to extract metal cations bound by metal-proton exchange and complexing agents as EDTA to release metals strongly retained on the cell surface by complexing competition.

Saavedra et al., (2019) achieved desorption percentages for Cu, Zn and As higher than 70% for *Chlorella vulgaris.* The maximum recovery was 89% achieved for As with 0.1 M HCl at 60 min. The recovery of Cu, Mn, and Zn increased slightly with the HCl concentration, with maximum desorption efficiencies of 81%, 80%, and 78% for Cu, Mn, and Zn, respectively, in 0.2 M HCl at 60 min. On the other hand, for *S. almeriensis*, HCl mediated recoveries of Cu, Zn and As above 90%, on the contrary, increasing the acid concentration up to 0.2M, produced a drastic drop in the percentage recovery of the metals below 50%, probably due to damage in the cell wall structure caused by the high acid concentration (Abdolali et al., 2015; Kołodyńska et al., 2017)

NaOH 0.1M provided 93% arsenic desorption of for *Chlorella vulgaris* in 20 minutes and up to 40%, for Cu and Zn. These alkaline solvents showed lower desorption efficiencies for *S. almeriensis* compared to *Chlorella vulgaris*, 70% for As and less than 50% for the other metals. Finally, desorption efficiencies using $CaCl_2$ increased with contact time in all the experiments. Increasing $CaCl_2$ concentration only significantly affected As desorption from *Chlorella vulgaris*, with a maximum recovery of 77% at 60 min, when 0.2 M $CaCl_2$ was used. On the other hand, a low recovery of all elements was obtained from *S. almeriensis*. Only 0.2 M $CaCl_2$ supported a relevant boron desorption efficiency of 38% at 10 min, the highest achieved for this element. From this study, it can be concluded that the elution of the biomass with 0.1M HCl gave the best desorption performance of the target metals in both microalgae species. It was reported by Oliveira et al., (2023) that up to 95% of the Zn concentration in swine wastewater was bioavailable, thus posing a high environmental risk of increased toxicity (Lu *et al.*, 2014). At the end of the experiment for an initial Zn concentration of 70 mg/L, fractionation reported 2% Zn in the soluble fraction, 30% as acid soluble forms, 25% as oxidizable fraction, 28% as reducible metal fraction and 18% in the residual unavailable fraction. All the above indicates that the prevalence of available metal fractions in the different studies favors the reuse of the harvested biomass, as the redissolution of metals can be easily performed with mild washing reagents, as noted above. Similar studies have been conducted with *Neochloris oleoabundans* (Gu and Lan, 2021), *Desmodesmus sp*. (Liu et al., 2021), *Scenedesmus sp.* (Oliveira et al., 2023), *Chlorella beijerinck* (Heidarpour et al., 2019).

Chapter 2:

Aim and Scope of the Thesis

2. Aims and Scope of the Thesis

2.1 Justification

Livestock farming is an economic and social sector of great relevance in Castilla y León, and the generation of slurry is inherently linked to this activity. The transformation of this waste, which generates a serious environmental problem, into nutrients and energy, is a renewable resource of great economic potential and represents an important challenge in our environment, given the need to import basic components and the scarcity of natural resources. In recent years, the serious problem of inadequate management of agro-industrial waste, especially piggery wastewater, due to its high content of organic matter, nitrogen and phosphorus as well as other micropollutants, has become evident.

Among all the categories of micropollutants, TTEs (heavy metals and certain metalloids) are particularly dangerous because they are non-biodegradable substances that accumulate in living organisms and can alter cellular processes with toxic effects, ultimately reaching the human food chain. In addition to posing a health and environmental risk, TTEs affect the microbiology of the photopurification systems, the treatment capacity and the biomass recovery alternatives and processes. Specifically, the bioelimination of Cu(II), Zn(II), As(V), As(III), DMA and Cd, has been studied. These toxic trace elements are present in swine manure either as part of the diets during the rearing or fattening stage or because they are found in the drinking water of these animals through leaching processes such as arsenic. Copper (Cu) and Zinc (Zn) are present in mineral additives which are commonly used in animal feed diet as feed growth-promoting. Arsenic (As) comes from water used in animal feed on farms in regions where groundwater or other matrices are polluted with this metalloid. Cadmium (Cd) is widely used in the livestock industry because of its presence in inorganic mineral supplements together with Pb and Hg salts (sulphates and phosphates) containing Cd impurities. In addition, uranium is a geogenic trace element naturally present in surface and groundwater in Castilla y León and can be incorporated into the diet of animals through drinking water.

As a eco-sustainable solution to this issue, treatments using consortia of microalgae and bacteria have shown great potential for the recovery of nutrients and contaminants from this type of wastewater. *S. almeriensis* has been chosen for experimentation throughout the thesis work, due to its demonstrated robustness to contaminants and high nutrient concentrations, as well as high tolerance to metals and changing environments. In addition, it is a microalgae species with a high growth rate. For its part, aerobic bacteria were chosen due to robustness and easy accessibility from wastewater treatment plants.

2.2 Objectives

The principal objective is to study and optimize the removal of metals and nutrients in piggery wastewater treatment processes in photobioreactors with microalgae and activated sludge made up of aerobic bacteria. Specific objectives were stablished as follows:

Objective 1: To study the effect of the main factors involved in Cu and Zn biosorption process with living microorganisms as well as to perform an adsorption isotherm study (**Chapter 4**). It will be a preliminary study to establish the main factors affecting the retention of metals by 3 different biomasses: Microalgae *S. almeriensis* pure, *S. almeriensis*-bacteria consortia and activated sludge.

Objective 2: To investigate the strength of the metal-microorganisms bonds, the retention mechanisms, and the mobility and bioavailability of the retained metals in subsequent biomass valorization procedures. 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 **(Chapter 5)**.

Objective 3: Investigate biosorption capacity and removal efficiency of three arsenic species: As(III), As(V) and the organometallic specie dimethylarsonic acid or cacodylic acid (DMA) (**Chapter 6**). In addition, adsorption isotherms, fitted to Langmuir and Freundlich models, and biomass growth assays were also carried out for the three arsenic species. In addition, structural changes in biomass surface were studied through FTIR and ESEM techniques.

Objective 4: To evaluate biosorption capacity of four less prevalent TTE (toxic trace elements) in piggery wastewater: Cd(II), Pb(II), Hg(II) and U(VI). In addition, adsorption isotherms, fitted to Langmuir and Freundlich models, and biomass growth assays were also carried out for the four species (**Chapter 7**)

Objective 5: To evaluate the effect of operational parameters on photobioreactor performance and metal removal (Cu, Zn, As and Cd) treating piggery wastewater by using microalgae-bacteria consortia and activated sludge through a Taguchi orthogonal array approach (**Chapter 8**).

Objective 6: To test the performance of a microalgae-bacteria based technology on a pilot plant scale photobioreactor for metal and nutrient removal. Removal of copper, zinc and arsenic as well as TOC, TN and NH_4^+ were assessed (**Chapter 9**).

Chapter 3:

Materials and Methods

3.1 Biomass characterization

3.1.1 Total and volatile solids determination

For the calculation of biosorption capacities expressed in mg of metal per gram of dry biomass, it is necessary to know the percentage of total and volatile solids of the biomass. To determine the total solids (TS) of the different biomasses, approximately 3 grams of previously freeze-dried biomass were dried at 105 ºC until constant weight. Then, by calcination of the biomass at 550 ºC. for 24h, the volatile solids (VS) content was determined by Equation 3.1

$$Dry\ residue\ (\%) = \frac{m_{dry\ biomass}}{m_{wet\ biomass}} * 100 \quad (3.1)$$

3.1.2 Protein characterization

The protein content was related to the organic N content of the samples, using a conversion factor of f=5.95 for protein content (Martín-Juárez et al., 2019) and f=6.25 for activated sludge (Zhong et al., 2012). Organic N was determined by the Kjeldahl method, as follows, by Equation 3.2:
A portion of dry biomass is accurately weighed on a filter paper. The paper is wrapped and placed inside the digestion tube, together with a dielectric piece, 6 mL of 96% H_2SO_4 and a Kjeldahl catalyst tablet. The tubes are placed in the digester, together with the fume collector, and a three-stage heating programme is established; 20 min ramp up to 150ºC, another 20 min up to 270ºC, and finally 1 h up to 370ºC. After completion, the solutions are allowed to cool to room temperature.
The digests are then distilled with 6 M NaOH. The distilled ammonia is collected in an Erlenmeyer flask containing an excess of the orthoboric acid solution and an indicator (a mixture of methyl red and methylene blue), where the ammonia reacts to give a stoichiometric amount of borate ions (the solution turns green) which are titrated with a standard sulphuric acid solution (the solution turns purple at the end point).

$$\%Proteins = f * \frac{2\ C(M)\ H_2SO_4 *\ V\ H_2SO_4 * 14.007g}{1000 * m_{muestra}(g)} * 100 \quad (3.2)$$

3.1.3 Lipid content

Lipid determination was carried out according to the following protocol: 100 mg of biomass and 100 mg of alumina (Al_2O_3) previously dried to remove moisture are weighed into a 50 mL Erlenmeyer. Add 20 mL of a 2:1 (v/v) $CHCl_3$-MeOH solution and cover with aluminium foil, taking care to tightly it so that the solvent does not vaporize. It is then placed in the incubator at 600^{0}C for one hour with constant shaking at 250 rpm.

After this time, the solution is transferred to a 50 mL falcon without a skirt and a 0.1M HCl solution is added. Vortex for 1 min and then centrifuge for 10 min at 10000 rpm. Protein precipitation is observed at the interface between the immiscible liquids. Add 1 mL of 0.5% aqueous $MgCl_2$ solution to separate the proteins. Vortex for 1 minute and centrifuge under the same conditions as in the previous step. After this step, the precipitation of the proteins at the interface, the sedimentation of the alumina at the bottom and two liquid phases; a clear one at the top and a darker one at the bottom, are observed in the falcon.

Finally, with the help of a micropipette, the organic phase (dark) in which the lipids are dissolved is collected on a glass vial previously dried in the oven at 40ºC and weighed. It is placed in the oven at low temperature overnight in order to evaporate the solvents and then the vial is weighed. The percentage of lipids contained in the sample is obtained from Equation 3.3:

$$\text{Lipids (\%)} = \frac{m_{sample\ vial}(g) - m_{vial\ mass}(g)}{m_{dried\ biomass}(g)} \cdot 100\% \tag{3.3}$$

3.2 Adsorption isotherms

Adsorption isotherms are a tool that relates the adsorbed amount of molecules on a solid surface to the concentration of these molecules in a medium that is in contact with the solid surface at a constant temperature. This study can provide important information about the biosorption process and better understand it for optimization.

An example of this type of isotherm is the Langmuir adsorption isotherm, which assumes that all adsorption positions are equivalent, so that all sites on the surface have the same probability of being occupied; that only one molecule is adsorbed per position, forming a monolayer of adsorbed molecules; and that the adsorbed molecules do not interact with each other. Consequently, this isotherm has certain limitations, since most surfaces are heterogeneous, not all positions are equal and there are interactions between the adsorbed molecules and physisorption layers can be formed.

The Langmuir equation is given by the following expression:

$$q = \frac{Q_{máx} * K_L * C}{1 + K_L * C_{eq}} \tag{3.4}$$

When linearizing this model, two possible equations can result, depending on what is to be represented: on the one hand, there is the representation of $\frac{C_{eq}}{q_{eq}}$ vs C_{eq} and $\frac{1}{q_{eq}}$ vs $\frac{1}{q_{máx}}$, where the value of the Langmuir constant K_L can be obtained. The two linearized equations 3.5 and 3.6 are as follows:

$$\frac{C_e}{q_e} = \frac{1}{q_{max}\,K_L} + \frac{C_e}{q_{max}} \tag{3.5}$$

$$\frac{1}{q_{eq}} = \frac{1}{q_{max} \cdot K_L \cdot C_{eq}} + \frac{1}{q_{max}} \tag{3.6}$$

Where, K_L is the Langmuir adsorption constant (L/mg), q_{eq} is the equilibrium adsorption capacity (mg/g) and Q_m is the saturation adsorption capacity or theoretical maximum capacity (mg/g). On the other hand, the adsorption adjusted to the Langmuir model can be expressed as a function of what is called the separation factor or dimensionless equilibrium parameter, R_L, which expresses the type of isotherm in question according to the following expression 3.7:

$$R_L = \frac{1}{1 + K_L \cdot C_O} \tag{3.7}$$

Where C_O is the initial concentration of the metal ions. The value of this dimensionless separation parameter gives information on the nature of adsorption, indicating irreversible adsorption if R_L=0, favorable if 0<R_L<1, linear if R_L=1 and unfavorable if R_L>1.

One of the adsorption isotherms for this type of heterogeneous surfaces with sites having different adsorption energies is the Freundlich adsorption isotherm, which attempts to incorporate substrate-substrate interactions on the surface. This model is not valid for high pressures but is more accurate than the Langmuir model for intermediate pressures and is used to describe the adsorption of solutes from liquid solutions onto solids. The Freundlich isotherm has the following expression 3.8:

$$q_{eq} = K_f \cdot C_{eq}^{1/n} \tag{3.8}$$

The linearized equation (3.9) is:

$$\log q_{eq} = \log K_f + \frac{1}{n} \cdot \log C_{eq} \tag{3.9}$$

Where Kf is the Freundlich adsorption constant relative to the adsorption capacity of the adsorbent (L/g), q_{eq} is the amount of adsorbate at equilibrium (mg/g), C_{eq} is the concentration of the adsorbate at equilibrium (mg/L) and n is a dimensionless constant that can explain the adsorption intensity of the system.

This expression relates the logarithm of the retention capacity of each species (q_{eq}) versus the logarithm of its equilibrium concentration (Ce). The K_f and n values can be calculated from the ordinate at the origin and the slope of the fit. Values of n between 1 and 10 represent favorable adsorption.

For the calculation of the biosorption of these heavy metals by the biomass of the microalgae and bacteria, the following equation was used experimentally (3.10) when calculated from the analysis of the liquid phase of the resulting suspension and from equation (3.11) when calculated from the solid phase resulting from the suspension:

$$q = \frac{V \cdot (C_i - C_f)}{W} \tag{3.10}$$

Where q is the adsorption capacity of the biomass at a specific contact time in units of mg heavy metal/g biomass, Ci and Cf are the initial and final concentrations of the heavy metals in units of mg heavy metal/L, V is the volume of the suspension in liters and W is the amount of dry biomass used in grams.

$$q = \frac{V \cdot C_f}{W} \tag{3.11}$$

Where q is the uptake capacity of the biomass at the specific contact time studied (mg toxic element/g of biomass); C_f is the final metal concentrations in solid dry phase, for each metal (mg toxic element/ L), V is the volume of suspension (L) and W is the amount of dry microalgae (g).

The removal efficiencies (RE) for TOC, TN, NH_4^+ and PO_4^{3-} along with Cu, Zn, As, and Cd from liquid medium were calculated using Equation (3.12). using the following expression 3.12:

$$(RE, \%) = \frac{(C_i - C_f)}{C_i} \cdot 100\% \tag{3.12}$$

where C_i represents the initial concentration added of the analyte, and C_f denotes the final concentration found of the analyte in the liquid fraction in mg/L.

3.3 Microwave-assisted digestion of solid samples

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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 180ºC for 20 min followed by 10 min at 180ºC (Bakircioglu et al., 2011). After cooling, the resulting solution was diluted with the appropriate amount of deionized water.

3.4 Analytical determination of heavy metal

The analytical techniques of ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy) and ICP-MS (Inductively Coupled Plasma Mass Spectrometry) are fundamental tools for the measurement of heavy metals in liquid samples. In the case of ICP-OES, a high-temperature plasma generates excited ions in a torch, and the radiation emitted by these ions, upon returning to ground states, is analyzed to determine the concentration of the metals. On the other hand, in ICP-MS, the ions generated in the plasma are separated by their mass-to-charge ratio in a mass spectrometer, allowing highly sensitive and selective detection of heavy metals in liquid samples. Both techniques offer complementary advantages, such as the ability to measure multiple elements simultaneously (ICP-OES) and exceptionally high sensitivity with low isobaric interference (ICP-MS).

3.5 Surface analysis by FTIR

Fourier transform infrared spectroscopy, known as FTIR (Fourier Transform Infrared Spectroscopy), is an analytical technique used to study the chemical composition of a sample as a function of the absorption of infrared radiation. The theoretical foundation of FTIR is based on the principles of infrared spectroscopy, which explores the molecular vibrations of chemical bonds.

When a sample is irradiated with infrared light, molecules absorb energy at specific frequencies associated with the vibrations of bonds between atoms. These vibrations include stretching and deformation of the bonds, providing unique information about the functional groups present in the sample. The Beer-Lambert law applies in this context, stating that the amount of light absorbed is proportional to the concentration of the components in the sample.

The key component of FTIR is the Fourier interferometer, which is used to collect interferometric data. Infrared light that has passed through the sample is split into two beams: one that interacts with the sample and one that serves as a reference. These beams are combined and subjected to interferometry, generating an interference pattern. By applying the Fourier transform to these data, the infrared spectrum of the sample is obtained. The resulting spectrum is presented as a graph, where the absorbance is plotted as a function of wavenumber (inverse of wavelength). Each peak in the spectrum corresponds to a specific molecular vibration, and the position and intensity of these peaks provide information about the identity and quantity of the chemical components in the sample.

In summary, FTIR takes advantage of the interaction between infrared light and molecular vibrations to provide detailed information about the chemical composition of a sample, making this technique a valuable tool in fields such as chemistry, biochemistry, materials science and environmental research. The use of an ATR (Attenuated Total Reflectance) detector in an infrared (FTIR) instrument has several advantages. This approach simplifies sample preparation by allowing direct analysis without additional treatment. The versatility of ATR covers different types of samples, from solids to liquids and powders, facilitating the analysis of a wide range of materials. In addition, the ATR technique excels in its ability to analyze small sample quantities, its high sensitivity that detects low concentrations, the reduction of spectral noise for sharper spectra and the ability to minimize environmental interferences. In summary, the ATR detector offers efficiency, versatility and improved data quality, making it a valuable choice in a variety of analytical applications.

A series of samples of the two biomasses used after biosorption of the different heavy metals at pH 7.5 were analyzed by FTIR to determine their interactions with the functional groups present in the cell wall of the biomasses, as well as their possible transformations. The samples were freeze-dried prior to FTIR analysis. The Telstar LyoQuest lyophilizer was used for subsequent FTIR measurement. In addition, control samples were prepared using fresh biomass prior to treatment with metals. Spectra were recorded between 4000-500 cm^{-1} with a Bruker Tensor 27 FTIR spectrometer equipped with a Golden gate ATR (attenuated total reflectance) detector, a resolution of 1 cm^{-1} and OPUS software, Optics User Software, belonging to the Department of Condensed Matter Physics, Crystallography and Mineralogy (University of Valladolid).

3.6 Surface analysis by ESEM-EDX

In order to observe the surface differences that may arise in the biomass prior and subsequent bioelimination of the different metal species by the biomass, the technique of environmental scanning electron microscopy has been used. (ESEM).

Environmental Scanning Electron Microscopy (ESEM) is an advanced electron microscopy technique that allows the observation of samples under environmental conditions, such as the presence of water or gases. Unlike conventional scanning electron microscopy, ESEM does not require the coating of samples with a conductive layer, which facilitates the observation of non-conductive or biological materials without altering their structure.

The theoretical base of ESEM is based on the incorporation of an environmental chamber into the microscope system. This chamber allows control of the pressure and composition of the surrounding gas around the sample, which facilitates the observation of samples in conditions close to those of the natural environment. The ESEM uses a backscattered electron detector to generate high-resolution images of the sample, similar to conventional scanning electron microscopy.

The main innovation of ESEM is the ability to operate in wet conditions, which is especially useful for studying biological materials, polymers, gels, and other materials sensitive to drying. This technique has significantly expanded the possibilities of microscopic observation by providing detailed images of samples that would not be readily accessible with other techniques. ESEM is applied in a variety of fields, including biology, geology, materials science, and nanotechnology, allowing the visualization of structures in their natural environment and providing valuable information about the morphology and composition of samples.

The analysis was carried out with an Environmental Scanning Electron Microscope (ESEM), model FEI - Quanta 200FEG with a low vacuum LFD secondary electron detector. They were performed by the technician in charge at the Advanced Microscopy Unit of the Uva Innova R&D Building at the Miguel Delibes Campus of the University of Valladolid.

3.7 Statistical analysis

Designs of experiments (DoE) are systematic and statistical approaches to efficiently plan, conduct and analyze scientific or industrial experiments. These experiments are designed with the purpose of understanding and optimizing processes, identifying the factors that affect the results and how they interact with each other. To do so, the objective of the experiment and the results to be improved or optimized must be identified. On the other hand, the factors that can affect the outcome of the experiment must be identified. Factors are controllable variables such as temperature, time, concentration, etc., as well as defining the possible levels for each factor. The levels are the specific values assigned to each factor during the experiment. Finally, the type of experimental design that best suits the problem must be selected. During the development of this doctoral thesis, some of these approaches have been used for the optimization of some of the processes studied in this thesis. This is the case of full factorial designs and Taguchi's design of experiments.

Full factorial experimental designs are a statistical tool that allows studying the effect of multiple factors and their interactions in a process. In the context and object of study of this doctoral thesis, the factors can be variables such as biomass concentration and type, heavy metal concentration, contact time, etc. Each factor is evaluated at different levels, and all possible combinations of levels are tested to understand how they affect the final result. In this way, the most influential factors and their interactions can be identified, allowing conditions to be adjusted to maximize biosorption. On the other hand, Taguchi approaches, developed by Genichi Taguchi, seek to minimize process variability, and improve the robustness of the design to small variations in operating conditions. Taguchi uses orthogonal arrays to evaluate multiple factors simultaneously with a minimum number of experiments. In addition, Taguchi distinguishes between two types of factors to be studied, on the one hand, control factors and noise factors (non-controllable). From this, Taguchi's designs attempt to identify controllable factors (control factors) that minimize the effect of the noise factors. During the experiment (Chapter 8), the noise factors are varied to cause variability and then the optimal configuration of the control factors is determined to make the process robust or resilient to the variation caused by the noise factors. A process designed with this approach will produce a more consistent output and will perform independently of the environment in which it is used. Contextualised to the present work, Taguchi allows experiments to be designed efficiently, with a minimum number of experimental runs needed to obtain meaningful results. This saves time and resources compared to other optimisation methods. It allows the identification of critical factors as Taguchi will help in this case to identify the most influential factors in the metal and nutrient removal process in the photobioreactors. This enables optimisation efforts to be focused on the most important aspects of the process. Taguchi methodology is robust to experimental noise, i.e. random variations that can affect the results of the experiments. This ensures that the conclusions obtained are more reliable and reproducible under real conditions. Furthermore, it can be used to optimise multiple process variables simultaneously, since it handles a very large number of responses to analyse and interpret. This allows solutions to be found that maximise the removal of metals and nutrients at the same time.

Additionally, Principal Component Analysis (PCA) is a statistical technique that reduces the complexity of data by finding patterns and relationships between variables. It transforms the original variables into a smaller set of uncorrelated variables called principal components. These components capture most of the variability in the data. In this thesis, PCA is used to simplify the interpretation of the data and explore relationships between relevant variables.

Chapter 4:

Effect of operational conditions on Cu and Zn bioelimination by *Scenedesmus almeriensis* and *activated sludge* in photobioreactor systems

Effect of operational conditions in Cu and Zn bioelimination by *Scenedesmus almeriensis* and *activated sludge* in photobioreactor systems

Abstract

Nowadays, wastewater from livestock facilities has high amounts of heavy metals, which are a severe socio-sanitary problem. Thus, strategies for treating those residues are vital to prevent contamination. Three different types of biomass were tested in order to study the biosorption capacities of Cu(II) and Zn(II): a pure *Scenedesmus almeriensis* strain, a bacterial sludge, and a consortium of *S. almeriensis* and bacteria grown in slurry water. Besides, different factors, such as contact time, light exposure, initial metal concentration, presence of organic matter and CO_2 were taken into account to establish optimized conditions for the metal uptake. For these two metals, a full complete factorial design of 144 experiments was carried out to determine significant factors and their interactions. The growth study showed that the consortia and the pure strain had significant growth. In terms of biosorption capacities, for Cu(II), the maximum values found were; 104.52 mg/g, 81.50 mg/g, and 67.71 mg/g, for pure strain culture, consortium, and activated sludge respectively at an initial metal concentration of 100 mg/L and an initial pH of 7.5. For Zn(II) maximum biosorption capacities were 121.65 mg/g, 96.71 mg/g, and 73.52 mg/g respectively at an initial metal concentration of 100 mg/L at an initial pH of 7. Hence, the type of biomass is central to achieve high retention capacities. Additionally, from a preliminary isotherm study, a good fit for the pure strain and the activated sludge was attained. The results are promising as high values were reached for bioelimination, which makes it possible to consider the treatment of wastewater in photobioreactors.

Keywords: Activated sludge, biosorption, heavy metal, microalgae, pig manure, wastewater

4.1 Introduction

Water pollution by toxic heavy metals is a severe socio-sanitary problem that requires efficient, environmentally friendly, and economically viable solutions. Heavy metal classification includes metals and metalloids with a density greater than 5 g/cm^3 in which we can find Arsenic (As), Chromium (Cr), Lead (Pb), Iron (Fe), Mercury (Hg), Silver (Ag), Copper (Cu) and Zinc (Zn) among others (Sarode et al., 2019). The contamination by metals and other pollutants today is an issue that especially affects wastewater, whose exposure to microorganisms, plants, and humans can be harmful due to their toxicity, which can be expressed by different pathways: displacing essential metal ions from biomolecules and other biologically functional groups, blocking essential functional groups of biomolecules, including enzymes, modifying the active conformation of biomolecules, such as enzymes and polynucleotides and disrupting the integrity of biomolecules and modifying some other biologically active agents. The contamination of water bodies and so many different types of wastewaters by metalloids and heavy metals have been a major environmental issue for the last few years, whose study is on increase. The origin of this contamination can be natural or anthropogenic, since wastewater, industrial activities such as mining, pigment manufacturing, and livestock production release a large quantity of these trace toxic elements daily into the environment. By their non-biodegradable nature, they accumulate and persist in nature in a way that leads to bioaccumulation in the food chains and causes severe environmental and health issues (Yang et al., 2015).

Livestock farming is a very important economic and social sector in Spain and Castile and Leon, and the generation of manure and in special pig manure is inherently linked to this activity. The transformation of this waste, which generates a serious environmental problem, into nutrients and energy, is a renewable resource with great economic potential and represents an important challenge in our environment, given our need to import basic components and the scarcity of natural resources. However, typical pig diets have a high content of phytates, which reduces the availability of Cu and Zn (Selle and Ravindran, 2008), essential at low concentration levels. Regarding the feed, to ensure animal health and productivity among other factors, pig diet is supplemented with Cu and Zn and other toxic trace elements (TTE), that are partially released to the atmosphere through urine and feces. Nevertheless, Cu and Zn can be toxic when prolonged exposure to concentrations higher than required takes place (Vardhan et al., 2019). Thus, these residues represent an alarming problem nowadays, so it is crucial to develop an effective treatment of the swine generated, which not only prevents contamination but also allows the recovery of organic matter and nutrients present in them. Among the conventional physicochemical methods for wastewater treatment whose use is most widespread are the ion exchange, membrane technologies, chemical precipitation, adsorption on activated carbon, electrochemical treatment

(Fu and Wang, 2011), their main disadvantages are that they are expensive methodologies or that require high maintenance or expensive operational cost, therefore, are not feasible in practice. On the other hand, they are only effective when the target pollutant to be removed is in a concentration greater than 100 mg/L (Suresh Kumar et al., 2015, Jaafari and Yaghmaeian, 2019). Hence, it is necessary to develop and implement new technologies that allow the removal of these trace toxic elements from these resources so they can be reused as biofertilizers and other high added value products. In contrast to conventional methods, bioremediation is becoming more and more widespread as an alternative methodology, which consists of the use of living or non-living microorganisms such as microalgae, bacteria, yeasts, fungi, etc. These microorganisms have been widely applied due to their environmentally friendly nature and their low-cost application, in addition to their high tolerance to the toxicity that these metals can generate in the environment, to the existence of extreme environmental conditions such as high salinity, stress due to the presence of nutrients, in addition to a large amount of active binding sites and large surface area (Cameron et al., 2018). Thus, in recent years, this type of treatment has been chosen for the removal of these metalloids from this type of matrix. This is the case of microalgae, which are considered to be eukaryote microscopic unicellular, photoautotrophic organisms that coexist in freshwater and marine ecosystems (Cooper and Smith, 2015) and have high growth rates and low nutrient content compared to other biomass organisms. Moreover, besides adsorption mechanisms, due to the hydrophilic nature of metals and the lipophilic nature of the cell membrane, diffusion processes into the cell are mediated by proteins, creating communication channels between the extracellular and intracellular spaces resulting in bioaccumulation processes, which involves irreversible equilibriums (Monteiro et al., 2011). Besides, this is a type of biomass that does not generate toxic substances unlike others such as fungi and bacteria. Cases of bioremediation with microalgae have been reported, obtaining a biosorption capacity for Cu (II) of 124.4 mg/g with *Scenedesmus Obliquus* with a contact time of 1 hour and at pH 7 (Kumar et al., 2014), 16.16 mg/g with *Chlorella minutissima UTEX2341* with a contact time of 3 hours and at pH 4 (Yang et al., 2015) or 14.48 mg/g with *Chlorella vulgaris* with a contact time of 30 min and pH 3.5 (Mehta and Gaur, 2001a). In the case of Zn (II), biosorption capacities of 21.1 were achieved for *Spirogyra insignis* with a contact time of 2 hours and at pH 6 (Romera et al., 2007), 123.46 mg/g for *Chlorella minutissima UTEX2341* with a contact time of 3 hours and at pH 6 (Yang et al., 2015) and 1.14 mg/g for *Sargassum sp*. with a contact time of 1 hour and at pH 5 (Sheng et al., 2004). Removals of 88% and 91.9% for Cu (II) and Zn (II) were achieved with *Chlorophyceae spp*. at pH 7 and 5.5 and 10 min and 3h respectively (Saavedra et al., 2018a). On the other hand, bacteria have been widely used in wastewater treatment, due to its great accessibility, low cost, and its abundance in the environment. They take the O_2 produced by the microalgae, giving rise to interesting interactions such as cooperative interactions (Gonçalves et al., 2017), association, symbiosis, or mutualism (Ramanan et al., 2016), although competitive or antagonistic interactions like parasitism or commensalism may also occur (Liu et al., 2017). There are plenty of studies on the bioelimination of Cu and Zn using bacterias, for example, *Pseudomonas aeruginosa* AT18 showed a removal of 87.7% of Zn and 95% of Cu (Pérez Silva et al., 2009), and *SRB Desulfovibrio* display removal values of 94.60% and 98.90% for Zn and Cu, respectively (Kiran et al., 2017). In the microalgae-bacteria consortiums, they take the O_2 produced by the microalgae, giving rise to interesting interactions to study, such as association, symbiosis, or mutualism, although competitive or antagonistic interactions may also occur (Gonçalves et al., 2017; Liu et al., 2017; Ramanan et al., 2016). Microalgae have been scarcely studied, very few examples can be found in literature, so that, the importance of this research. For instance, a mixed culture of algae-bacteria biomass composed by bacteria, cyanobacteria, *Chlorophycae,* and *Diatoms* was used as a biofilter for Cu (II) removal, achieving removal capacities of 24 mg/g for Cu (II) (Loutseti et al., 2009). Removals of 62% and 90% for Copper and Zinc respectively (Safonova et al., 2004) and other types of mixed cultures ensured >95% removal of toxic elements such as Mn, Cu, Al, Ti, Si, S, and Fe (Sahoo et al., 2020).

On account of this problematic, in this work, Cu (II) and Zn (II) uptake in a multimetallic solution has been studied in order to optimize the biosorption capacities by a complete factorial design of 144 experiments by three different types of biomass with promising potential in biosorption (Saavedra et al., 2018b) (a pure strain of *Scenedesmus almeriensis,* its microalgae-bacteria consortium and sludges). As there is few information about adsorption of Cu (II) and Zn (II) by the microalgae-bacteria consortium, and to elucidate which microorganism and factors are conditioning the process, microalgal viability, growth, and biosorption capacity tests were conducted for the three species in multimetallic solutions, evaluating the influence of the factors such as the pH, contact time from 1h to 72h, initial metal concentration, presence of light, organic matter and CO_2.

4.2 Materials and methods

4.2.1 Innoculum and reagents

Microalgae biomass of *Scenedesmus almeriensis* (provided by the University of Almeria, Spain) grown in synthetic medium and in liquid fraction of piggery wastewater as well as activated sludge from WWTP (Wastewater treatment plant) of Valladolid, was used for all biosorption studies. The biomass was stored in darkness conditions at 4°C for quality assurance purposes. For Cu and Zn, stock solutions were prepared using $CuCl_2 \cdot 2H_2O$, and $ZnCl_2$ (Sigma Aldrich, Germany). All stock solutions were prepared in ultra-pure water. Multimetallic removal experiments were daily arranged by a stock solution containing 4000 mg/L of Cu and 5000 mg/L of Zn in ultra-pure water at acid-medium (pH < 3). The stock solution was periodically analyzed and stored in darkness at 4°C. The culture growth medium used was a Bristol medium (UTEX S.A). LED lamps at 1200 μE/m^{2}s were used in a 12:12 h:h photoperiod. NaOH (0.1 M) and HCl (0.1 M) were used for pH adjustment. All the chemicals employed in this study were analytical grade (Sigma Aldrich, Germany). All plastic and glass containers were washed in dilute HNO_3 (10% v/v) for 24 h and rinsed 3 times with Milli-Q water (R > 18 MΩ cm) before use. The toxic metals and the concentration range of synthetic solutions used in this study were selected based on the composition of the liquid fraction of pig manure (ASAE, 2003).

4.2.2 Multimetallic biosorption experiments design (DoE)

Adsorption experiments were performed batch-wise for each biomass species at different conditions. Microalgae culture was centrifuged at 7800 rpm for 10 min, washed with Milli-Q water to remove the growth medium, and centrifuged again before the determination of the total solids (TS) concentration. To perform the multimetallic biosorption experiments, a complete factorial design was developed (see **Table 4.2**). Factor and its levels are summarized in **Table 4.1**. The factors proposed to affect biosorption process were the type of biomass, the contact time, the presence or absence of intense light, simulating periods of light-dark 12:12h, the initial concentration of metal, the presence or absence of organic matter in the form of peptone and CO_2. The pH value was adjusted to 7.5 to simulate the environmental conditions that exist in wastewater treatment photobioreactors (Posadas et al., 2015). The two levels of contact time were 72 h based on the typical hydraulic residence time used for wastewater treatment photobioreactors (Acién et al., 2012) and 1h. Three different MMS concentration were used for initial MMS concentration tests were: **MMS_1** (Cu: 15 mg/L, Zn: 40 mg/L), **MMS_2** (Cu: 60 mg/L, Zn: 70 mg/L), and **MMS_3** (Cu: 100 mg/L, Zn: 100 mg/L). Peptone selected concentration was 80 mg/L in all experiments (Saavedra et al., 2019).

Table 4.1. Matrix of the full factorial design of 48 experiments with the combinations of factors carried out for each of the tested biomasses.

Control factors	Level -1	Level 0	Level 1
Concentration (C)			
Cu (mg/L)	15	60	100
Zn (mg/L)	40	70	100
	Level -1		Level 1
Organic matter **(OM)**	Absence		80 mg/L
Light **(L)**	Ambient light-darkness 12h:12h		Light-darkness 12h:12h
Contact time **(T)**	1h		72h
CO_2	Yes		No supply

Table 4.2. Matrix of the full factorial design of 48 experiments with the combinations of factors carried out for each of the tested biomasses.

Run	OM	L	T	CO_2	(C)
E1	-1	-1	-1	-1	-1
E2	-1	-1	-1	-1	0
E3	-1	-1	-1	-1	1
E4	-1	-1	-1	1	-1
E5	-1	-1	-1	1	0
E6	-1	-1	-1	1	1
E7	-1	1	-1	-1	-1
E8	-1	1	-1	-1	0
E9	-1	1	-1	-1	1
E10	-1	1	-1	1	-1
E11	-1	1	-1	1	0
E12	-1	1	-1	1	1
E13	1	-1	-1	-1	-1
E14	1	-1	-1	-1	0
E15	1	-1	-1	-1	1
E16	1	-1	-1	1	-1
E17	1	-1	-1	1	0
E18	1	-1	-1	1	1
E19	1	1	-1	-1	-1
E20	1	1	-1	-1	0
E21	1	1	-1	-1	1
E22	1	1	-1	1	-1
E23	1	1	-1	1	0
E24	1	1	-1	1	1
E25	-1	-1	1	-1	-1
E26	-1	-1	1	-1	0
E27	-1	-1	1	-1	1
E28	-1	-1	1	1	-1
E29	-1	-1	1	1	0
E30	-1	-1	1	1	1
E31	-1	1	1	-1	-1
E32	-1	1	1	-1	0
E33	-1	1	1	-1	1
E34	-1	1	1	1	-1
E35	-1	1	1	1	0
E36	-1	1	1	1	1
E37	1	-1	1	-1	-1
E38	1	-1	1	-1	0
E39	1	-1	1	-1	1
E40	1	-1	1	1	-1
E41	1	-1	1	1	0
E42	1	-1	1	1	1
E43	1	1	1	-1	-1
E44	1	1	1	-1	0
E45	1	1	1	-1	1
E46	1	1	1	1	-1
E47	1	1	1	1	0
E48	1	1	1	1	1

Studies were made with a liquid volume of 200 mL in borosilicate glass bottles of 500 mL. The biomass concentration was 1 g/L for experiments carried out with the pure microalgae, the microalgae-bacteria consortia and the activated sludge, maintained during all the experiments at 23°C, in a stirring of 250 rpm and pH 7.5 as mentioned before. After the stirring time has elapsed, the solid phase is separated from the liquid phase via centrifugation and washed repeatedly with Mili-Q water. Once the solid phase is completely dry, microwave-assisted acid digestion is performed, and then the concentration of Cu and Zn is determined by ICP-OES. Metal uptake experiments were carried out to study the removal capacity of the three selected microalgae under the different conditions for each experiment. The uptake of toxic metals by microalgae biomass was calculated in terms of the experimental uptake capacity (q) as follows in Equation 3.11 of Materials and Methods. On the other hand, the metal removal efficiency (RE) was also another parameter for evaluation. It is expressed in percentage as defined by Equation 3.12 in Material and Methods

4.2.3 Equilibrium isotherm studies

Langmuir isotherm has been the most widely used model to the sorption of a solute from a liquid solution. Using Langmuir's model, adsorption isotherms associated with the process has been used to understand the mechanisms of interaction through adsorption present among the three different types of biomass used as biosorbents; pure microalgae, sludge, and a microalgae-bacteria consortium; as well for the two metals of the multi-metal solution used, Cu and Zn. This may bring out valuable information that can help us to better understand what is happening in the biosorption process and to optimize it. Solutions with concentration of Cu (II) and Zn (II) ranging from 10 to 200 mg/L were prepared and used in thus study in a fixed dose of biomass of 1g/L at a contact time of 72h at 23ºC in presence of organic matter in the form of peptone in a concentration of 80 mg/L of peptone. The procedure carried out was as follows, once the contact time has elapsed, a 20 mL aliquot of the suspension is taken, centrifuged at 7800 rpm for 10 minutes and passed through a 0.45 µL filter. The biosorption capacity of Cu(II) and Zn(II) by the biomass (q) was calculated according to Equation 3.10. Fowler-Langmuir and Freundlich Isotherm model were performed according to Equations 3.4-3.9 of Materials and Methods Thesis Chapter. Analytical procedures are described in section 4.2.4 of this Chapter.

4.2.4 Analytical procedures

The concentrations of Cu and Zn of the digested solid were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (VARIAN 725) according to the internal procedures of the Instrumental Techniques Laboratory (LTI – UVa). For quality assurance, two reference water materials were included in the ICP-OES analysis as quality control (QC) samples: ICP multielement Calibration Standard Solution, 100 mg/L Scharlau (26 elements in HNO_3 5%), and a Certified Reference Material (Environment Canada TMDA-64.2 LOT 0313, HNO_3: 0.2%) as trace element fortified calibration standard. QC samples were measured every 10 samples, considering a range within 10% of the true value for valid acceptance. The pH was measured using a pH-meter Basic 20+ (Crison, Spain). Determination of TS and TSS concentrations were performed according to standard methods (E.W. Rice, R.B. Baird, A.D. Eaton, 2017).

Solid samples from biosorption experiments and the final sample from the last step of the sequential extraction procedure were digested for Cu and Zn analysis by microwave-assisted acid digestion. About 0.1 g of dried biomass was accurately weighed and digested with 10.0 mL of 69% nitric acid for analysis 69% (Panreac AppliChem, Spain) in a Mileston Ethos Plus microwave oven. Digestion was carried out with a temperature ramp from 25ºC to 180ºC for 20 min followed by 10 min at 180ºC (Bakircioglu et al., 2011). The digestion is controlled with EasyWave 3 software. After the digestion, the resulting liquid was diluted to 30 g with deionised water.

4.2.5 Statistical analysis

Experimental data analysis for multimetallic biosorption experiments was performed using Statgraphics 18 software. An analysis of variance (ANOVA) was made at a confidence level of 95% ($\alpha = 0.05$) to establish an interpretation of the results.

4.3 Results and discusion

The results showed that all three types of biomass species studied were viable regardless of the metal tested. Results for biosorption capacities for three biomass are shown in **Table S4.1** of supplementary material. ANOVA results are also shown in Suplementary material in **Tables S4.2-10.** In a first screening, clear differences were seen between the different types of biomass in terms of biosorption capacities, with the following being the decreasing order of efficiency in the removal of these metalloids; the pure strain of *S. almeriensis*, microalgae-bacteria consortium, and sludges in the last place. Furthermore, to study the major interactions between microalgae and bacteria when they are in a consortium, a growth or variation study was also executed for the 3 types of biomass for all possible combinations of factors for a contact time of 72 hours, finding clear synergies for the case of the consortium, which is the one that reported the highest growth factors, followed by bacteria and finally the *S. almeriensis*. Finally, to clarify or have a first approach of the possible mechanisms involved in the process of biosorption of Cu and Zn in a multimetal solution, Langmuir and Freundlich isotherms have been performed. On the other hand, experimental metal concentrations were chosen to operate under unfavourable conditions, but within the range of concentrations found in literature for these metals in piggery wastewater: **MMS_1** (Cu: 15 mg/L, Zn: 40 mg/L), **MMS_2** (Cu: 60 mg/L, Zn: 70 mg/L), and **MMS_3** (Cu: 100 mg/L, Zn: 100 mg/L).

4.3.1 Biomass growth experiments

The biomass growth experiments for the different types of biomasses were carried out in photobioreactors at contact times of 72 hours, with a total of 72 experiments, 24 per biomass. Biomass growth experiments were conducted with the runs in which contact times of 72h were involved, due to the possibility of growth of the biomass. In 1h contact times experiments, biomass growth were not expected. Thus, an experimental design was implemented, and the results were interpreted by a multifactor ANOVA, determining the effects and interactions. To try to see other effects, a multi-factor ANOVA was performed for each type of biomass in 6 subddesigns per biomass per concentration and per metal tested. The findings were: the significant factors for the pure microalgae species were the concentration (0.0006) as well as for the activated sludge (0.0000). Nevertheless, for the consortium, the concentration (0.0000) and the light intensity (0.0000), were significant factors. Results for biomass growth are shown in **Figure 4.1.**

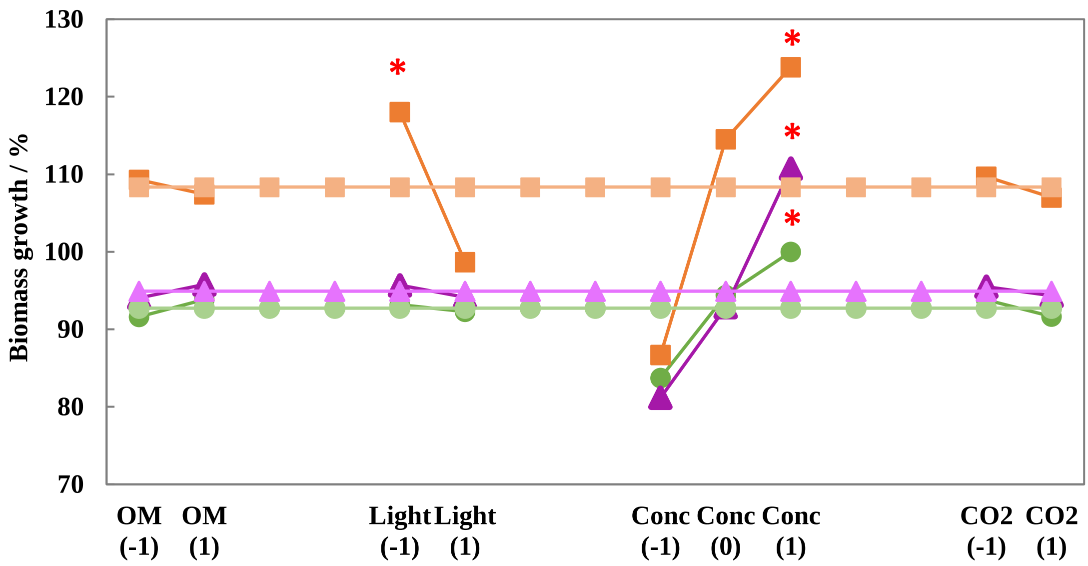

Figure 4.1. Means plot for biomass growth (—●—) *S. almeriensis*, (—■—) S. almeriensis-bacteria consortia, (—▲—) Activated sludge and (—●—) for *S. almeriensis* grand mean, (—■—) *S. almeriensis-bacteria* grand mean, and (—▲—) activated sludge grand mean. Red Asterisks show significant differences found (5% significance level).

Greater factors of growth are obtained with the presence of ambient light at cycles of 12:12h, Another clear tendency to remark is that higher concentrations of heavy metal lead to higher growth, which again shows that the greater concentrations of Cu(II) and Zn(II) do not suppose a toxic effect in the metabolism of these microorganisms producing their death but the growth.

As it can see in the previous **Figure 4.1**, the microalgae-bacteria consortium is the case for which the greatest growth factors are achieved, and therefore symbiotic and synergetic interactions must be crucial for this type of biomass. It is followed by bacteria and pure microalgae strain, which remain at average values of cell death (<100%) for low metal concentration. In the same way, attending to the significant factors for the process, the light is defined as a vital factor for the growth of the consortium, since with the use of the ambient light in cycles of 12:12h, growth factors of almost 120% are obtained, on the other hand, with the use of the artificial LED light also during cycles of 12:12h, On the other hand, it was found hardly any death but also no increase in the amount of biomass, as higher concentrations of Cu(II) and Zn(II) were reached, higher growth factors were achieved, which are proving not to affect negatively the metabolism of the microorganisms, at least with these types of biomass tested, as no toxic effect was observed, growth factors acquire values of almost 130% for the consortium and 110% for activated sludge.

4.3.2 Multimetallic biosorption experiments

In a first approach from the ANOVA analysis, factors that showed a significant effect (p-value < 0.05) were the biomass (0.0000), the initial metal concentration (0.0000), and the contact time for Cu (II) (0.0247) and for Zn (II) (0.0040) (Data not shown). Thus, from the results obtained, the influence of the type of biomass used, the initial concentration added, and the contact time is clear. In terms of biosorption capacities, for Cu (II), the maximum values found were; 104.52 mg/g, 81.50 mg/g, and 67.71 mg/g, for pure strain culture, consortium, and sludges respectively at an initial concentration of 100 mg/L and an initial pH of 7. For Zn (II) maximum biosorption capacities of 121.65 mg/g, 96.71 mg/g, and 73.52 mg/g at an initial concentration of 100 mg/L at an initial pH of 7. Since the level of concentration is a very influential factor, in a more exhaustive analysis, 6 sub-designs were implemented, that is, one for each level of initial concentration and metal, to be able to interpret the meaning of these effects with the help of means plots, and thus be able to discern statistically the most favorable conditions for the bioelimination of these metalloids in wastewater.

4.3.2.1 Low-level concentration

As mentioned above, mean plots of the 6 sub designs will address the direction of the effects to be studied and its significance. Results for low level of initial metal concentration are shown in **Figure 4.2.**

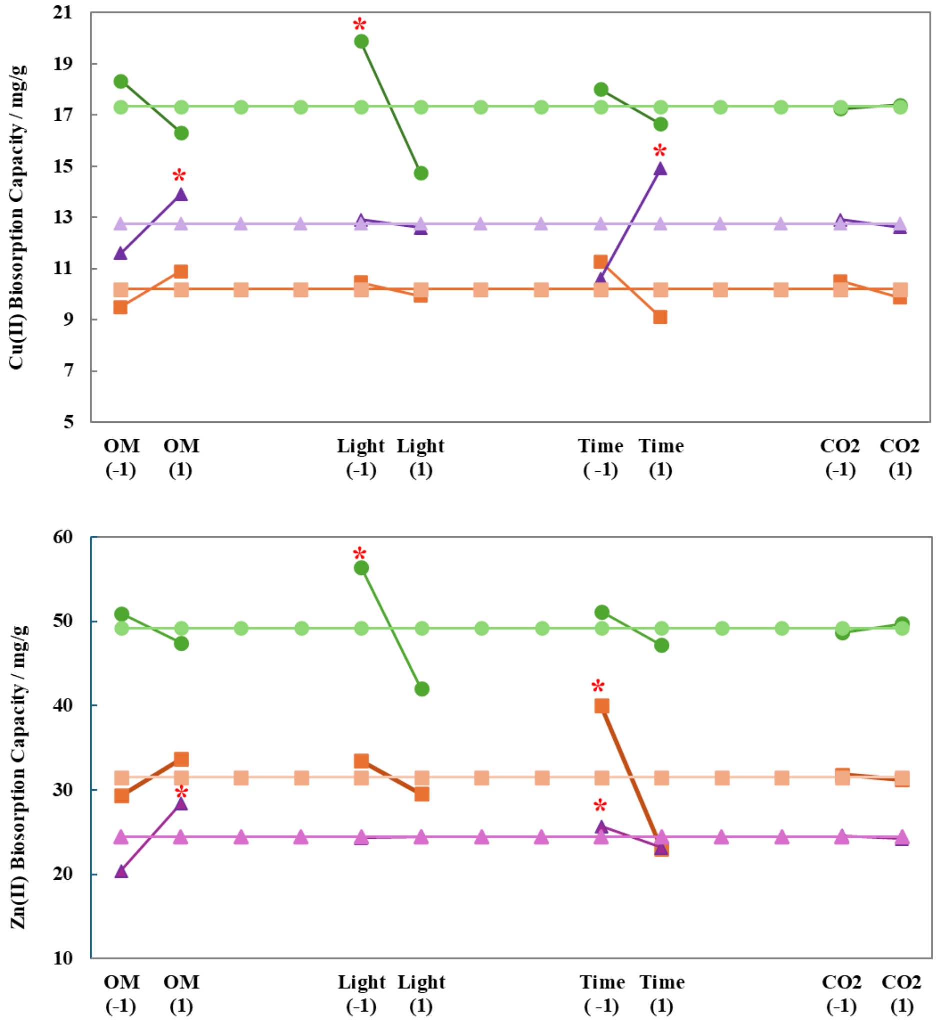

Figure 4.2. Means plot for biosorption capacity results for Cu and Zn al low level: **MMS$_1$** (Cu: 15 mg/L, Zn: 40 mg/L), (─●─) *S. almeriensis*, (─■─) S. almeriensis-bacteria consortia, (─▲─) Activated sludge and (─●─) for *S. almeriensis* grand mean, (─■─) *S. almeriensis-bacteria* grand mean, and (─▲─) activated sludge grand mean. Asterisks show significant differences found (5% significance level).

As can be seen in the **Figure 4.2**, for Cu (II), the key factor, in this case, is the presence of ambient light at 12:12h light-dark cycles, with which greater biosorption capacities are achieved. This may be an explanained by the fact that at low metal concentrations, the metabolism of the microalgae would be mainly driven by photosynthetic functions, with the milder ambient light conditions being more favorable for growth and therefore metal assimilation capacity. On the contrary, an effect in the opposite direction is evident in the case of the consortium, in which intense light conditions resulted in higher retention capacities matching with the conditions of lower growth of this type of biomass. Meanwhile, bacteria remain practically unaffected by these factors, including light intensity, as they are not photosynthetic microorganisms. The pure strain of microalgae presented higher values of biosorption capacities, while the grand mean of the other two types of biomass is significantly lower. For Zn (II), the light intensity remains as a key factor for the biosorption of metals, especially for microalgae. In contrast, it seems that contact time is also a key factor for the consortium and the bacteria, so shorter contact times and more specifically, those tested at 1 hour, promote higher capacities.

4.3.2.2 Medium level concentration

For experiments at medium concentration, 60 mg/L for Cu (II) and 70 mg/L for Zn (II), the statistically most significant factors become the contact time, obtaining greater retention capacities at shorter contact times, in this case, at 1 hour. This is even more significant in the case of microalgae. On the other hand, the biosorption of these biomasses decrease in the same order as that observed for the experiments at low concentration; first, the microalgae, followed by the consortium and finally the bacteria (**Figure 4.3**).

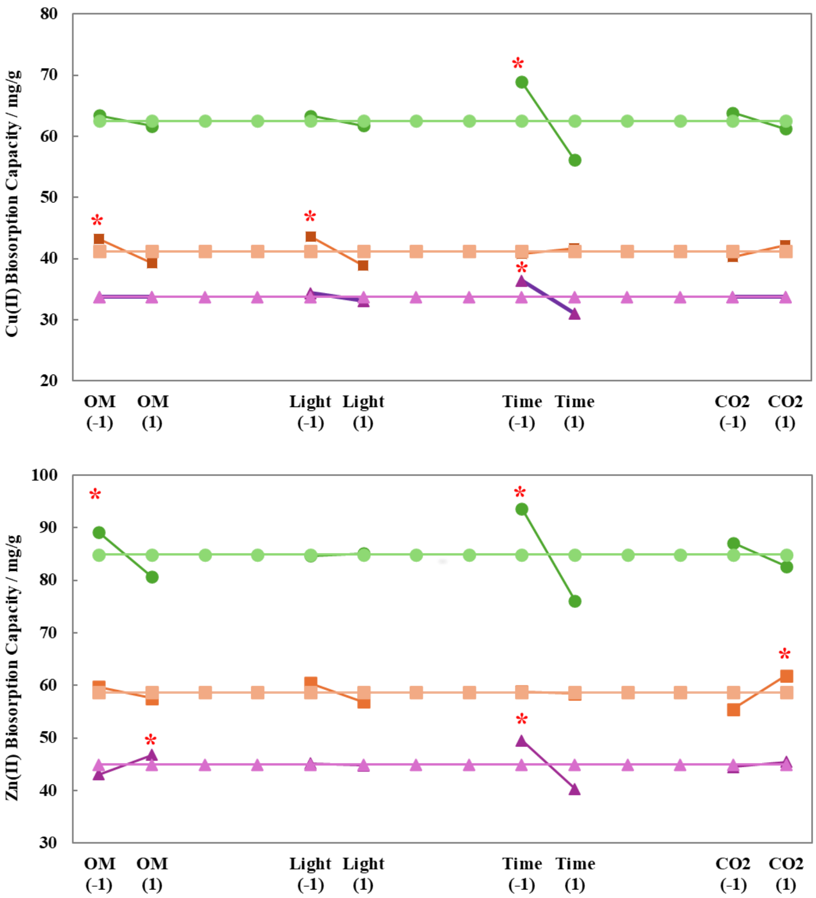

Figure 4.3. Means plot for biosorption capacity results for Cu and Zn al medium level: **MMS$_2$** (Cu: 60 mg/L, Zn: 70 mg/L). (–●–) *S. almeriensis*, (–■–) S. almeriensis-bacteria consortia, (–▲–) Activated sludge and (–●–) for *S. almeriensis* grand mean, (–■–) *S. almeriensis-bacteria* grand mean, and (–▲–) activated sludge grand mean. Asterisks show significant differences found (5% significance level).

In the case of Zn (II), a new significant factor appears, and this is the presence of OM in the medium. For example, the presence of OM lowers the retention capacity of the pure microalgae. This phenomenon could be explained by the interactions between the metal ion and the organic matter (Saavedra et al., 2019).

4.3.2.3 High-level concentration

For Cu at high concentrations of 100 mg/L, can be observed that contact time once again becomes the dominant effect, to the detriment of the biosorption capacities of the microalgae and bacteria at contact times of 72 hours, while, for the consortium, the opposite behaviour is showed: greater capacities for the longer contact times are achieved. It is also noteworthy that there is an effect not detected until now: the increase in the capacity of the pure microalgae strains in the presence of organic matter (**Figure 4.4**). Finally, for Zn(II), certain effects are not observed. The trend observed so far in the behaviour of higher capacities is fully maintained.

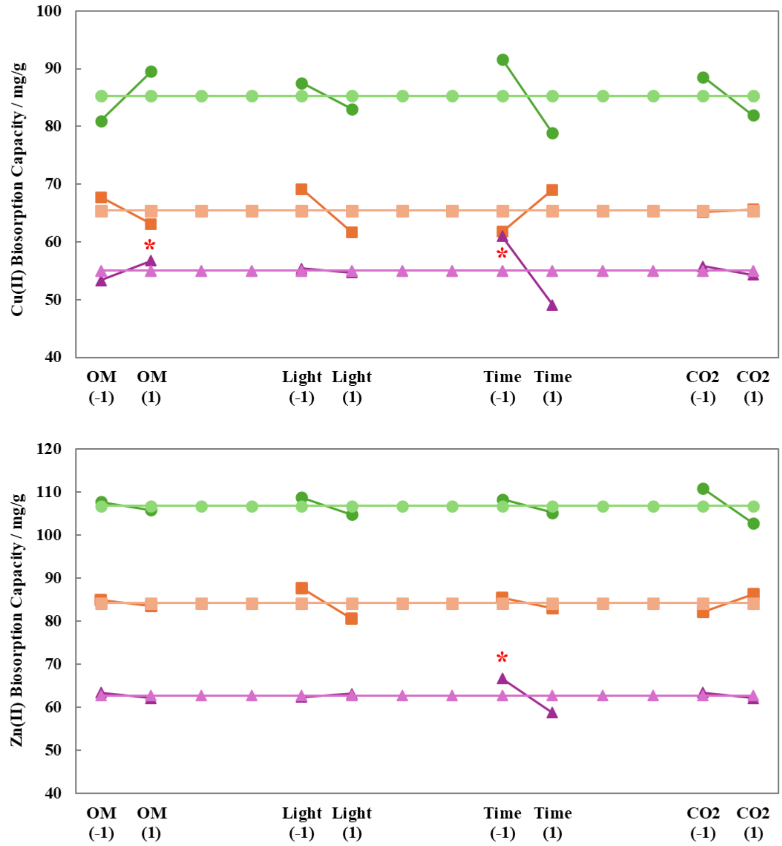

Figure 4.4. Means plot for biosorption capacity results for Cu and Zn al medium level: **MMS$_3$** (Cu: 100 mg/L, Zn: 100 mg/L). (—●—) *S. almeriensis*, (—■—) S. almeriensis-bacteria consortia, (—▲—) Activated sludge and (—●—) for *S. almeriensis* grand mean, (—■—) *S. almeriensis-bacteria* grand mean, and (—▲—) activated sludge grand mean. Asterisks show significant differences found (5% significance level).

Regarding organic matter content**,** Saavedra et al., (2019b*)*, evaluated the influence of organic matter (OM) and CO_2 addition on the bioremediation potential of two microalgae typically used for wastewater treatment: *Chlorella vulgaris* and *Scenedesmus almeriensis*, pointing out a decrease in the biosorption capacity of these biomasses as the concentration of organic matter in the medium increases. The presence of OM decreased the total biosorption capacity, especially in As (from 2.2 to 0.0 mg/g for *C. vulgaris* and from 2.3 to 1.7 mg/g for *S. almeriensis*) and Cu (from 3.2 to 2.3 mg/g for *C. vulgaris* and from 2.1 to 1.6 mg/g for *S. almeriensis*) and Zn (from 2.7 to 2.3 mg/g for *C. vulgaris* and by contrast from 2.0 to 2.4 mg/g for *S. almeriensis*)

Regarding initial concentration, Pourang and Rezaei, (2021), reported a positive significant effect observed between the initial concentration and copper biosorption. This can be justified because of initial concentration act as the necessary driving force to dominate the mass transfer resistance of metal ions between the aqueous phase and the biosorbent surface, hence a higher initial concentration of metal ion may lead to an increase in metal ion uptake. Also, an increasing of the initial ions concentration may also lead to a more intensive interaction between the adsorbates and adsorbents, hence enhance the availability of the adsorptive sites on the surface of the sorbents. Regarding contact time, also a positive significant effect was found in Cu biosorption, although, values of q_{bio} for different contact times are not shown in that study. It was also reported that the longer contact time led to a higher copper removal from aqueous environment. The plausible explanation of this is that at the beginning of the adsorption process, many vacant adsorption sites on the biosorbent surface were available for copper binding. In the studies in which the range of contact time was wider compared to that of current research, over time, as the binding sites were occupied, the rate of metal ion biosorption decreased to saturation.

Zhou et al., (2012) after 8 days of culturing, the algae *C. pyrenoidosa* and *S. obliquus* removed 72.8–95.6% and 72.8–99.7% of zinc, respectively. The copper removal efficiency by *C. pyrenoidosa* increased gradually during the first four days and thereafter remained nearly constant throughout the experiment. However, the copper removal efficiency by *S. obliquus* increased gradually during the whole experimental period. After 8 days of culturing, the algae *C. pyrenoidosa* and *S. obliquus* removed 79.3–90.9% and 75.9–91.4% of copper, respectively.

The high and easy removal of heavy metals found in this work, working with intermediate-high concentrations of Cu and Zn, enables the application of wastewater treatments based on microalgae as a promising technology for the treatment wastewaters contaminated with heavy metals of different types possible to a great extent. Furthermore, these Cu and Zn concentrations were used in other studies of the research group, allowing comparison of results (Antolín et al., 2024; Collao et al., 2022).

4.3.3 Adsorption Isotherms. Langmuir and Freundlich model.

Results from Langmuir and Freundlich adjustment are shown in **Table 4.3**. Comparing the calculated q_{max} with the $q_{biosorption}$ from the obtained experimental data, it is obtained that in major cases $q_{max} < q_{biosorption}$, so it is quite probable that other multiple mechanisms may be involved in the process of Cu(II) and Zn(II) biosorption by these 3 types of biosorbents, among them microprecipitation or bioaccumulation, In this case, it would be necessary to perform speciation of the metals involved in the metal uptake process to get to know how the process has been carried out. The following **Table 4.3** shows the main parameters of the linear adjustments made. Higher values of q_{sat} are obtained for the case of Cu (II) in the majority of cases. The highest q_{max} values are obtained for Cu(II) vary from 147.06 to 227.27 mg/g and for Zn(II) from 75.76 to 108.70 mg/g. The maximum uptakes for Cu(II) were found in the consortium and for Zn(II) in *Scenedesmus almeriensis*. In all cases it is observed that q_{max}(Cu)> q_{max}(Zn)

Table 4.3. Compilation of Langmuir and Freundlich isotherms parameters and regression parameters for Cu(II) and Zn(II)

	Metal ion	Regression parameters			Isotherm Parameters	
Langmuir approach		Slope	Intercept	R^2	q_{max} (mg/g)	K (L/mg)
Scenedesmus almeriensis	*Cu (II)*	0.0056	0.4411	0.9462	178.57	0.01
	Zn (II)	0.0096	0.2582	0.9878	104.17	0.04
Activated sludge	*Cu (II)*	0,0059	0,4713	0,9426	169.49	0.01
	Zn (II)	0,0128	0,2383	0,9900	78.13	0.05
Microalgae-bacteria consortium	*Cu (II)*	0.9756	0.9788	0.9944	227.27	0.001
	Zn (II)	0.009	0.9663	0.9462	75.76	0.01
Freundlich approach						
	Metal ion	Regression parameters			Isotherm Parameters	
		Slope	Intercept	R^2	*kf*	*n*
Scenedesmus almeriensis	*Cu (II)*	0,6656	0,6662	0,9897	4,64	1,502
	Zn (II)	0,6395	0,6936	0,9432	4,94	1,564
Activated sludge	*Cu (II)*	0,5822	0,7700	0,9860	5,89	1,718
	Zn (II)	0,4066	0,9834	0,9856	9,62	2,459
Microalgae-bacteria consortium	*Cu (II)*	0,4303	0,7604	0,9990	5,76	2,324
	Zn (II)	1,0880	-0,9247	0,9691	0,12	0,919

For the linear adjustments of both metals in the pure microalgae, good adjustment values have been obtained. The linear trend of these isotherms is remarkable, which means that a great contribution to the uptake process of metals comes from adsorption phenomena. In the same way, it is remarkable the existence of a better linearization of the isotherm for activated sludge, existing a better adaptation to it for Zn(II). Finally, for Freundlich isotherm, good adjustments have also been collected. In the study of Yang et al., (2015) adsorption isotherms with microalgae *Chlorella minutissima* were performed, for Langmuir isotherm, values of 16.16 and 123.46 mg/g were achieved for maximum biosorption capacities and 0.25 and 0.03 L mg^{-1} for K_l for Cu(II) and Zn(II) respectively. Regarding Freundlich adjustment, values of 4.08 and 3.55 were achieved for K_f, while the values of n, were 0.5 and 1.14 for Cu(II) and Zn(II), being a non-favorable process for Cu. On the other hand, the maximum adsorption capacities of Zn are similar to those obtained in this study, while those of Cu are much higher in the microalga S. *almeriensis* in the present study. On the other hand, different maximum biosorption capacities of Cu and Zn have been found in other species. 26.6 and 37.45 mg/g on *Saccharomyces cerevisiae* for Cu and Zn respectively, with n values greater than 1, showing the feasibility of the process at pH 5, 4 g/L sorbent dosage, 23 ºC and contact time of 60 minutes (Savastru et al., 2022). *Jayakumar et al., (2021)* with *Caulerpa scalpelliformis*, at a pH 5.7, sorbent dosage 1.5 g/L, agitation speed 150 rpm and contact time of 60 minutes, a maximum biosorption capacity of 83.33 mg/g was obtained. *Gu et al., (2021)* for its part, in single-metal-ion aqueous solution of 50 ppm Cu and Zn ions with 0.3 g /L of *N. oleoabundans* biomass at pH 7.0, 25 °C and 30 min of contact time, obtained q_m of 78 and 78.5 mg/g for Cu and Zn respectively. *Pourang and Rezaei, (2021)* performed adsorption isotherm studies were carried out using five different initial metal ion concentrations between 30–170 mg/L, obtaining q_m 69.93 and 156.25 mg/g of for Cu adsorption in *Rutilus kutum and Cerastoderma glaucum,* being values similar to those achieved in the present work. In general, in this study, q_m higher than those found in the literature were found, which positions microalga *S. almeriensis* as a robust alga to metal treatment, capable of assimilating a large amount of these pollutants.

4.4 Conclusions and future perspectives

S. almeriensis resulted in better performance than activated sludge in the bioelimination of metals from synthetic wastewater prepared for the experiments. Hence, microalgae are more contributive to the biosorption capacity of metals in the context of actual wastewater. In addition, together with the growth study in the 72h batch experiments, it can be stated that a greater biomass growth is achieved when microalgae and bacteria are in the consortium and in high heavy metal concentration conditions. Besides, a study of isotherms determination has been made. Future research needs to consider the use of real pollutant water and scale it to outdoor cultivation for determining the feasibility and operational cost of microalgae and bacteria-based technologies, as well as to determine the different retention mechanisms of these metals by the biomass and assessing the feasibility of biomass treatment with mild solvents in order to enable heavy metal lixiviation for further valorization processes of the biomass.

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Effect of operational conditions in Cu and Zn bioelimination by *Scenedesmus almeriensis* and *activated sludge* in photobioreactor systems

Supplementary material

Table S4.1. Matrix of biosorption capacities results for Cu(II) and Zn(II) in *S. almeriensis* for the different combination factors (A1 to A48)

	S. almeriensis		*S. almeriensis-bacteria consortia*		*Activated sludge*	
Run	Cu q_{bio}	Zn q_{bio}	Cu q_{bio}	Zn q_{bio}	Cu q_{bio}	Zn q_{bio}
A1	18.60	52.71	10.82	33.38	11.02	24.67
A2	69.53	99.65	42.85	56.31	39.62	49.08
A3	86.83	103.29	72.15	95.14	59.97	69.24
A4	24.61	67.01	13.75	52.67	11.86	25.21
A5	55.36	79.65	46.90	65.71	39.71	49.74
A6	90.58	104.21	52.54	70.39	60.37	68.27
A7	19.02	52.57	8.18	24.87	11.38	24.32
A8	65.44	98.68	36.08	47.00	38.12	47.57
A9	94.39	113.65	57.64	68.80	57.77	67.28
A10	18.25	49.65	9.45	33.37	10.36	23.90
A11	68.84	97.31	37.46	51.29	37.76	47.58
A12	93.88	110.31	72.81	96.45	58.56	68.43
A13	16.52	46.16	13.95	49.93	9.88	26.19
A14	79.70	102.11	41.67	58.19	34.22	50.32
A15	94.22	110.89	68.79	94.80	62.07	60.87
A16	16.91	51.69	10.49	39.50	10.22	27.05
A17	78.32	99.24	43.22	69.84	35.77	52.14
A18	96.89	113.22	59.10	89.07	61.80	61.27
A19	13.06	39.19	13.15	49.40	10.42	27.95
A20	65.05	84.06	37.83	53.22	35.66	53.73
A21	90.44	108.19	51.23	71.84	67.71	73.52
A22	17.07	50.35	10.45	37.11	9.62	26.11
A23	68.91	88.61	40.63	69.06	30.31	46.19
A24	86.27	102.46	60.00	96.71	59.92	64.99
A25	21.27	60.92	9.23	26.13	12.52	16.52
A26	60.21	83.90	46.34	60.01	27.51	36.39
A27	71.55	112.14	71.52	85.35	47.79	58.49
A28	17.96	53.57	5.10	14.45	12.40	16.42
A29	55.67	77.41	48.03	65.58	30.04	38.78
A30	67.09	102.67	81.50	96.33	48.00	60.94
A31	13.95	34.77	10.02	26.10	11.59	15.73
A32	68.90	91.16	44.44	66.23	29.65	38.92
A33	67.66	101.28	70.34	87.03	47.51	57.47
A34	13.03	36.19	9.41	23.59	11.76	16.58
A35	63.09	85.09	43.30	65.69	27.40	36.55
A36	76.16	113.80	63.27	79.45	46.87	57.17
A37	23.73	66.23	10.16	25.07	18.11	29.70
A38	60.84	75.90	38.73	52.08	32.40	39.06
A39	99.18	115.47	72.24	84.34	53.74	62.03
A40	19.56	52.57	10.15	26.58	17.30	28.83
A41	46.94	59.50	40.74	55.74	35.65	45.51
A42	94.09	108.15	75.32	86.47	49.40	57.90
A43	11.83	36.63	8.59	19.51	18.31	31.63
A44	40.96	61.38	33.81	50.49	32.49	40.64
A45	104.52	121.65	57.56	69.81	49.77	58.37
A46	11.85	36.71	10.17	22.45	17.39	29.74
A47	52.81	74.47	37.54	51.94	33.07	46.72
A48	50.88	66.58	60.58	75.34	49.61	57.99

Table S4.2. ANOVA of the results of Cu and Zn biosorption capacity in Scenedesmus almeriensis in medium concentration experiments

	Factor	Sum Of Squares	DoF	Mean Square	F	p-value
Cu	A:CO_2	0.099225	1	0.099225	0.02	0.8958
	B:Light	105.576	1	105.576	20.18	0.0064
	C:OM	16.3216	1	16.3216	3.12	0.1376
	D:Contact time	7.37123	1	7.37123	1.41	0.2885
	AB	0.731025	1	0.731025	0.14	0.7239
	AC	0.0361	1	0.0361	0.01	0.9370
	AD	20.295	1	20.295	3.88	0.1060
	BC	1.3924	1	1.3924	0.27	0.6279
	BD	31.979	1	31.979	6.11	0.0564
	CD	19.5364	1	19.5364	3.73	0.1111
	Total error	26.1571	5	5.23143		
	Total (corr.)	229.495	15			
Zn	A:CO_2	4.5796	1	4.5796	0.14	0.7201
	B:Light	823.69	1	823.69	25.86	0.0038
	C:OM	48.5112	1	48.5112	1.52	0.2720
	D:Contact time	62.9642	1	62.9642	1.98	0.2187
	AB	7.4529	1	7.4529	0.23	0.6490
	AC	0.342225	1	0.342225	0.01	0.9215
	AD	141.491	1	141.491	4.44	0.0889
	BC	3.29423	1	3.29423	0.10	0.7608
	BD	249.482	1	249.482	7.83	0.0381
	CD	106.296	1	106.296	3.34	0.1273
	Total error	159.255	5	31.8509		
	Total (corr.)	1607.36	15			

Table S4.3. ANOVA of the results of Cu and Zn biosorption capacity in *Scenedesmus almeriensis* in medium concentration experiments

	Factor	Sum Of Squares	DoF	Mean Square	F	p-value
Cu	A:CO_2	26.7548	1	26.7548	1.19	0.3256
	B:Light	9.87531	1	9.87531	0.44	0.5372
	C:OM	11.4075	1	11.4075	0.51	0.5086
	D:Contact time	646.812	1	646.812	28.71	0.0030
	AB	139.772	1	139.772	6.20	0.0551
	AC	29.0252	1	29.0252	1.29	0.3079
	AD	1.05576	1	1.05576	0.05	0.8372
	BC	252.572	1	252.572	11.21	0.0204
	BD	17.5771	1	17.5771	0.78	0.4176
	CD	391.347	1	391.347	17.37	0.0088
	Total error	112.662	5	22.5325		
	Total (corr.)	1638.86	15			
Zn	A:CO_2	79.0321	1	79.0321	2.04	0.2126
	B:Light	0.7225	1	0.7225	0.02	0.8967
	C:OM	285.441	1	285.441	7.37	0.0421
	D:Contact time	1233.77	1	1233.77	31.84	0.0024
	AB	195.72	1	195.72	5.05	0.0745
	AC	65.2056	1	65.2056	1.68	0.2512
	AD	0.912025	1	0.912025	0.02	0.8841
	BC	223.951	1	223.951	5.78	0.0613
	BD	46.854	1	46.854	1.21	0.3216
	CD	264.388	1	264.388	6.82	0.0476
	Total error	193.773	5	38.7547		
	Total (corr.)	2589.77	15			

Table S4.4. ANOVA of the results of Cu and Zn biosorption capacity in *Scenedesmus almeriensis* in high concentration experiments

	Factor	Sum Of Squares	DoF	Mean Square	F	p-value
Cu	A:CO_2	175.231	1	175.231	1.18	0.3266
	B:Light	82.0383	1	82.0383	0.55	0.4905
	C:OM	291.983	1	291.983	1.97	0.2195
	D:Contact time	654.976	1	654.976	4.42	0.0896
	AB	136.247	1	136.247	0.92	0.3818
	AC	284.85	1	284.85	1.92	0.2244
	AD	199.022	1	199.022	1.34	0.2990
	BC	291.641	1	291.641	1.97	0.2197
	BD	53.1077	1	53.1077	0.36	0.5756
	CD	256.56	1	256.56	1.73	0.2455
	Total error	741.5	5	148.3		
	Total (corr.)	3167.16	15			
Zn	A:CO_2	265.364	1	265.364	1.50	0.2748
	B:Light	64.4809	1	64.4809	0.37	0.5720
	C:OM	13.5792	1	13.5792	0.08	0.7926
	D:Contact time	37.4544	1	37.4544	0.21	0.6644
	AB	90.6304	1	90.6304	0.51	0.5058
	AC	275.726	1	275.726	1.56	0.2667
	AD	179.024	1	179.024	1.01	0.3602
	BC	268.796	1	268.796	1.52	0.2721
	BD	90.8209	1	90.8209	0.51	0.5053
	CD	28.4622	1	28.4622	0.16	0.7046
	Total error	882.806	5	176.561		
	Total (corr.)	2197.14	15			

Table S4.5. ANOVA of the results of Cu and Zn biosorption capacity in *Scenedesmus almeriensis*-bacteria consortia in low concentration experiments

	Factor	Sum Of Squares	DoF	Mean Square	F	p-value
Cu	A:CO_2	1.64481	1	1.64481	0.27	0.6284
	B:Light	1.11831	1	1.11831	0.18	0.6886
	C:OM	7.77016	1	7.77016	1.25	0.3137
	D:Contact time	18.9443	1	18.9443	3.06	0.1408
	AB	1.10776	1	1.10776	0.18	0.6900
	AC	1.02516	1	1.02516	0.17	0.7010
	AD	0.0915062	1	0.0915062	0.01	0.9080
	BC	0.0189063	1	0.0189063	0.00	0.9581
	BD	8.02306	1	8.02306	1.29	0.3067
	CD	0.0175562	1	0.0175562	0.00	0.9596
	Total error	30.9834	5	6.19668		
	Total (corr.)	70.7448	15			
Zn	A:CO_2	1.36306	1	1.36306	0.01	0.9088
	B:Light	61.2698	1	61.2698	0.65	0.4559
	C:OM	76.5188	1	76.5188	0.82	0.4080
	D:Contact time	1161.96	1	1161.96	12.38	0.0170
	AB	0.262656	1	0.262656	0.00	0.9599
	AC	63.4811	1	63.4811	0.68	0.4483
	AD	13.7085	1	13.7085	0.15	0.7181
	BC	2.31801	1	2.31801	0.02	0.8813
	BD	56.8139	1	56.8139	0.61	0.4718
	CD	50.091	1	50.091	0.53	0.4979
	Total error	469.4	5	93.8801		
	Total (corr.)	1957.18	15			

Table S4.6. ANOVA of the results of Cu and Zn biosorption capacity in *Scenedesmus almeriensis*-bacteria consortia in medium concentration experiments

	Factor	Sum Of Squares	DoF	Mean Square	F	p-value
Cu	A:CO_2	16.1403	1	16.1403	5.41	0.0675
	B:Light	87.3758	1	87.3758	29.30	0.0029
	C:OM	60.9571	1	60.9571	20.44	0.0063
	D:Contact time	2.47276	1	2.47276	0.83	0.4043
	AB	0.400056	1	0.400056	0.13	0.7291
	AC	1.05576	1	1.05576	0.35	0.5777
	AD	0.761256	1	0.761256	0.26	0.6349
	BC	4.29526	1	4.29526	1.44	0.2838
	BD	3.89076	1	3.89076	1.30	0.3051
	CD	61.4264	1	61.4264	20.60	0.0062
	Total error	14.91	5	2.982		
	Total (corr.)	253.685	15			
Zn	A:CO_2	164.609	1	164.609	9.91	0.0254
	B:Light	50.9082	1	50.9082	3.07	0.1404
	C:OM	18.6192	1	18.6192	1.12	0.3381
	D:Contact time	0.511225	1	0.511225	0.03	0.8676
	AB	5.3361	1	5.3361	0.32	0.5953
	AC	12.0409	1	12.0409	0.73	0.4334
	AD	60.2176	1	60.2176	3.63	0.1152
	BC	2.44922	1	2.44922	0.15	0.7167
	BD	57.836	1	57.836	3.48	0.1210
	CD	373.069	1	373.069	22.47	0.0052
	Total error	83.0305	5	16.6061		
	Total (corr.)	828.627	15			

Table S4.7. ANOVA of the results of Cu and Zn biosorption capacity in *Scenedesmus almeriensis*-bacteria consortia in high concentration experiments

	Factor	Sum Of Squares	DoF	Mean Square	F	p-value
Cu	A:CO_2	0.832656	1	0.832656	0.01	0.9216
	B:Light	222.98	1	222.98	2.87	0.1510
	C:OM	85.3314	1	85.3314	1.10	0.3426
	D:Contact time	210.758	1	210.758	2.71	0.1604
	AB	81.5861	1	81.5861	1.05	0.3524
	AC	2.81401	1	2.81401	0.04	0.8565
	AD	12.9061	1	12.9061	0.17	0.7004
	BC	65.7316	1	65.7316	0.85	0.3998
	BD	89.9178	1	89.9178	1.16	0.3311
	CD	1.50676	1	1.50676	0.02	0.8947
	Total error	388.328	5	77.6656		
	Total (corr.)	1162.69	15			
Zn	A:CO_2	68.4756	1	68.4756	0.47	0.5245
	B:Light	199.233	1	199.233	1.36	0.2960
	C:OM	6.9696	1	6.9696	0.05	0.8359
	D:Contact time	22.7529	1	22.7529	0.16	0.7097
	AB	287.642	1	287.642	1.96	0.2200
	AC	26.2656	1	26.2656	0.18	0.6895
	AD	7.53503	1	7.53503	0.05	0.8295
	BC	40.6406	1	40.6406	0.28	0.6208
	BD	39.8792	1	39.8792	0.27	0.6241
	CD	181.172	1	181.172	1.24	0.3166
	Total error	732.178	5	146.436		
	Total (corr.)	1612.74	15			

Table S4.8. ANOVA of the results of Cu and Zn biosorption capacity in activated sludge in low concentration experiments

	Factor	Sum Of Squares	DoF	Mean Square	F	p-value
Cu	A:CO_2	0.3364	1	0.3364	1.88	0.2289
	B:Light	0.3844	1	0.3844	2.15	0.2028
	C:OM	21.0681	1	21.0681	117.64	0.0001
	D:Contact time	74.909	1	74.909	418.26	0.0000
	AB	0.497025	1	0.497025	2.78	0.1566
	AC	0.265225	1	0.265225	1.48	0.2779
	AD	0.0676	1	0.0676	0.38	0.5658
	BC	0.540225	1	0.540225	3.02	0.1429
	BD	0.0004	1	0.0004	0.00	0.9641
	CD	46.6489	1	46.6489	260.47	0.0000
	Total error	0.895475	5	0.179095		
	Total (corr.)	145.613	15			
Zn	A:CO_2	0.514806	1	0.514806	1.23	0.3179
	B:Light	0.117306	1	0.117306	0.28	0.6192
	C:OM	254.801	1	254.801	608.60	0.0000
	D:Contact time	25.6289	1	25.6289	61.22	0.0005
	AB	0.869556	1	0.869556	2.08	0.2091
	AC	1.32826	1	1.32826	3.17	0.1350
	AD	0.0826562	1	0.0826562	0.20	0.6754
	BC	2.21266	1	2.21266	5.29	0.0699
	BD	0.581406	1	0.581406	1.39	0.2916
	CD	129.106	1	129.106	308.38	0.0000
	Total error	2.09333	5	0.418666		
	Total (corr.)	417.337	15			

Table S4.9. ANOVA of the results of Cu and Zn biosorption capacity in activated sludge in medium concentration experiments

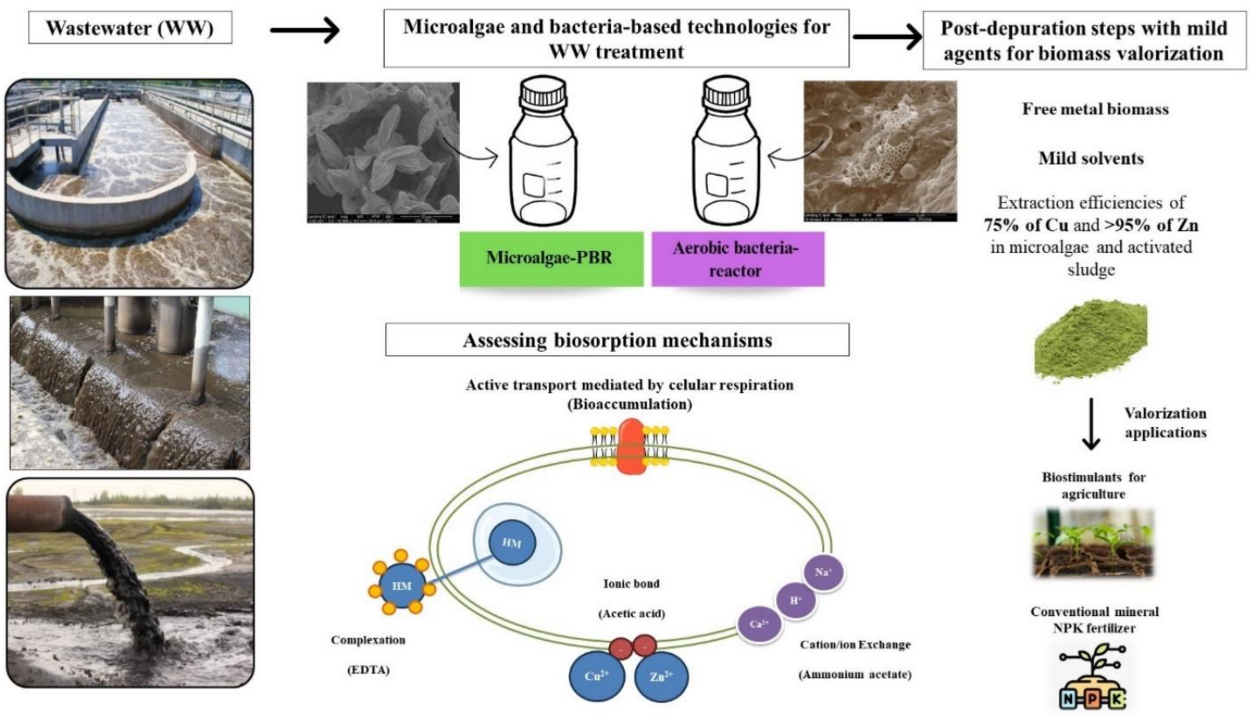
	Factor	Sum Of Squares	DoF	Mean Square	F	p-value
Cu	A:CO_2	0.0001	1	0.0001	0.00	0.9943
	B:Light	6.83823	1	6.83823	3.79	0.1090
	C:OM	0.0036	1	0.0036	0.00	0.9661
	D:Contact time	115.348	1	115.348	63.97	0.0005
	AB	13.69	1	13.69	7.59	0.0401
	AC	0.000025	1	0.000025	0.00	0.9972
	AD	4.18202	1	4.18202	2.32	0.1883
	BC	0.4096	1	0.4096	0.23	0.6538
	BD	1.2544	1	1.2544	0.70	0.4423
	CD	91.4892	1	91.4892	50.74	0.0008
	Total error	9.01638	5	1.80328		
	Total (corr.)	242.231	15			
Zn	A:CO_2	3.51562	1	3.51562	0.49	0.5149
	B:Light	0.6084	1	0.6084	0.08	0.7824
	C:OM	55.1306	1	55.1306	7.70	0.0392
	D:Contact time	340.218	1	340.218	47.49	0.0010
	AB	14.3262	1	14.3262	2.00	0.2165
	AC	2.3409	1	2.3409	0.33	0.5923
	AD	19.36	1	19.36	2.70	0.1611
	BC	0.819025	1	0.819025	0.11	0.7490
	BD	5.40563	1	5.40563	0.75	0.4248
	CD	10.3684	1	10.3684	1.45	0.2828
	Total error	35.8216	5	7.16433		
	Total (corr.)	487.914	15			

Table S4.10. ANOVA of the results of Cu and Zn biosorption capacity in activated sludge in high concentration experiments

	Factor	Sum Of Squares	DoF	Mean Square	F	p-value
Cu	A:CO_2	8.7025	1	8.7025	1.68	0.2515
	B:Light	1.83602	1	1.83602	0.35	0.5775
	C:OM	46.172	1	46.172	8.91	0.0306
	D:Contact time	569.777	1	569.777	110.00	0.0001
	AB	0.9025	1	0.9025	0.17	0.6937
	AC	11.0889	1	11.0889	2.14	0.2033
	AD	0.235225	1	0.235225	0.05	0.8397
	BC	1.83602	1	1.83602	0.35	0.5775
	BD	1.5129	1	1.5129	0.29	0.6121
	CD	0.3844	1	0.3844	0.07	0.7962
	Total error	25.8998	5	5.17996		
	Total (corr.)	668.347	15			
Zn	A:CO_2	6.64351	1	6.64351	0.78	0.4170
	B:Light	2.41026	1	2.41026	0.28	0.6171
	C:OM	6.69516	1	6.69516	0.79	0.4154
	D:Contact time	252.095	1	252.095	29.67	0.0028
	AB	2.10976	1	2.10976	0.25	0.6394
	AC	14.0063	1	14.0063	1.65	0.2554
	AD	1.95301	1	1.95301	0.23	0.6519
	BC	23.4983	1	23.4983	2.77	0.1572
	BD	32.8616	1	32.8616	3.87	0.1064
	CD	13.6715	1	13.6715	1.61	0.2605
	Total error	42.4829	5	8.49658		
	Total (corr.)	398.427	15			

Chapter 5:

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



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

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 their 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, *S. 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 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 oxidisable 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.

Keywords: *Bioremediation, heavy metals, activated sludge, Scenedesmus almeriensis, metal fractionation, metal availability.*

5.1 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.

5.2 Materials and methods

5.2.1 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 ºC under continuous agitation, applying an LED irradiance of 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a 12-hour 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 4ºC 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.

5.2.2

5.2.2 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 be 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 ºC under continuous stirring at 250 rpm in a multi-point magnetic stirrer, irradiated with 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ using LED lamps arranged on top working in 12-hour 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 ºC 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:

$$\% \, RE_m = \frac{q_m \cdot C_{b-72}}{C_{m-0}} \times 100 \qquad (5.1)$$

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

5.2.3 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.

$$Metal\ extraction\ (\%) = \frac{C_{m,e} \cdot V_e}{q_m \cdot m_b} \times 100 \tag{5.2}$$

Where V_e is the volume of extractant (L), and $C_{m,e}$ 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.

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 (**See Table S5.1**). 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.

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 4 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 S5.2**). 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 4ºC 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.

5.2.4 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 ºC 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 72h was calculated as:

$$\text{Biomass growth ratio} = \frac{C_{b-72}}{C_{\mathrm{b}-0}} \tag{5.3}$$

where C_{b-72} ($g \cdot L^{-1}$) is the concentration of dry biomass in the photobioreactor after 72h of contact time and $C_{\mathrm{b}-0}$ ($g \cdot L^{-1}$) 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 180ºC for 20 min followed by 10 min at 180ºC(Bakircioglu et al., 2011). 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 4000-500 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.

5.2.5 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.

5.3 Results and discussion

5.3.1 Copper and zinc uptake

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 our 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 *S. 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 72h 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 chosen to operate under the most unfavorable conditions within the range of values found in 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 5.1,** are the average of the performed duplicates; the given uncertainties have been estimated as their standard deviation.

Table 5.1. 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

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).

5.4 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 (**Figure 5.1**).

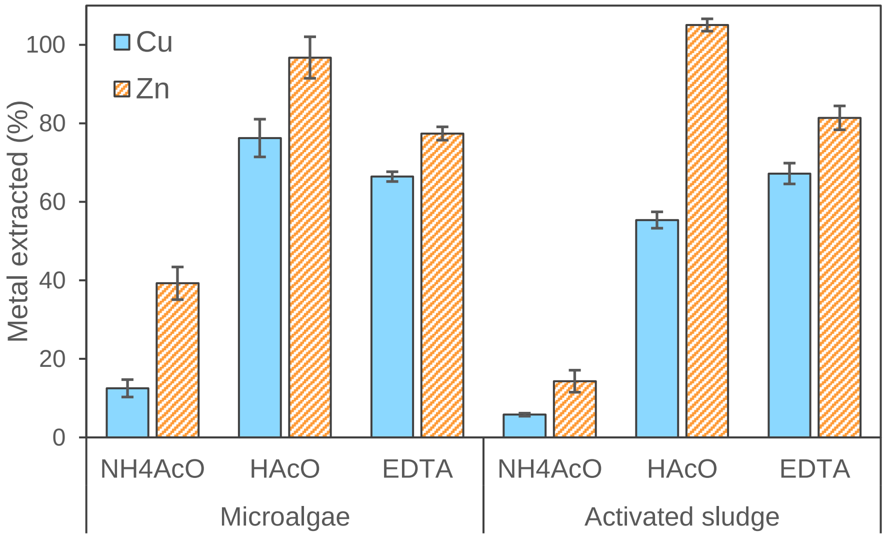

Figure 5.1. Percentage of Cu(II) and Zn(II) extracted from metal loaded biomasses of microalgae *S. almeriensis* and activated sludge (aerobic bacteria) using reagents with different chemical properties at 0.1 M concentration. The vertical error bars are the standard deviations of three replicates.

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 Fisher 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 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*, 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 5.2**, in mg metal per g of dry biomass, and in **Figure 5.2**, in percent fraction. The extraction scheme was applied in duplicate, and results shown are the mean values.

Table 5.2. 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+F4+res.)	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

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 5.2**). 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.

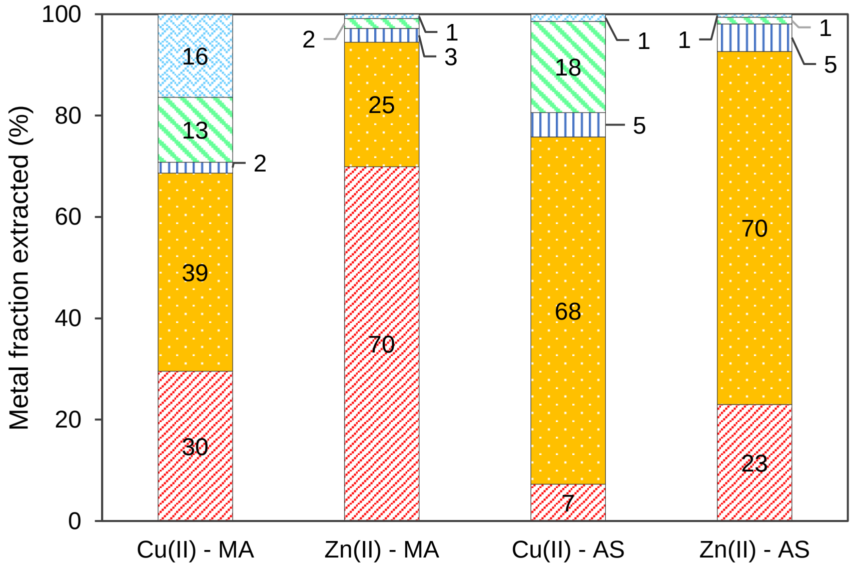

Figure 5.2. Metal fractionation results, in percentage fraction, of the sequential extraction of bioaccumulated metals Cu and Zn by microalga *S. almeriensis*, MA, and by activated sludge rich in aerobic bacteria, AS. F1: $MgCl_2$, F2: acetate buffer; F3: $NH_2OH \cdot HCl$; F4: H_2O_2; Residual fraction

Figure 5.2 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 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 ºC) 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 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.

5.5 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 **Figure 5.3A** and 5.**3B**, respectively. The biomasses show similar adsorption patterns, indicating that functional groups and molecular composition of microalgae and bacteria cell walls are alike.

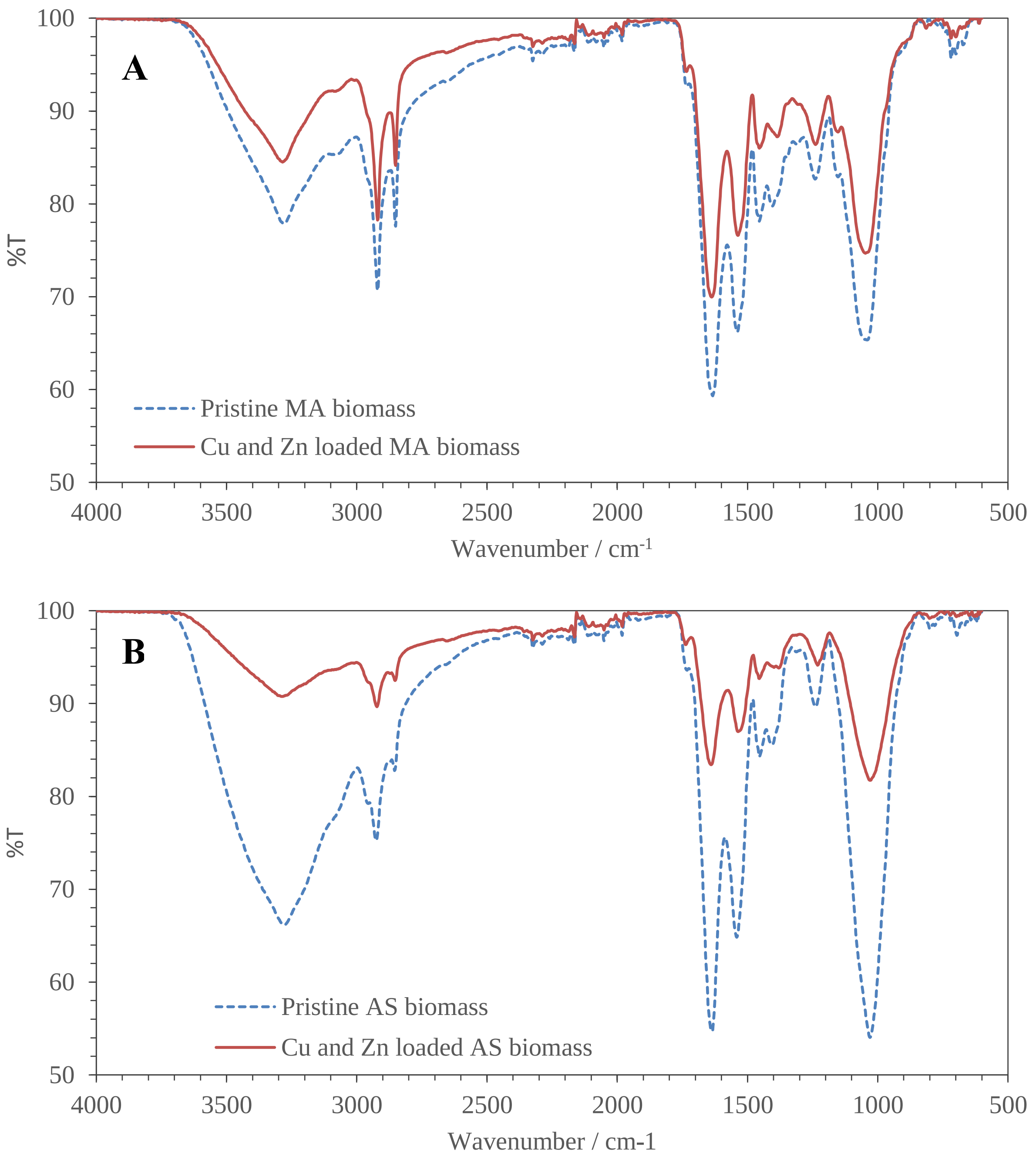

Figure 5.3. FTIR spectra of pristine and metal loaded biomass for (A) green microalga *S. almeriensis* and (B) activated sludge

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 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 3280-3284 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 1633-1637 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 1454-1455 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 1028-1047 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 **Figures 5.3A** and 5.**3B**, biosorption of Cu and Zn causes a noticeable decrease of characteristic FTIR bands, this attenuation being more significant in activated 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 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 **Figure 5.4** (microalga) and **Figure 5.5** (activated sludge). According to ESEM imaging, pristine microalgae presents a structure of small, almond-shaped striated globules (**Figures 5.4A1** and 5.**4A2**). After biosorption, these domains are observed to be in closer contact, forming a more compact group of globules (**Figures 5.4B1** and 5.**4B2**). **Figure 5.5** depicts ESEM images taken from freeze dried activated sludge before (**Figures. 5A1** and **5A2**) and after Cu and Zn biosorption (**Figures. 5.5B1** and 5.**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.

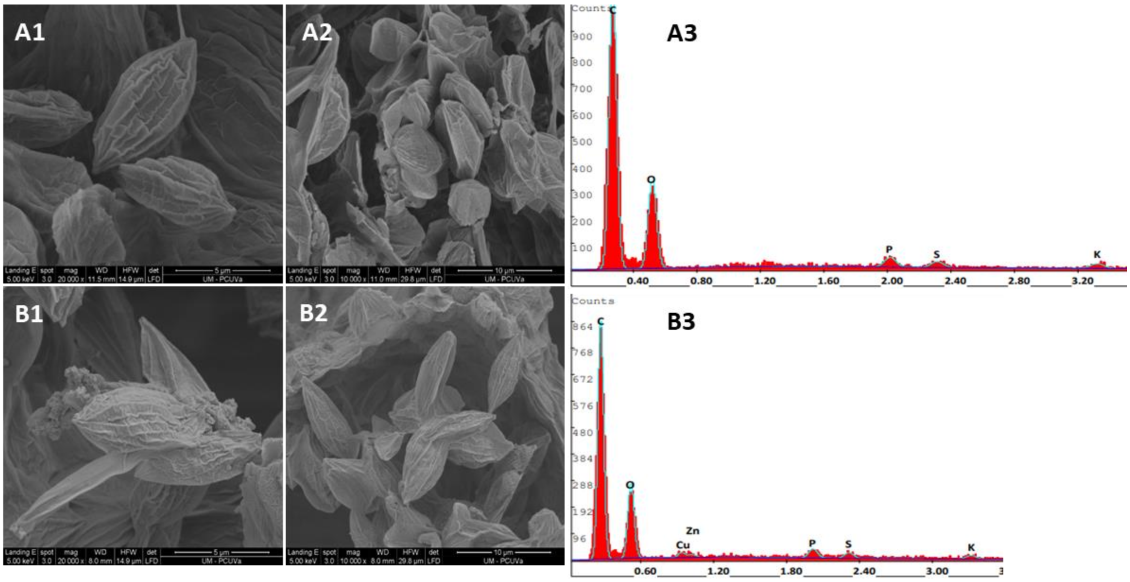

Figure 5.4. ESEM images of pristine (A1, A2) and metal exposed (B1, B2) biomass of microalga *S. almeriensis* recorded at 20,000x (A1, B1) and 10,000x (A2, B2) magnification. ESEM-EDX spectra confirm the biosorption of Cu and Zn on the biomass cell membrane after wastewater treatment (A3, B3).

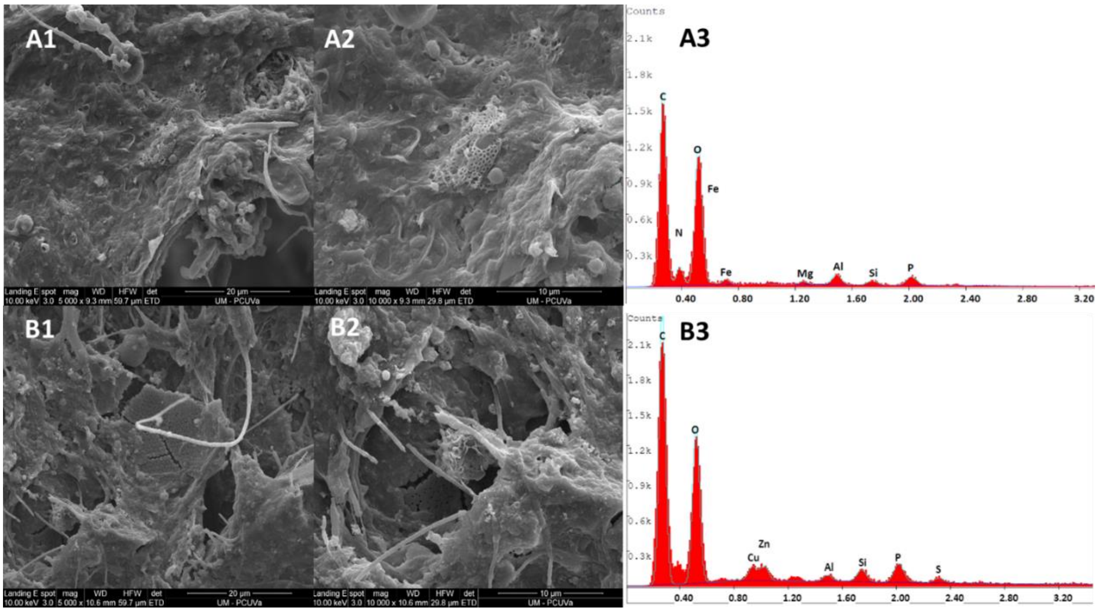

Figure 5.5. ESEM images of pristine (A1, A2) and metal exposed (B1, B2) biomass of activated sludge (aerobic bacteria) recorded at 5,000x (A1, B1) and 10,000x (A2, B2) magnification. ESEM-EDX spectra confirm the biosorption of Cu and Zn on the biomass cell membrane after wastewater treatment (B3).

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 (**Figures 5.4A3** and 5.**5A3** for microalgae and activated sludge, respectively), and after the contact with metal containing wastewater (**Figures 5.4B3** and 5.**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 (**Figures** 5.**4B3** and 5.**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 (**Figures 5.5A3** and 5.**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.

5.4 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.

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

Supplementary material

Supplementary Material

Table S5.1. Experimental conditions for single extraction procedure of Cu and Zn in microalgae *S. almeriensis* and activated sludge.

Extractant agent	pH	T (ºC)	Time (h)
0.1 M ammonium acetate[1]	7	rt	0.5
0.1 M acetic acid[2]	2-3	rt	0.5
0.1 M EDTA[3]	10-11	rt	0.5

[1] Prepared from ammonium acetate 98% (Scharlab, Spain)
[2] Prepared from acetic acid 99.8% (Scharlab, Spain)
[3] Prepared from EDTA·2H_2O (Panreac, Spain)

Table S5.2. Experimental conditions for sequential extraction procedure of Cu and Zn in microalgae *S. almeriensis* and activated sludge.

Extractant agent	pH	T (ºC)	Time (h)
0.1 M magnesium chloride[1] (F1)	7	rt	1
0.5 M acetate buffer[2] (F2)	5	rt	1
0.1 M hydroxylamine hydrochloride[3] (F3)	2	rt	2
Hydrogen peroxide 30% w/v[4] (F4)	2	85	2

[1] Prepared from $MgCl_2 \cdot 6H_2O$ (Panreac, Spain)
[2] Prepared from anhydrous sodium acetate and glacial acetic acid from (Merck, Germany) and (Scharlab, Spain) respectively.
[3] Prepared from hydroxylamine hydrochlorohydrate (Panreac, Spain)
[4] From (Panreac, Spain)
F1…F4 symbolize the metal fractions extracted; rt, room temperature

Table S5.3. Frequencies (cm^{-1}) of FTIR absorption bands of microalgae *S. almeriensis* biomass before and after bioaccumulation of Cu(II) and Zn(II).

Functional group	Pristine microalgae biomass	After Cu – Zn accumulation
Surface -OH and stretching N-H	3280.86	3284.11
Aliphatic C-H stretching	2919.83-2851.24	2919.46-2851.17
Amide group (N-H and C=O stretching vibrations)	1633.81	1637.32
C-N amide	1539.38	1537.22
Carboxylate anion	1454.66-1404.61	1454.19-1385.41
$-SO_3$ stretching groups	1240.14	1238.48
Phosphate group	1040.48	1047.43

Table S5.4. Frequencies (cm^{-1}) of FTIR absorption bands of activated sludge biomass before and after bioaccumulation of Cu(II) and Zn(II).

Functional group	Pristine bacteria biomass	After Cu – Zn accumulation
Surface -OH and N-H stretching	3280.90	3284.98
Aliphatic C-H stretching	2925.48	2922.72
Amide group (N-H and C=O stretching vibrations)	1636.06	1638.63
C-N amide	1540.95	1536.49
Carboxylate anion	1454.32- 1405.52	1455.12-1379.04
$-SO_3$ stretching groups	1238.96	1230.36
Phosphate group	1029.65	1028.96

Chapter 6:

Bioelimination of As(III), As(V) and DMA by microalgae and activated sludge grown in nutrient rich wastewaters

Bioelimination of As(III), As(V) and DMA by microalgae and activated sludge grown in nutrient rich wastewaters

Abstract

The treatment and management of wastewater from the livestock industry, particularly pig manure, is one of the greatest environmental challenges. The use of microalgae grown in photobioreactors for wastewater treatment is an environmentally friendly alternative to the traditional wastewater treatment plants. In this work, the bioelimination of the species of Arsenic, As(III), As(V) and dimethylarsonic acid (DMA), is studied using two biomasses: a pure microalgae *Scenedesmus almeriensis* and an activated sludge. To analyze the influence of time on the As bioelimination by both biomasses, as well as the effect of As on biomass growth, experiments were carried out at different contact times ranging from 1h to 15 days. Removal efficiencies close to 80% were achieved for As(III) and As(V) by activated sludge in the first 24 h, while for microalgae removal efficiencies of 80% were achieved for DMA at a contact time of 10 days. Speciation studies were performed to verify the capacity of biotransformation of As species to less toxic species by the biomasses. FTIR and ESEM-EDX surface studies were performed to elucidate the main mechanisms involved in the biosorption process. Finally, the experimental data of biosorption of As by both biomasses were fitted to Langmuir and Freundlich isotherm models.

Keywords: *Activated sludge, arsenic, biosorption, isotherms, microalgae, speciation*

6.1 Introduction

Livestock farming is a very important economic and social sector, especially the pig farming, which has experienced a significant increase in global demand in recent years. This has triggered intense activity on pig farms, resulting in the production of large quantities of wastewater with high loads of organic matter, nitrogen (N) and phosphorus (P), leading to eutrophication and other environmental problems (López-Sánchez et al., 2022; Vardhan et al., 2019). Therefore, this waste represents an alarming problem at present, and it is crucial to develop an effective treatment of the swine manure generated, which not only avoids contamination, but also allows the recovery of the organic matter and nutrients present in the manure. In addition, these wastes contain some toxic trace elements (TTE) from their use as food additives, or in the case of arsenic, which may stem from feed water used on farms located in regions where groundwater or other matrices are polluted with this metalloid (Ubando et al., 2021; Wang et al., 2022). The presence of arsenic in swine manure can lead to its inclusion in soil, water or even the food chain. Arsenic concentrations in the liquid fraction of swine manure have been reported up to 690 μg/L (Gao et al., 2016). For this reason, the management of the liquid fraction of pig manure is a problem that requires an urgent, effective and sustainable solution due to its high content of N, P, C and other emerging pollutants such as traces of antibiotics or their metabolites, as well as high concentrations of metals such as Cu, Zn, Fe, Cd, Pb and As among others (ASAE, 2003; Wang et al., 2023). Regarding arsenic, exposure to this metalloid is linked to cardiovascular disease, diabetes or cancer (Mohan and Pittman, 2007). It also accumulates in tissues such as hair, skin, and bones (Ghayedi et al., 2019). There are several species of arsenic, firstly, the inorganic species As(III) and As (V), in the form of arsenite and arsenate, and secondly, the organic species such as mainly monomethylarsonic acid (MMA) and dimethylarsonic acid (DMA). There are also other organic species of arsenic, like arsenonbetaine or arsenocoline which are considered non-toxic (Papry et al., 2021; Patel et al., 2023). Both inorganic species have the ability to inhibit metabolic energy functions generated in the mitochondria (Singh et al., 2015), although As(V) is absorbed more slowly than its trivalent analogue in biological systems (Hussain et al., 2021). The anionic form of As(V) has the capability to compete with phosphates in cellular metabolic reactions by uncoupling oxidative phosphorylation processes to break the highly energetic bonds of ATP. Whereas trivalent arsenic has a high affinity for the thiol groups of proteins, such as cysteine, preventing the formation of disulphide bridges, which are very important for protein structures, and contributing to the deactivation of enzymes. In summary, trivalent arsenic is the most toxic arsenic species, followed by pentavalent arsenic and organic species such as MMA and DMA. According to the US Environmental Protection Agency, the Maximum Concentration Limits (MCL) for As is 10 μg/L in potable water (U.S. EPA, 2009), as well as in the Spanish regulation (Ministerio de Agricultura Alimentación y Medio Ambiente, 2015). Conventional physicochemical methods for wastewater treatment include techniques such as ion exchange with resins, membrane filtration, chemical precipitation, and electrochemical methods. These methods are expensive due to high maintenance and operational cost, which makes them not very feasible in practice. Furthermore, they are not very effective at metal concentrations below 100 mg/L (Jaafari and Yaghmaeian, 2019; Suresh Kumar et al., 2015). For this reason, the use of bioremediation is emerging as a sustainable alternative in the manure treatment, as it uses live organisms, mainly plants and microorganisms, to eliminate pollutants from wastes, water or contaminated soils, with the aim of reducing the impact of industrial or other anthropogenic activities (Priya et al., 2022). It should be noted that bioremediation falls within the framework of Green Chemistry due to the low use of reagents, solvents and energy in these processes (Leong and Chang, 2020). Moreover, wastewater treatments based on microalgae and bacteria have become popular and widely studied in recent years with promising outcomes, although to the best of our knowledge, there are few studies that address the problem presented in our work. (Aguilar et al., 2020; Altowayti et al., 2020; Arora et al., 2017; Cameron et al., 2018; Monteiro et al., 2011; Podder and Majumder, 2017; Saavedra et al., 2018b; Tabaraki and Heidarizadi, 2018). Microalgae and bacteria exhibit several metal uptake mechanisms, such as physical adsorption, complexation, coordination, microprecipitation, ion exchange or a combination of these (Zoroufchi Benis et al., 2020) (Bădescu et al., 2018) (Sahmoune, 2016). These processes occur due to the existence of numerous functional groups in the cell wall such as carboxyl, hydroxyl, amino groups, amides, thiols, etc. (Jebelli et al., 2017). Moreover, methylation is also a mechanism of detoxification by microorganisms such as microalgae and bacteria. Methylation involves arsenic methyltranferases, which act as active catalysts (Zhang et al., 2013). In addition, redox reactions are possible during cellular respiration since As(V) is a final electron acceptor and could be reduced to As(III) (Yin et al., 2011).

In this work, the bioelimination capacity of three arsenic species, As(III), As(V) and the organometallic species dimethylarsonic acid (DMA), were studied by two different biomasses grown in a synthetic wastewater medium. Biomasses based on the green microalga *Scenedesmus almeriensis and* activated sludge from wastewater treatment plant (WWTP) were selected to evaluate the influence of biomass type on the bioelimination of the arsenic species. The bioelimination of each of the chosen arsenic species was studied, as well as the growth of the biomass in the presence of these metalloid species at a metal concentration of 0.10 and 0.50 mg/L for 15 days in a synthetic medium simulating piggery wastewater. In addition, a speciation study of the liquid fraction of the bioelimination experiments was carried out to study the possible existence of biotransformation mechanisms by the microorganisms and the medium. Finally, experimental data of biosorption of the three arsenic species for a range of concentrations from

0.01 to 500 mg/L and at contact times of 1 and 72 hours were fitted to Langmuir and Freundlich models to determine the maximum adsorption capacity of the biomasses used.

6.2 Materials and methods

All the reagents described in the different experiments were of analytical grade and the solutions prepared using deionized (ultrapure) water. All reagent to prepare As(III), As(V), DMA standard solutions and to make up synthetic wastewater, $Na_2HAsO_4\ 7{\cdot}H_2O$, As_2O_3, $(CH_3)_2AsO_2H$, KNO_3, $CaCl_2{\cdot}2H_2O$, NaCl, $MgSO_4$, $CO(NH_2)_2$, peptone, meat extract and NaOH), were purchased from Sigma Aldrich (Germany). NaOH to pH adjustment was from Panreac. All plastic and glass vessels were washed in dilute HNO_3 (10% v/v) for 24 h and rinsed 3 times with Milli-Q water (R > 18 MΩ cm) before use. Biosorption experiments were carried out in 500 mL Pyrex glass bioreactors operating in batch mode at room temperature and constant stirring of 250 rpm using a magnetic stirrer. In order to simulate real operating conditions of photobioreactors and aerobic reactors, microalgae-based biosorption experiments were irradiated with 1200 $\mu E{\cdot}m^{-2}{\cdot}s^{-1}$ in 12-12h light-dark periods and activated sludge experiments were conducted with ambient light.

6.2.1 Microalgae and activated sludge

Biomasses based on a green microalgae and aerobic bacteria activated sludge were used as inoculum in the bioreactors for the arsenic bioelimination experiments in synthetic wastewater. The green microalgae *Scenedesmus almeriensis* from monoculture grow in a synthetic medium was provided by the Department of Chemical Engineering, University of Almeria (Spain), while the activated sludge was collected from the aerobic bioreactor of the municipal wastewater treatment plant located at Valladolid (Spain). The chemical composition of the fresh biomasses was determined by protein, lipid and water content. The nitrogen content was determined by the Total Kjeldahl Nitrogen method (applying a nitrogen-protein factor of f = 5.95 for microalgal biomass (Martín-Juárez et al., 2019) and of f = 6.25 for activated sludge (Zhong et al., 2012)), the lipid content was analyzed using the Bligh and Dyer method (Breil et al., 2017) and the water content was determined using a gravimetric method described in Materials and Methods section. The protein and lipid content of microalgal biomass was 43.9% and 11.7%, respectively, while for activated sludge it was 51.9% and 12.6%, respectively (all percentages on a dry-weight ash-free biomass). The water content of the fresh biomasses was 93.1% and 99.1% for microalgae and activated sludge, respectively. Prior to the biosorption experiments in order to avoid chemical and thermal degradation of biomasses, the microalgae-based biomass were kept in Bristol medium under continuous agitation and at a temperature of 21ºC with light-dark periods of 12-12 h, irradiated with LED light (1200 $\mu E{\cdot}m^{-2}{\cdot}s^{-1}$) to simulate sunlight periods, while the activated sludge was kept in the darkness with aeration at 4ºC in order to avoid their chemical and thermal degradation. Fresh activated sludge was observed under the microscope (Leica DM 4000 B). No microalgae or cyanobacteria have been detected. Fresh samples of inoculum were freeze-dried for subsequent FTIR and ESEM-EDX analysis.

6.2.2 Biosorption experiments

Experimental arsenic removal as As(III), As(V) and DMA species was carried out using. the microalga *Scenedesmus almeriensis* and activated sludge in a synthetic wastewater medium simulating piggery wastewater. This synthetic wastewater was prepared dissolving in deionized water: 30 mg urea, 32.5 mg KNO_3, 4 mg $CaCl_2 \cdot 2H_2O$, 7 mg NaCl, 2 mg $MgSO_4 \cdot 7\ H_2O$, 110 mg peptone and 160 mg of meat extract per liter of final solution, according to the method described by Alcántara *et al*. (Alcántara et al., 2015), resulting in 120 mg/L for total organic carbon (TOC), 45 mg/L for inorganic carbon (IC) and 60 mg/L of total nitrogen (TN). 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 be impossible to study independently the heavy metals uptake by pure microalgae and by aerobic bacteria. All experiments were carried out in bioreactors with a total suspension volume (biomass/wastewater) of 200 mL and a final biomass concentration of 2 and 4 g/L for microalgae and activated sludge, respectively.

The initial pH value of the biomass suspension was adjusted to 7.5 with diluted NaOH, which is the usual pH value for the operation of photobioreactors and aerobic reactors in wastewater treatment (Posadas et al., 2015). Finally, in order to assess the adsorption of each of arsenic species by the two biomasses, the species As(III), As(V), and DMA were added to the biomass suspensions at different initial concentrations, depending on the experiment to be analyzed. Bioelimination experiments were carried out at initial arsenic concentrations of 0.10 and 0.50 mg/L. The concentration range of the arsenic species used in this study were selected based on the typical composition of the piggery wastewaters (ASAE, 2003; Collao et al., 2022). Stock solutions of As(III), As(V) and DMA were prepared by dissolving the appropriate amount of Na_2HAsO_4, As_2O_3 and $(CH_3)_2AsO_2H$, respectively, in deionized (ultrapure) water. To follow biomass growth, the initial and final amounts of biomass at each corresponding contact time were determined by measuring of total and volatile solids content according to standard methods (E.W. Rice, R.B. Baird, A.D. Eaton, 2017). Moreover, to analyse the influence of the time on arsenic bioelimination by both biomasses, samples were taken at different times ranging from 8 h to 15 days in the rest of the experiments. The uptake of toxic metals by microalgae biomass was calculated in terms of the experimental uptake capacity (q) as follows in Equation 3.11 of Materials and Methods. On the other hand, the metal removal efficiency (RE) was also another parameter for evaluation. It is expressed in percentage as defined by Equation 3.12 in Material and Methods.

6.2.3 Adsorption isotherm models

In order to determine the maximum adsorption capacity of the studied biomasses, the experimental data were fitted to the Langmuir isotherm model and the Freundlich isotherm model (Atkins, 1998). The adsorption isotherm studies were performed at initial concentration of the arsenic species ranged from 0.01 to 500 mg/L. Moreover, to analyse the influence of the time on arsenic bioelimination by both biomasses, samples were taken at 1 and 72 hours. Fowler-Langmuir and Freundlich Isotherm model were performed according to Equations 3.4-3.9 of Materials and Methods Chapter.

6.2.4 Speciation studies

A speciation study was carried out with the aim of studying the possible biotransformation of arsenic species. speciation studies were performed on the liquid fraction resulted in the bioelimination experiments of the arsenic species by activated sludge at an initial As concentrations of 0.1 and 0.5 mg/L and at contact times of 3, 7 and 15 days. In order to compare the behavior of the bioelimination of As species by the two biomasses at higher concentrations, speciation studies were also carried out on the liquid fractions resulting from biosorption experiments with an initial concentrations of As species of 10 and 50 mg/L and at a contact time of 72 hours

6.2.5 Analytical methods

For all experiments, a sample of 5 mL of the liquid fraction of the biomass suspension was taken. Then, it was filtered using a 0.45 µm pore-size nylon filter, and the As total content was determined by inductively coupled plasma spectrometry (ICP). Samples with high arsenic content greater than 1 mg/L were analysed by ICP together with an optical emission spectrophotometer (ICP-OES) (Varian 725-ES, Agilent, Santa Clara, CA, USA). The emission lines at 188.980 nm and 193.696 nm, using the mean of both for quantification, were used for arsenic measurement. On the other hand, samples with low arsenic content were measured using an inductively coupled plasma source mass spectrometer (ICP-MS) with an octopolar reaction system (HP 7500c, Agilent, USA). For quality assurance, two water reference materials were included in the ICP-

MS analysis as quality control (QC) samples: ICP multielement Calibration Standard Solution, 100 mg/L Scharlau (26 elements in HNO_3 5%), and a Certified Reference Material (Environment Canada TMDA-64.2 LOT 0313, HNO_3: 0.2%) as a trace element enriched calibration standard. QC samples were measured every 10 samples, considering a range within 10% of the true value for valid acceptance. The individual concentrations of the arsenic species in the filtered supernatant were determined by HPLC-ICP-MS (Agilent 2200 HPLC system coupled to Agilent 7800 ICP-MS). Species separation was carried out with a Hamilton PRP x100 ion exchange column (4.1 x 250 mm x 10mm) in isocratic regime with a mobile phase of 10mM dibasic ammonium phosphate adjusted to a pH of 8.25. The pH was measured using a pH-meter Basic 20+ (Crison, Spain). The analytical procedure was in accordance with the guidelines proposed by the FDA for As speciation in food and biological matrices (Gray et al., 2015). Infrared spectra of the initial biomass (prior to As addition) and of the biomass after arsenic biosorption were analyzed using a Fourier-Transform Infrared spectrometer (FTIR). Biomass samples were centrifuged at 7800 rpm for 10 minutes and freeze-dried in a Telstar LyoQuest freeze-dryer. The FTIR spectra were recorded between 1000-500 cm^{-1} 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 changes of biomass morphology before and after arsenic 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 FEI274 Quanta200FEG with a secondary electron detector LFD low vacuum.

6.3 Results and Discussion

6.3.1 Adsorption isotherm

The adsorption of the arsenic species, As (III), As(V) and DMA, by the two biomasses used were analyzed at an initial concentrations of arsenic species ranging from 0.01 to 500 mg/L a contact time of 1 and 72 hours. The fitting parameters of Langmuir and Freundlich isotherm models together with their adjusted R^2 obtained for the adsorption of arsenic species by both biomasses at the different contact times are displayed in **Table 6.1**. Comparing the R^2 values obtained, both isotherm models showed a good fit to the experimental data.

Table 6.1. Langmuir and Freundlich parameters for As adsorption by *Scenedesmus almeriensis* (SA) and activated sludge (Act. Sl.) at different contact times.

Time	Arsenic species	Biomass	Langmuir model				Freundlich model		
			q_{max} (mg/g)	K_L (L/mg)	R_L	R^2	K_f (L/g)	n	R^2
1 h	As(III)	*SA*	0,40	$2{,}08\cdot10^{-2}$	0,12	0,9341	$1{,}01\cdot10^{-2}$	1.36	0.9881
		Act. Sl.	1,90	$2{,}30\cdot10^{-2}$	0,50	0,9654	$4{,}69\cdot10^{-3}$	1.72	0.9876
	As(V)	*SA*	0,18	$3{,}18\cdot10^{-2}$	0,81	0,9897	$5{,}48\cdot10^{-3}$	1.18	0.9854
		Act. Sl.	2,46	$1{,}17\cdot10^{-1}$	0,59	0,9785	$2{,}61\cdot10^{-1}$	1.53	0.9707
	DMA	*SA*	0,39	$9{,}56\cdot10^{-3}$	0,93	0,9956	$5{,}51\cdot10^{-3}$	1.22	0.9794
		Act. Sl.	0,58	$1{,}56\cdot10^{-1}$	0,90	0,9725	$3{,}04\cdot10^{-2}$	1.31	0.9960
72 h	As(III)	*SA*	9,52	$9{,}51\cdot10^{-3}$	0,60	0,9445	$9{,}33\cdot10^{-2}$	1.14	0.9441
		Act. Sl.	23,1	$2{,}04\cdot10^{-3}$	0,82	0,9986	$5{,}16\cdot10^{-2}$	1.06	0.9992
	As(V)	*SA*	4,85	$2{,}87\cdot10^{-3}$	0,78	0,9856	$3{,}58\cdot10^{-2}$	1.19	0.9837
		Act. Sl.	34,7	$3{,}67\cdot10^{-4}$	0,95	0,9999	$2{,}48\cdot10^{-2}$	1.16	0.9789
	DMA	*SA*	37,0	$6{,}53\cdot10^{-4}$	0,92	0,9858	$2{,}34\cdot10^{-2}$	1.00	0.9697
		Act. Sl.	3,16	$3{,}82\cdot10^{-3}$	0,74	0,9948	$1{,}24\cdot10^{-1}$	2.28	0.9861

Regarding the results of the adsorption experiments at a contact time of 1 hour, the highest q_m values were obtained for the adsorption of all arsenic species studied by activated sludge. While for the experiments at 72 hours contact time, the highest maximum adsorption capacities were achieved for the adsorption of the inorganic arsenic species by activated sludge and of the organic species, DMA, by the microalgal biomass.

As shown in ***Table 6.1***, all the dimensionless separation coefficient, R_L, calculated from Langmuir linear fit ranged from 0 to 1, and the heterogeneity factor of the adsorption, n, calculated from Freundlich linear fit were higher than 1 in all cases, showing that the adsorption is favorable according to both models. Langmuir constant (K_L) is related to the strength of adsorbate. Higher values of K_L were obtained for the adsorption experiments at 72 hours, indicating the need for a longer time to produce the equilibrium necessary for higher arsenic removal. The same trend was observed for the values obtained for Freundlich constants (K_F), that indicates the adsorption capacity of the adsorbent. Similar results in terms of maximum adsorption capacity, R_L and n values were obtained by other authors using *Chlorella vulgaris* (Ghayedi et al., 2019, Alharbi et al., 2023) which is a green microalgae like *S. almeriensis*. In contrast, different results to those obtained in the present study have been found for other species of algae and bacteria (Podder and Majumder, 2017; Tabaraki and Heidarizadi, 2018). This indicates that the species of microorganism has a great influence on the maximum adsorption capacities of arsenic.

6.3.2 Biosorption experiments

The biosorption of the three arsenic species (As(III), As(V) and DMA) by the two biomasses selected were performed at initial concentrations of arsenic species of 0.10 and 0.50 mg/L and at different contact time ranging from 8h to 15 days. The metal removal efficiency (*%RE*) obtained for the arsenic species by the microalga *S. almeriensis* and activated sludge are shown in **Figure 6.1**. As it can observed in **Figures 6.1A** and 6.**1B**, the activated sludge-biomass showed a higher affinity for the most toxic species of the metalloid (As(III) and As(V)), reaching the highest values of *%RE*, close to 80%, at a contact time of 1 day for both initial inorganic arsenic concentrations. At longer contact times, an expulsion of the metal into the medium is observed for both initial As concentrations.

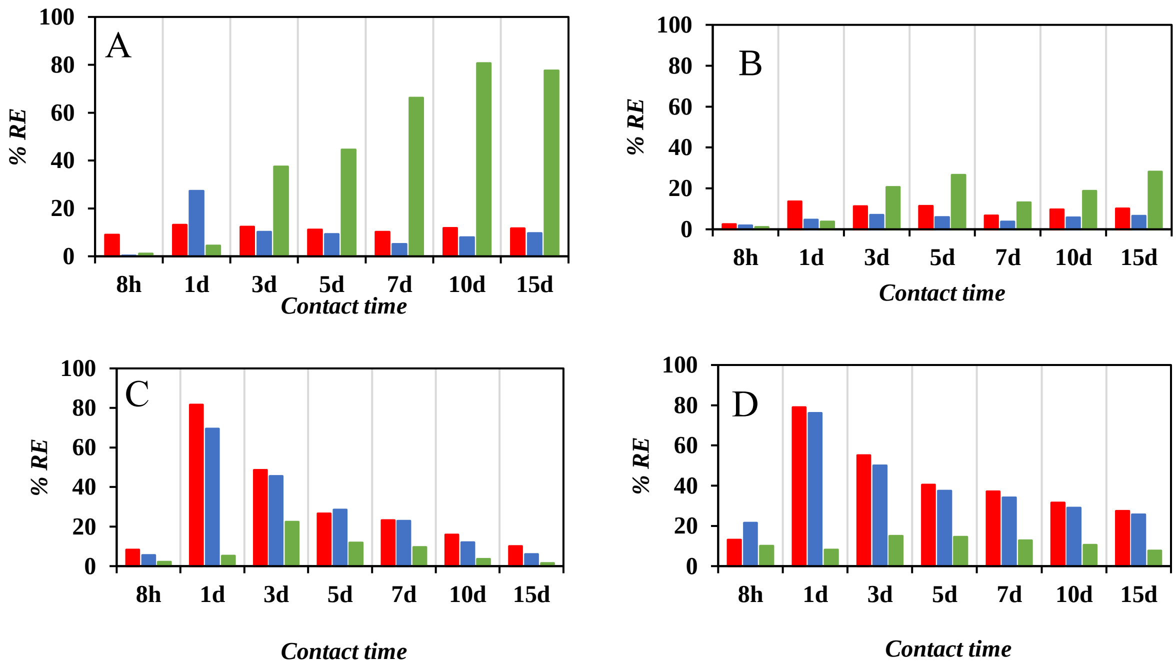

Figure 6.1. Removal efficiencies (%RE) by the two biomasses tested, *Scenedesmus almeriensis* (A and B) and activated sludge (C and D) for the arsenic species at the different initial concentrations. (■) As (III), (■) As (V) and (■) DMA.

This behavior is more pronounced at lower initial As concentrations, which could indicate detoxification mechanisms by the cells present in the activated sludge biomass. In contrast, the biomass formed by the microalgae showed a higher larger biosorption of the less toxic As species (DMA), requiring longer contact times to achieve the highest *RE* values. The optimum time to bioeliminate DMA species for low initial concentrations was 10 days (*RE* = 80.6) while for higher initial concentrations, the highest bioelimination efficiency (*RE* = 28.1) was obtained at contact time of 15 days. This behavior may be due to a possible development of acclimatization processes (López-Pacheco et al., 2021). Moreover, comparing the *RE* values obtained in this work with other reported studies with similar initial concentrations of arsenic species, similar results has been published by *Shakya and Ghosh* using a mixed bacterial community grown in anaerobic sludge (Shakya and Ghosh, 2019). The results obtained for the metal removal efficiency (*%RE*) are consistent with those obtained in terms of biosorption capacities (*q*) which are shown in ***Figure 6.2***.

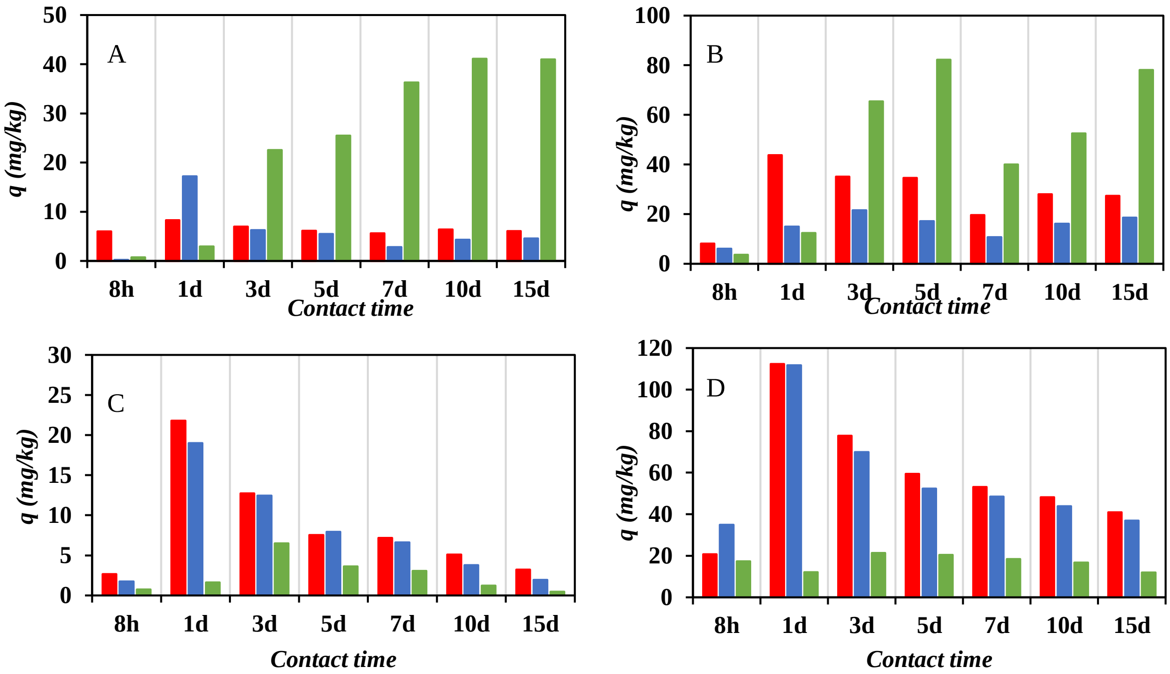

Figure 6.2. Adsorption capacities (q) calculated for the two biomasses, activated sludge (A and B) and *Scenedesmus almeriensis* (C and D) for the arsenic species at the different initial concentrations of 0.1 and 0.5 mg/L. (■) As (III), (■) As (V) and (■) DMA.

For the experiments using the activated sludge, maximum values of *q* for the low initial concentration of the metal species (0.1 mg/L) were obtained for the inorganic arsenic species, being 21.8 and 19.0 mg/kg for As(III) and As(V), respectively, at a contact time of 1 day. Whereas, for the higher initial metal species concentrations (0.5 mg/L), the maximum biosorption capacities of 112.3 and 111.7 mg/kg were obtained for As(III) and As(V), respectively, at a contact time of 1 day. Regarding the behavior of the organic species DMA, lower *q* values were obtained for both initial concentrations, obtaining the highest values of 6.5 and 21.3 mg/kg at a contact time of 3 days. Regarding the biosorption capacities using microalgae *Scenedesmus almeriensis*, higher values of biosorption capacities were obtained for the organic As species from the contact time of 3 days, achieving the maximum *q* value of 41.1 for low initial concentrations at a contact time of 10 days and 82.2 mg/kg for higher DMA concentrations at a contact times of 5 days. Microalgal biomass showed a poor affinity for the inorganic As species.

In summary, the results of As removal, both in terms of *%RE* and *q*, indicated that the biomass composed by activated sludge showed higher removal of inorganic As species at a contact time of 1 day, whereas the microalgal biomass presented a higher affinity for the organic As species requiring longer contact times for appreciable arsenic removal. Low removal of 19% were obtained by a *Chlorella sp*.-bacteria based photobioreactor fed with real swine wastewater doped with 0.5 mg/L of As(V) (Collao et al., 2022)

Comparable results of biosorption capacities have been found in the literature for other species of microalgae or bacteria species as it shown in **Table 6.2**.

Table 6.2. Biosorption data reported in literature of different arsenic species by different microalgal and bacterial biomass.

Metal species	***Biomass species***	***pH***	***Concentration***		***T (ºC)***	***Contact time***	***Uptake q (mg/g)***	***Ref.***
			Initial metal (mg/L)	***Biomass (g/L)***				
	Microalgal biomass							
As(III)	*Nannochloropsis sp.*	--	0.5	--	--	--	2	(Upadhyay et al., 2016)
	Spirulina platensis	7	0.1	--	28	7 days	14	(Samal et al., 2004)
		7	0.5	--	28	7 days	12	
	Scenedesmus bijuga	7	0.1	--	28	7 days	32	
		7	0.5	--	28	7 days	30	
	Chlorella vulgaris	7	0.1	--	28	7 days	30	
		7	0.5	--	28	7 days	26	
	mixed bacterial community grown in anaerobic sludge	--	0.2	--	--	40 min	90	(Shakya and Ghosh, 2019)
	Bacterial biomass							
	Acidithiobacillus ferrooxidans DLC-5	7	0.5	2	25	*48 h*	*(35)*	(Xu et al., 2019)
	Microalgal biomass							
As(V)	*Chalmydomonas reinhardtii*	7	0.5	--	25	144 h	40	(Ramírez-Rodríguez et al., 2019)
	Chlorella vulgaris grown in piggery wastewater	7.5	0.5	1	r.t.	5 days	(180)	(Collao et al., 2022b)
		7.5	0.5	1	r.t.	5 days	(208)	
	Spirulina platensis	7	0.1	--	28	7 days	18	(Samal et al., 2004)
		7	0.5	--	28	7 days	17	
	Scenedesmus bijuga	7	0.1	--	28	7 days	36	
		7	0.5	--	28	7 days	35	
	Chlorella vulgaris	7	0.1	--	28	7 days	34	
		7	0.5	--	28	7 days	30	
	Bacterial biomass							
	Pseudomonas stutzeri WS9 + Micrococcus yunnanensis WS11 + Bacillus thuringiensis WS3	7	9.00	0,6	37	6	98 (14.66)	(Aguilar et al., 2020b)
	Bacillus arsenicus MTCC 4380	7	100	2	30	1,5	96 (894.13)	
	Bacillus cereus P1C1Ib	7	432.54	--	28	72	84	
	Bacterial biomass							
DMA	Acidithiobacillus ferrooxidans DLC-5	7	0.5	2	25	48	(120)	(Xu et al., 2019)

*r.t.: means room temperature; --: not available; values in brackets indicate removal efficiencies in mg/kg.

In summary, the differences found between our work and the existing literature can be attributed to a combination of factors related to sampling methods, sample preparation and experimental conditions including initial biomass and toxic element concentration as well as biomass death of growth during bioremediation treatment. These factors should be taken into account when interpreting the results and comparing studies from different authors.

6.3.3 Biomass viability

The evolution of the biomass growth in the biosorption experiments at different initial concentrations of the three arsenic species at a contact time ranging from 8h to 15 days, as well as the growth of each biomass without the metal addition, is shown in **Figure 6.3**. As can be observed from this figure, maximum biomass concentrations of 2.65 g/L were reached for the biomass based on microalgae with an initial As(V) concentration of 0,1 mg/L and at a contact time of 15 days. Whereas for activated sludge, maximum biomass concentrations of 4.79 g/L were reached with an initial As(III) concentration of 0,5 mg/L and at a contact time of 3 days. Since the initial biomass concentration was of 2 and 4 g/L for microalgae and activated sludge, respectively, these results show a growth of up to 30% for microalgal biomass and of 20% for activated sludge. Regarding biomass composed by the microalgae, a progressive growth of the biomass is observed throughout the contact time, parallel to the progressive biosorption of the metalloid species, without causing apparent cell death after 15 days of experimentation. Acclimatization is a very common strategy for microalgae and bacteria-based biomass when they are exposed to an unusual medium or environment according to the literature. In addition, livestock wastewater may be toxic to some microalgae strains (López-Sánchez et al., 2022). In agreement with these results, in Saavedra et al., (2018) work, no inhibition was detected from initial and final values of microalgae biomass growing in all the control and multimetallic experiments with Cu and Zn among others such as As. In that work, the measured growth resulted lower than 15% (w/w) in all the tests for a contact time of 72h. On the other hand, Collao et al., (2022) addressed the effect of Cu, Zn and As, three toxic elements frequently present in piggery wastewater tested on the performance and microbiome of a *Chlorella Vulgaris*-bacteria consortia. A *Chlorella sp*.-bacteria based photobioreactor fed with real swine wastewater were doped with Zn (100 mg/L), Cu (100 mg/L), and As (0.5 mg/L) separately (Collao et al., 2022). Measurements of TSS in As-spiked PBR were similar than those observed in the control. No statistical differences were found between them. Despite its higher toxicity, As had a lower impact on the reactor microbiome due to its lower uptake from biomass, resulting in an increase in microalgae growth. In addition, this fact is supported by the decrease in soluble TOC and TN in the medium, assimilated by the microorganisms. Removals of 92 y 47% were reached for TOC and TN respectively (Collao et al., 2022).

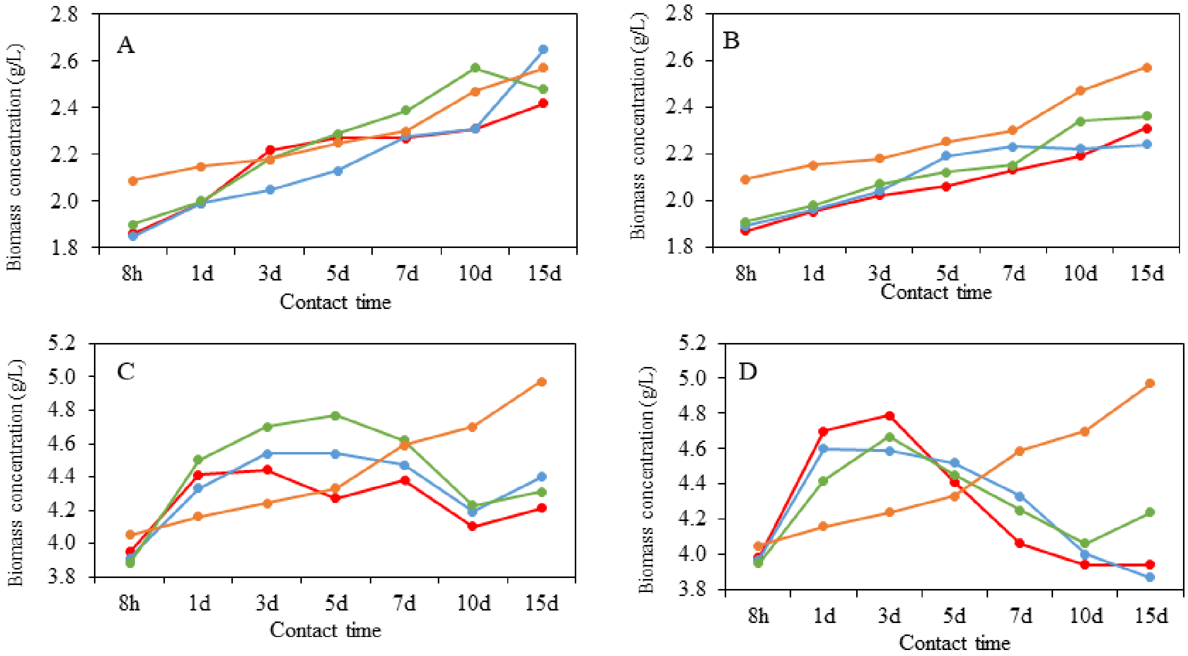

Figure 6.3. Evolution of biomass concentrations (mg/L), Scenedesmus almeriensis (A and B) and activated sludge (C and D), over time in the presence of arsenic species at initial concentrations of 0.1 and 0.5 mg/L. (-●-) As (III), (-●-) As (V), (-●-) DMA and (-●-) control.

The biomass of activated sludge showed a much faster growth, not needing an acclimatization time as the microalgae exhibits. Progressive growth is observed up to a contact time of 3 days, followed by a decrease in biomass concentration. This behavior is consisted with the metal removal process, since the decrease in biomass concentration occurs from day 3, after the maximum removal of the metal from the medium occurs **(Figure 6.1)**. Furthermore, it is observed that the decrease in biomass concentration of activated sludge is influenced by the initial arsenic concentration as the decrease in biomass growth is much more pronounced in experiments with higher concentrations of the metalloid than in experiments with lower concentrations of the species. This behavior is not observed when the biomass is formed by the microalgae *S. almeriensis* which is in concordance with the results reported by *Levy et al., (2005)* in which reported that As(III) has no effect on algal growth at normal environmental concentrations for the green microalgae *Chlorella sp.*

6.3.4 <u>Arsenic speciation</u>

Given the pronounced profile obtained for the bioelimination of the As species by activated sludge (***Figures 6.2C*** and 6.***2D***), speciation studies were performed on the liquid fraction resulted in the bioelimination experiments of the arsenic species by activated sludge at an initial As concentrations of 0.1 and 0.5 mg/L and at contact times of 3, 7 and 15 days (**Figure 6.4**).

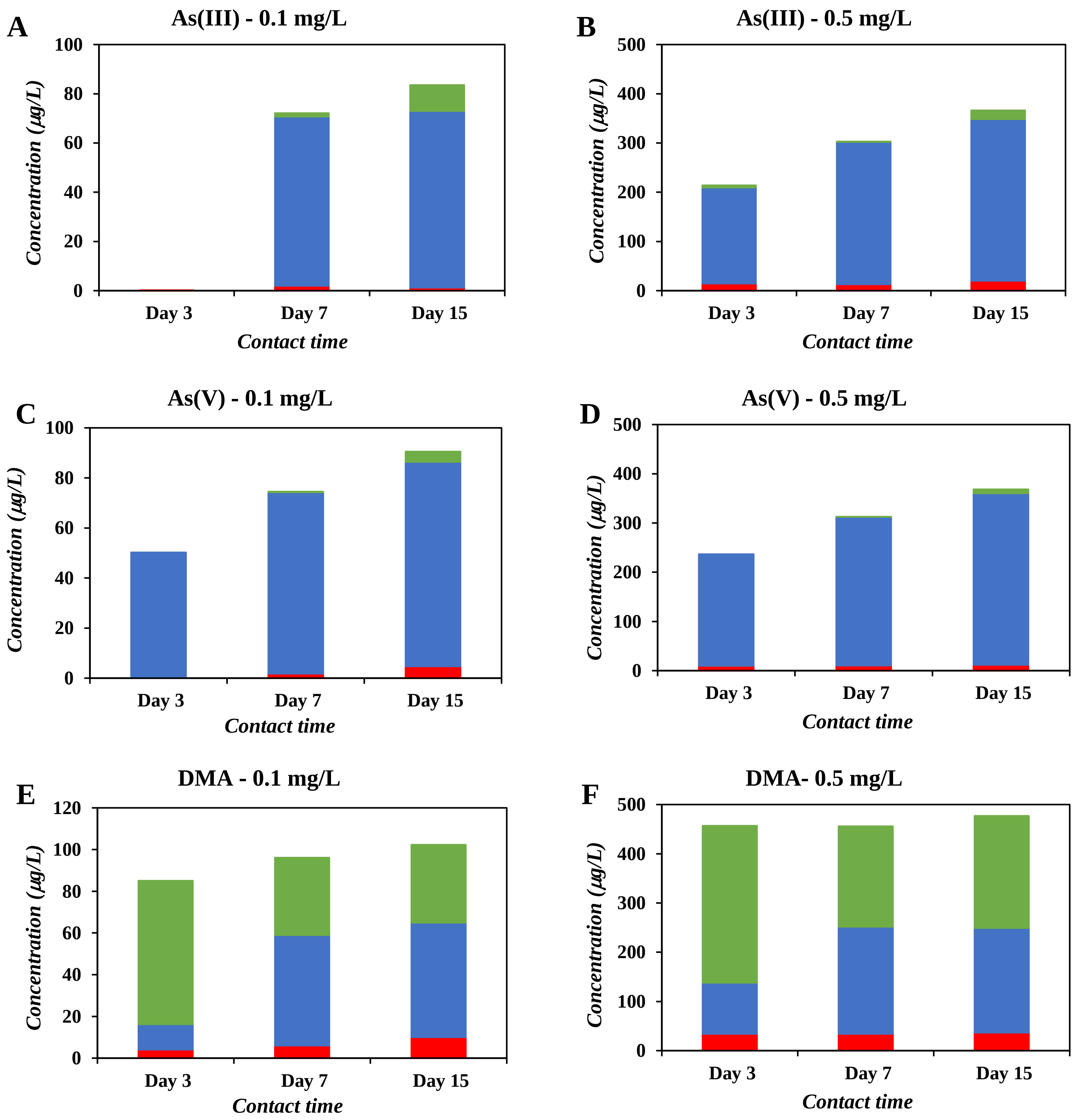

Figure 6.4. Arsenic speciation results performed from the liquid fraction of the bioelimination experiments of the three As species, at initial concentrations of 0.1 and 0.5 mg/L, by activated sludge and at a contact times of 3, 7 and 15 days. (■) As (III), (■) As (V) and (■)DMA.

As can be drawn from ***Figure 6.4***, for both initial As concentrations, there is a clear transformation of the initial As(III) added to the less toxic species of the metalloid. At initial concentrations of 0.1 mg/L, it is observed that the major species after As(III) biosorption is the oxidized As(V) species after a contact time of 7 days. At longer contact times, a higher biotransformation of As(III) is observed in the organic species DMA, although in much lower proportion than in As(V). Similar behavior was observed at the initial As(III) concentration of 0.5 mg/L but from contact times of 3 days. When the initial species is As(V) for both concentrations studied, it can be concluded that As(V) hardly undergoes biotransformation to other As species during the analyzed contact times as can be seen in ***Figures 6.3B*** and 6.***3C***. A slight transformation to As(III) and DMA can be observed, but it is negligible compared to the detected As(V) concentrations. Finally, from ***Figures 6.3E*** and 6.***3D***, it is possible to observ that approximately 20% of the added DMA was biotransformed into As(V) within 3 days of contact, increasing to about 45% at longer contact times for both initial concentrations of added DMA. This is in agreement with the results of biosorption of As species by activated sludge over time, since the maximum bioelimination of As(III) and As(V) is reached at a contact time of 1 day after which the species are returned to the aqueous medium, this fact may be due to their biotransformation.

The remarkable accumulation potential of toxic semimetals clearly reflects the detoxification capacity of microalgae, which can be achieved through adsorption on the cell surface, reduction, methylation, and thiol-mediated sequestration in the vacuole (Wang et al., 2013). The large accumulation of As can be attributed to the presence of various functional groups such as hydroxyl, carboxyl, and sulfate in the cell wall, which endow its surface with a negative charge, thereby electrostatically attracting toxic elements to the algal surface and increasing the accumulation capacity (Monteiro et al., 2012). In addition, higher accumulation of As in biomass is probably due to the simultaneous accumulation and adsorption processes in the solution (Sun et al., 2015).

The adsorption of As is facilitated by the functional groups in the cell wall of microalgae. Multiple functional groups, such as carboxyl, hydroxyl and amides conjugated to As(V) and As(III). After adsorption, the As species enter the cell through the absorption process. As(III) absorption requires little or no energy, while As(V) absorption has high energy demands. As(III) is absorbed by Aquaglyceroporin and As(V) via the phosphate transport system (Wang et al., 2015).

In order to compare the behavior of the bioelimination of As species by the two biomasses at higher concentrations, speciation studies were carried out on the liquid fractions resulting from biosorption experiments with an initial concentrations of As species of 10 and 50 mg/L and at a contact time of 72 hours (**Table 6.3**).

Table 6.3. Arsenic speciation results performed from the liquid fraction of the bioelimination experiments of the three As species, at initial concentrations of 10 and 50 mg/L, by activated sludge and the microalga at a contact time of 72 hours.

Biomass	***Initial As concentration (mg/L)***		***As species detected in the liquid fraction***			
			As (III) (mg/l)	***As (V) (mg/L)***	***DMA (mg/L)***	***MMA (mg/L)***
Scenedesmus almeriensis	As(III)	10	nd	9.22	0.04	nd
		50	nd	53.72	0.03	0.16
	As(V)	10	nd	10.01	0.03	nd
		50	nd	52.34	0.03	0.73
	DMA	10	nd	0.01	10.41	0.02
		50	nd	nd	54.95	0.10
Activated sludge	As(III)	10	nd	8.27	0.27	0.01
		50	0.14	47.00	0.68	0.02
	As(V)	10	0.05	8.71	0.85	nd
		50	0.05	48.85	2.62	nd
	DMA	10	0.41	0.15	9.44	0.01
		50	0.01	0.24	55.62	nd

*nd: means not detected

As it can be observed from **Table 6.3**, when the initial species added is As(III), the main compound detected after the biosorption process is its oxidized form, As(V), for both microalgal biomass and activated sludge. Besides, a slight part of As(III) is methylated and detected as MMA and DMA. Therefore, these microorganisms reduce the toxicity of the medium as As(III), the most toxic species, was transformed into other less toxic species. The biotransformation of As(III) to As(V) as reported in the literature could be mainly due to microbial oxidation of As(III) to As(V) since the chemical oxidation reaction has much slower kinetics (Stolz et al., 2006) and in most microalgal species, the oxidation of As(III) occurs outside the plasma membrane and is carried out by extracellular enzymes (Papry et al., 2022).

In the experiments in which the initial species added was As(V), it can be observed from **Table 6.3** that most of the As(V) remains unchanged after the selected contact time for both biomasses. Although it is possible to observe that a small part of As(V) was transformed into MMA and DMA by microalgal biomass and activated sludge, respectively. It should be noted that a very small amount of As (III) was detected in the liquid fraction of the activated sludge experiments, which is an unusual transformation. The rare conversion of As(V) to As(III) could be explained as reported by *Meng et al.* by the entry of As(V) specie as arsenate into the cell via phosphate transporter channels through a competitive and metabolism-dependent process in which is reduced to As(III) via the As(V) reductase ArsC and subsequently excreted out of the cell via the ArsB pump (Meng et al., 2004). Finally, when DMA was the initial As species, no biotransformation was observed since the majority species detected was the organic species DMA and the presence of As(V) and MMA was insignificant for both biomasses. Although it should be noted that DMA (at initial concentration of 10 mg/L) has been transformed in a small proportion to As(III) in the experiments in which the biomass was composed by activated sludge. Therefore, it can be said that As(V) and DMA species have not undergone any redox or complexation reaction involving electron exchange. These results agree with those reported for other microalgae species by Mao et al., (2022). On the other hand, a possible explanation of the low detection of MMA species in some of cases analyzed is probably due to the fact that the conversion to the dimethylated species is very fast and this intermediate is not detected as a monomethylated species (Papry et al., 2021).

6.3.5 Effect of As species on biomass cell wall

Infrared spectra of the initial biomass and the biomass after the biosorption of the different arsenic species were analysed to elucidate the interactions between the As species with the different functional groups present in the cell wall of the microorganisms, as well as their possible transformations. FTIR spectra of the microalgae *S. almeriensis* before and after the addition of the arsenic species are shown in **Figure 6.5.** As can be observed**,** the most notable frequencies are those generated by the broad band at 3280 cm^{-1} and the steep band at 1663 cm^{-1}, corresponding to the stress vibrations of the O-H and C=O bonds, respectively. These signals reveal the presence of acidic groups ($R\text{-}CO_2H$) and most probably alcohols (R-OH) on the microalgal biomass surface, which can be mistaken for conjugated ketones (present at frequencies slightly below 1700 cm^{-1}). In addition, because the broad O-H signal is not very intense and is obtained very close to the signals in the proximity of 3000 cm^{-1} (corresponding to saturated and unsaturated C-H), it is difficult to glimpse and confirm the presence of amide or amine groups, since they have this form of doublet and are quite frequent in this type of microalgae. There are also very intense bands characteristic of the harmonics of aromatic groups (as benzenes) around 2000 cm^{-1}. It is also interesting to note the presence of a C=C voltage vibration band above 1539 cm^{-1}, which confirms the presence of unsaturated groups in this biomass and the unsaturated hydrogens present at frequencies above 3000 cm^{-1}. Finally, and within the fingerprint zone (1500 -1000 cm^{-1}), a series of intermediate intensity signals can be observed corresponding to vibrations of C-O bonds of the acid groups (1340 cm^{-1}) and to CH_2d type bonds (1464 cm^{-1}). However, among all the signals there is the strongest one around 1040 cm^{-1}, which, due to its shift in the spectrum, is most likely due to S=O or S-O vibrations caused by the presence of sulfoxide or sulfonate groups. Although, according to the literature, this band could also correspond to phosphate-type groups. All these bonds are possible by complex formation or ion exchange reactions and are possible at near neutral pH (Mandal et al., 2011) (Giri et al., 2013). This shift in FTIR spectra of microalgae indicated that amide, carboxyl, and hydroxyl functional groups were involved in the adsorption of arsenic by algae according to the study reported by Mao et al., (2022).

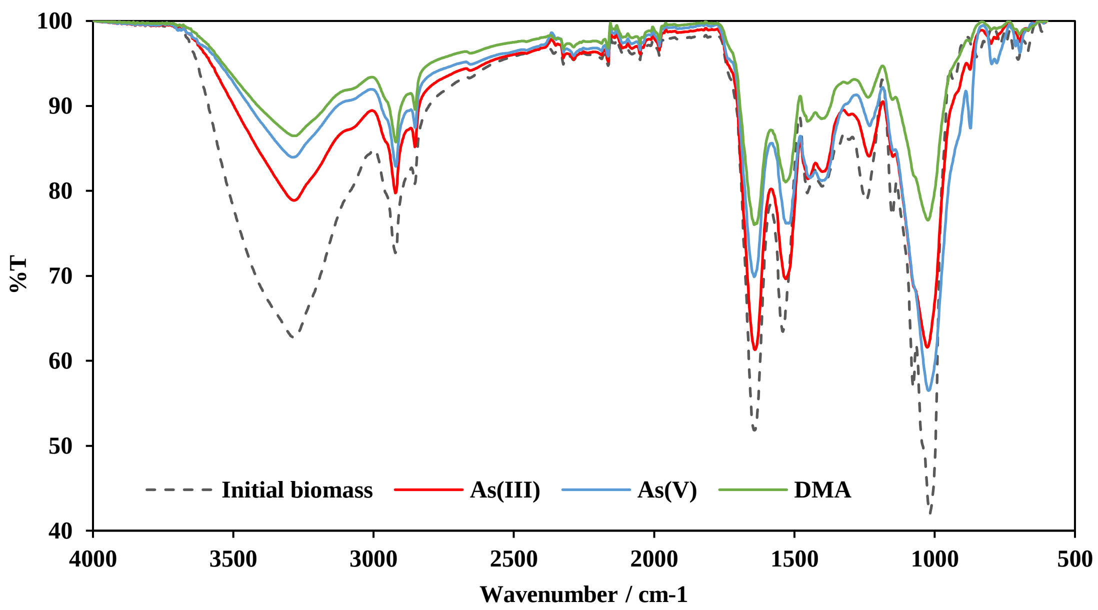

Figure ***6.5.*** FTIR spectra of microalgae *Scenedesmus almeriensis* (Initial biomass) and loaded with the arsenic species.

The FTIR spectra of biomass based on activated sludge, before and after arsenic addition, are presented in **Figure 6.6.** The most remarkable frequencies are those generated by the broad band at 3280 cm^{-1} and the steep band at 1636 cm^{-1}, corresponding to the stress vibrations of the O-H and C=O bonds, respectively. These signals reveal the presence of acid groups (R-CO_2H) and most probably alcohols (R-OH) on the biomass surface. Characteristic harmonics bands of aromatic groups (such as benzenes) around 2000 cm^{-1} are also noticeable. The presence of a C=C voltage vibration band, around 1540 cm^{-1}, is remarkable and confirms the presence of unsaturated groups in this biomass and the unsaturated hydrogens present at frequencies above 3000 cm^{-1}. Among all the signals, the most intense signal appears around 1029 cm^{-1}, most probably referred to S=O vibrations due to its displacement within the spectrum. In summary, and also in agreement with the available literature, it is found that both biomasses present practically the same functional groups on their surface and in a similar proportion. In other reported studies, FTIR and X-ray spectroscopy (XPS) revealed the interactions of inorganic arsenic species with the C-O-C, C-O-

H and $-NH_2$ functional groups of proteins, as binding sites between the cell membrane of the microorganisms constituting of the biomass and the metal species (Zhang et al., 2020).

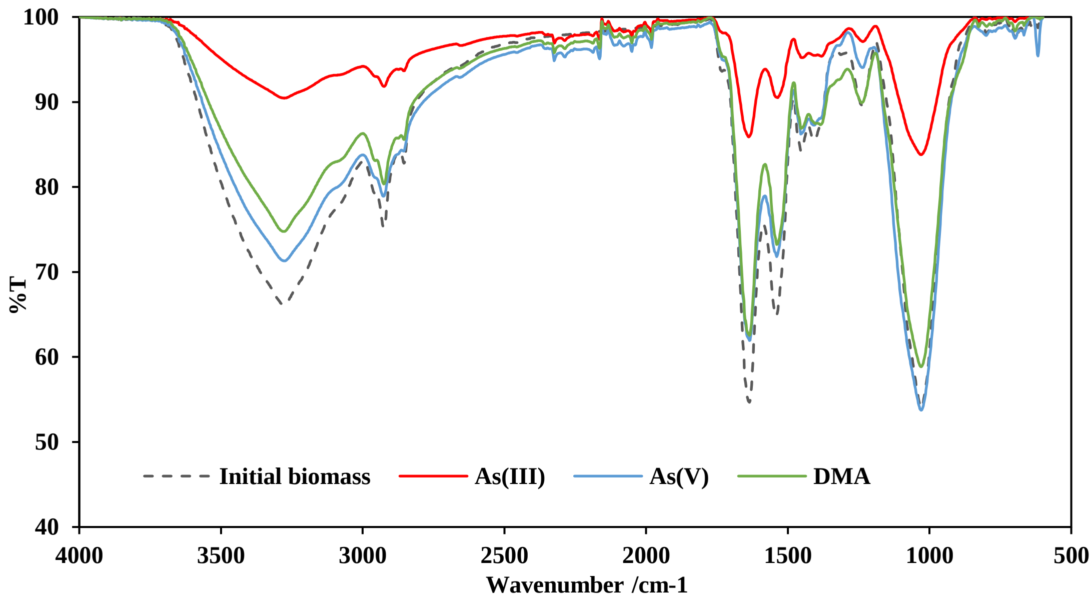

Figure 6.6. FTIR spectra of activated sludge (Initial biomass) and loaded biomass with the three arsenic species.

Finally, the morphology of the surfaces of both biomasses before and after metal biosorption was analyzed by ESEM-EDX, the corresponding images are shown in ***Figure 6.7***. As can be seen, the morphology of the two biomasses hardly undergoes any changes after the biosorption of the different arsenic species. ***Figure 6.7A1*** shows that the morphology of activated sludge is composed by circular and filamentous structures, which could correspond to different bacterial strains present in the biomass. This morphology hardly changes after the biosorption of the different arsenic species (***Figures 6.7A2-A4***). However, due to the mixture of bacteria, sludge and other components present in the biomass, a change in the surface structure may not be visible. On the other hand, the initial microalgal biomass presents a structure of small almond-shaped striated globules (***Figure 6.7B1***). After biosorption, the biomass acquires a structure in which the component cells appear in a much more aggregated form than in the initial biomass as can be observed in ***Figures 6.7B2-B4***. These results are in agreement with those reported in literature, which confirm, by ESEM images of freeze-dried biomass, that no cell disruption occurs during the biosorption of arsenic species, thus preserving cell structure and constituents (Chen et al., 2015; Hosseinizand et al., 2018).

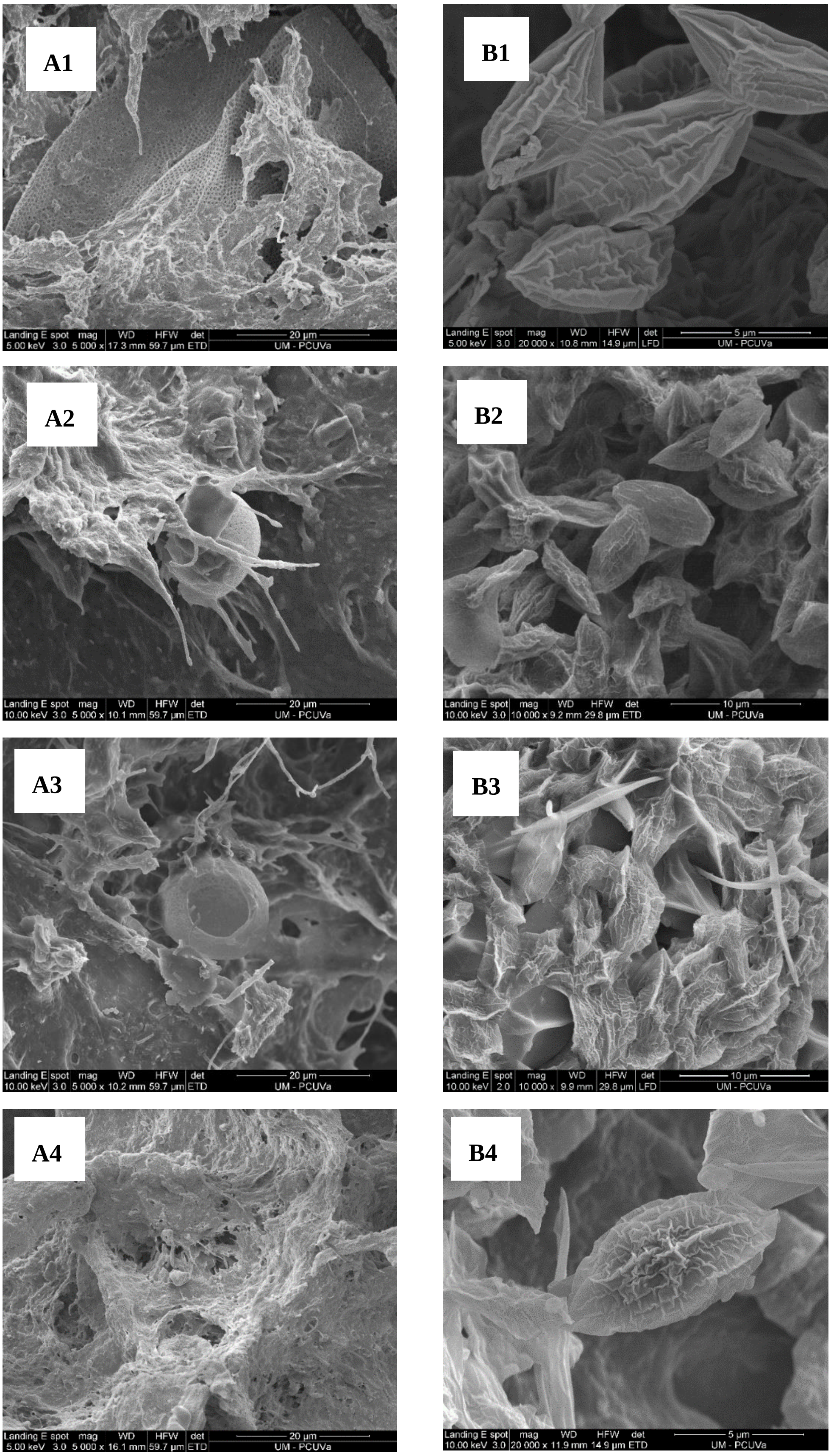

Figure 6.7. ESEM images of freeze-dried solid samples of activated sludge (A) and *Scenedesmus almeriensis* (B): before biosorption (A1, B1) and after biosorption of As(III) (A2, B2), As(V) (A3, B3) and DMA (A4,B4).

6.4 Conclusions and future perspectives

Green microalgae *S. almeriensis* has demonstrated a good ability for bioelimination of low concentrations (0.10 mg/L) of the organic As species DMA, with removal efficiencies of 80%, but poorer performance towards inorganic As species. The presence of As species in the culture media does not restrain biomass growth. In contrast, activated sludge is much more efficient at removing moderate concentrations (0.50 mg/L) of inorganic As species within the first 24-72 hours, being able to bioeliminate about 80% of the inorganic species, As(III) and As(V). For longer contact times, the toxicity of the inorganic As species inhibits biomass growth and the retained As species are excreted back into the solution. Through speciation studies, As(III) appears to be biotransformed into less toxic As(V) and DMA during the biosorption of As species by the biomass formed by activated sludge. The maximum bioelimination percentages were obtained for the inorganic species by the activated sludge at a contact time of 1 hour while for the microalgae, the maximum elimination percentages were obtained for the organic species DMA at a contact time of 10 days. In view of the results obtained, it can be affirmed that this type of microorganisms are promising in terms of the bioelimination of these Toxic Trace Elements (TTE), opening up the possibility of using microalgae grown in wastewater to test the effectiveness of microalgae-bacteria consortia and their subsequent elution for the biomass valorization processes and the applications of the obtained bioproducts applying the biorefinery and circular economy concepts.

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Chapter 7

Effect of organic matter in toxic trace metal and nutrient removal by Scenedesmus almeriensis and activated sludge

Effect of organic matter in toxic trace metal and nutrient removal by *Scenedesmus almeriensis* and activated sludge

Abstract

The study investigated the removal efficiency (RE) and uptake capacities (q) of four toxic trace elements (TTE), cadmium (Cd), lead (Pb), mercury (Hg), and uranium (U) by the microalga *Scenedesmus almeriensis* and activated sludge. Results indicate that S. *almeriensis* exhibited high elimination percentages exceeding 70% for all TTE within the first hour of contact time. However, there were fluctuations in removal efficiencies over time, particularly for U(VI). Cd showed a maximum removal efficiency of 97% in 24 h of contact time, while Pb and Hg reached 98% and 99% removal efficiencies respectively by day 6 of contact time. U showed a removal efficiency of 96% at 8 hours of contact time. Conversely, activated sludge showed consistent higher elimination percentages exceeding 95% for all TTE throughout the contact time. Total organic carbon (TOC) removal was significant in both *S. almeriensis* and activated sludge, reaching maximum values of 92% and 88% respectively for Pb and U. However, total nitrogen (TN) removal was lower, with negative removals observed from day 10 for activated sludge. Biomass growth analysis revealed a decrease in biomass from day 6 for *S. almeriensis* and from day 3 for activated sludge, particularly in tests with higher TTE concentrations.

Keywords: *Activated sludge, biosorption, heavy metal, microalgae, wastewater, toxic trace elements (TTE)*

7.1 Introduction

Heavy metals are considered a group of metals, metalloids and transition metals with a density between 4 and 9 g/cm^3 and a molecular mass of between 63.55 and 200.59 g/mol (Leong and Chang, 2020). Cadmium (Cd), Lead (Pb), Mercury (Hg) and Uranium (U) are considered toxic heavy metal in terms of environmental regulation by the USEPA. Cd, Pb, Hg are trace elements incorporated into pig feed through trace impurities in feed. U(VI) is a geogenic trace element naturally present in surface and groundwater in Castilla y León and can be incorporated into the diet of animals through drinking water. Exposure to high levels of these toxic trace metals (TTE) may have adverse effects on human health and the environment. The source of these TTE as well as its possible adverse effects on human health are described in **Table 7.1**.

Table 7.1. Source of Cd, Pb, Hg and U in drinking water and its possible adverse effects on human health

Metal	Source	Potential health effects from long term exposure in human	Common sources of contaminant in drinking water
Cd(II)	Fertilizers, mining, pesticides, plastic industry, refining, welding	*Kidney damage*	*Corrosion of galvanized pipes; erosion of natural deposits; discharge from metal refineries; runoff from waste batteries and paints*
Pb(II)	Coal combustion, electroplating, battery manufacturing, mining, pigments, paints, etc.	*Infants and children: Delays in physical or mental development; children could show slight deficits in attention span and learning abilities; Adults: Kidney problems; high blood pressure*	*Corrosion of household plumbing systems, erosion of natural deposits*
Hg (II)	Earth's crust, volcanic activity, fossil fuels, thermoelectric power plants, mining	*Kidney damage*	Erosion of natural deposits; discharge from refineries and factories runoff from landfills and croplands
U(VI)	Soil, sediments.	*Increased risk of cancer, kidney toxicity*	*Erosion of natural deposits*

Due to the possibility of this trace metal incorporation into the food chain, the control of food, water and soils is currently being closely monitored to prevent any type of toxin from entering the food chains and indirectly affecting humans. (Alam and McPhedran, 2019). Different national and international organizations impose limits for discharge water, inland water and water for human consumption (**Table 7.2**).

Table 7.2. Maximum contaminant level in mg/g for heavy metals according to (Ministerio de Agricultura Alimentación y Medio Ambiente (2015) and USEPA

Metal	**DMA (mg/L)** (Ministerio de Agricultura Alimentación y Medio ambiente, 2015)	**MCL (mg/L)** (U.S. EPA, 2009)
Cd(II)	0.005	0.005
Pb(II)	0.005	0.015
Hg (II)	0.001	0.002
U(VI)	0.030	0.03

Given the urgent need to establish a solution to this emerging global problem, the treatment of these wastewaters with living microorganisms such as microalgae, bacteria or its consortia, has attracted a great deal of attention due to its high effectiveness and their ability to interact with metal ions (Zoroufchi Benis et al., 2020). With the possibility of the recovery of the resulting biomass after a clean-up process in terms of circular economy (Bădescu et al., 2018).

Microalgae and bacteria-based technologies are cost-effective, have a high growth rate, are cheap for the value, do not generate toxic residues and the biomass grown can be valorized due to its high protein and lipid content. Moreover, microalgae and bacteria have several mechanisms by which biosorption of different metal species occurs, such as physical adsorption, complexation, coordination, microprecipitation, ion exchange or a combination of these. (Sahmoune, 2016). These processes occur due to the existence of numerous functional groups in the cell wall such as carboxyl, hydroxyl, amino groups, amides, thiols, etc. (Jebelli et al., 2017); (Asere et al., 2019). Several studies reported toxic trace element removal by microorganisms. This is the case of (Gu and Lan, 2021a), that reported maximum values for biosorption capacities of Cd, Pb and Hg of 73.1, 213 and 183 mg/g by *Neochloris oleoabundans* for a contact time of 30 min. On the other hand, (Vogel et al., 2010) studied uranium (VI) biosorption by the green microalgae *Chlorella vulgaris* varying uranium concentrations from 5 µM to 1 mM, and in the environmentally relevant pH range from 4.4 to 7.0. Living cells uptake in a 0.1 mM uranium solution at pH 4.4 was 14.3 mg U/g dry biomass in a contact time of 5 minutes. Dead cells reached a biosorption uptake of 28.3 mg U/g dry biomass. This corresponds to 45% and 90% of total uranium in solution, respectively. Zheng et al., (2018) studied the bioelimination viability of U(VI) by *Saccharomyces cerevisiae* in a biomass dose of 1g/L, 250 rpm, 30ºC and initial concentration of U(VI) of 10 mg/L. A maximum biosorption of 66% was achieved at pH 5.5. Above this pH, biosorption capacity drops sharply. In the work of Cheng et al., (2023) *Ankistrodsemus* sp. has been used to treat the uranium-contaminated water, and more than 98% of uranium in the solution can be removed by the alga, when the initial uranium concentration ranges from 10 to 80 mg/L When the initial uranium concentration is 10, 20, 40 and 80 mg/L, the maximum uranium removal efficiency of *Ankistrodsemus* sp. will reach 99.2%, 98.3%, 98.2% and 90.9% respectively at 96 h. Uranium removal remains constant throughout the 4 days of contact, but from this day on, a decrease in the RE of this metal is observed, due to an inhibition of the growth of the biomass with the nutrients already consumed. However, combined heavy metal and nutrient removal is still nuclear and further studies are needed. In this work, the effect of time and initial metal concentration on the bioelimination of Cd, Pb, Hg and U in a synthetic wastewater with moderate content of nutrients such as organic carbon, nitrogen and organic matter has been studied. In addition, an isotherm study was carried out using Langmuir and Freundlich models in order to estimate the maximum adsorption capacity of these metal species for these two types of biomasses, and the simultaneous removal of heavy metals and nutrients from the medium was evaluated.

7.2 Materials and methods

All the reagents described in the different experiments were of analytical grade and the solutions prepared using deionized (ultrapure) water. All reagent to prepare Cd(II), Pb(II), Hg(II) and U(VI) solutions and to make up synthetic wastewater, $CdCl_2$, $PbNO_3$, $HgCl_2$, UO_2NO_3, KNO_3, $CaCl_2 \cdot 2H_2O$, NaCl, $MgSO_4 \cdot 7H_2O$, peptone, meat extract were purchased from Sigma Aldrich, Germany. NaOH to pH adjustment was from Panreac. All plastic and glass containers were washed in dilute HNO_3 (10% v/v) for 24 h and rinsed 3 times with Milli-Q water (R > 18 MΩ cm) before use.

7.2.1 Inoculum and synthetic wastewater

Monocultures of S. *almeriensis* microalgae in a synthetic culture medium were provided by the Department of Chemical Engineering of University of Almeria (Spain) and maintained in home-made Bristol freshwater medium at 21-23 ºC under continuous agitation, applying an LED irradiance of 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a 12-hour 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 4ºC 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.

All tests were performed in a nutrient rich synthetic wastewater to simulate the typical composition of piggery wastewater in the different adsorption and biosorption experiments. The concentration of the components of the synthetic wastewater in the bottles, as 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).

7.2.2 Adsorption isotherms

To estimate maximum biosorption capacities, it is necessary to know the amount of metal adsorbed as a function of the metal concentration in solution. For this purpose, two different adsorption isotherm models, Langmuir and Freundlich, were applied. Fowler-Langmuir and Freundlich Isotherm model were performed according to Equations 3.4-3.9 of Materials and Methods section. The biosorption of the 4 TTE was studied for a contact time of 24 and 72 hours at an adjusted pH of 7.5 in order to simulate the pH and residence time conditions used in this type of bioreactors (Posadas et al., 2015) (Acién et al., 2012) (Gola et al., 2020), at a temperature of 21-23°C, with 12:12 h light and dark periods at a light intensity of 1200 µE $m^{-2}s^{-1}$. for different initial concentrations of the different metals, from 1 ppm to 200 ppm at an agitation speed of 250 rpm. The experimental procedure is detailed below and is identical for the 2 biomasses used. First, the different standard solutions of Cd(II), Pb(II), Hg(II) and U(VI) of 1000 ppm for each were prepared from the corresponding salts indicated in the previous section, from which the volume necessary to prepare each of the suspensions at the different initial metal concentrations was taken. Next, the necessary amount of microalgae or sludge is added to a 500 mL glass bottle according to the percentage of total dry matter it contains to obtain a final concentration of 2 and 4 g/L of dry biomass respectively on a dry matter basis up to a final volume of 250 mL. 1, 10, 25, 50, 100 and 200 ppm solutions were prepared respectively. Once all the suspensions are prepared, the pH of all the suspensions is adjusted to 7.5 using a NaOH solution. Once the time has elapsed, 15 mL of the suspensions are taken in Falcon tubes, centrifuged for 10 min at 7000 rpm using the SIGMA Fisher Bioblock Scientific 2-16P Centrifuge and the supernatant liquid is filtered with 0.45 µm pore diameter filters. Finally, the filtrate is refrigerated at 4°C for subsequent determination of metals by ICP-MS.

The uptake of toxic metals by microalgae biomass was calculated in terms of the experimental uptake capacity (q) as follows in Equation 3.10 of Materials and Methods.

7.2.3 Metal and nutrient removal experiments

Cd, Pb, Hg, U bioremoval experiments were carried out batch-wise in borosilicate glass bottles of 500 mL capacity containing 250 mL of suspensions prepared as follows. The appropriate volumes of 1000 mg/L standard solutions of Cd(II), Pb(II), Hg(II) and U(VI) as well as synthetic wastewater were then added to achieve the desired concentration of each element, similar to the typical concentrations reported in piggery wastewaters (ASAE, 2003). In addition, the initial concentrations chosen are two and ten-fold the maximum concentration allowed in drinking water according to EPA (see **Table 7.3**). Finally, microalgae or bacteria inoculum was added to achieve a concentration of 2 and 4 g/L respectively, expressed on a dry matter basis.

Table 7.3. Initial concentrations for Cd(II), Pb(II), Hg(II) and U(VI) experiments according to maximum concentration allowed in drinking water (EPA).

Metal	2X (μg/L) C1	10X (μg/L) C2
Cd(II)	10	50
Pb(II)	10	50
Hg (II)	2	10
U(VI)	60	300

The pH of the resulting suspension was adjusted to 7.5 with 0.1 M NaOH (Panreac, Spain) to simulate the environmental conditions existing in photobioreactors (Posadas et al., 2015c); (Acién et al., 2012) and aerobic bioreactors (Gola et al., 2020) treating nutrient rich wastewaters. Finally, the total volume of the suspension was made up to 500 mL with deionized water. The bioreactors were kept at 25 ºC under continuous stirring at 250 rpm in a multi-point magnetic stirrer (ThermoFisher Scientific), irradiated with LED lamps with an irradiance of 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ arranged on top of the stirring plate and programmed to work in 12-hour photoperiod. Samples were taken al contact times of 1h, 8h, 1 day, 3 days, 6 days, 10 days and 15 days. After the contact time had elapsed, the suspensions were centrifuged at 7800 rpm for 10 min. Quantification of the metal content of the supernatant is performed by ICP-MS. The biosorption capacity of toxic metals by microalgae and activated sludge biomass was calculated in terms of the experimental uptake capacity (q) as follows in Equation 3.11 of Materials and Methods. On the other hand, metal and nutrient removal efficiency (RE) were calculated according to Equation 3.12 in Material and Methods section.

Biomass growth was determined gravimetrically by desiccation of a portion of the biomass in the oven at 105±1 ºC for at least 24 h until constant weight and referred to the total suspension.

7.2.4 Analytical methods

The process for heavy metals analysis, as well as TOC, TN and pH measurement, were performed collecting a 15 mL volume of suspension after the contact time had elapsed in BRs. Subsequently, the samples underwent centrifuged at 7800 rpm for 10 min, and then the supernatants were filtered using a 0.45 mm nylon membrane filter. Total organic carbon (TOC) and total nitrogen (TN) concentrations were measured using a TOC-V CSH analyzer equipped with a TNM-1 chemiluminescence module (Shimadzu, Kyoto, Japan). The concentration of Cd(II), Pb(II), Hg(II) and U(VI) was determined by inductively coupled plasma spectrometry coupled plasma source mass spectrometer (ICP-MS) in an octopolar reaction system (HP 7500c, Agilent, USA) employed according to the internal procedures of the Instrumental Techniques Laboratory of the University of Valladolid (LTI – UVa). For quality assurance, two reference water materials were included in the ICP-MS analysis as quality control (QC) samples: ICP multielement Calibration Standard Solution, 100 mg/L Scharlau (26 elements in HNO_3 5%), and a Certified Reference Material (Environment Canada TMDA-64.2 LOT 0313, HNO_3: 0.2%) as trace element fortified calibration standard. QC samples were measured every 10 samples, considering a range within 10% of the true value for valid acceptance. The removal efficiencies (%RE) of As were calculated according to Equation (2) and the uptake of As by the biomass (q) was calculated according to Equation (1). The samples of the different biomasses after biosorption of the different species at pH 7.5 were analyzed by FTIR in order to determine the interactions between the As species with the different functional groups present in the cell wall, as well as their possible transformations. The FTIR spectra were recorded between 4000-500 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}. High sensitivity DLATGS and RockSolidTM high stability interferometer, with a resolution of 1 cm^{-1} and using OPUS software. To observe the surface differences of the biomass before and after the bioelimination of the different TTE treatment, a dry and freeze-dried portion of each biomass was prepared before and after the biosorption of each metal species studied. The surface study by ESEM-EDX with an Environmental Scanning Electron Microscope (ESEM), model FEI - Quanta 200FEG with a secondary electron detector LFD low vacuum.

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 ºC for at least 24 h until constant weight. The biomass concentration after contact time of bioremoval experiments was obtained gravimetrically, determining the amount of dry biomass in a 25 mL aliquot of the suspension taken at the end of the contact period.

7.3 Results and discussion

7.3.1 Adsorption isotherms

The results of the isotherms that have been applied are shown in **Table 7.4**.

Table 7.4. Equilibrium adsorption parameters obtained using Langmuir and Freundlich models for Cd(II), Pb(II), Hg(II) and U(VI)

		Langmuir		Freundlich	
		q_{max} (mg/g)	K_L (L/mg)	Kf	n
$Cd(II)_{24h}$	*S. almeriensis*	*32.68*	0.281	*5.54*	*1.449*
	Activated sludge	*100.00*	0.101	*7.22*	*1.089*
$Pb(II)_{24h}$	*S. almeriensis*	11.10	0.052	0.02	0.913
	Activated sludge	79.37	0.02	1.57	0.984
$Hg(II)_{24h}$	*S. almeriensis*	81.30	0.011	9.15	1.271
	Activated sludge	43.29	0.531	14.82	1.109
$U(VI)_{24h}$	*S. almeriensis*	166.67	0.129	6.05	0.484
	Activated sludge	27.10	0.08	1.70	1.022
$Cd(II)_{72h}$	*S. almeriensis*	*53.19*	0.079	*4.20*	*1.072*
	Activated sludge	*88.50*	0.278	*17.25*	*1.375*
$Pb(II)_{72h}$	*S. almeriensis*	11.79	0.055	0.67	0.899
	Activated sludge	22.62	0.087	1.78	0.920
$Hg(II)_{72h}$	*S. almeriensis*	53.19	0.298	8.16	1.128
	Activated sludge	53.48	0.645	21.58	1.390
$U(VI)_{72h}$	*S. almeriensis*	270.27	0.018	2.39	0.641
	Activated sludge	14.16	3.548	5.80	1.693

In 24 h the best q_{max} for Cd and Pb in activated sludge, 100 and 79 mg/g and for Hg and U in *S. almeriensis*, 81 and 167 mg/g were obtained. At 72 hours, the highest q_{max} for Cd and Pb are still in activated sludge, 89 and 23 mg/g while for Hg it remains at 53 mg/g for both biomasses. Finally, the highest q_{max} for U is obtained for *S. almeriensis*, 270 mg/g. Regarding contact time, at 72 hours of contact time, in general, lower q_{max} values are obtained than for 24 hours of contact time, possibly due to a greater cell death of the microorganism as the contact time with the toxic elements has increased. Similar results were found in the literature. Tüzün et al., (2005) reached maximum biosorption capacity of 42.6, 96.3 and 72.2 mg/g for Cd(II), Pb(II) and Hg(II) onto *Chlamydomonas reinhardtii* for a contact time of 120 min and 0.8 g/L of biomass concentration in the case of monometallic experiments. Differences between the results obtained in the present work and those found in the literature are due to changes in the experimental conditions under which the investigations were carried out, e.g. biomass concentration, growth or death that the biomass has undergone during treatment.

For mixed metal systems, maximum capacities of 16.8, 61.5 and 36.7 mg/g were obtained respectively, reducing the biosorption of these metals by 61, 34 and 49% with respect to the monometallic system. On the other hand, effect of Pb(II), Hg(II) and Cd(II) on green alga *Neochloris oleoabundans* were investigated by Gu and Lan, (2021). Maximum biosorption capacities were 73, 213 and 183 mg/g. Adsorption capacity of *N. oleoabundans* biomass to the tested two-valence metal ions is proportional to the electronegativity and inversely proportional to the radius of the metal ions. Similar results were obtained for these toxic trace metals for $q_{max.}$ A compilation of maximum biosorption capacities found in the literature is shown in the following table (**Table 7.5**).

Table 7.5. Maximum biosorption capacities found in the literature for Cd(II), Pb(II), Hg(II) and U(VI) and its conditions

Metal	Microorganism	pH	Contact time (min)	Q_{max} (mmol/g)	Q_{max} (mg/g)	K_L L/mg	Reference
Cd(II)	*Sargassum sp.*	5.5		1.07	120.28	8.679	(Cruz et al., 2004)
	Sargassum sp.	4	-	0.0205	2.30	0.373	(Mahmood et al., 2017)
	Sargassum sp.	5		0.706	79.36	13.049	(Nessim et al., 2011)
	Codium vermilara	6	120	-	21.8	0.10	(Romera et al., 2007)
	Spirogyra insignis	6	120	-	22.9	0.12	
	Asparagpsis armata	6	120	-	32.3	0.09	
	Chondrus crispus	6	120	-	75.2	0.06	
	Ascophyllum nodosum	6	120	-	87.7	0.15	
	Fucus spiralis	6	120	-	114.9	0.11	
	Sargassum sp.	5	-	0.086	9.67	2.203	(Babu Rao et al., 2016b)
	Sargassum sp.	5	-	0.76	85.43	11.34	(Sheng et al., 2007)
	Chlorella Vulgaris		-	0.098	11.06	9.83	(Hockaday et al., 2022)
	Chlorella Vulgaris	7	-	1.33	149.9	0.013	(Edris et al., 2014)
	Scenedesmus olbliquus		-	0.22	24.72	0.42	(Hockaday et al., 2022)
Hg (II)	*Sargassum sp.*	3	-	0.435	87.25	2.206	(Vijayaraghavan and Joshi, 2012)
	Sargassum sp.	5	-	0.727	145.83	4.413	(Saravanan et al., 2010)
Pb(II)	*Sargassum sp.*	5	-	1.46	302.51	132.84	(Chen and Yang, 2005)
	Sargassum sp.	7	-	1.25	259	30.6	(Martins et al., 2006)
	Codium vermilara	6	120	-	63.3	0.11	(Romera et al., 2007)
	Spirogyra insignis	6	120	-	51.5	0.57	
	Asparagpsis armata	6	120	-	63.7	0.04	
	Chondrus crispus	6	120	-	204.1	0.01	
	Ascophyllum nodosum	6	120	-	178.6	0.09	
	Fucus spiralis	6	120	-	204.1	0.13	
	Sargassum sp.	5	-	0.574	118.93	4.144	(Nessim et al., 2011)
	Sargassum sp.	6	-	0.945	195.80	-	(Perumal et al., 2007)
	Sargassum sp.	5	-	1.16	240.35	14.23	(Sheng et al., 2004)
	Sargassum sp.	6	-	1.032	213.83	2.9	(Vijayaraghavan et al., 2009)
	Chlorella vulgaris	7	-	0.86	178.5	0.0009	(Edris et al., 2014)

7.3.2 TTE bioelimination

The TTE (toxic trace element) bioelimination results obtained are shown in the following figures, **Figure 7.1** (*S. almeriensis*) and **Figure 7.2** (Activated sludge)

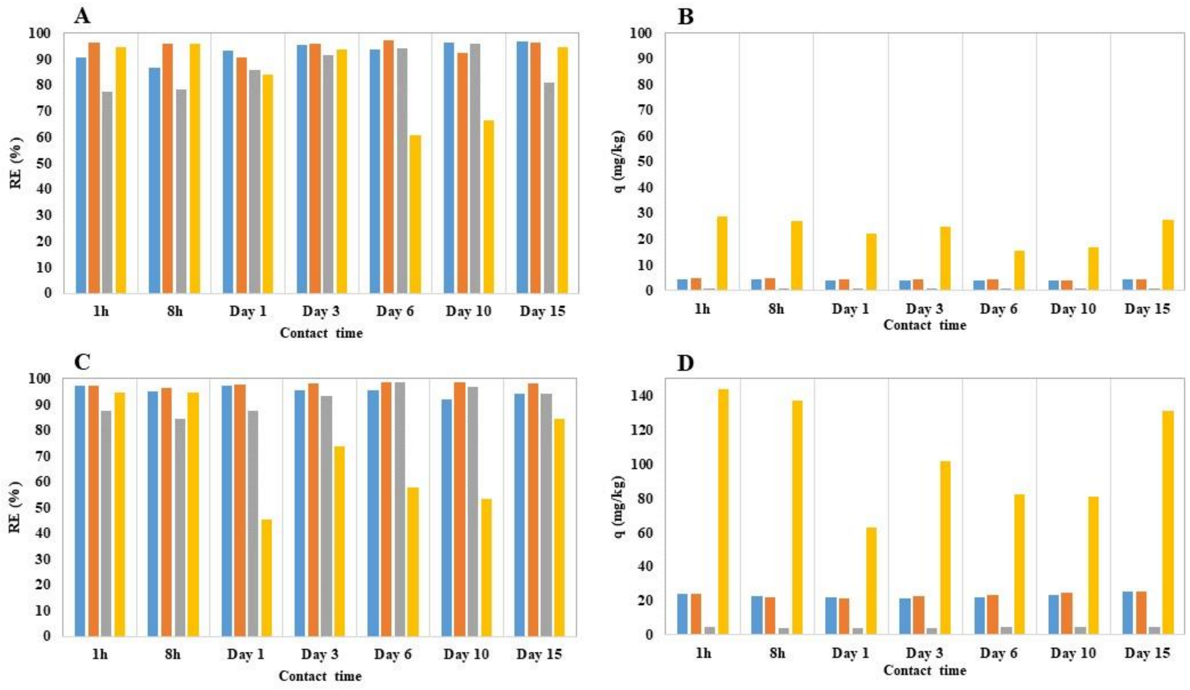

Figure 7.1. Metal removal efficiency (RE) and biosorption capacity (q) for microalgae-bioreactors doped with (■) Cd(II) (■) Pb(II), (■) Hg(II) and (■) U(VI) respectively for C1 (A and B) and C2 (C and D) metal concentrations tested

For the microalgae *S. almeriensis*, percentages of elimination of the four metals, above 70 % are obtained from the first hour of contact time. For C1 (low) concentrations only a significant decrease in the percentage of elimination is observed for U(VI), which recovers from day 10 to day 15 of the contact time. This decrease in the U removal occurs from day 6, while for C2 (high) concentration tests this decrease occurs on day 1. For Cd, a maximum removal efficiency of 97 % and an uptake capacity of 23 mg/kg is obtained at day 1, for Pb 98 % and uptake capacity of 25 mg/kg at day 6. For Hg 99 % of RE is obtained and 5mg/kg uptake capacity at day 6 is observed for C2 concentration. Finally, for U, 96% of RE and 143 mg/kg uptake capacity at 8h of contact time is obtained for C1 and C2 concentration tests respectively.

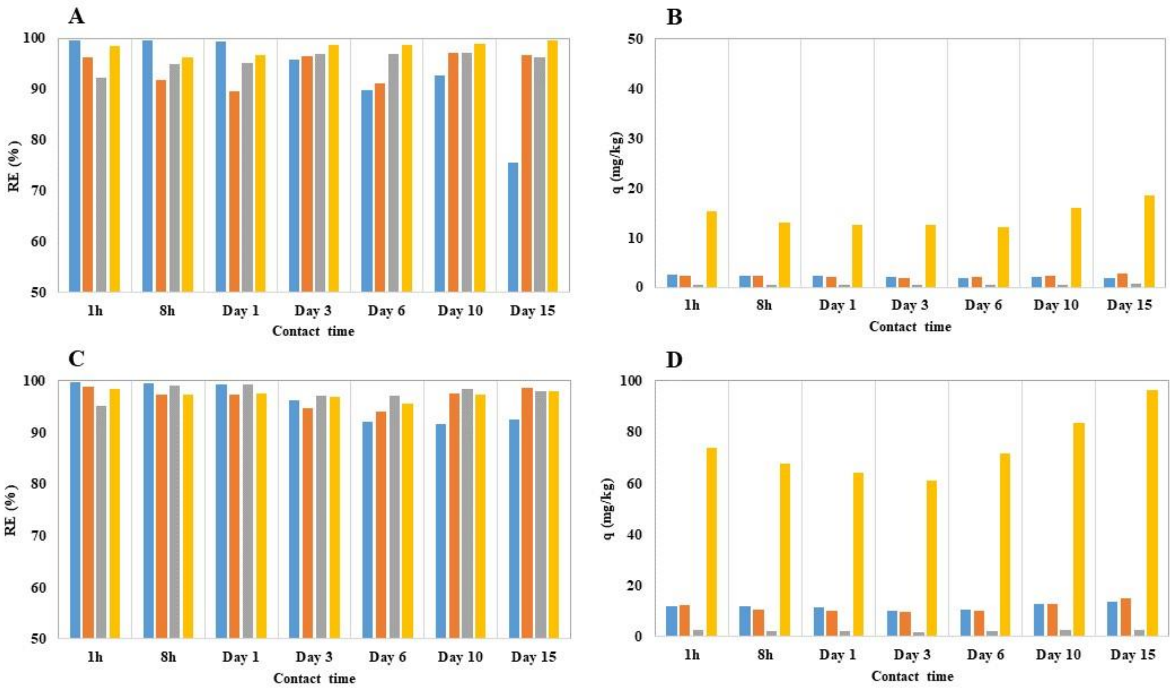

Figure 7.2. Metal removal efficiency (RE) and biosorption capacity (q) for bacteria-bioreactors doped with (■) Cd(II) (■) Pb(II), (■) Hg(II) and (■) U(VI) respectively for C1 (A and B) and C2 (C and D) metal concentrations

For Activated sludge, better results are obtained in general than for *S. almeriensis*. For all the TTE, elimination percentages higher than 95 % are obtained, some of them very close to 100%, which do not decrease throughout the days of contact time, except for cadmium at C1 concentrations of the metal speice. As for the uptake capacities (q), maximum values of 13, 15, 2.8 and 96 mg/kg were obtained for Cd, Pb, Hg and U respectively. According to similar results from literature, Torres et al., (2017a) performed experiments with similar metal concentration 1X (0.015, 0.054 and 0.01 mg/L), 10X (0.15, 0.54 and 0.1 mg/L) and 40 X (0.6, 2.16 and 0.4 mg/L) for Cd, Pb and Hg respectively with *Nannochloropsis salina*. Hg sorption was not tested based on results from other researchers indicating that minimal amounts of Hg remain in the biomass or media due to volatilization (Napan et al., 2015). In general, the microalgae were able to sorb a higher mass of contaminant ions with higher initial contaminant concentrations. The average Cd sorption percentage pointed out by the authors was 75%, 111%, and 112% at the 1X, 10X, and 40X concentrations, respectively. The average sorption percentages by Pb at the 1X, 10X, and 40X concentrations were 86%, 102%, and 96%.

For U(VI) biosorption tests, was hard to find comparable studies regarding initial metal concentrations. Zheng et al., (2018) tested Saccharomyces cerevisiae for U biosorption. At pH 2.4, only 32.6% of U(VI) was removed by S. cerevisiae. Between pH 2.4 and pH 6.0, uranium biosorption increased quickly. With the increase in pH, the number of positive potential adsorption sites that compete with uranyl ions on the cell surface decreased. The biosorption efficiency reached a peak at 66.3% at pH 5.5 for an initial concentration of 10 mg/L, 1g/L of biomass and 4 h of contact time. A compilation of metal uptake and RE (removal efficiency) can be seen in **Table 7.6** for Cd(II), Pb(II), Hg(II) and U(VI) for different microorganisms.

Table 7.6. Performance of Cd, Pb, Hg and U biosorption by different microorganisms

Metal specie	Microorganisms	pH	Initial metal conc.	Biomass conc.	T	Contact time	Uptake (RE)	References
			mg/L	g/L	ºC	h	mg/g (%)	
Cd(II)	*Scenedesmus quadricauda*	5	10-50	0.20	rt	1	135.1	(Mirghaffari et al., 2014)
	Chlamydomonas sp.	7.5	-	1	30	1	23.3	(Zheng et al., 2016)
	Chlorella sp.	7.5	-	1	30	1	25.5	
	Coelastrum sp.	7.5	-	1	30	1	32.8	
	Chlorella minutissima	6	-	4	28	20 min	303	(Yang et al., 2015b)
	Chlorella sp.	6	10	1.3	-	-	15.51 (93)	(Shen et al., 2018)
	Neochloris oleoabundans	7	-	0.3	25	30 min	73.1	(Gu and Lan, 2021a)
	C. reinhardtii	7	1	-	25	6	(90)	(Piña-Olavide et al., 2020)
	PRO2	7	1	-	25	6	(70)	
	FACHB-12	6	3	0.8	rt	2	60 (62)	(Ma et al., 2021)
	FACHN-12 biofilm with luffa sponge	6	3	0.8	rt	2	79 (73)	
	FACHN-12 biofilm with K3	6	3	1-2	rt	2	133 (93)	(Wang et al., 2021)
	Didymogenes palatina XR	6	2	1-2	-	-	7.41 (88)	
Pb(II)	*Neochloris oleoabundans*	7	-	0.3	25	30 min	213.4	(Gu and Lan, 2021a)
	Nannochloropsis oculata	-	0.5	cell density 1×105 cell/mL	rt	7 days	(28)	(Waluyo et al., 2020)
	Nannochloropsis oculata	-	0.7	cell density 1×105 cell/mL	rt	7 days	(29)	
	Nannochloropsis oculata	-	0.9	cell density 1×105 cell/mL	rt	7 days	(38)	
	Nannochloropsis oculata	-	1.1	cell density 1×105 cell/mL	rt	7 days	(48)	
	Nannochloropsis oculata	-	1.3	cell density 1×105 cell/mL	rt	7 days	(55)	
	Aphanothece sp.	-	18.6	-		30 min	186 (99)	(Keryanti and Mulyono, 2021)
Hg(II)	*Neochloris oleoabundans*	7	-	0.3	25	30 min	182.5	(Gu and Lan, 2021a)
	Scenedesmus sp.	6.9	0.007		25	20 days	(64)	(Vela-García et al., 2019)
	Chlorella sp.	6.9	0.007		25	20 days	(83)	
	Pleurococcus sp.	6.9	0.007		25	20 days	(86)	
	Consorcio	6.9	0.007		25	20 days	(81)	
	M1-RD	4	1		30	2	(96.7)	(Peng et al., 2018)
	M2-RD	4	1		30	2	(91.1)	
	M3-RD	4	1		30	2	(84.4)	
	Native biomass	5	1		30	2	(48.5)	
	RD: unmodified residual microalgae	5	1		30	2	(57.6)	
U(VI)	*Chlorella vulgaris**	4.4	23.8	0.76	-	0.08	14.3	(Vogel et al., 2010)
	C. vulgaris	4.4	23.8	0.76	-	96	26.6	(Vogel et al., 2010)

7.3.3 TOC and TN removal

The nutrient (TOC and TN) removal results in the presence of TTE (toxic trace element) are shown in the following figures, **Figure 7.3** (*S. almeriensis*) and **Figure 7.4** (Activated sludge)

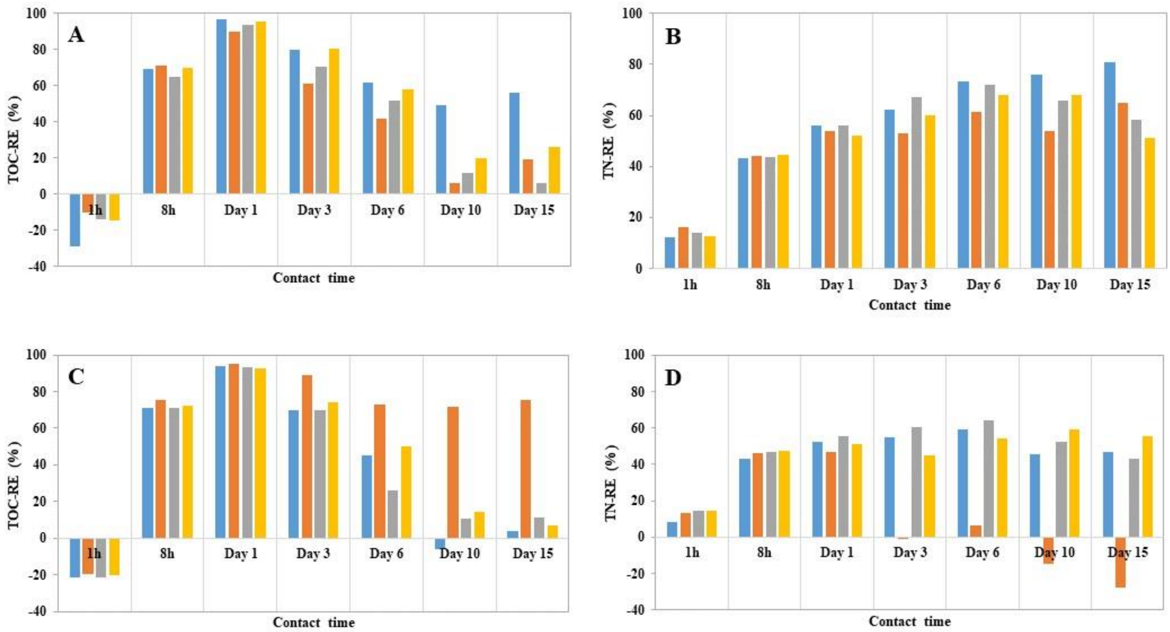

Figure 7.3. Nutrient removal efficiency for *microalgae*-bioreactors doped with (■) Cd(II) (■) Pb(II), (■) Hg(II) and (■) U(VI) respectively for C1 (A and B) and C2 (C and D) metal concentrations tests

For S. *almeriensis*, TOC elimination is progressive throughout the contact time until day 1, where eliminations greater than 90 % are reached in the four tests, and from which elimination decreases for both C1 and C2 concentration tests. Furthermore, the elimination of TN is progressive and constant throughout contact time, and there is no decrease as in the case of TOC-RE for experiments with C1 concentrations. Total nitrogen removal for experiments with higher concentrations (C2) is lower, not exceeding 60% TN removal for any of the metals. For C2 from day 10 of contact time, there is a negative TN removal of -15 and -28% for days 10 and 15 respectively, which is explained by the degradation of the biomass, dissolving EPS in the medium, and showing the non-viability of the biomass from day 10 of contact time. In addition, in the TOC elimination at contact time, a dissolution in the medium of organic matter is observed, giving rise to negative percentages of TOC elimination. The results of nutrient removal for Activated sludge are shown below (**Figure 7.4**).

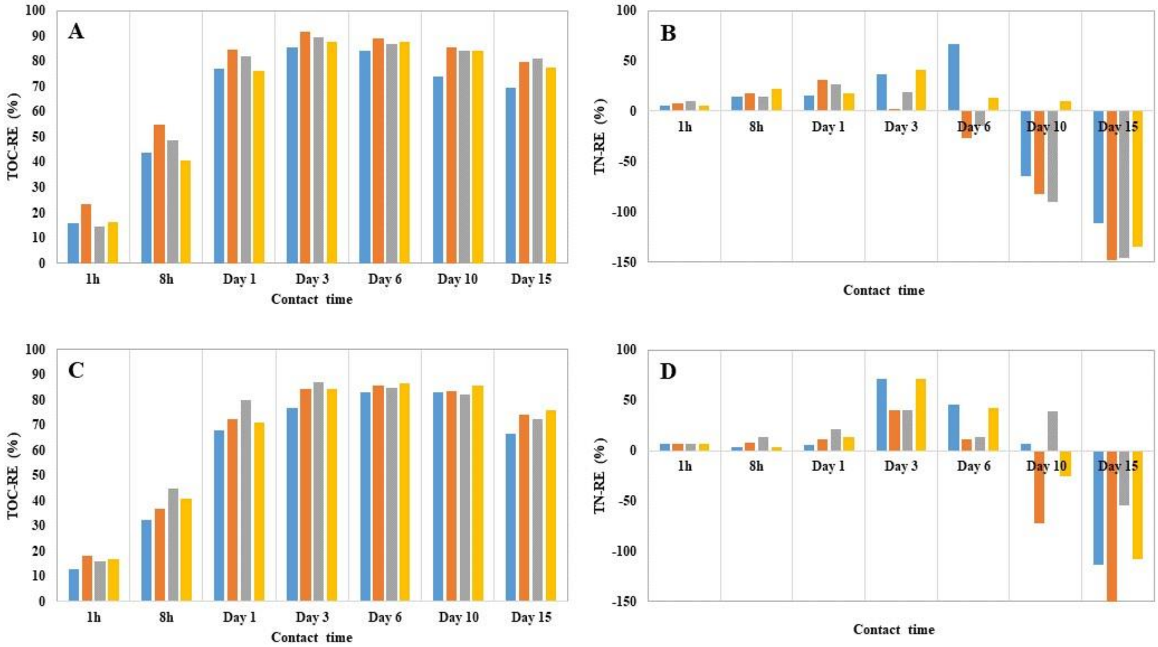

Figure 7.4. Nutrient removal efficiency for *bacteria*-bioreactors doped (■) Cd(II) (■) Pb(II), (■) Hg(II) and (■) U(VI) respectively for C1 (A and B) and C2 (C and D) concentrations

As for the activated sludge, a pronounced increase in total organic carbon removal is observed for both C1 and C2 concentration tests, from the 1h to day three of contact time. Thus, the maximum TOC-RE is reached from day three and remains constant throughout the contact time, reaching maximum TOC removals of 85, 92, 89 and 88% of for Cd, Pb, Hg and U, respectively.

On the contrary for TN, since for the activated sludge much lower eliminations of TN are achieved than for the microalgae *S. almeriensis* and from day 10 of contact time for both C1 and C2 concentration tests, negative eliminations of total nitrogen were determined, demonstrating a destruction or degradation of the biomass, reflecting the non-viability of this biomass in the conditions in which it has been tested and in the presence of these TTE at least from day 6 of contact time. These results will also be compared with the growth of the biomass, in terms of biomass concentration that has been achieved throughout the contact times. In addition, nutrient depletion in the medium, as shown in **Figures 7.3** and **7.4**, may be another reason why biomass stops growing from day 7-10 of contact.

Similar results can be found in literature. According to Barreiro Vescovo (2019), during the first 24 h, cultures containing activated sludge bacteria exhibited the best COD removal, with no differences between the different initial ratios of microalgae to bacteria. Percent reductions between 77.4% and 88.5% were achieved, corresponding to removals between 101.8-117.3 mg COD /L·day. In microalgae culture, there was a COD reduction of 32.2% after 24 h of incubation.

During the following days, there was an increase in COD concentration, and it reached a concentration higher than the initial concentration at the end of the test (154 vs. 132.3 mg/L initial COD), resulting in a negative removal rate (-16.4 %) as in the present study. The increase in the concentration of organic components in the culture may be due to the production of extracellular polymeric substances (EPS) by the microalgae.

7.3.4 Biomass viability

The biomass growth in terms of biomass concentration in the presence of TTE (toxic trace element) are shown in the following **Figure 7.5** for microalgae *S. almeriensis* and activated sludge.

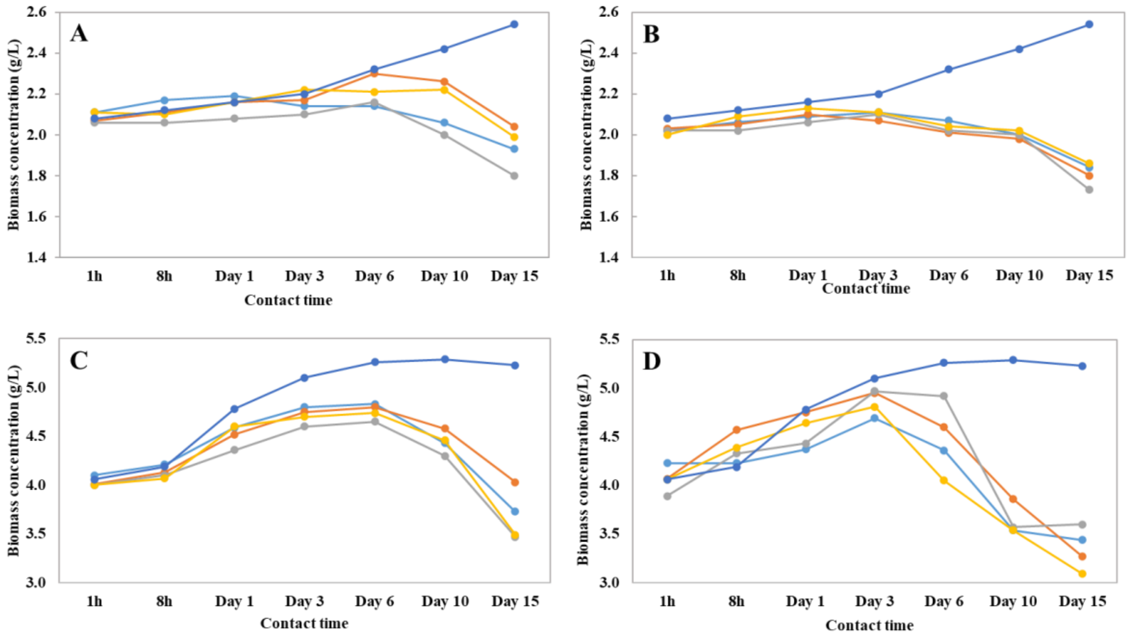

Figure 7.5. Biomass growth in control bioreactors () and spiked with Cd(II) (), Pb(II) (), Hg(II) () and U(VI) () respectively for microalgae (A and B) and bacteria-bioreactors (C and D) and for C1 (A and C) and C2 (B and D) concentrations tests

While for the control microalgae culture, a constant linear growth can be observed throughout the contact time, for the tests performed with the toxic trace metals, a linear growth similar to that of the microalgae control culture is observed until day 6 in the case of C1 concentration tests, after which the biomass concentration began to decrease. For the experiments with a C2 metal concentration this decrease in biomass concentration started earlier, from day 3 of contact time. In the case of activated sludge, an exponential growth of biomass was observed from day 1, which stabilized from day 6 onwards. In the case of the tests with C1 metal concentration, the growth profile of the cultures was similar to that of the control culture but with biomass concentrations below this. In the case of the tests with TTE in C2 concentration, the pattern was not so similar to that of the control, and a sharp decrease in biomass concentration was observed from day 6 onwards, due to the toxicity of these species.

Torres et al., (2017b) observed that Hg and Cd had the least impact on growth at all tested conditions, the average growth with Hg at the 1X concentration was 95.1% and only decreased to 89.8% at the 40X concentration. Similarly, average growth in the presence of Cd at the 1X concentration was 94.2% compared to 88.6% at 40X. The average growth in the presence of 1X Pb under all three light levels was 90.5% compared to 84.1% at the 40X concentration. The effects of Pb on microalgal cells have been shown to drastically change mitochondrial structure, nuclear content and cell volume reduction. Thus, for the present work is supported the fact that from day 3 of contact time, the viability of these cultures, both microalgae and activated sludge, is compromised by the presence of these TTE. On the other hand, it should be noted that the metabolic activity of these microorganisms is maintained until at least 72 hours of contact time, which is important to take into account when planning larger-scale experiments.

7.3.5 Effect of TTE on biomass

Both biomasses show a similar biosorption pattern of the TTE, thus, indicating that similar functional groups are involved in the retention of heavy metals on the cell surface of microalgae and bacteria. 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 **Figure 7.6** and **Table 7.7** and **7.8** for microalgae and activated sludge respectively. Once the metal is biosorbed, a significant decrease in band intensity is observed. This can be explained by the fact that metals have a large mass compared to common atoms in biomolecules (C, O, H, N, S…) and their incorporation into the membrane leads to a reduction in the vibration frequency of the membrane functional groups. Substituted groups, resulting in significantly lower signal intensity compared to unsubstituted groups. For both biomasses, a broad absorption band is observed between 3278-3282 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 2920-2926 cm^{-1} correspond to vibrations of aliphatic C-H bonds. The intense absorption bands between 1635-1643 cm^{-1} and 1537-1546 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 1384-1455 cm^{-1} are due to a weaker, symmetric type of vibration of C-O bonds of carboxyl groups. In the 1238-1242 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 1018-1151 cm^{-1} can be assigned to C-O bonds of alcohols and C-N and P-O bonds. These results suggest that new bonds are formed during metal biosorption that displace lighter ions or molecules, resulting in the vibrations observed in the raw or pristine biomass (**Table 7.7** and **7.8**).

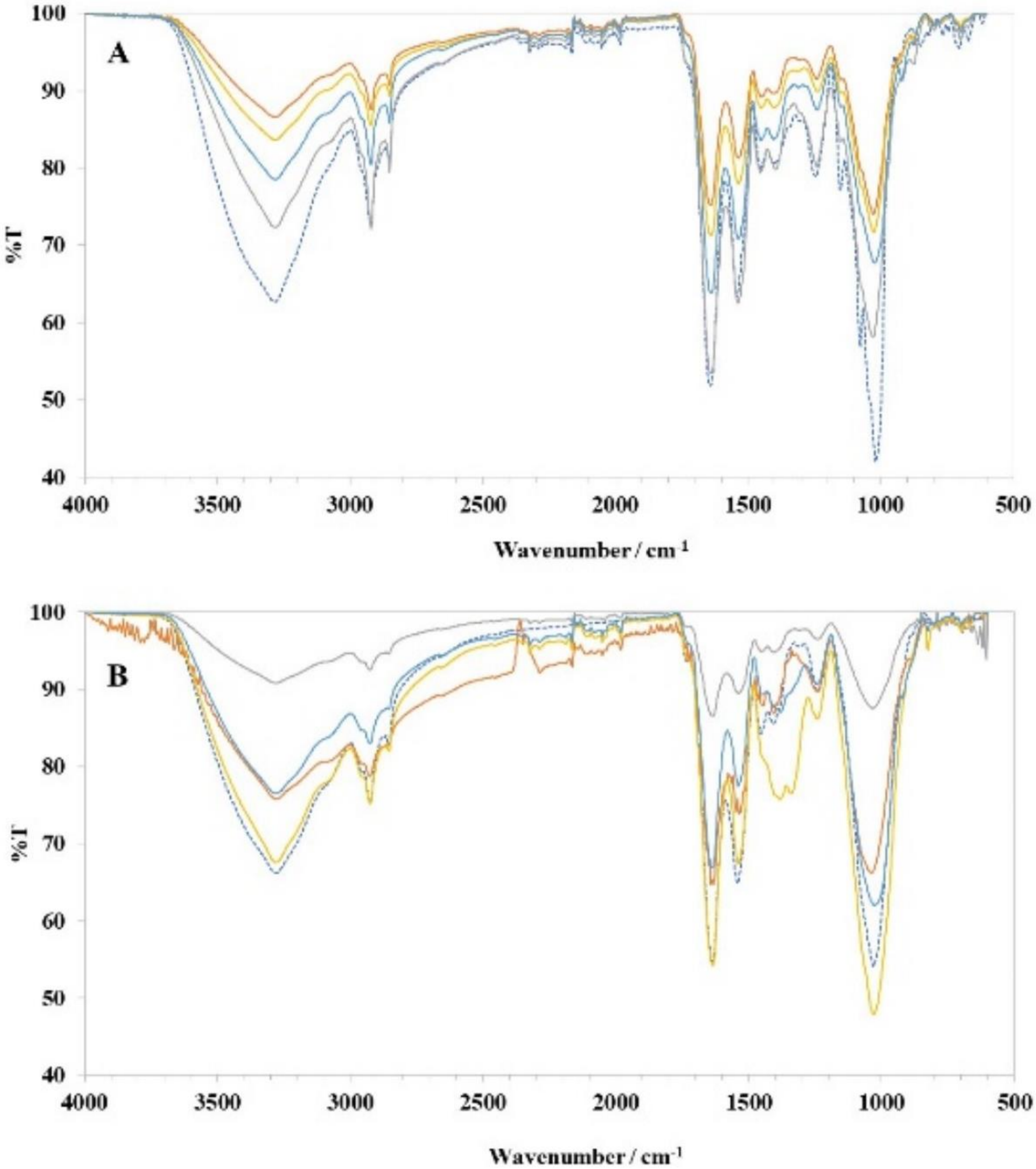

Figure 7.6. FTIR spectra of native and metal sorbed biomass for green microalga (A) *Scenedesmus almeriensis* and activated sludge (B). (----) Native biomass (——) Cd loaded (——) Pb loaded (——) Hg loaded (——) and U loaded biomass.

Similarly, the observation of shifted wavenumbers in certain bands suggests the formation of complexes between Cd, Pb, Hg and U ions and functional groups like hydroxyl and carboxyl groups. Consequently, the identification of O-H and C=O groups from carbonyl compounds, as well as N-H and C-O-C groups from esters, amino acids, and phosphates, highlights their involvement in bonding with the metal ions. These findings provide evidence for the presence of lipids, amino acids (proteins), and polysaccharides within the cell membrane. The significant intensity of O-H, C=O, and N-H signals in both spectra further supports the notion that microorganism cell membranes are predominantly composed of lipids and proteins, potentially in the form of lipoproteins (Vargas, 2019). Hence, despite the complexity of the biological samples and the possibility of various mechanisms like bioaccumulation, it is reasonable to conclude that metal biosorption primarily occurs through ion exchange and complex formation reactions, particularly involving protonable groups. Furthermore, the discrepancy between the intensity of the FTIR bands and the bioelimination obtained in microalgae and bacteria suggests that other mechanisms, such as bioaccumulation, may be involved in the removal of these trace toxic elements by bacteria.

Table 7.7. Frequencies (cm^{-1}) of FTIR absorption bands of microalgae *S. almeriensis* biomass before and after bioaccumulation of Cd, Pb, Hg and U

Functional group	Native biomass (cm^{-1})	Cd(II) loaded biomass (cm^{-1})	Pb(II) loaded biomass (cm^{-1})	Hg(II) loaded biomass (cm^{-1})	U(VI) loaded biomass (cm^{-1})
Surface -OH and stretching N-H	3282.73	3282.73	3278.87	3280.80	3280.80
Aliphatic C-H stretching	2922.05	2920.13	2920.13	2922.05	2922.05
	2852.52	2850.69	2852.52	2852.52	2852.52
Amide group (N-H stretching and C=O stretching vibrations)	1643.29	1641.37	1639.44	1639.44	1639.44
Secondary amine groups	1541.07	1537.21	1537.21	1537.21	1537.21
Carboxylate anion	1454.28-1402.20	1452.35-1402.20	1454.28-1396.41	1452.35-1402.20	1454.28-1402.20
$-SO_3$ stretching groups	1244.04	1240.19	1240.19	1238.26	1240.19
Phosphate group	1151.46-1018.38	1147.61-1026.09	1149.53-1029.95	1149.53-1026.09	1149.53-1022.24

Table 7.8. Frequencies (cm^{-1}) of FTIR absorption bands of activated sludge biomass before and after bioaccumulation of Cd, Pb, Hg and U

Functional group	Native biomass (cm^{-1})	Cd(II) loaded biomass (cm^{-1})	Pb(II) loaded biomass (cm^{-1})	Hg(II) loaded biomass (cm^{-1})	U(VI) loaded biomass (cm^{-1})
Surface -OH and stretching N-H	3280.80	3280.80	3280.80	3278.87	3280.80
Aliphatic C-H stretching	2925.91	2925.91	2925.91	2925.91	2925.91
Amide group (N-H stretching and C=O stretching vibrations)	1635.58	1641.37-1631.72	1639.43-1633.65	1635.58	1635.58
Secondary amine groups	1541.07	1546.85-1535.28	1544.93-1537.21	1539.14	1539.14
Carboxylate anion	1461.99-1407.99	1384.8-1444.6	1402.2-1450.4	1375.19-1336.62	1442.70-1392.56
$-SO_3$ stretching groups	1238.26	1242.11	1238.26	1242.11	1242.11
Phosphate group	1029.95	1037.67	1033.81	1029.95	1026.09

The ESEM-EDX spectra registered using energy dispersive X-ray spectroscopy coupled to ESEM on gold-coated biomass samples allowed the identification of chemical elements present in the biomass surface, before and after the contact with TTE containing wastewater. ESEM images were registered at different magnifications and are shown in **Figure 7.8** (microalgae) and **Figure 7.9** (activated sludge). According to ESEM imaging, raw microalgae presents a structure of small, almond-shaped striated globules. After TTE biosorption, these structures are observed to be in closer contact, forming more compact group of cells. **Figure 7.9** depicts ESEM images taken from freeze dried activated sludge before and after TTE biosorption. The observed circular and filamentous structures could correspond with different bacteria strains present in the material.

The EDX spectra evidenced the occurrence of TTE on the biomass surface after contact time. 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 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.

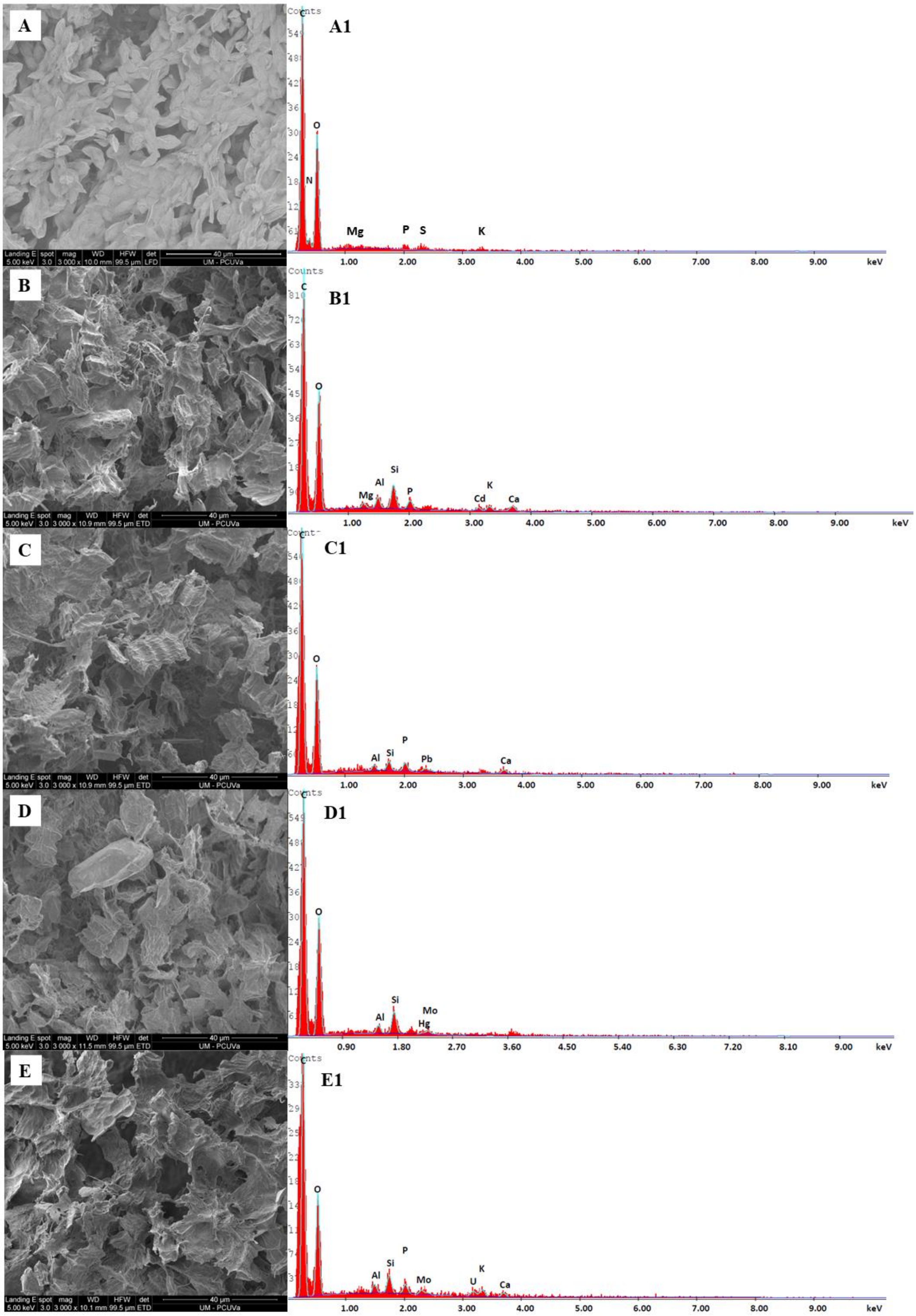

Figure 7.8. ESEM images of pristine (A) and metal exposed (B, C, D and E) biomass of microalga *Scenedesmus almeriensis* recorded at 3,000× magnification. ESEM-EDX spectra confirm the biosorption of Cd, Pb, Hg and U on the biomass cell membrane after wastewater treatment (A1, E1).

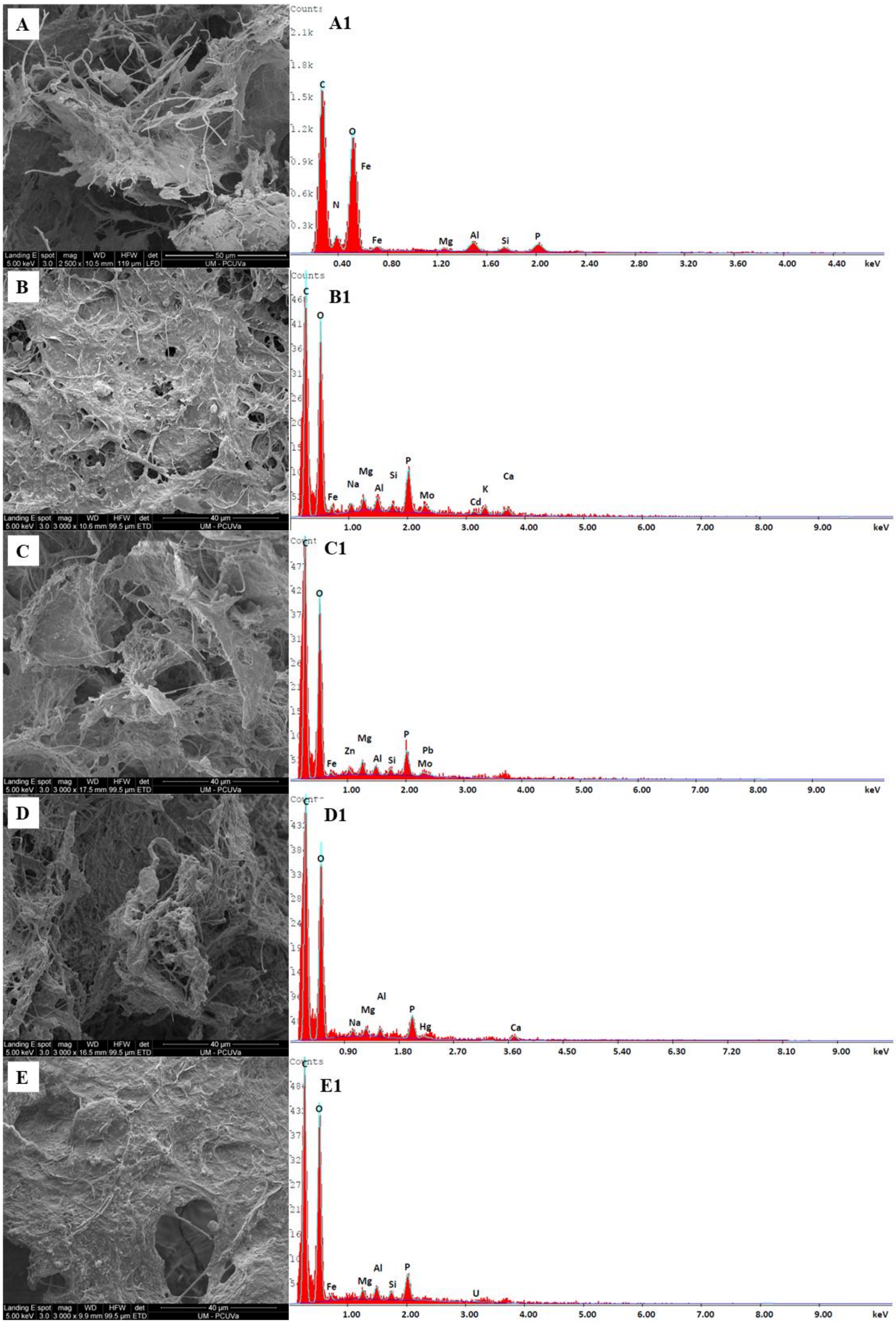

Figure 7.9. ESEM images of pristine (A) and metal exposed (B, C, D and E) biomass of activated sludge recorded at 3000× magnification. ESEM-EDX spectra confirm the biosorption of Cd, Pb, Hg and U on the biomass cell membrane after wastewater treatment (A1-E1).

7.4 Conclusions

In summary, this study provides a valuable insight into the efficacy of *S. almeriensis* microalgae and activated sludge for removing toxic trace elements (TTE) from aqueous solutions. *S. almeriensis* displayed swift elimination of TTE, with percentages exceeding 70% within the first hour of contact time. However, activated sludge consistently achieved even higher elimination percentages, surpassing 95% for all TTE throughout the contact period. Notably, activated sludge exhibited stable elimination percentages without significant declines over time, highlighting its robustness in TTE removal. Nevertheless, the emergence of negative total nitrogen eliminations from day 10 in activated sludge experiments suggests potential biomass degradation, indicating the need for further investigation into the long-term effects of TTE exposure on microbial communities. Additionally, biomass concentration analysis revealed a decline in biomass for both *S. almeriensis* and activated sludge, particularly pronounced under higher TTE concentrations, indicating the toxic impact of these elements on microbial growth. These findings underscore the promising potential of both biological systems for TTE removal in wastewater treatment. However, they also emphasize the importance of monitoring biomass viability and growth under toxic conditions to ensure the sustainability and effectiveness of treatment processes. By shedding light on the capabilities of different organisms in TTE removal, this study contributes to the development of more efficient and environmentally friendly wastewater treatment technologies. Future research should focus on optimizing conditions to maximize TTE removal while minimizing potential adverse effects on microbial communities and biomass integrity.

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Chapter 8:

Effect of operational parameters on metal and nutrient removal from piggery wastewater using biological treatments with microalgae and bacteria

Effect of operational parameters on metal and nutrient removal from piggery wastewater using biological treatments with microalgae and bacteria

Abstract

The management and treatment of swine wastewater represent a pressing global environmental concern due to elevated concentrations of organic matter and nutrients, particularly nitrogen, along with the presence of toxic elements such as Cu(II), Zn(II), As(V), and Cd(II). A sustainable solution to this challenge involves the biological treatment of these wastes using photobioreactors and aerobic bacteria bioreactors. In this study, a Taguchi´s Design of Parameters was implemented to optimize operational parameters within both types of bioreactors and enhance the removal of toxic elements. Controllable factors such as total dissolved nitrogen (TN), contact time (T), and biomass concentration (B) were investigated, while photoperiod (L), PWW C/N ratio (P), and toxic metal concentrations (M) were considered as noise factors. The bacteria bioreactor achieved higher metal and nutrient removal efficiencies (98% Cu, 97% Zn, 72% As, 99% Cd, 88% total organic carbon (TOC), 63% TN, 89% NH_4^+) than the photobioreactor (81% Cu, 96% Zn, 98% As, 93% Cd, 83% TOC, 56% TN, 63% NH_4^+). Among the critical factors influencing bioreactor performance are TN and metal concentrations at the inlet, which inversely impact TOC removal (TOC-RE) and TN removal (TN-RE) in contrast to biomass growth. Furthermore, no significant results were found in the signal-to-noise ratio of the design, indicating robustness in the performance of the systems. The findings were further analyzed using Principal Component Analysis (PCA) to provide comprehensive insights.

Keywords: *Bacteria; heavy metal; microalgae; nutrient removal; piggery wastewater (PWW)*

8.1 Introduction

The development of intensive and factory farming, such as pig farming, has significantly increased manure production all over the world. The problem with these wastes is their high load of organic carbon, nitrogen, ammonia and phosphorus (López-Pacheco et al., 2021). These nutrients cause serious problems when untreated swine manure is discharged into the environment and can lead to eutrophication problems (Cheng et al., 2019). On the other hand, mineral additives are commonly used as feed growth-promoting in animal feed and heavy metals are also present in wells used as source of drinking water in farms. Copper (Cu) is an essential trace element because Cu-rich diets accelerate pig growth (Oliveira et al., 2021). However, Cu excess can also be detrimental to both livestock and humans. Zinc (Zn) is another essential trace metal used to prevent enteric infection and diarrhea, and it serves as an antibiotic growth promoter (Zhang and Guo, 2009). Arsenic (As) is a metalloid that is essential for animal growth. Cadmium (Cd) is widely used in the livestock industry due to its presence in inorganic mineral supplements, often in conjunction with Zn salts (sulphates and phosphates) that may contain Cd impurities. However, only a small fraction (10-20%) is assimilated by the animal, with the majority being discharged with the manure. The concentrations of heavy metals in wastewater from the livestock industry vary. For Cu, the values range between 0.28 and 4.7 mg/L (Collao et al., 2022; Zhang et al., 2011). Zn concentrations are reported between 0.98 and 12 mg/L (Zeng et al., 2021), and As levels can reach up to 670 μg/L (Gao et al., 2018). In the case of Cd(I), concentrations range from 0.022 (Zhang et al., 2011) to 0.35 mg/L (Moral et al., 2008), with moderate values of 0.16 mg/L (Creamer et al., 2010). For this reason, it is necessary to evaluate and eliminate these contaminants from wastewater, as they can pose further potential risks to human health and the ecological environment. The use of biological treatment, which involves highly efficient, low-cost, and are environmentally friendly technologies, have focused attention in recent years. Microalgae and bacteria have demonstrated to be highly effective in removing heavy metals and other pollutants, such as Cu, Zn, As, and other trace toxic elements such as Cd, from different types of wastewaters (Wollmann et al., 2019). The biomass can later be valorized as animal feed or fertilizer, provided that the bioaccumulated metals are absent in the recovered by-products. The treated water can be reused as irrigation water. Among them, the use of photobioreactors (PBRs) and aerobic bioreactors (BRs) in wastewater treatment plants represents a sustainable and green solution for managing livestock waste, such as piggery wastewater (PWW) (Sepúlveda-Muñoz et al., 2022). This robust system efficiently addresses the challenges of cleaning this highly contaminant waste, simultaneously removing organic matter, nutrients, and toxic trace elements. The resulting biomass comprises a consortium of microalgae and bacteria, each exhibiting different biosorption performance towards metalloid species. The biological depuration process of heavy metal-containing wastewater involves many detoxification mechanisms: (a) biosorption on the cell surface through electrostatic interactions. Since the cell surface is negatively charged, positive cations exhibit easier electrostatic interaction. This process may involve complexation or microprecipitation, (b) biodegradation facilitated by enzymes released into the culture medium, (c) photodegradation, which occurs under light and photosynthetic oxidation conditions, especially in open ponds where microalgae are cultivated, (d) biotransformation into other nontoxic or easily removable metabolites, and (e) Bioaccumulation in organelles such as chloroplasts, vacuoles, and mitochondria in microalgae (Nagarajan et al., 2020). Once inside the cell, the metal ion induces a stress response that counteracts its toxic effects, such as oxidative stress, or sequesters the metal ion to metal-binding proteins called metallothioneins (MT). The ability of microalgae and bacteria to remove both nutrients and contaminants from the medium is supported by several studies. For example, Cheng et al. (2017) showed that the microalgae *Chlorella pyrenoidosa* removed ammoniacal nitrogen (NH_4^+-N), total phosphorus (TP), and Chemical Oxygen Demand (COD) by 75.9%, 68.4%, and 74.8%, respectively. Notably, Zn(II) and Cu(II) were removed from wastewater at rates of 65.71% and 53.64% respectively with initial metal concentrations of 2.8 for Zn(II) and 2.2 mg/L for Cu(II). On the other hand, a proper initial dilution of raw piggery wastewater allows many microalgae species to grow and tolerate concentrations of other pollutants. (Ayre et al., 2017). Gao et al., (2018), working with wastewater with 0.78 mg/L of Cu (II), 0.92 mg/L of Zn (II), and 690 µg/L of As (V), achieved with a *Chlorella vulgaris*-bacteria consortium removals of 42%, 45% and 35%, respectively (Gao et al., 2018). However, these percentages increased to 59%, 55%, and 48%, respectively, when the *Chlorella vulgaris* culture was in contact with an activated sludge medium (Gao et al., 2018). The same bioelimination trend was observed with the *Chlorella vulgaris*-bacteria consortia for initial concentrations of 100 mg/L for Cu(II) and Zn(II) and 0.5 mg/L for As, resulting in removal rates of 83%, 81% and 19%, respectively (Collao et al., 2022). In addition, moderate to high nutrient removals from the medium were achieved, with 76% removal of TOC and 48% removal of TN for initial swine wastewater concentrations of 713 mg/L and 256 mg/L for TOC and TN, respectively. This work aims to optimise the purification of piggery wastewater by investigating the effect of operational parameters in photobioreactors and aerobic bioreactors on the removal of organic carbon, total nitrogen, ammonia and toxic elements (Cu(II), Zn(II), As(V), and Cd(II)). For this purpose, a Taguchi Design of Parameters has been implemented for each of the biomasses to be studied, i.e. the microalgae *Scenedesmus almeriensis (microalgae-bacteria consortia)* and aerobic bacteria from activated sludge. This approach, widely applied in various studies (Zolfaghari et al., 2011; Googerdchian et al., 2018), aims to optimise experimental conditions. In this work, two real piggery wastewater (PWW) matrix doped with toxic elements were employed for batch experiments, focusing on both metal and nutrients removal. The influence of controllable factors such as the initial total nitrogen concentration, contact time and initial biomass concentration on metal and nutrient removal in bioreactors will be studied and discussed.

On the other hand, non-controllable factors, including the light:dark photoperiod, the C/N ratio of the PWW to be treated, and the initial metal concentration, were also investigated. The results of this work will contribute to the practical assessment of pilot plant applications and the overall monitoring of environmental factors, particularly those related to bioremediation using microalgae-bacteria consortia.

8.2 Materials and methods

8.2.1 *Reagents*

All the reagents employed in the different experiments were of analytical grade, and the solutions were prepared using deionized (ultrapure) water. Stock solutions for Cu(II), Zn(II), As(V), and Cd(II) were prepared using $CuCl_2{\cdot}2H_2O$, anhydrous $ZnCl_2$, Na_2HAsO_4 $7{\cdot}H_2O$, and $CdCl_2$ (obtained from Sigma Aldrich, Germany) and stored at 4 ºC. NaOH, utilized for pH adjustment, was sourced from Panreac.

8.2.2 *Piggery wastewater and inoculum*

Two different PWW from Estación Experimental Las Palmerillas (Almería) (P1) and from a farm in Narros, Segovia (Spain) (P2) were collected just before the start of each experiment (**Table 8.1**). The differences in PWW characteristics arise from their diverse origins, along with distinct treatment and management practices influenced by varied temperatures and storage conditions. Throughout the lab-experiments, both PWWs were stored under darkness conditions at 4 °C to minimize degradation. Subsequently, the raw PWW was appropriately diluted to achieve the selected concentrations of TN in the feed. Dilution of PWW is necessary to prevent microbial inhibition by high ammonium concentration (García et al., 2017; Posadas et al., 2017).

Table 8.1. Chemical composition of raw piggery wastewater used in experiments in batch bioreactors.

Parameter	PWW Almería (P1)	PWW Segovia (P2)
pH	8.5	7.9
TOC (mg/L)	707	34559
IC (mg/L)	890	11689
TN (mg/L)	1244	8290
NH_4^+ (mg/L)	516	2190
PWW C/N	0.57	4.17
Cu(II) (mg/L)	0.3	3.2
Zn(II) (mg/L)	2.7	7.2
As(V) (µg/L)	0.004	0.03
Cd(II) (µg/L)	0.002	0.005

Monocultures of *S. almeriensis* microalgae were provided by Department of Chemical Engineering of the University of Almeria (Spain). The microalgae were maintained in a homemade Bristol freshwater medium at 21-23 ºC under continuous agitation. LED irradiance of 1200 µmol·m^{-2}·s^{-1} was applied in a 12-hour photoperiod (Zambrano et al., 2023). The activated sludge used in the experiments was collected from the aerobic bioreactor of the municipal urban wastewater treatment plant located at Valladolid (Spain). The collected sludge was stored in the dark under aeration at 4 ºC until its use. Microscopic observation of fresh activated sludge using a Leica DM 4000 B microscope revealed no presence of microalgae or cyanobacteria.

8.2.3 *Experimental set-up and optimization of operational conditions*

The experiments were carried out in 500 mL Pyrex glass bioreactors, operating in batch mode with a temperature of 23 ± 1 °C. A constant agitation of 250 rpm was applied to a 200 mL suspension (see **Figure 8.1**). All experiments were carried out at an initial pH of 7.5 (Posadas et al., 2017) to simulate the environmental conditions existing in bioreactors (Posadas et al., 2015; Acién et al., 2012) and aerobic bioreactors (Gola et al., 2020) treating PWW. Experiments with microalgae were subjected to irradiation with 1200 µE·m^{-2}·s^{-1} at two different photoperiods outlined in **Table 8.2.**

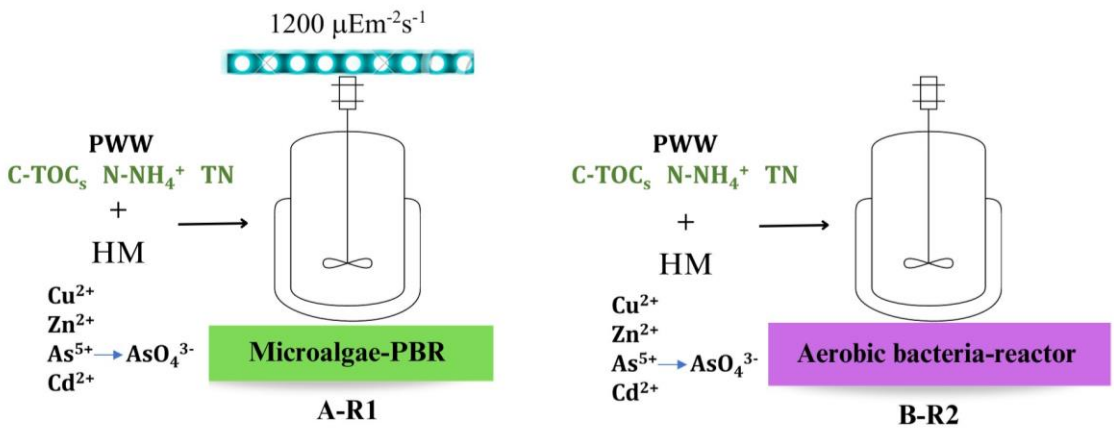

Figure 8.1. Experimental set up for bioreactors in batch mode for A-R1 (algae-PBR) and B-R2 (aerobic bacteria reactor)

Based on the objective of optimizing PWW cleaning and recognizing the significance of controllable factors on PBRs performance, along with the influence of non-controllable environmental factors inherent in such processes, a Taguchi Parameter Design approach was chosen. The objective was to identify optimal conditions for metal removal, as well as to determine the conditions that most significantly affect biomass growth and bioreactor performance. Therefore, a Taguchi Parameter Design incorporating both an inner orthogonal array $L_4(2^3)$ and a outer one $L_4(2^3)$, was employed to determine the optimal values of controllable factors that minimize the impact of non-controllable or noise factors (Hadiani et al., 2018). The study aimed to assess the impact of controllable factors, including the initial total nitrogen concentration, contact time, and initial biomass concentration, on the treatment efficiency of wastewater in photobioreactors and aerobic reactors. The objective was to identify optimal levels for these controllable factors to achieve maximum removal of organic matter, nitrogen, and metals present in Piggery Wastewater. Simultaneously, the study aimed to work with controllable factor levels that are least sensitive to noise, representing uncontrollable influences, within the framework of Taguchi Parameter Design. Concurrently, in the orthogonal inner array, controllable factors were considered, while in the outer array, non-controllable factors, or noise factors, were addressed. These noise factors cannot be controlled on a real scale and those chosen for this experiment were the photoperiod of light chosen to simulate different seasons of the year with varying daily radiation levels, the C/N ratio of the PWW to be treated, and the initial metal concentration of Cu(II), Zn(II), As(V) and Cd(II) in the spiked feed inlet. For all six factors, including both controllable and non-controllable ones, two different levels were selected. For the controllable factors, the following two levels were chosen: 100 and 200 mg/L for the initial nitrogen concentration, aligning with typical concentrations at the inlet of the bioreactors to prevent the inhibition of microbial growth due to high ammonium concentrations in PWW (García et al., 2017; Posadas et al., 2017; Acién Fernández et al., 2018). These concentrations were obtained by diluting the PWW with tap water. For contact time, representative of typical residence times in aerobic reactors and bioreactors, 24 and 72 h were chosen for the bacteria-based reactor and microalgae-based PBR, respectively (Posadas et al., 2017). Regarding biomass concentration, different levels were chosen for microalgae compared to activated sludge biomass. The two levels of inoculum concentration were 1 g/L and 2 g/L for microalgae, and 2 g/L and 4 g/L for bacterial biomass. This difference is attributed to the lower inoculum concentration required for microalgae to prevent the inhibition of photosynthetic activity. As for the non-controllable or noise factors, the following two levels were selected for each: photoperiod, simulating periods of higher and lower solar intensity, and the number of daylight hours per day (8 hours of light and 16 hours of darkness, and 16 hours of light and 8 hours of darkness); C/N ratio of the piggery wastewater (0.57 and 4.17); and initial metal concentration of metals in the reactors (10 mg/L for Cu(II) and Zn(II), 0.1 mg/L for As(V) and Cd(II) at the low concentration level, and 50 mg/L for Cu(II) and Zn(II), 0.5 mg/L for As(V) and Cd(II) at the high concentration level) (ASAE, 2003b). These concentrations were selected to operate under the most unfavorable conditions within the range of values reported in the literature for these metals in PWW (Gao et al., 2018a; Zeng et al., 2021a) Additionally, these concentrations were previously used in other studies of our research group, allowing the comparison of results (Collao et al., 2022). Detailed information is presented in **Table 8.2.** The order of execution of each set of 16 experiments was randomized.

The experimental data were analyzed through an ANOVA test for means and signal-to-noise (S/N) ratios and factorial plots to determine the optimal conditions for metal and nutrient removal, and their effect on biomass growth. An analysis of the signal-to-noise (S/N) ratio was performed to evaluate the optimal levels of control factors that makes the process robust or resistant to variation caused by noise factors. In the present study, the goal has been to maximize the removal efficiency of metals (Cu(II), Zn(II), As(V) and Cd(II) ions) and nutrients (TOC, TN and NH_4^+) present in PWW. For this response, the S/N ratio is of the 'bigger is better' type. Therefore, the mean S/N ratio for each controllable factor at any level was determined by averaging the S/N ratios of the responses at each level according to the expression in equation (8.1).

$$\frac{S}{N} = -10 \log \left[\frac{\sum_{i=1}^{n} \left(\frac{1}{y_i} \right)^2}{n} \right] \tag{8.1}$$

where n is the number of replicates under the same combination of factors and experimental levels, and y_i represents the response obtained for each replicate.

Table 8.2. Control and noise factors and levels proposed for the orthogonal arrays $L_4(2^3)$ x $L_4(2^3)$

Control factors	Level 1	Level 2
TN at inlet (mg/L) **(N)**	100	200
Time contact (h) **(T)**	24	72
Biomass concentration **(B)**		
Microalgae (g/L)	1.0 ± 0.1	2.0 ± 0.1
Activated sludge (g/L)	2.0 ± 0.1	4.0 ± 0.1
Noise factors	**Level 1**	**Level 2**
Light photoperiod **(L)**	1200 $\mu Em^{-2}s^{-1}$ at 8h:16h	1200 $\mu Em^{-2}s^{-1}$ at 16h:8h
PWW C/N **(P)**	0.57	4.17
HM concentration **(M)**		
Cu (mg/L)	10	50
Zn (mg/L)	10	50
As (mg/L)	0.1	0.5
Cd (mg/L)	0.1	0.5

It should be noted that the initial concentrations of Cu(II), Zn(II), As(V) and Cd(II) in the diluted PWW used as feed were very low, therefore, their influence on the final concentration of the doped feed was negligible (see **Table 8.1**). The two $L_4(2^3)$ orthogonal arrays mentioned above are combined to give rise to the following experimental matrix of **Table 8.3**. Runs E1-E16 refers to bioelimination experiments in A-R1 (with *S. almeriensis*) and to bioelimination experiments with B-R2 (with bacteria from activated sludge).

Table 8.3. Experimental matrix of the inner $L_4(2^3)$ and outer $L_4(2^3)$ orthogonal arrays with respective levels for control and noise factor for microalgae (A-R1) and activated sludge (B-R2) tests.

	$L_4(2^3)$	**M**	M1	M2	M2	M1
		P	P1	P2	P1	P2
$L_4(2^3)$		**L**	L1	L1	L2	L2
N	**T**	**B**	**R**	**R**	**R**	**R**
N1	T1	B1	y_{E1}	y_{E2}	y_{E3}	y_{E4}
N1	T2	B2	y_{E5}	y_{E6}	y_{E7}	y_{E8}
N2	T1	B2	y_{E9}	y_{E10}	y_{E11}	y_{E12}
N2	T2	B1	y_{E13}	y_{E14}	y_{E15}	y_{E16}

Metal and nutrient removal efficiency (RE) were calculated according to Equation 3.12 in Material and Methods section.

8.2.4 *Analytical procedure*

The process for heavy metals and arsenic analysis, as well as TOC, TN, NH_4^+, and pH measurement, involved collecting a 10 mL volume of suspension after the contact time had elapsed in BRs. Subsequently, the samples underwent centrifuged at 7800 rpm for 10 min, and then the supernatant was filtered using a 0.45 mm nylon membrane filter. Total organic carbon (TOC) and total nitrogen (TN) concentrations were measured using a TOC-V CSH analyzer equipped with a TNM-1 chemiluminescence module (Shimadzu, Kyoto, Japan). The concentration of ammonia nitrogen (NH_4^+) was determined using an ammonium selective electrode (Orion 9512 HPWBNWP ammonia, Thermo Scientific, Waltham, MA, USA). The concentration of Cu(II) and Zn(II) was determined by inductively coupled plasma spectrometry combined with an optical emission spectrophotometer (ICP-OES) (Varian 725-ES, Agilent, Santa Clara, CA, USA). As(V) and Cd(II) concentrations was measured using an inductively coupled plasma source mass spectrometer (ICP-MS) in an octopolar reaction system (HP 7500c, Agilent, USA). All plastic and glass containers were washed in diluted HNO_3 (10% v/v) for 24 h and were rinsed three times with Milli-Q water before and after use for heavy metals and metalloids analysis.

It is noteworthy that pH was not a controlled variable throughout the operation of the batch bioreactors but was initially set at pH 7.5, a reference value in the development of bioreactors for wastewater treatment (Posadas et al., 2017). After the contact time had elapsed, the final pH was also measured. In terms of biomass viability, the amounts of initial and final biomass after the contact time were measured through the determination of total solids (TS). 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 ºC for at least 24 h until constant weight.

8.2.5 *Statistical analysis*

The analysis of the experimental metal removal data was performed using Statgraphics Centurion 19 (Statgraphics Technologies, Inc.) and Excel® 2019 (Microsoft). Analysis of variance (ANOVA) for the factors and their interactions was conducted at a confidence level of 95% ($\alpha = 0.05$) to interpret the results. In addition, IBM SPSS Statistics 26 (IBM Corporation) was employed for Principal Components Analysis (PCA). PCA is a statistical procedure or technique for analyzing large datasets with a high number of dimensions per observation, increasing data interpretability while preserving the maximum amount of information. It allows the visualization of multidimensional data, and many studies use the first two principal components to plot data in two dimensions and to visually identify clusters of closely related data points.

8.3 Results and discusion

A total of 16 experiments were carried out for each of the inoculum employed (A1-A16) for microalgae-bacteria consortia and B1-B16 for bacteria-based), involving three control factors and three non-controllable (noise factors) at two levels. The results of both sets of experiments are shown in **Table 8.4**. These results will be discussed throughout this results section.

Table 8.4. Results for metal removal efficiency (Cu-RE, Zn-RE, As-RE and Cd-RE) nutrient removal (TOC-RE, TN-RE and NH_4^+-RE) and biomass growth (%) for A-R1 and B-R2.

	Run	Cu-RE	Zn-RE	As-RE	Cd-RE	TOC-RE	TN-RE	NH_4^+-RE	Biomass Growth (%)
		%	%	%	%	%	%	%	%
	A1	57.9	74.83	52.2	81.7	15.8	4.5	-2.2	6.9
	A2	73.9	86.0	98.2	84.2	11.4	13.2	10.3	8.0
	A3	72.6	88.7	95.0	90.8	-42.9	5.4	6.5	15.6
	A4	51.0	72.5	59.5	85.4	3.8	17.3	25.5	-2.2
	A5	75.4	90.6	6.6	92.0	37.4	6.5	14.7	-4.8
	A6	77.4	82.3	87.9	88.8	67.0	27.4	12.2	7.7
	A7	81.4	82.7	87.3	89.2	24.5	-5.6	18.4	9.2
A-R1	A8	16.9	96.6	48.8	93.8	75.2	39.6	34.4	12.1
	A9	51.3	84.1	27.0	86.5	16.7	3.9	27.5	-0.7
	A10	53.2	84.1	83.2	89.5	-7.3	12.2	33.4	8.1
	A11	56.7	82.2	76.0	87.7	-32.6	8.1	35.1	6.2
	A12	46.7	85.6	30.8	93.4	-0.01	19.0	45.6	1.1
	A13	53.7	76.8	34.7	82.6	17.1	3.2	42.2	-3.7
	A14	70.9	70.3	87.7	76.9	59.9	27.7	45.0	8.0
	A15	65.2	79.7	83.0	85.6	7.7	6.6	42.2	14.4
	A16	15.8	96.5	50.4	91.3	83.0	56.8	63.3	12.7
	B1	91.8	84.6	<1	90.8	0.2	13.0	65.6	-1.6
	B2	92.0	95.1	66.9	95.8	66.5	39.3	46.4	3.3
	B3	94.9	92.0	66.0	93.9	-17.9	9.8	53.9	10.7
	B4	95.6	88.0	7.4	93.9	84.2	50.5	48.0	-5.5
	B5	97.9	74.3	<1	92.9	44.5	18.2	89.2	-4.3
	B6	96.5	92.3	47.6	94.5	77.1	58.0	94.6	0.5
	B7	94.6	86.7	41.0	92.6	-16.0	21.5	78.0	2.9
B-R2	B8	34.5	97.9	18.3	98.8	86.1	62.8	55.4	-8.9
	B9	89.2	83.4	<1	91.8	10.6	3.8	24.9	-2.7
	B10	86.5	91.2	40.2	93.9	21.1	25.7	55.9	-4.4
	B11	78.3	86.2	42.0	91.4	-94.2	-3.5	27.1	6.3
	B12	94.6	88.2	15.5	97.1	48.6	21.1	34.6	-3.9
	B13	85.8	26.9	7.7	76.1	39.5	10.5	77.3	9.3
	B14	88.9	89.7	72.2	93.2	83.0	39.9	47.8	4.2
	B15	86.4	78.9	70.3	87.7	0.1	7.9	51.3	5.1
	B16	97.0	93.2	33.3	96.8	87.6	55.7	50.6	-2.3

8.3.1 *ANOVA and hypothesis tests*

An ANOVA study was carried out on heavy metal removal efficiency for each of the factors tested, whose results are shown in the following table (**Table 8.5 and 8.6**). The main results, together with the mean plots will be discussed throughout following sections.

Table 8.5. ANOVA for metal removal efficiency (Cu, Zn, As and Cd-REs) for A-R1 and B-R2 in terms of mean and signal to noise ratio (S/N).

S. almeriensis (A-R1)								
	Cu-RE p-value		Zn-RE p-value		As-RE p-value		Cd-RE p-value	
Factor	Mean	S/N	Mean	S/N	Mean	S/N	Mean	S/N
N	$2.48 \cdot 10^{-4}$	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	$6.46 \cdot 10^{-4}$
T	>0.05	$2.46 \cdot 10^{-2}$	$2.45 \cdot 10^{-2}$	>0.05	>0.05	>0.05	>0.05	>0.05
B	>0.05	>0.05	$4.83 \cdot 10^{-2}$	>0.05	$2.57 \cdot 10^{-2}$	>0.05	$2.44 \cdot 10^{-3}$	$6.34 \cdot 10^{-5}$
L	$1.62 \cdot 10^{-4}$	-	>0.05	-	>0.05	-	$8.22 \cdot 10^{-3}$	-
P	$1.57 \cdot 10^{-4}$	-	>0.05	-	>0.05	-	>0.05	-
M	$3.31 \cdot 10^{-5}$	-	>0.05	-	$9.50 \cdot 10^{-7}$	-	>0.05	-
Activated sludge *(B-R2)*								
	Cu-RE p-value		Zn-RE p-value		As-RE p-value		Cd-RE p-value	
Factor	Mean	S/N	Mean	S/N	Mean	S/N	Mean	S/N
N	>0.05	>0.05	>0.05	>0.05	$3.31 \cdot 10^{-3}$	>0.05	$2.19 \cdot 10^{-2}$	>0.05
T	>0.05	>0.05	>0.05	>0.05	$1.36 \cdot 10^{-3}$	>0.05	>0.05	>0.05
B	>0.05	>0.05	>0.05	>0.05	$2.58 \cdot 10^{-4}$	>0.05	$2.42 \cdot 10^{-2}$	>0.05
L	>0.05	-	>0.05	-	$1.13 \cdot 10^{-3}$	-	$3.04 \cdot 10^{-2}$	-
P	>0.05	-	$4.96 \cdot 10^{-2}$	-	$7.05 \cdot 10^{-4}$	-	$1.75 \cdot 10^{-3}$	-
M	>0.05	-	>0.05	-	$2.76 \cdot 10^{-5}$	-	>0.05	-

Table 8.6. ANOVA of mean and signal-to- noise ratio (S/N) for nutrient removal efficiency (TOC, TN, and NH_4^+ REs) and biomass growth (%) in A-R1 and B-R2.

S. almeriensis (A-R1)								
	TOC-RE p-value		TN-RE p-value		NH_4^+-RE p-value		Biomass growth p-value	
Factor	Mean	S/N	Mean	S/N	Mean	S/N	Mean	S/N
N	$1.93 \cdot 10^{-2}$	>0.05	>0.05	0.027	$8.56 \cdot 10^{-9}$	>0.05	>0.05	>0.05
T	$9.20 \cdot 10^{-7}$	>0.05	$9.20 \cdot 10^{-3}$	>0.05	$6.24 \cdot 10^{-7}$	>0.05	>0.05	>0.05
B	>0.05	>0.05	>0.05	0.021	$1.05 \cdot 10^{-2}$	>0.05	>0.05	>0.05
L	$8.75 \cdot 10^{-4}$	-	>0.05	-	$7.33 \cdot 10^{-7}$	-	$2.24 \cdot 10^{-3}$	-
P	$1.04 \cdot 10^{-5}$	-	$2.34 \cdot 10^{-5}$	-	$8.60 \cdot 10^{-7}$	-	>0.05	-
M	$8.79 \cdot 10^{-5}$	-	$4.60 \cdot 10^{-2}$	-	$1.48 \cdot 10^{-5}$	-	$2.18 \cdot 10^{-4}$	-
Activated sludge *(B-R2)*								
	TOC-RE p-value		TN-RE p-value		NH_4^+-RE p-value		Biomass growth p-value	
Factor	Mean	S/N	Mean	S/N	Mean	S/N	Mean	S/N
N	$6.27 \cdot 10^{-3}$	>0.05	$2.02 \cdot 10^{-4}$	>0.05	$8.87 \cdot 10^{-4}$	>0.05	$2.34 \cdot 10^{-2}$	>0.05
T	$1.30 \cdot 10^{-3}$	>0.05	$1.66 \cdot 10^{-4}$	>0.05	$2.93 \cdot 10^{-4}$	>0.05	>0.05	>0.05
B	$3.80 \cdot 10^{-3}$	>0.05	>0.05	>0.05	>0.05	>0.05	$8.57 \cdot 10^{-5}$	>0.05
L	$3.86 \cdot 10^{-3}$	-	>0.05	-	$1.43 \cdot 10^{-2}$	-	>0.05	-
P	$3.03 \cdot 10^{-4}$	-	$7.80 \cdot 10^{-8}$	-	>0.05	-	$3.57 \cdot 10^{-5}$	-
M	$1.32 \cdot 10^{-3}$	-	>0.05	-	>0.05	-	$1.38 \cdot 10^{-5}$	-

8.3.2 *Effect of operating factors on metal removal in bioreactors (BRs)*

In this and subsequent sections, the main significant results obtained for the tested factors will be presented, as well as the most significant and contributing interactions obtained between control and noise factors relevant to choosing the optimal combination of factors to obtain the best signal-to-noise ratio and to mitigate the variation of noise factors, which are uncontrollable at large scale. In addition, at the beginning of each section, the factors that contribute most to each of the responses to be optimised will be described.

Microalgae-bacteria bioreactors (A-R1)

Regarding contributions, the metal concentration (M factor) was found to be the most important contributor to Cu-RE, accounting for 37 %. In the same way, M factor accounted for 79% of the As removal efficiency. On the other hand, B was the most contributing factor to Zn and Cd removal, accounting for 13 and 35%.

Regarding control factors, in microalgae-bacteria consortia PBRs (A-R1), the initial TN concentration (N factor) emerged as a significant factor only for Cu removal, with higher removal efficiencies observed at low initial TN concentrations of 100 mg/L. However, this factor has no significant influence on the removal of Zn, As and Cd. Thus, removals efficiencies ranged from 63 to 52, 84 to 82, 67 to 59 and 88 to 87% for Cu(II), Zn(II), As(V), and Cd(II) respectively, for this factor at the two levels tested (**see Figure 8.2**).

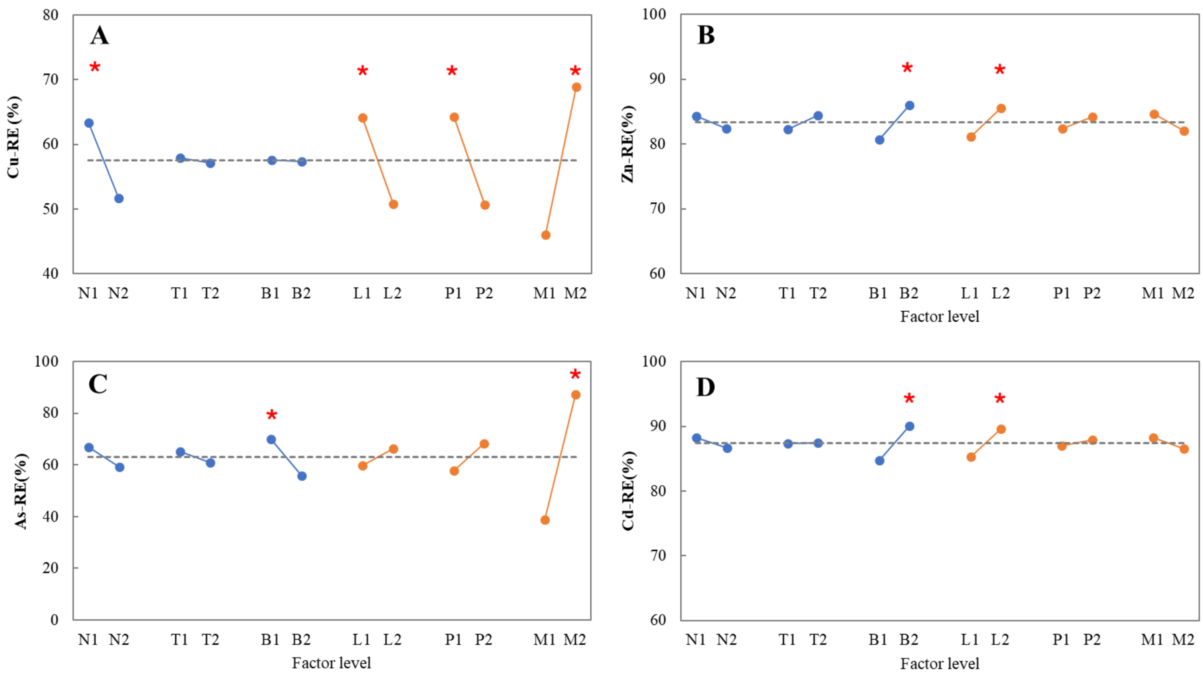

Figure 8.2. Means plot for removal efficiencies (REs) for Cu (A), Zn (B), As (C) and Cd (D) in microalgae-bacteria bioreactor (A-R1). (—●—) control factor, (—●—) noise factor and (-----) grand mean. Red asterisks show differences that are significant at the 95% confidence level.

Contact time (T factor) is not significant for the RE of these four metals in A-R1. The maximum mean values for these variables were 58, 84, 65, and 88% for Cu, Zn, As, and Cd, respectively. Related to literature, García et al. (2019) observed a decrease in Zn elimination as the hydraulic retention time (HRT) decreased from 10 to 6 days, from 98 to 91% in terms of RE. On the other hand, regarding B factor (biomass concentration), a higher initial biomass concentration (B2 for the B factor) resulted in significantly higher REs of the cationic species Zn and Cd, while for As, significantly higher REs were obtained with lower initial biomass concentrations (B1), possibly due to a higher photosynthetic performance of the culture, resulting in a better performance of the depuration/cleanup process. For A-R1, the best values of 58, 86, 70 and 90% of metal RE were achieved for Cu, Zn, As, and Cd respectively. In the same way, for Zn, Cd and As, the biomass concentration (B factor) played a significant role, contributing 13 %, 35 % and 7%, respectively, to their removal efficiencies. All contribution percentages can be consulted in supplementary material in **Table S8.2.**

Regarding noise factors, the 16:8h irradiation photoperiod (L factor) has a significant positive effect on the removal of Cu, Zn and Cd by A-R1, where L1 photoperiods favoured Cu removal and L2 Zn and Cd removal. The type of swine wastewater used in relation to the C/N load (P factor) significantly influences the bioelimination of metals, being higher for swine wastewater with an organic load of a C/N ratio 4.17 (P2 level), except for Cu elimination by A-R1, which is promoted by P1 swine wastewater with a lower C/N ratio (**Figure 8.2**). Supporting these results for Zn, As and Cd removal from PWW (Zheng et al., (2019), the authors tested different C/N ratio, obtaining better PBR performance for 5/1, 25/1 and 125/5 than for ratios of 17/20, supporting the fact that wastewater with a higher proportion of C favours the viability of the crops and thus the removal of metals from the medium through assimilation by micro-organisms, which, unlike the other metals, Cu removal is more efficient with manures with a C/N ratio of less than 1.

As for the noise factor M (metal concentration), for Cu and As-RE, there is a significant effect, since for the level of the M2 factor, higher ERs are obtained than for M1.Examples with similar concentrations of metals and nutrients to those used in this study can be found in the study by Collao et al. (2022) where a consortium of microalgae *Chlorella vulgaris-bacteria* was used to treat swine wastewater with an initial concentration of 100 mg/L for Cu(II) and Zn(II), and 500 μg/L of As, resulting in removal rates of 83, 81, and 19%, respectively. These results are in agreement with those obtained in the present study, obtaining much lower RE for the anionic species in solution. The low removal efficiency of anionic species (such as arsenite and arsenate) may be largely due to the negatively charged cell surface, resulting in more effective electrostatic interactions with positively charged or cationic species. In fact, metal binding to anionic functional groups on the cell surface has been reported as a general mechanism of metal resistance and a protective mechanism (Álvarez et al., 2012). On the other hand, few studies have been found that investigate the removal of Cd from the medium during the wastewater purification process. This is exemplified in a study by (Yang et al., 2015), where the removal of Cu, Zn, and Cd by *Chlorella minutissima* UTEX2341 in a synthetic wastewater matrix was studied. The concentrations tested were for Cu 12, 25 and 64 mg/L, obtaining bioelimination percentages of 84, 82, and 30%, respectively, with an initial biomass concentration ranging between 7 and 8 g/L. For Zn, metal concentrations of 131, 262, and 392 mg/L were tested, obtaining bioelimination percentages of 62, 46, and 38%, respectively, with initial biomass concentrations ranging between 6 and 7 g/L. For Cd, metal concentrations of 22, 45, and 67 mg/L were examined, yielding bioelimination percentages of 74, 55, and 39%, respectively, with initial biomass concentrations between 7 and 8 g/L. Both biosorption and bioaccumulation mechanisms were reported, and the presence of high metal concentrations in the medium did not inhibit biomass growth, except for high Zn concentrations over a contact time of 7 days (Yang et al., 2015). On other study by (Oliveira et al., 2021a) the average removal of Cu and Zn from PWWs was 75% and 89%, respectively, with concentrations ranging between 0.5-3 mg/L for Cu and 5-25 mg/L for Zn in all possible combinations.

Regarding interactions between control and noise factors, the TxL interaction is the most significant for Cu-RE (9%). For this interaction, working al a level T1 of the control factor T, will make the system more robust or invariant to the effect of the noise factor L (uncontrollable in a real process). For Zn-RE TxM interaction has a high contribution (35%) as well as for Cd-RE (12%), where shorter operating times (T1) will favor the lower effect of the metal concentration factor (M), which is uncontrollable in practice.

Bacteria bioreactors (B-R2)

The most important contributing factor for metal removal in B-R2 were as follows. No significant factors were found for Cu-RE. P factor was the most contributing to Zn and Cd removal, accounting for 23 and 35% respectively. Finally, M factor contributes with 78%, As in the case of A-R1 experiments, the initial metal concentration is a key factor in its removal, with As removal being more effective at As concentrations corresponding to the M2 level.

Regarding control factors, in bacteria BRs (B-R2), the initial TN concentration (N factor) is a significant factor in As and Cd REs by bacteria. For the cationic specie Cd, its bioelimination is favored at 100 mg/L of TN (N1 level), while for As(V) in solution as arsenate (anionic specie), its elimination is favored at a higher TN concentration (N2 level) of 200 mg/L, reaching elimination rates of 94% and 35% for Cd(II) and As(V), respectively (see **Figure 8.3**). Regarding contact time (T factor), in B-R2 no significances were found except in the case of As-RE, where higher percentages of removal of this species are obtained at longer contact times (T2 level) (36%). This may be related to a greater contribution of metabolic processes in As elimination by the bacterial cells present in the activated sludge, which are of the nitrifying type, this suggests that media rich in nitrogen would favor their viability (Wang et al., 2017). Additionally, an interaction between the control factors, initial TN concentration and contact time (NxT), affects As uptake. Specifically, at the high level of contact time (72h), there is a significant increase in As uptake when TN concentration is also high. However, for low contact time, the increase in TN does not produce the same effect on As bioelimination. The averaged maximum RE for Cu, Zn, As and Cd for this factor N in B-R2 were 90, 89, 36, and 94%, respectively.

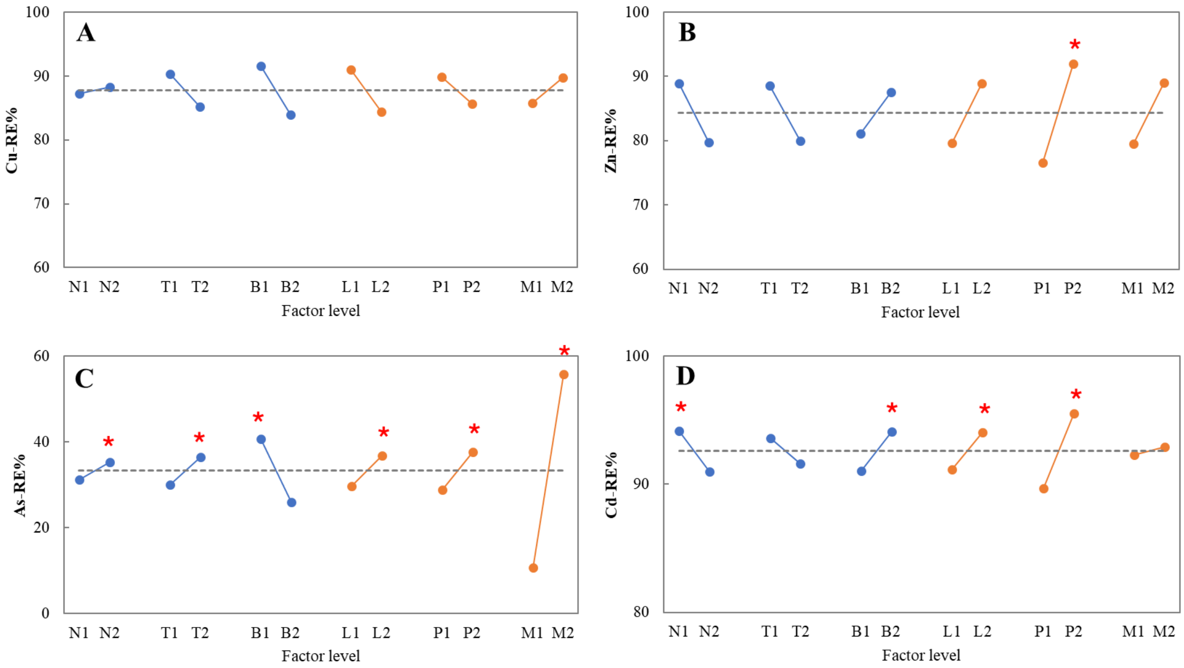

Figure 8.3. Means plot for removal efficiencies (REs) for Cu (A), Zn (B), As (C) and Cd (D) in bacteria bioreactors (B-R2). (—●—) control factor, (—●—) noise factor and (-----) grand mean. Red asterisks show differences that are significant at the 95% confidence level.

Initial biomass concentration (B factor) significantly affected metal RE by B-R2 and was favored by lower initial biomass concentration (B1 level), reaching removal efficiencies of 92, 88, 41 and 94% for Cu, Zn, As, and Cd respectively. Removal efficiency was not affected significantly by biomass concentration for Cu and Zn, but it was for As and Cd, being favored by lower (B1 level) and higher initial biomass concentration (B2 level), respectively. In general, B-R2 exhibited a better performance and tolerance towards metals in terms of RE for Cu, Zn, and Cd, achieving higher values of these variables. Conversely, A-R1 demonstrated better performances in the removal of As, indicating improved tolerance to the toxicity of this species for the microalgae. Furthermore, significant differences exist in the optimal removal conditions for cationic metal species (Cu, Zn, and Cd), which exhibit enhanced bioelimination with short contact times (T1 level), low initial nitrogen loads (N1 level), and low biomass concentrations (B1 level). On the other hand, arsenic bioelimination in aerobic reactors is favored by high nitrogen loads (N2 level), long contact times (T2 level), and low initial inoculum concentrations (B1 level).

In the case of B-R2, regarding noise factors, which are not controllable in a real scaling-up application, it has been observed and remarked that the 16:8h irradiation photoperiod (L2 level) has a significant positive effect on the removal of Zn, As, and Cd by B-R2. The initial metal concentration (M factor), as mentioned before, has a clear effect on metal elimination, with the high level of the factor positively influencing the bioelimination of these metals, only in the case of As(V), leading to increased RE with higher concentrations of this metal species in the medium. The rest of the variations of the RE with metal concentration result from random error. This demonstrates an advantage, as these biomasses exhibit capacity to assimilate and remove metals from the medium. As the initial metal concentration increases (M2 level), uptake by both biomasses significantly increases.

Not many studies have been found that work with PWW treatment with mixtures of microalgae and activated sludge inoculum to enhance their performance. This is the case of Gao et al., (2018), for the PBR treatment of wastewater from PWW with an initial concentration of nutrients of 744, 138 and 88 mg/L of TOC, TN and TP, respectively. For metals, at initial concentrations of 0.78 mg/L of Cu 0.92 mg/L of Zn and 690 μg/L of As, bioeliminations of 42, 45 and 35% respectively are achieved for a consortium of C*hlorella vulgaris* with activated sludge. On the other hand, when the consortium used is that of *Scenedesmus obliquus* with activated sludge, the results of nutrient removal are maintained, while those of metal bioelimination increase to removal percentages of 46, 50 and 38% for Cu, Zn and As, respectively.

Regarding control and noise factor interactions, no significant interactions were found for Cu and Zn-RE. For As-RE, significant but little contribution from the BxM interaction (5%) was obtained, resulting that for B2, working conditions with less fluctuation of the M-factor are obtained. For Cd-RE the significant interactions with the highest contribution are NxP (7%), TxP (7%) and BxM (7%), for which the level of the controllable factor for which working conditions with less fluctuation of the noise factor are obtained are N1, T1 and B2 respectively. These interactions, along with the rest of the interactions plots and the ANOVA table, are available in the supplementary material of this Chapter.

8.3.3 Effect of control and noise factors, and their interactions on bioreactors performance and biomass growth

Microalgae-bacteria bioreactors (A-R1)

The main contributions of the factors to the removal of different nutrients and biomass growth were as follows. In microalgae-bacteria-based BRs (A-R1), the noise T factor (contact time) contributes 53 % to TOC-RE, P (PWW C/N ratio) contributes with 54% to TN-RE, N (initial TN) contributes with 63% to NH_4^+-RE and M (metal concentration) contributes with 31% to biomass growth.

Regarding the initial TN concentration in A-R1, higher TN concentrations significantly negatively influence TOC-RE, but positively affect NH_4^+-RE removal. The mean values and their variation at the two concentration levels were 21-18% and 15-42% for removal of TOC and NH_4^+, respectively (see **Figure 8.4**). The effect of NH_4^+ concentration on nutrient removal efficiency and cell viability of *C. vulgaris* cultivated in Southwest China was investigated by Zheng et al., (2019), they found that an optimal NH_4^+ concentration of 110 mg/L (low ammoniacal concentration for the experiment) resulted in 89% cell viability and 96% COD removal, along with complete NH_4^+ removal, and 91% TP removal. However, when higher concentrations (220 mgL^{-1}) were used, cell viability dropped to 61%, and removal efficiency decreased to around 50% in all cases. Additionally, Ciardi et al. (2022) noted that NH_4^+ concentrations should remain below 180 mg/L at the inlet of the PBRs to ensure the viability of *S. almeriensis* cultures. To avoid this inhibition and ensure sufficient removal efficiency, various strategies can be implemented, such as diluting manure or adding a carbon source to achieve a suitable C/N ratio. So, in this way, our best results for lower initial TN concentration are in agreement with found in literature for A-R1.

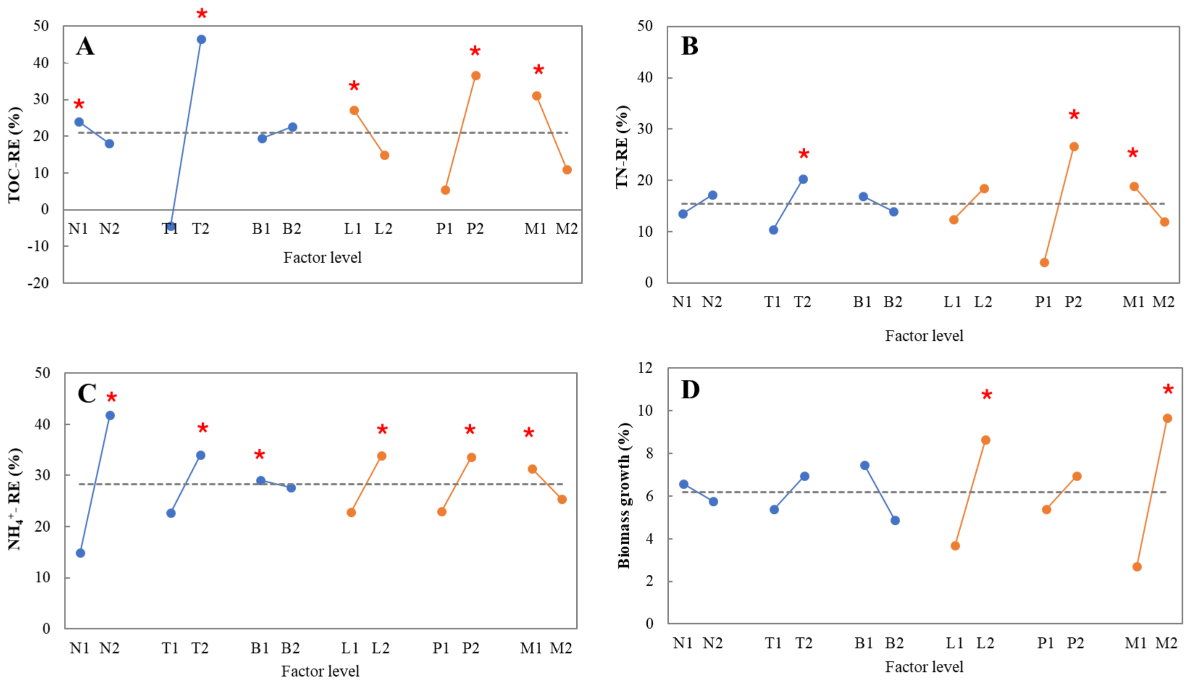

Figure 8.4. Means plot for removal efficiencies (REs) of TOC (A), TN (B) and NH_4^+(C) and biomass growth (D) in microalgae-bacteria bioreactors (A-R1). (—●—) control factor, (—●—) noise factor and (-----) grand mean. Red asterisks show differences that are significant at the 95% confidence level.

Regarding to Time factor (T), contact times of 72 hours (T2 level) exhibited a significant positive effect on the removal of TOC, TN, and NH_4^+ in A-R1. Specifically, the mean removal values at low and high factor levels were -4-46%, 10-20%, and 22-34% for TOC, TN, and NH_4^+, respectively. Negatives removal have been reported in numerous works (García et al., 2017b; Marín et al., 2019). Negative REs are explained by the solubilization of biomolecules and EPS from biomass surface. According to this behavior with contact time, Hernández et al., (2013) treated the liquid fraction from pig slurry in 5L photobioreactors with the microalgae *Chlorella sorokiniana* and activated sludge at a higher HRT of 10 days. Removal values of 58.1, 82.7 and 58% of sCOD, NH_4^+ and SP (soluble phosphorus) were obtained.

The initial biomass concentration (B factor), in terms of TS (g/kg), has a significant but not strong effect in terms of p-value on the removal of NH_4^+ from the medium. The mean values of removal at low (B1) and high (B2) levels were 19-22%, 16-13%, and 29-27% for TOC, TN, and NH_4^+, respectively. Maximum removal efficiencies were also achieved in other studies for microalgae initial concentration of 1.1 g/L (Nguyen et al., 2022).

In relation to noise factors, the 16:8 photoperiods (L2 level) significantly favored NH_4^+ removal and biomass growth, while short photoperiods (L1) favored TOC removal. As for the C/N ratio of PWW, the 4.17 ratio of P2 level significantly favored TOC, TN and NH_4^+ removal.

Besides, the metal concentration in the bioreactors in relation to their performance in terms of TOC, TN and NH_4^+ removal from the medium, will be discussed, as well as biomass growth during the purification process (see **Figures 8.4 and 8.5**). For A-R1, the initial metal concentration has a significant effect on nutrient removal, favored by a low concentration of metal (M1) for all of them. Removals of 31-11%, 18-11%, 31-25% were obtained for TOC, TN and NH_4^+ respectively. Li et al., (2018) showed that the presence of Cu(II) can reduce the removal of NH_4^+ by *Coelastrella sp*.by growth inhibition. In terms of growth, an opposite trend was observed, in which the growth rate increased significantly under conditions of higher presence of these metals from an average growth of 2.7 to 9.7%. On the other hand, as Collao et al., (2022) obtained, heavy metal presence did not affect NH_4^+ removal. Finally, the biomass growth was also significantly favoured for conditions of higher metal concentration (M2), reaching maximum values of 4% growth in terms of TS. As seen in the literature, a wide variety of microalgae have proven to be able to tolerate certain concentrations of the most toxic metal species such as Cd and As (Shanab et al., 2012). As(V) was reported to improve the growth of cyanobacterium *Nostoc minutum* and microalgae *Chlorella salina* and *Chlorella* sp, (Ferrari et al., 2013; Leong and Chang, 2020). The results of Oliveira et al. (2021) showed that the addition of 0.5 mg Cu/L and 25 mg Zn/L provided the greatest increase of VSS. On the other hand, negative nutrient removal rates or low nutrient removal rates have in some cases been compared with existing literature, e.g. the work of Oliveira et al., (2021), with HRAPs doped with Cu and Zn at different concentrations, CODs concentrations, increased with respect to the initial concentration of soluble COD (200 mg/L). These values increased from the first days of operation and occurred both at low concentrations of these metals and at high concentrations. The HRAP with low concentration of these metals reached a CODs concentration of up to 795.0 mg/L and in the HRAPs higher Cu and Zn concentrations, values of CODs reached 753.3 and 682.5 mg/L, respectively. These results may reflect that organic matter degradation would have stopped due to bacteria inhibition responsible for this degradation.

In account of control and noise factors interactions, the TxP interaction is the most significant for TOC-RE (7%). For this interaction, working al a level T1 of the control factor T, will make the system more robust or invariant to the effect of the noise factor P (uncontrollable in a real process). In contrast, poorer results are obtained.

For TN-RE, TxP interaction has a higher contribution (17%) but the same occurs as for TOC-RE for T1, where a lower interference of the noise factor P. For NH_4^+-RE. no significant factors were found. For biomass growth, TxL interaction has a higher contribution (19%), For this interaction, working al a level T1 of the control factor T, will make the system more robust or invariant to the effect of the noise factor L., where for 24h contact time the L factor has no effect, while for 72h contact time, biomass growth is favored by 16:8 photoperiods as expected in photosynthetic systems.

Bacteria bioreactors (B-R2)

In bacteria-based BRs (B-R2), the noise P factor (PWW C/N ratio) contributes 56 % to TOC-RE, 69 % to TN-RE. The influence of T contributes 36 % to NH_4^+-RE and M contributes with 31% to biomass growth.

Regarding control factors, initial TN concentrations of 200 mg/L (N1 level) significantly and positively affect the removal of TOC, TN and NH_4^+. Specifically, the mean values and their variation at the two concentration levels of N factor were 41-24%, 34-20%, 66-46% and -0.35-1.5% for RE-TOC, RE-TN, and NH_4^+-RE and biomass growth, respectively (**Figure 8.5**).

Similarly, in B-R2, contact times of 72 hours (T2 level) demonstrated a significant positive effect on the removal of TOC, TN, and NH_4^+except for biomass growth, consistent with the results observed in A-R1 experiments. Specifically, the mean removal values at low and high levels of T factor were 15-50%, 20-34%, 45-68% and 0.29-0.82% for TOC, TN, NH_4^+ and biomass growth respectively. The initial biomass concentration B1 significantly positively influenced TOC-RE. and biomass growth in B-R2. The removal efficiencies were, 42-22%, 28-25%, 55-57% and 3-1.8% for TOC, TN, NH_4^+ and biomass growth respectively. In this way, nutrients could be better assimilated by the biomass at a lower concentration, resulting in higher growth under these conditions.

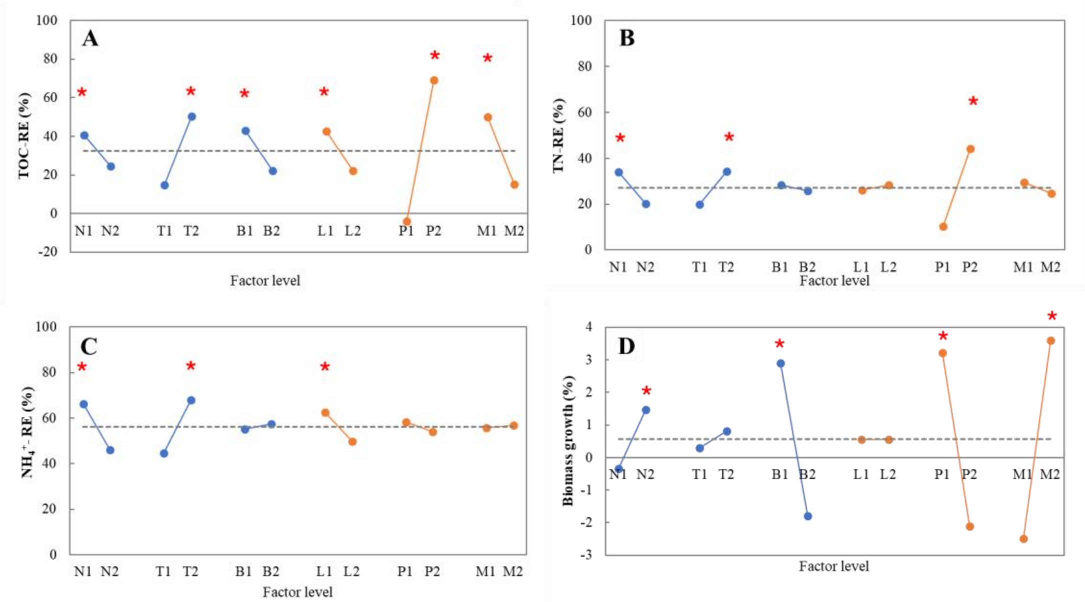

Figure 8.5. Means plot for removal efficiencies (REs) of TOC (A), TN (B) and NH_4^+(C) and biomass growth (D) in bacteria bioreactors (B-R2). (—●—) control factor, (—●—) noise factor and (-----) grand mean. Asterisks show differences that are significant at the 95% confidence level.

In relation to L factor, TOC and NH_4^+ removal was promoted by L1 level of the factor, this may be due to the presence of photosynthetic bacteria like cyanobacteria in PWW, as a treatment with aerobic bacteria would not be expected to have a significant effect in L factor.

In relation to P factor in relation to noise factors in B-R2, at P2 level significantly increases TOC and TN removal from the medium, while P1 strongly promotes biomass growth, due to strong nitrificant profile of the activated sludge employed in B-R2 experiments. According to Zheng et al., (2019) in PBRs cultivated with microalgae, higher final biomass concentrations were found for a C/N ratio of 25:1, achieving maximum COD removal efficiency of 99%, complete removal of NH_4^+, and 96% removal of TN.

Finally, regarding HM concentration as noise factor, TOC-RE were the only significantly favored nutrient removal by a low concentration of metal (M1). Removals of 50-15%, 29-25%, 55-56% and -2.5-3.5% were obtained for TOC, TN, NH_4^+ and biomass growth, respectively.

From literature, Saavedra et al., (2019), observed a remarkable protective effect against heavy metal toxicity in *S. almeriensis* cultures when organic material was present in the culture media. Collao et al. (2022) conducted a study in a continuous photobioreactor fed with real swine manure spiked with 100 mg/L of Cu and 100 mg/L of Zn and found statistically different values of total suspended solids (TSS) compared to the photobioreactor without metal doping. Interestingly, Cu doping led to higher TSS levels than in the control, comparable to the increase of TS with increasing metal concentration in the medium in the present work. Besides, the doped photobioreactors of Collao et al., (2022) maintained metabolic activity, achieving steady states with removal rates of 83% and 81% for TOC, and 46% and 58% for TN, in the case of Cu and Zn, respectively.

As previous reported in various studies, a release of TOC from the biomass is observed, increasing TOC concentration due to hydrolysis of EPS and acidogenesis (Kiran and Thanasekaran, 2011). Fewer studies with only bacteria were found in the literature. On the other hand, for *Chlorella vulgaris* cultured PBRs in the presence of higher concentrations of Cu and Zn, up to 100 mg/L (higher than in the present work), the growth, viability, and quality of the biomass generated were compromised to a high degree, as well as reducing the amount of heterotrophic bacterial communities capable of oxidising organic matter, resulting in a decrease in the percentage of soluble TOC removal from the medium. (Collao et al., 2022)

In account of control and noise factors interactions, the BxM interaction is the most significant for TOC-RE (2%). For this interaction, working al a level B1 of the control factor B, will make the system more robust or invariant to the effect of the noise factor M (uncontrollable in a real process). For TN-RE, no significant factors were found.

For NH_4^+-RE., BxM interaction has a higher contribution (9%), For this interaction, working al a level B1 of the control factor B, will make the system more robust or invariant to the effect of the noise factor M. Finally for biomass growth, NxM interaction was the most significant, where for N2, the system will be more resistant to noise factor fluctuation in a real scenario. These interactions as well as the rest of the interactions plots along with the ANOVA table are available in the supplementary material of this Chapter.

8.3.4 S/N ratio

In this section, signal to noise ratio will be discussed. By this way, signal-to-noise ratio, in our work, a higher S/N ratio indicates the system is more robust in relation to noise factors variability, due to the fact that they are not controllable in real scenarios. This means that the higher the signal-to-noise ratio, the lower the influence of noise and the higher the contribution of the signal to the final result. Significant results are shown un **Figures 8.6** and **8.7** marked with red asterisks.

In general terms, few significant S/N ratios have been obtained, which indicates that our system is quite robust to the variability that may be contributed by environmental noise factors that are not controllable in real applications.

For A-R1, only significant S/N responses were obtained for the combination factor-level T1 for Cu-RE, N1 and B2 for Cd-RE and B2 for TN-RE. T factor is not significant for Cu-RE, thus, T1 would be the best choice for contact time. In the same way, N is neither a significant factor in Cd-RE and N1 would be the best option for initial TN in the feed of the PBR. Finally, regarding B factor, its optimal level for S/N ratio matches with optimal level for optimal response, so, B2 would be the level chosen for operation. For the rest of the responses of interest, it is sufficient to observe the optimal described in previous sections of this chapter, as the S/N ratio is not significant. However, it is important to take into account that, in some cases, it may happen that there is a disparity between the best S/N ratio and the optimal or desired result.

On the other hand, for B-R2, no significant differences are obtained in terms of S/N ratio for HM removal. This may be due to the more robust operation of bacteria-based bioreactors compared to microalgae-based bioreactors. This gives a wider range of options for the choice of control factor levels to obtain the optimal operating values in the bioreactors, due to the no significant difference of noise factors in the S/N ratio. Related to nutrient removal, the only significant difference found is for TOC-RE for B factor, where level B2 is the best choice in terms of S/N ratio. On the contrary, optimal conditions for TOC-RE is the combination of factor-level B1. For the rest of the responses of interest, it is sufficient to observe the optima described in previous sections of this chapter, as the S/N ratio is not significant.

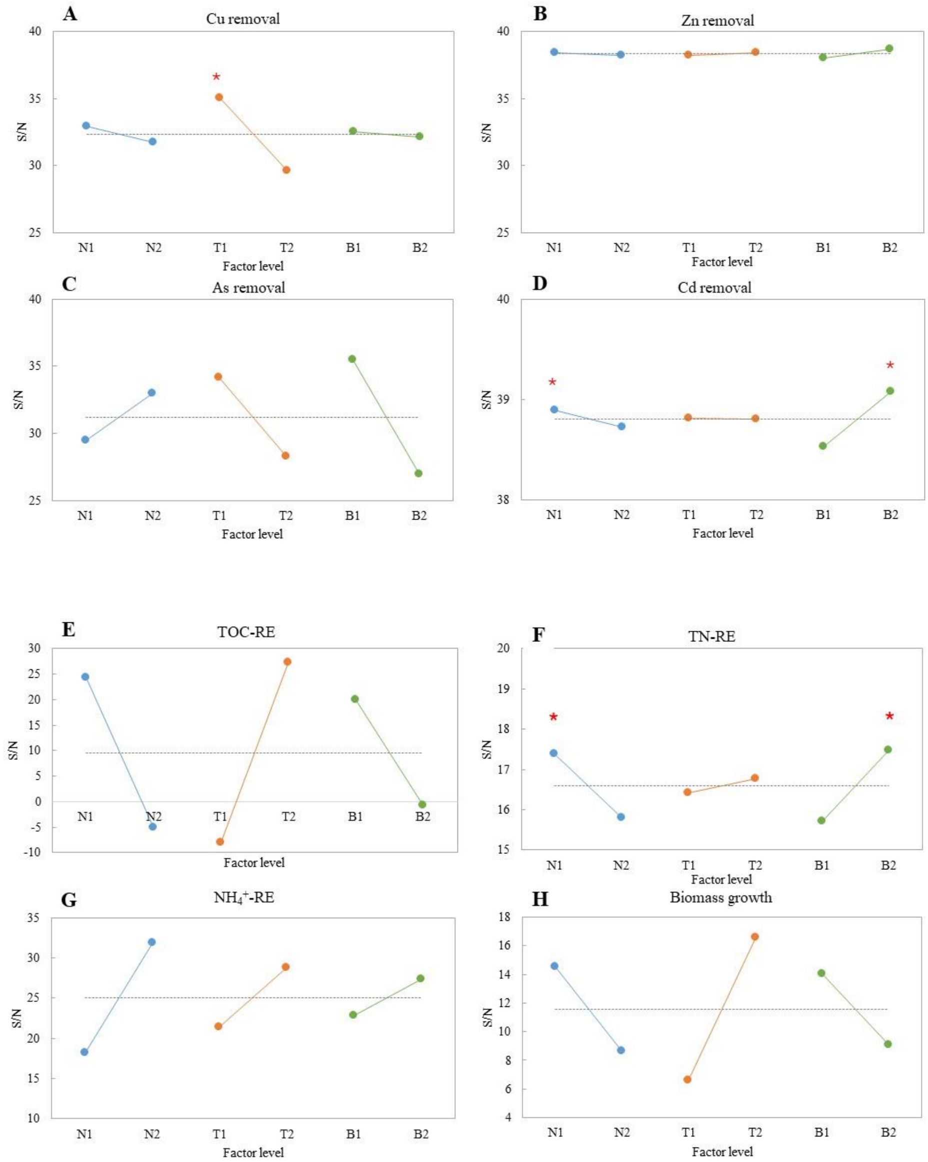

Figure 8.6. S/N ratio for heavy metal (A, B C and D), nutrient removal, and biomass growth (E, F, G and H) for A-R1

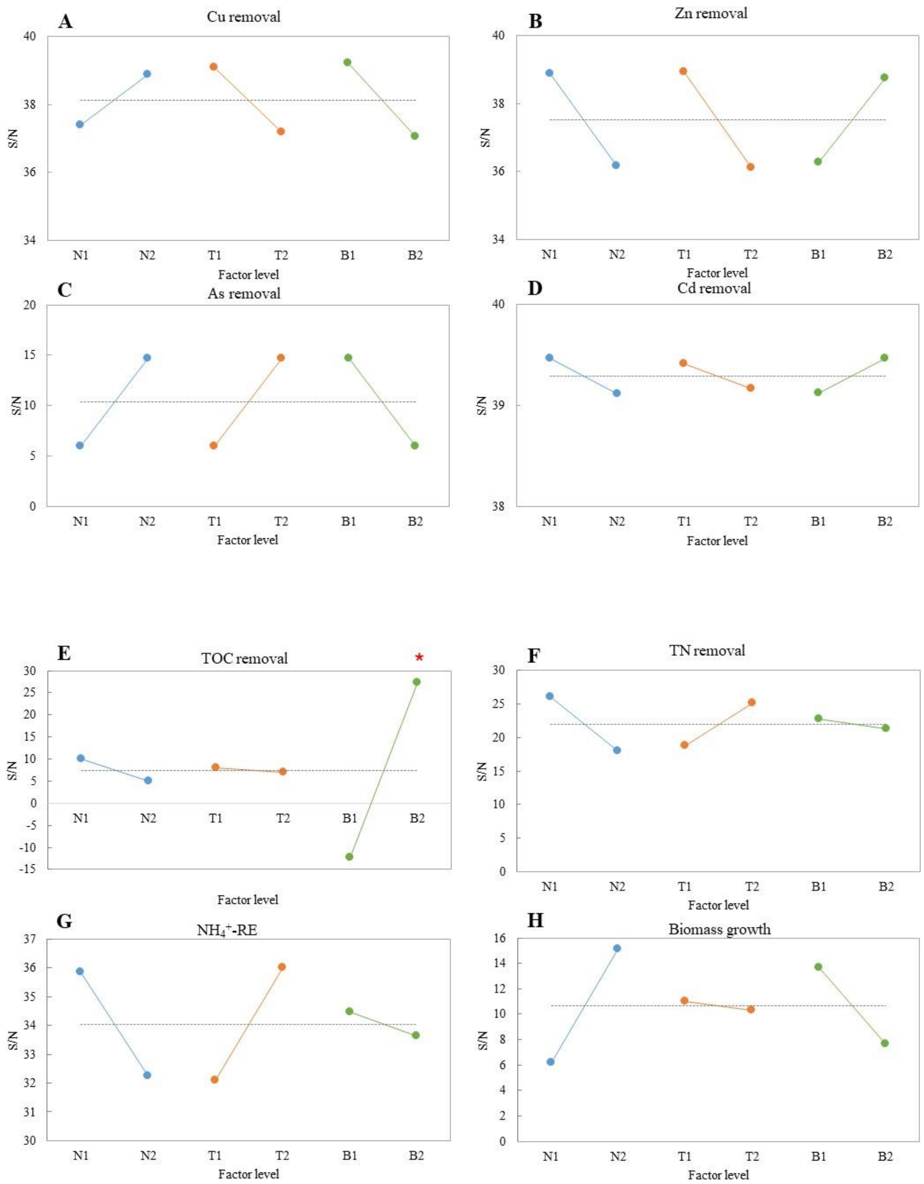

Figure 8.7. S/N ratio for heavy metal (A, B C and D), nutrient removal, and biomass growth (E, F, G and H) for B-R2

8.3.5 Principal Component Analysis (PCA)

Due to the large number of variables in this study to be optimised, a multivariate analysis method was applied to reduce the dimensionality by applying Principal Component Analysis (PCA) to the analytical results of the 16+16 experiments. Only the first two principal components, PC1 and PC2, explain more variance than with the original responses (73%) and allow a much more visual interpretation. Results are shown in **Figure 8.8.**

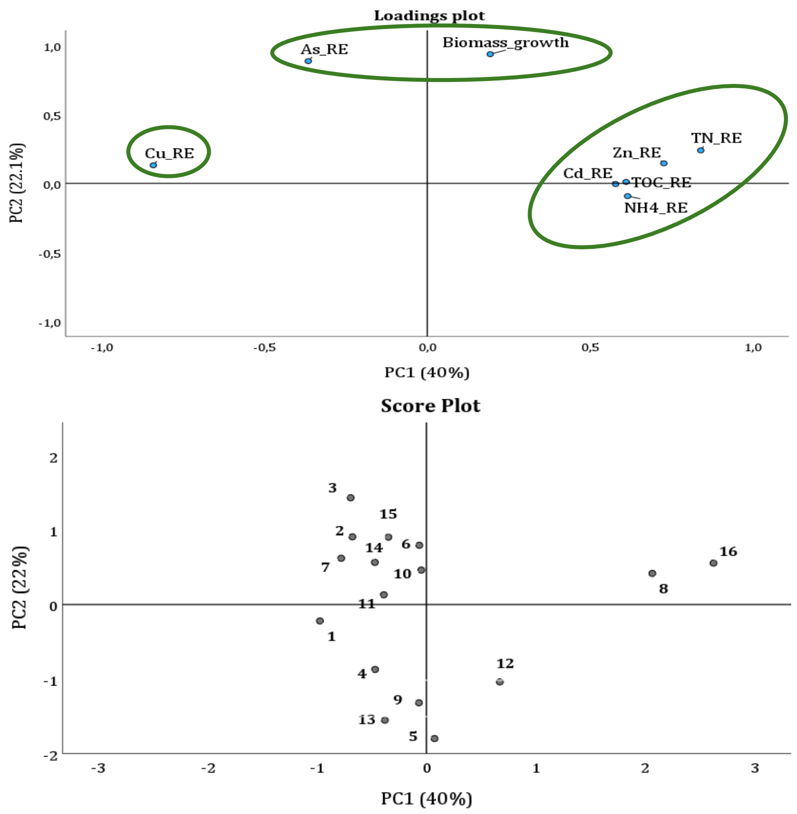

Figure 8.8. Loadings and Score Plots from PCA regarding different studied variables and experiments for A-R1 (A) and (B),

In the case of A-R1, a clear distribution of the variables Cd-RE, Zn-RE, corresponding to cationic metal species, and the elimination of the nutrients TOC, TN and NH_4^+, were observed all of them in the positive part of PC1. In the same way, in the scores graph, it can be seen that the experiments/run that have a greater weight in this PC1 component are 8 and 16, corresponding to experiments with a combination of control factors that coincide in contact time of 72 hours and noise factors that coincide in exposures to solar radiation over a longer period of time; 16:8, PWW with a C/N ratio of 4.17 and a lower initial load of metals. All this would make these variables cluster around the same group. On the contrary, other variables, such as Cu removal, seem to have a completely opposite behavior to those mentioned above, while arsenic removal and growth have a considerable load on PC2.

For B-R2 (**Figure 8.9**), something similar occurs with the removal of cationic species with the exception of Cu and As as for the microalgae. For the removal of TOC and TN, in relation to the loadings plot, they continue to cluster around the high positive loadings of PC1, favored by conditions 8 and 16, analogous to those of R1-A. In summary, a clear trend towards cationic metal removal (except for Cu), and nutrient removal, is favored by long contact times and a lower initial nitrogen loading up to 100 mg/L.

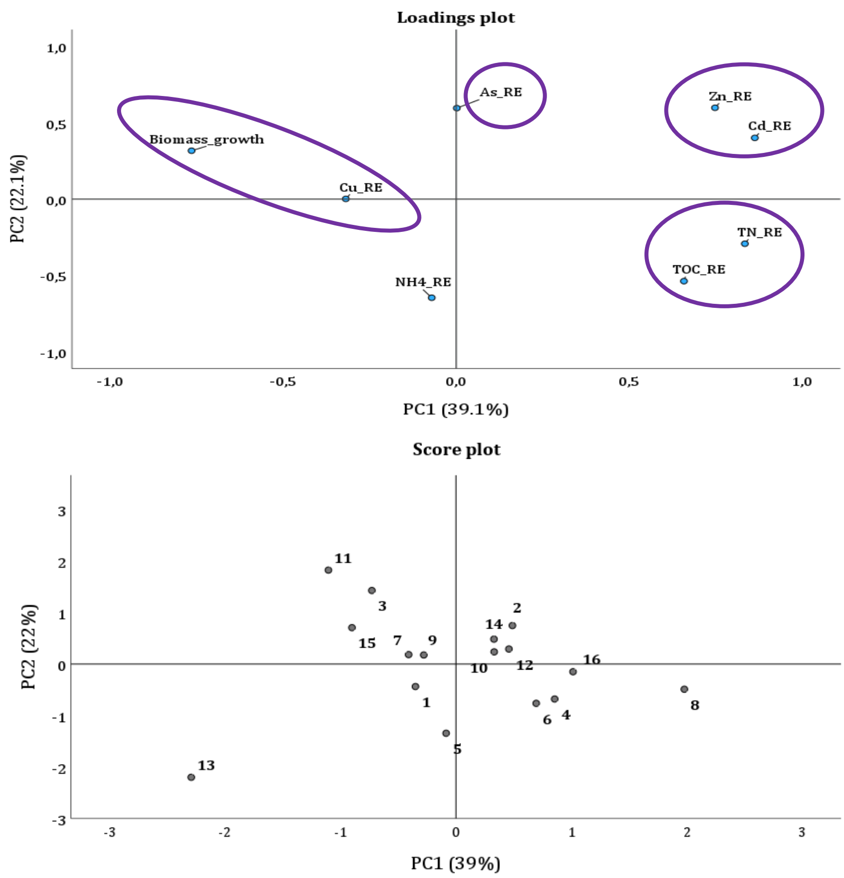

Figure 8.9. Loadings and Score Plots from PCA regarding different studied variables and experiments for B-R2 (A) and (B)

8.4 Conclusions

In this study, Cu(II), Zn(II), As(V) and Cd(II) were effectively removed during the biological treatment with microalgae-bacteria consortia and activated sludge-based treatments in metal spiked bioreactors, as well as the nutrients (TOC, TN and NH_4^+) present in the suspension. Nutrient removal was clearly reduced with increasing concentration of metals in the suspension, while the increase in the initial concentration of these metals does not condition the increase of the uptake of these metals, also increasing the uptake capacity of these metals. Best REs of Cu(II), Zn(II), As(V), and Cd(II) ranged from 81-98%, 96-97%, 98-72%, and 93-99% respectively. For TOC, TN and NH_4^+ removal, values from 83-88%, 56-63%, and 63-89% were reached for A-R1 and B-R2 respectively. B-R2 shows a better performance respect to A-R1 in terms of heavy metals and nutrient removal in swine wastewater treatment, except for As(V) bioelimination. Higher growth was observed for higher and lower C/N ratio PWW for A-R1 and B-R2 system respectively. Both A-R1 and B-R2 cultures growth, were significantly enhanced by high initial metal concentrations. On a whole, it is difficult to favor the simultaneous elimination of metals and nutrients due to high metal removal, limits or compromises nutrient removal.

Acknowledgments

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Effect of operational parameters on metal and nutrient removal from piggery wastewater using biological treatments with microalgae and bacteria

Supplementary material

ANOVA for metal, nutrient removal and biomass growth for A-R1

Table S8.1. ANOVA for metal removal (Cu, Zn, As and Cd REs) and nutrient removal (TOC, TN, NH_4^+ REs) and biomass growth for A-R1.

Cu removal (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	[N]	542.17	1	542.17	426.95	2.48E-04	9.61
T	[Y]	2.66	1				
B	[Y]	0.21	1				
L	[N]	721.02	1	721.02	567.79	1.62E-04	12.78
P	[N]	735.33	1	735.33	579.06	1.57E-04	13.03
M	[N]	2084.22	1	2084.22	1641.30	3.31E-05	36.94
NxL	[N]	19.92	1	19.92	15.69	2.87E-02	0.35
NxP	[N]	48.73	1	48.73	38.38	8.47E-03	0.86
NxM	[N]	40.75	1	40.75	32.09	1.09E-02	0.72
TxL	[N]	492.31	1	492.31	387.69	2.86E-04	8.73
TxP	[N]	408.89	1	408.89	321.99	3.77E-04	7.25
TxM	[N]	435.86	1	435.86	343.24	3.43E-04	7.73
BxL	[Y]	0.94	1				
BxP	[N]	67.73	1	67.73	53.34	5.30E-03	1.20
BxM	[N]	41.30	1	41.30	32.53	1.07E-02	0.73
e		**3.81**	**3**	**1.27**			**0.07**
Total		**5642.0**	**15**				**100.00**
Zn removal (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	[Y]	13.877	1				
T	[Y]	19.417	1				
B	[N]	114.537	1	114.54	8.90	2.45E-02	13.29
L	[N]	78.668	1	78.67	6.11	4.83E-02	9.13
P	[Y]	12.477	1				
M	[N]	28.339	1	28.34	2.20	1.88E-01	3.29
NxL	[N]	30.117	1	30.12	2.34	1.77E-01	3.50
NxP	[Y]	11.042	1				
NxM	[N]	63.182	1	63.18	4.91	6.86E-02	7.33
TxL	[N]	78.778	1	78.78	6.12	4.82E-02	9.14
TxP	[Y]	19.488	1				
TxM	[N]	301.101	1	301.10	23.40	2.89E-03	34.95
BxL	[N]	34.678	1	34.68	2.69	1.52E-01	4.03
BxP	[Y]	0.915	1				
BxM	[N]	54.945	1	54.94	4.27	8.43E-02	6.38
e		77.2	6	12.87			8.96
Total		861.6	15				100

Pool: pooled or not pooled. SS: Sum of Squares. DoF: freedom degrees. MS: Mean Square. F: Fisher number. p: p-value. e: error (residual)

Table S8.*1*. *continued*

As removal (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	[*Y*]	245.18	1				
T	[*Y*]	80.23	1				
B	[*N*]	799.90	1	799.90	6.34	2.57E-02	6.74
L	[*Y*]	177.11	1				
P	[*Y*]	448.65	1				
M	[*N*]	9422.51	1	9422.51	74.70	9.50E-07	79.43
NxL	[*Y*]	91.17	1				
NxP	[*Y*]	29.82	1				
NxM	[*Y*]	13.01	1				
TxL	[*Y*]	167.46	1				
TxP	[*Y*]	107.90	1				
TxM	[*Y*]	31.51	1				
BxL	[*Y*]	33.30	1				
BxP	[*Y*]	32.53	1				
BxM	[*Y*]	181.88	1				
e		1639.7	13	126.13			13.82
Total		11862.1	15				100

Cd removal (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	[*Y*]	9.71	1				
T	[*Y*]	0.06	1				
B	[*N*]	111.35	1	111.35	14.60	2.44E-03	35.20
L	[*N*]	76.19	1	76.19	9.99	8.22E-03	24.09
P	[*Y*]	3.26	1				
M	[*Y*]	12.24	1				
NxL	[*Y*]	6.36	1				
NxP	[*Y*]	6.53	1				
NxM	[*Y*]	12.39	1				
TxL	[*Y*]	1.11	1				
TxP	[*Y*]	1.09	1				
TxM	[*N*]	37.25	1	37.25	4.88	4.73E-02	11.78
BxL	[*Y*]	25.63	1				
BxP	[*Y*]	10.15	1				
BxM	[*Y*]	3.00	1				
e		91.5	12	7.63			28.94
Total		316.3	15				100

Table S8.1. *continued*

Factor	Pool	SS	DoF	MS	F	p	%
TOC removal (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	[N]	142.44	1	142.44	11.55	1.93E-02	0.73
T	[N]	10346.81	1	10346.81	838.62	9.20E-07	53.03
B	[Y]	38.66	1				
L	[N]	616.88	1	616.88	50.00	8.75E-04	3.16
P	[N]	3884.44	1	3884.44	314.84	1.04E-05	19.91
M	[N]	1626.11	1	1626.11	131.80	8.79E-05	8.33
NxL	[N]	114.95	1	114.95	9.32	2.83E-02	0.59
NxP	[Y]	1.02	1				
NxM	[Y]	17.65	1				
TxL	[N]	857.34	1	857.34	69.49	4.06E-04	4.39
TxP	[N]	1358.34	1	1358.34	110.10	1.36E-04	6.96
TxM	[N]	183.17	1	183.17	14.85	1.20E-02	0.94
BxL	[Y]	2.09	1				
BxP	[N]	318.92	1	318.92	25.85	3.82E-03	1.63
BxM	[Y]	2.27	1				
e		61.69	5	12.34			0.32
Total		19511.10	15				100.00
TN removal (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	[Y]	53.468	1				
T	[N]	386.520	1	386.520	10.357	9.20E-03	10.235
B	[Y]	34.226	1				
L	[N]	146.818	1	146.818	3.934	7.54E-02	3.888
P	[N]	2038.746	1	2038.746	54.627	2.34E-05	53.986
M	[N]	193.564	1	193.564	5.186	4.60E-02	5.126
NxL	[Y]	92.354	1				
NxP	[Y]	3.220	1				
NxM	[Y]	0.038	1				
TxL	[Y]	17.179	1				
TxP	[N]	637.564	1	637.564	17.083	2.03E-03	16.883
TxM	[Y]	122.403	1				
BxL	[Y]	43.830	1				
BxP	[Y]	6.294	1				
BxM	[Y]	0.199	1				
e		373.212	10	37.321			9.883
Total		3776.424	15				100

Table S8.1. *continued*

			NH_4^+ removal (%)				
Factor	Pool	SS	DoF	MS	F	p	%
N	[N]	2875.07	1	2875.07	5471.22	8.56E-09	62.81
T	[N]	515.03	1	515.03	980.09	6.24E-07	11.25
B	[N]	8.35	1	8.35	15.90	1.05E-02	0.18
L	[N]	482.87	1	482.87	918.90	7.33E-07	10.55
P	[N]	452.74	1	452.74	861.57	8.60E-07	9.89
M	[N]	143.56	1	143.56	273.19	1.48E-05	3.14
NxL	[N]	8.47	1	8.47	16.12	1.02E-02	0.19
NxP	[Y]	1.41	1				
NxM	[Y]	0.30	1				
TxL	[Y]	0.01	1				
TxP	[N]	7.19	1	7.19	13.68	1.40E-02	0.16
TxM	[N]	41.58	1	41.58	79.13	2.99E-04	0.91
BxL	[Y]	0.73	1				
BxP	[N]	40.22	1	40.22	76.55	3.23E-04	0.88
BxM	[Y]	0.17	1				
e		**2.63**	**5**	**0.53**			**0.06**
Total		**4577.71**	**15**				**100**
			Biomass growth (%)				
Factor	Pool	SS	DoF	MS	F	p	%
N	[Y]	2.72	1				
T	[Y]	9.76	1				
B	[N]	26.68	1	26.68	4.84	5.53E-02	4.32
L	[N]	98.17	1	98.17	17.82	2.24E-03	15.90
P	[Y]	9.60	1				
M	[N]	194.31	1	194.31	35.27	2.18E-04	31.48
NxL	[Y]	2.19	1				
NxP	[N]	14.02	1				
NxM	[Y]	0.11	1				
TxL	[N]	114.92	1	114.92	20.86	1.35E-03	18.62
TxP	[N]	92.62	1	92.62	16.81	2.68E-03	15.01
TxM	[Y]	6.02	1				
BxL	[Y]	0.57	1				
BxP	[N]	40.97	1	40.97	7.44	2.33E-02	6.64
BxM	[Y]	4.59	1				
e		49.58	9	5.51			8.03
Total		617.24	15				100

Table S8.2. ANOVA for metal removal (Cu, Zn, As and Cd REs) and nutrient removal (TOC, TN, NH_4^+) and biomass growth for B-R2.

Cu removal (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	[Y]	5.03	1				
T	[N]	106.01	1	106.01	21.08	1.37E-01	3.08
B	[N]	226.90	1	226.90	45.12	9.41E-02	6.60
L	[N]	173.95	1	173.95	34.59	1.07E-01	5.06
P	[N]	69.07	1	69.07	13.73	1.68E-01	2.01
M	[N]	62.17	1	62.17	12.36	1.76E-01	1.81
NxL	[N]	258.42	1	258.42	51.39	8.82E-02	7.52
NxP	[N]	481.81	1	481.81	95.80	6.48E-02	14.02
NxM	[N]	449.79	1	449.79	89.44	6.71E-02	13.08
TxL	[N]	227.07	1	227.07	45.15	9.41E-02	6.61
TxP	[N]	242.30	1	242.30	48.18	9.11E-02	7.05
TxM	[N]	311.91	1	311.91	62.02	8.04E-02	9.07
BxL	[N]	436.25	1	436.25	86.75	6.81E-02	12.69
BxP	[N]	243.83	1	243.83	48.49	9.08E-02	7.09
BxM	[N]	143.00	1	143.00	28.44	1.18E-01	4.16
e		**5.03**	**1**	**5.03**			**0.15**
Total		**3437.51**	**15**				**100**

Zn removal (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	Y	333.93	1				
T	[Y]	295.14	1				
B	[Y]	167.64	1				
L	[Y]	339.14	1				
P	[N]	936.91	1	936.91	4.69	4.96E-02	23.18
M	[Y]	358.27	1				
NxL	[Y]	85.27	1				
NxP	[Y]	164.57	1				
NxM	[Y]	68.58	1				
TxL	[Y]	337.22	1				
TxP	[N]	505.80	1	505.80	2.53	1.36E-01	12.52
TxM	[Y]	76.58	1				
BxL	[Y]	90.54	1				
BxP	[Y]	124.52	1				
BxM	[Y]	157.41	1				
e		2598.80	13	199.91			64.30
Total		4041.51	15				100

Pool: pooled or not pooled. SS: Sum of Squares. DoF: freedom degrees. MS: Mean Square. F: Fisher number. p: p-value. e: error (residual)

Table S8.2. *continued*

As removal (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	[N]	67.65	1	67.65	300.82	3.31E-03	0.65
T	[N]	165.04	1	165.04	733.86	1.36E-03	1.58
B	[N]	871.68	1	871.68	3875.95	2.58E-04	8.32
L	[N]	198.12	1	198.12	880.94	1.13E-03	1.89
P	[N]	318.75	1	318.75	1417.35	7.05E-04	3.04
M	[N]	8151.38	1	8151.38	36245.41	2.76E-05	77.84
NxL	[N]	35.34	1	35.34	157.16	6.30E-03	0.34
NxP	[N]	5.15	1	5.15	22.88	4.10E-02	0.05
NxM	[N]	44.18	1	44.18	196.45	5.05E-03	0.42
TxL	[N]	9.89	1	9.89	43.96	2.20E-02	0.09
TxP	[N]	62.23	1	62.23	276.70	3.59E-03	0.59
TxM	[N]	23.47	1	23.47	104.35	9.45E-03	0.22
BxL	[Y]	0.26	1				
BxP	[Y]	0.19	1				
BxM	[N]	519.23	1	519.23	2308.76	4.33E-04	4.96
e		**0.45**	**2**	**0.22**			**0.00**
Total		**10472.55**	**15**				**100**

Cd removal (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	[N]	40.22	1	40.22	10.78	2.19E-02	10.11
T	[N]	16.02	1	16.02	4.29	9.30E-02	4.03
B	[N]	38.00	1	38.00	10.18	2.42E-02	9.55
L	[N]	33.41	1	33.41	8.95	3.04E-02	8.40
P	[N]	137.65	1	137.65	36.88	1.75E-03	34.60
M	[Y]	1.49	1				
NxL	[Y]	10.00	1				
NxP	[N]	28.34	1	28.34	7.59	4.00E-02	7.12
NxM	[Y]	1.08	1				
TxL	[N]	14.24	1	14.24	3.82	1.08E-01	3.58
TxP	[N]	27.95	1	27.95	7.49	4.09E-02	7.03
TxM	[Y]	0.21	1				
BxL	[Y]	5.89	1				
BxP	[N]	15.34	1	15.34	4.11	9.84E-02	3.86
BxM	[N]	28.00	1	28.00	7.50	4.08E-02	7.04
e		18.66	5	3.73			4.69
Total		397.84	15				100

Table S8.2. *continued*

TOC removal (%)

Factor	Pool	SS	DoF	MS	F	p	%
N	[N]	1032.05	1	1032.05	157.97	6.27E-03	2.69
T	[N]	4998.51	1	4998.51	765.10	1.30E-03	13.02
B	[N]	1708.02	1	1708.02	261.44	3.80E-03	4.45
L	[N]	1683.88	1	1683.88	257.74	3.86E-03	4.39
P	[N]	21574.53	1	21574.53	3302.31	3.03E-04	56.20
M	[N]	4951.75	1	4951.75	757.94	1.32E-03	12.90
NxL	[N]	226.93	1	226.93	34.74	2.76E-02	0.59
NxP	[N]	21.97	1	21.97	3.36	2.08E-01	0.06
NxM	[N]	314.58	1	314.58	48.15	2.01E-02	0.82
TxL	[Y]	4.73	1				
TxP	[N]	197.69	1	197.69	30.26	3.15E-02	0.51
TxM	[N]	187.08	1	187.08	28.64	3.32E-02	0.49
BxL	[N]	548.26	1	548.26	83.92	1.17E-02	1.43
BxP	[Y]	8.33	1				
BxM	[N]	930.33	1	930.33	142.40	6.95E-03	2.42
e		13.07	2	6.53			0.03
Total		38388.64	15				100

TN removal (%)

Factor	Pool	SS	DoF	MS	F	p	%
N	[N]	784.46	1	784.46	32.33	2.02E-04	11.75
T	[N]	824.54	1	824.54	33.99	1.66E-04	12.35
B	[Y]	22.63	1				
L	[Y]	19.25	1				
P	[N]	4616.20	1	4616.20	190.27	7.80E-08	69.12
M	[N]	85.68	1	85.68	3.53	8.96E-02	1.28
NxL	[Y]	13.48	1				
NxP	[Y]	37.34	1				
NxM	[Y]	1.57	1				
TxL	[Y]	40.18	1				
TxP	[N]	124.75	1	124.75	5.14	4.68E-02	1.87
TxM	[Y]	0.63	1				
BxL	[Y]	39.44	1				
BxP	[Y]	17.30	1				
BxM	[Y]	50.80	1				
e		242.61	10	24.26			3.63
Total		6678.24	15				100

Table S8.2. *continued*

NH_4^+ removal (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	[N]	1632.61	1	1632.61	21.77	8.87E-04	26.51
T	[N]	2203.86	1	2203.86	29.38	2.93E-04	35.78
B	[Y]	22.32	1				
L	[N]	657.48	1	657.48	8.77	1.43E-02	10.68
P	[Y]	72.21	1				
M	[Y]	5.41	1				
NxL	[Y]	20.76	1				
NxP	[Y]	160.86	1				
NxM	[Y]	24.56	1				
TxL	[Y]	122.83	1				
TxP	[Y]	231.88	1				
TxM	[Y]	7.49	1				
BxL	[Y]	81.70	1				
BxP	[N]	367.64	1	367.64	4.90	5.12E-02	5.97
BxM	[N]	547.17	1	547.17	7.30	2.23E-02	8.88
e		750.02	10	75.00			12.18
Total		6158.78	15				100
Biomass growth (%)							
Factor	Pool	SS	DoF	MS	F	p	%
N	[N]	13.10	1	13.10	7.81	2.34E-02	2.75
T	[N]	**1.13**	1				
B	[N]	88.87	1	88.87	52.96	8.57E-05	18.67
L	[N]	**0.00**	1				
P	[N]	113.55	1	113.55	67.67	3.57E-05	23.85
M	[Y]	147.36	1	147.36	87.83	1.38E-05	30.96
NxL	[N]	**0.37**	1				
NxP	[N]	**2.30**	1				
NxM	[N]	44.96	1	44.96	26.80	8.46E-04	9.45
TxL	[N]	41.76	1	41.76	24.89	1.07E-03	8.77
TxP	[N]	**0.79**	1				
TxM	[Y]	**7.21**	1				
BxL	[N]	12.99	1	12.99	7.74	2.38E-02	2.73
BxP	[N]	**1.38**	1				
BxM	[N]	**0.23**	1				
e		**13.42**	**8**	**1.68**			**2.82**
Total		**476.01**	**15**				**100.00**

Interactions plots

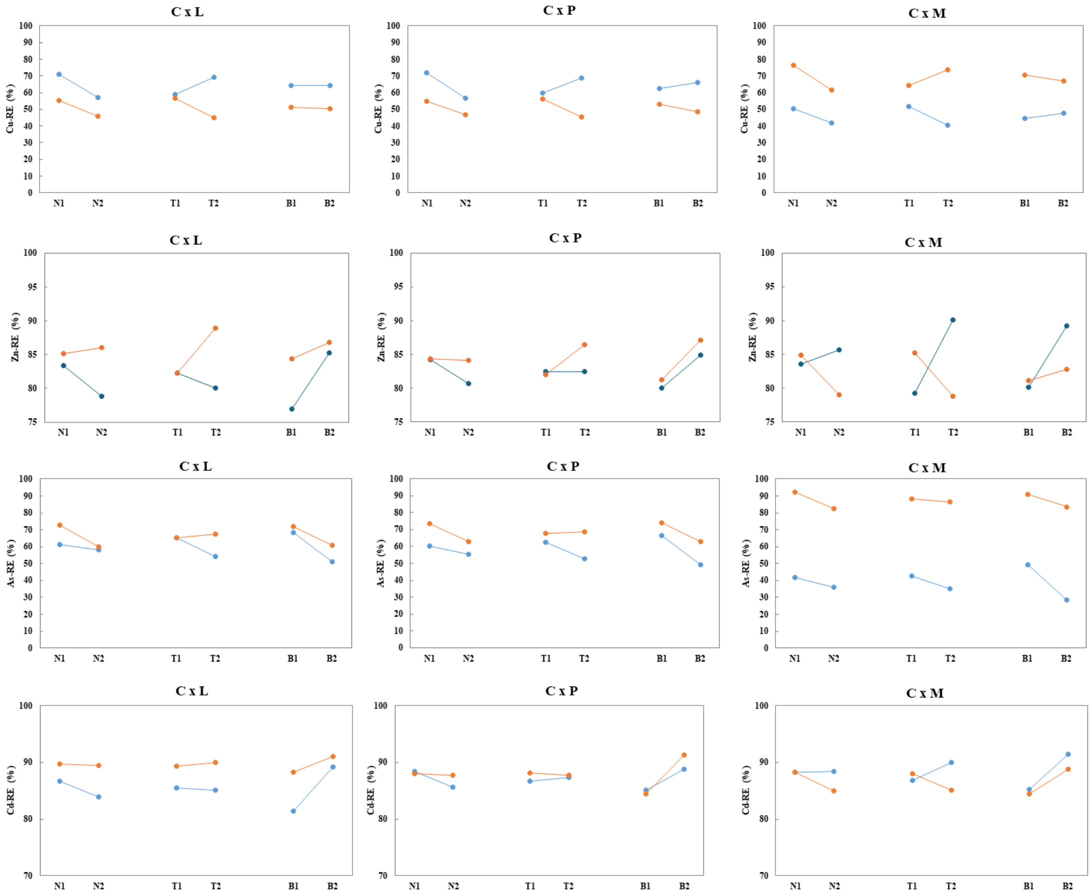


Figure S8.1. Control factor and noise factors interactions plot for removal efficiencies (REs) of Cu, Zn, As and Cd in microalgae-bacteria bioreactors (A-R1). (—●—) level 1, (—●—) level 2.

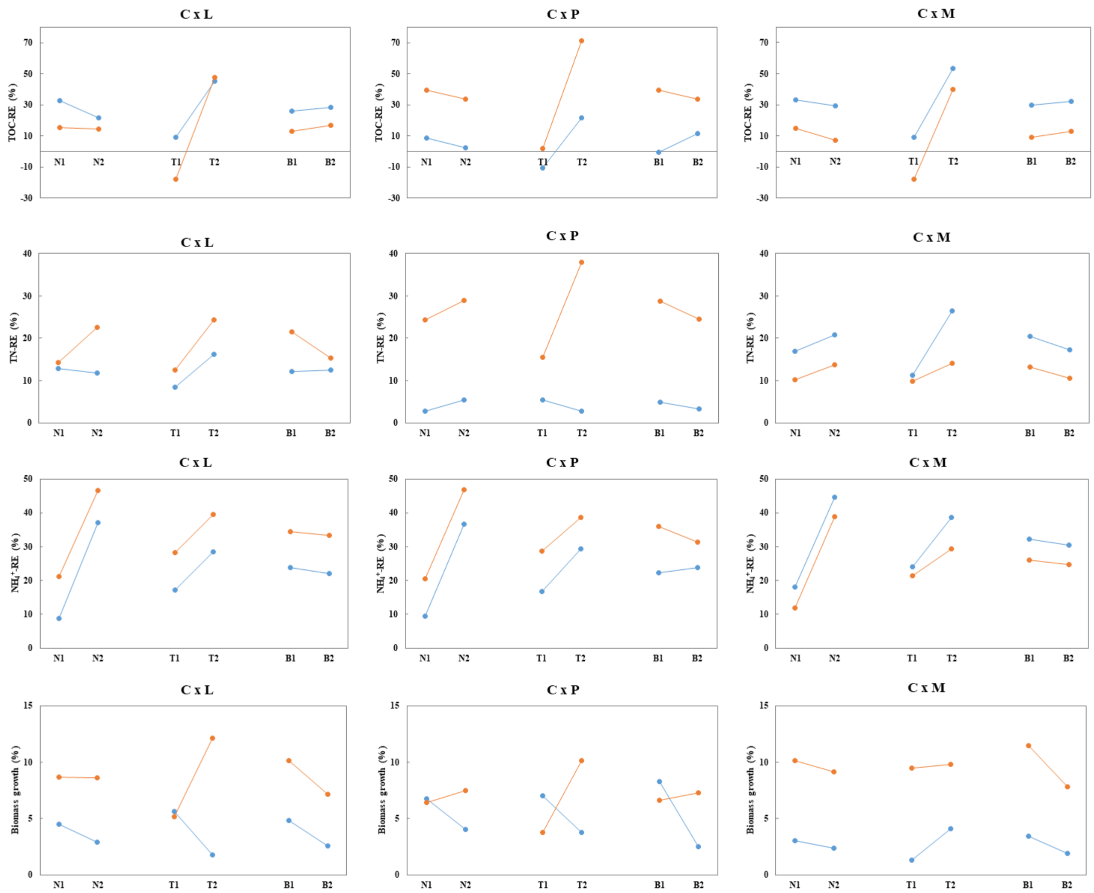


Figure S8.2. Control factor and noise factors interactions plot for removal efficiencies (REs) of TOC, TN and NH_4^+, and biomass growth in microalgae-bacteria bioreactors (A-R1). (—●—) level 1, (—●—) level 2.

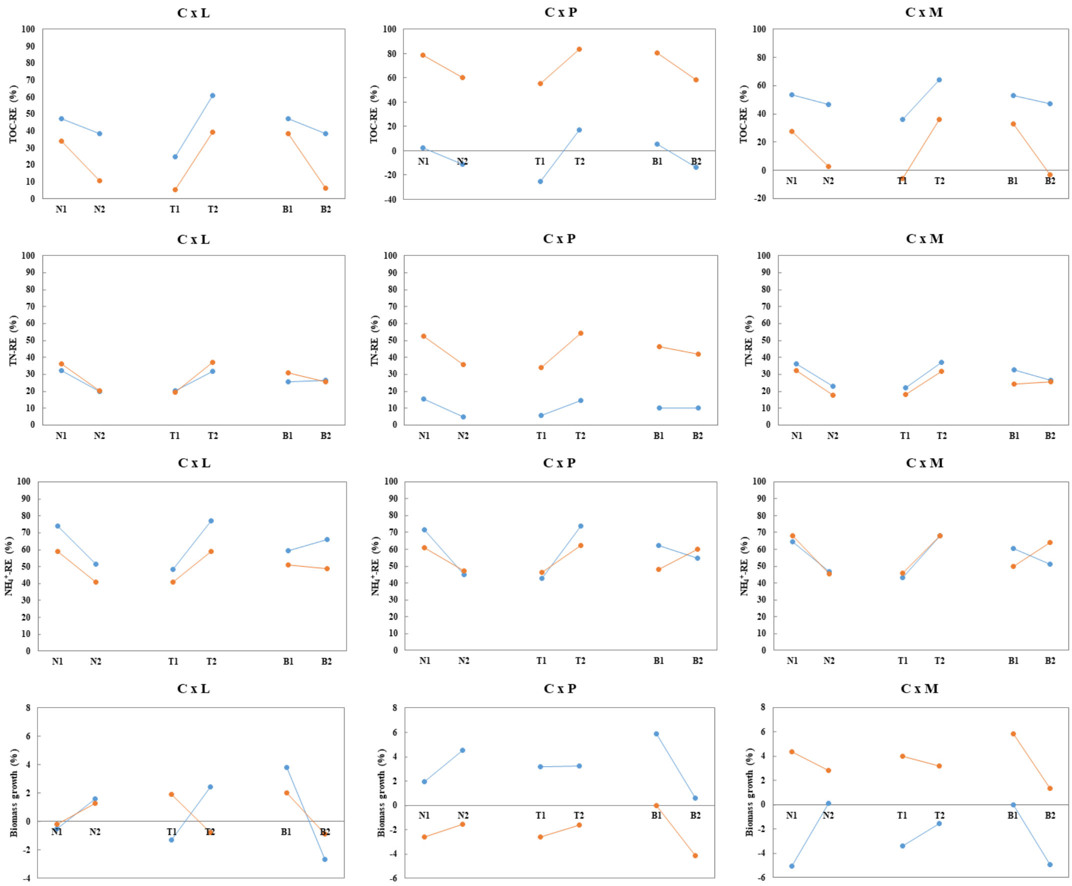


Figure S8.3. Control factor and noise factors interactions plot for removal efficiencies (REs) of Cu, Zn, As and Cd in bacteria bioreactors (B-R2). (—●—) level 1, (—●—) level 2.

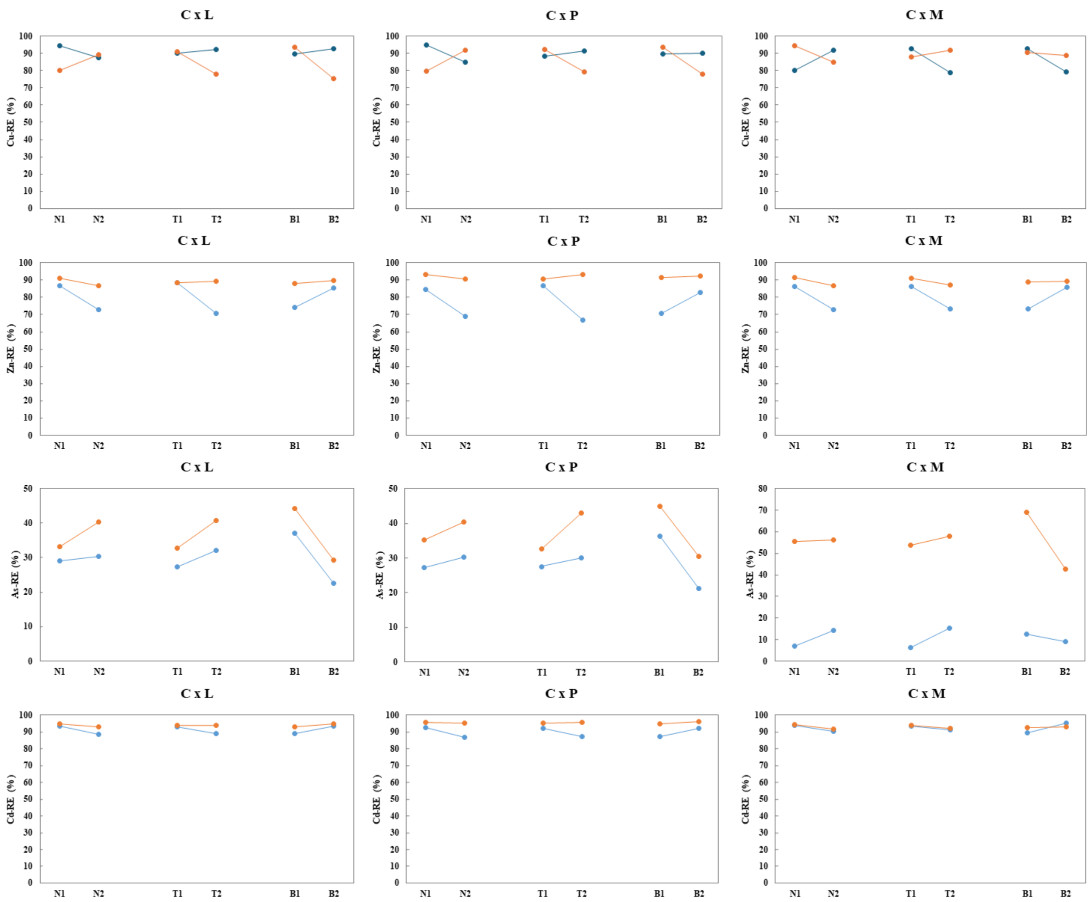


Figure S8.4. Control factor and noise factors interactions plot for removal efficiencies (REs) of TOC, TN and NH_4^+, and biomass growth in bacteria bioreactors (B-R2). (—●—) level 1, (—●—) level 2.

ANOVA S/N - A-R1

Table S8.3. ANOVA of S/N ratio for metal removal (Cu, Zn, As and Cd REs) and nutrient removal (TOC, TN, NH_4^+) and biomass growth for A-R1.

Factor	Pool	SS	DoF	MS	F	p	%
Cu removal (%)							
N	$[Y]$	1.35	1				
T	$[N]$	29.14	1	29.14	39.08	2.46E-02	95.13
B	$[Y]$	0.14	1				
e		1.49	2	0.75			4.87
Total		30.63	3				100
Zn removal (%)							
N	$[Y]$	0.038	1				
T	$[Y]$	0.026	1				
B	$[N]$	0.43	1	0.43	13.58	0.07	87.17
e		0.064	2	0.026			12.83
Total		0.49	3				100
As removal (%)							
N	$[Y]$	12.15	1				
T	$[Y]$	34.59	1				
B	$[N]$	72.16	1	72.16	3.09	0.22	60.69
e		12.15	2	23.37			39.31
Total		118.90	3				100
Cd removal (%)							
N	$[N]$	0.030	1	0.030	1545.302	6.46E-04	8.92
T	$[Y]$	3.83E-05	1				
B	$[N]$	0.302	1	0.302	15770.975	6.34E-05	91.07
e		3.83E-05	2	0.000			0.01
Total		0.332	3				100
TOC removal (%)							
N	$[Y]$	863.27	1				
T	$[N]$	1244.58	1	1244.58	1.93	0.30	49.07
B	$[Y]$	428.30	1				
e		1291.57	2	645.78			50.93
Total		2536.15	3				100
TN removal (%)							
N	$[N]$	2.48	1	2.48	36.19	0.027	43.08
T	$[Y]$	0.14	1				
B	$[N]$	3.14	1	3.14	45.82	0.021	54.54
e		0.14	2	0.07			2.38
Total		5.75	3				100
NH_4^+ removal (%)							
N	$[N]$	187.23	1	187.23	5.00	0.15	71.45
T	$[Y]$	53.83	1				
B	$[Y]$	21.00	1				
e		74.83	2	37.41			28.55
Total		262.06	3				100
Biomass growth (%)							
N	$[Y]$	34.91	1				
T	$[N]$	99.49	1	99.49	3.36	0.21	62.69
B	$[Y]$	24.31	1				
e		59.22	2	29.61			37.31
Total		158.71	3				100

ANOVA S/N B-R2

Table S8.4. ANOVA of S/N ratio for metal removal (Cu, Zn, As and Cd REs) and nutrient removal (TOC, TN, NH_4^+) and biomass growth for B-R2

			Cu removal (%)				
Factor	Pool	SS	DoF	MS	F	p	%
N	$[Y]$	2.20	1				
T	$[Y]$	3.58	1				
B	$[N]$	4.66	1	4.66	1.61	0.33	44.61
e		6.86	2	2.89			55.39
Total		10.44	3				100
			Zn removal (%)				
N	$[Y]$	7.47	1				
T	$[N]$	7.87	1	7.87	1.15	0.40	36.58
B	$[Y]$	6.17	1				
e		13.64	2	6.82			63.42
Total		21.51	3				100
			As removal (%)				
N	$[Y]$	76.03	1				
T	$[N]$	76.14	1	76.14	1.01	0.42	33.51
B	$[Y]$	75.03	1				
e		151.07	2	75.53			66.49
Total		227.20	3				100
			Cd removal (%)				
N	$[N]$	0.12	1	0.12	1.40	0.36	41.20
T	$[Y]$	0.06	1				
B	$[Y]$	0.12	1				
e		0.24	2	0.09			58.80
Total		0.30	3				100
			TOC removal (%)				
N	$[Y]$	24.03	1				
T	$[Y]$	0.93	1				
B	$[N]$	1572.96	1	1572.96	126.03	0.01	98.44
e		1596.99	2	12.48			1.56
Total		1597.92	3				100
			TN removal (%)				
N	$[N]$	64.90	1	64.90	3.08	0.22	60.60
T	$[Y]$	39.96	1				
B	$[Y]$	2.23	1				
e		67.13	2	21.10			39.40
Total		107.09	3				100
			NH_4^+ removal (%)				
N	$[Y]$	13.12	1				
T	$[N]$	15.41	1	15.41	2.23	0.27	52.68
B	$[Y]$	0.73	1				
e		13.84	2	6.92			47.32
Total		29.25	3				100
			Biomass growth (%)				
N	$[N]$	48.87	1	48.87	2.56	0.25	56.15
T	$[Y]$	20.42	1				
B	$[Y]$	17.75	1				
e		66.62	2	19.08			43.85
Total							100

Chapter 9:

Heavy metal and nutrient removal in piggery wastewater using a microalgae-bacteria consortia in a pilot scale photobioreactor

Heavy metal and nutrient removal in piggery wastewater using a microalgae-bacteria consortia in a pilot scale photobioreactor

Abstract

Pilot scale photobioreactor of *Scenedesmus almeriensis*-bacteria consortia were tested for the removal of Cu(II), Zn(II) and As(V) from liquid fraction of swine wastewater/piggery wastewater. After 15 days to achieve steady state of the PBR, it was doped with a mixture of different concentrations of Cu, Zn and As. PBRs ran for 20 additional days. Metal removal efficiencies were 77.1 %, 78.1 % and 77.4 % for Cu, Zn and As respectively. Metals found in the resulting biomass after 35 days of PBR operation were 20.06 mg/g, 16.01 mg/g and 23.82 μg/g for Cu(II), Zn(II) and As(V) respectively. Nutrient removal was also evaluated in this work, reaching removal efficiencies averages of 52, 91, 96 and 89% before doping PBR with toxic elements mixture and, 41, 70, 72 and 67% after doping for TOC, TN, NH_4^+ and PO_4^{3-} respectively. This work showed the good performance of this photobioreactor based on microalgae technology for the treatment of wastewater from piggery wastewater, not only in terms of removal of organic matter and nutrients, but also for the removal of heavy metals by the biomass. Moreover, the metals had a negative effect on the reactor biomass. Additionally, the present study is a validation of previous laboratory scale batch studies in heavy metal and nutrient and organic matter removal. media

Keywords, Bacteria, bioremediation, heavy metals, microalgae, piggery wastewater, swine manure

9.1 Introduction

Pork production is an industry that faces a number of challenges, including the sustainable use of water for pork production. Intensive pig farming requires large amounts of water for supplying the animals, cleaning and processing. It is estimated that 4000 L of water is used to process one kg of pork (Fresán et al., 2019). This can have a significant impact on local water resources and the environment. Water used in pork production can contain a variety of contaminants depending on its source, including antibiotics, ammonium, heavy metals and nutrients such as nitrogen, phosphorus and organic matter (Liu et al., 2023). Most common metals found in this type of wastewater are Copper (Cu), zinc (Zn) and arsenic (As) among others (López-Pacheco et al., 2021). Heavy metals (HM) not only affect aquatic life, but also accumulate in human tissues through the food chain, causing serious health problems ranging from neurological disorders to chronic diseases. Lack of effective regulation and insufficient environmental awareness exacerbate this situation, underscoring the urgent need for coordinated global action to preserve the quality and vitality of our water resources. This is why the United Nation Organization has stablished within the Sustainable Development Goal (SDG) its sixth objective to ensure availability and sustainable management of water and sanitation (Mentes, 2023).

Regarding heavy metals, Cu and Zn are found among the components of certain feedstuffs as a growth promoter for the animal, while As enters the diet of these cattle through the drinking water, depending on the area in which the livestock farm is established. These metals are not assimilated by the animal and are excreted and returned back to the environment through the urine and feces (Li et al., 2020). Arsenic (As) often comes from the water used on farms where this metalloid is present in the rocks that form the existing aquifers (Upadhyay et al., 2022). The most common concentrations of heavy metals found in this type of wastewater from livestock industry varies for Cu in values between 0.28 and 4.7, although high values were found up to 148 mg/L (Collao et al., 2022; Zhang et al., 2011). For Zn, values from 0.98 and 12 were reported (Zeng et al., 2021b), while for arsenic, values of up to 670 μg/L were found (Gao et al., 2018b). In the case of Cd(II), values between 0.022 (Zhang et al., 2011) and 0.35 mg/L (Moral et al., 2008), with moderate values of 0.16 (Creamer et al., 2010).

A great alternative of physical-chemical treatment of this PWW is biological treatment with microorganisms such as microalgae and bacteria. Photobioreactors (PBRs) emerge as a green technology, capable of providing a solution to one of the major environmental pollution problems. The microalgae photosynthesize, using sunlight as an energy source to convert carbon dioxide and nutrients into biomass. Bacteria degrades organic matter and nitrogenous compounds. In the work of *Collao et al., (2022)*, for a chlorella vulgaris-bacteria consortia PBR with an HRT of 22 days and an initial nutrient concentration of 713, 256 and 141 of $TOC,\ TN\ and\ NH_4^+$ respectively, achieves nutrient removals of 77, 53 and 24% respectively. As for metals, for an initial concentration of Cu, Zn and As of 100, 100 and 0.5 mg/L, eliminations of 83, 69, and 19%, respectively, are obtained (Collao et al., 2022). Gao et al., (2018) evaluated PBR performance for wastewater treatment of piggery wastewater with an initial concentration of nutrients of 744, 138 and 88 mg/L of TOC, TN and TP, respectively, where nutrient eliminations of 60 to 80% were achieved. For metals, at initial concentrations of 0.78 mg/L of Cu 0.92 mg/L of Zn and 690 μg/L of As, bioeliminations of 42, 45 and 35% respectively were achieved for a consortium of C*hlorella vulgaris* with bacteria from the manure. On the other hand, when the consortium used is that of Scenedesmus obliquus with bacteria, the results of nutrient removal are maintained, while those of metal bioelimination increase to removal percentages of 46, 50 and 38% for Cu, Zn and As, respectively (Gao et al., 2018).

In addition to the ability of PBRs to remove pollutants, PBRs offer other advantages. On the one hand, the biomass generated during the process can be reused in various applications, such as the production of fertilizers or bioenergy (Acién Fernández et al., 2018; Ayre et al., 2017). On the other hand, these systems have a low energy cost and do not generate toxic products. In summary, the sustainable use of water in pork production is a major challenge. PBRs with consortia of microalgae and bacteria offer a promising solution for treating wastewater from swine manure. These systems allow the removal of heavy metals and nutrients at a low energy cost and without the use of chemicals. In addition, they maximize biomass recovery for reuse in additional applications (Savio et al., 2020). Thus, the purpose of this study is to determine the removal efficiency of toxic trace elements, nutrients and organic matter in a semi-continuous reactor fed with a dilute liquid fraction of swine manure spiked with a mixture of Cu, Zn and As. Determination of metal removal in aqueous and solid phases for the entire process are also presented.

9.2 Materials and methods

9.2.1 Semi-continuous experiments

Semi-Continuous mode tests were conducted in a thin-layer cascade reactor with two-channels located at the Institute for Agricultural and Fisheries Research and Training (IFAPA) in Almería, Spain. A scheme of the process and a photo of the PBR is represented in **Figure 9.1**. The fiberglass thin layer photobioreactor had an area of 30 m^2 and a depth of 0.04 m. The reactor was inoculated with a microalgae-bacteria consortia, mainly composed of *S. almeriensis*, with a concentration of 1.0 g/L Initial pH in PBR was around 7.5 (Posadas et al., 2015). Composition regarding heavy metals of the initial biomass inoculum is described in **Table 9.1.** All these values are below the limits established by the legislation for animal feed.

Table 9.1. Heavy metal composition of *Scenedesmus almeriensis*-bacteria consortia and Maximum permitted values legislated in livestock feed

Zn	Pb	Cu	Cr	Ni	Cd	Hg	As
(mg/g)	(µg/g)	(mg/g)	(µg/g)	(µg/g)	(µg/g)	(µg/g)	(µg/g)
0.7	2.3	0.4	4.2	3.1	0.07	0.03	1.6
Maximum permitted values legislated in livestock feed							
(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
150*	10*	25	-**	-**	1*	0.1*	2*

* values calculated on a feed with a moisture content of 12%.
** non-legislated metals

The reactor was operated in semi-continuous mode at 5 days HRT. It was fed twice a day with 240 L/d of a diluted liquid fraction of pig slurry at 20%. After 15 days, steady state was achieved, and the diluted liquid fraction of pig manure in the inlet PBR was doped with a mixture of Cu, Zn and As to a final concentration selected for each metal (**see Table 9.2**), which was fed to the reactor for 20 more days. The doped feed was prepared at nominal concentrations of Cu(II), Zn(II), As(V) and Cd(II) using $CuCl_2{\cdot}2H_2O$, anhydrous $ZnCl_2$, Na_2HAsO_4 $7{\cdot}H_2O$ and $CdCl_2$ (Sigma Aldrich) and kept at 4ºC in darkness. It should be noted that the initial concentrations of Cu(II), Zn(II), and As(V) in the diluted PWW used as feed were very low, therefore, their influence on the final concentration of the doped feed was negligible (see **Table 9.1).** Ultrapure water was obtained from Mili-Q Advantage Ultrapure Water purification system from Merck Milipore (Billercia, USA). Copper and zinc were selected since they are introduced in the diet of the pigs. On the other hand, arsenic is present as a contaminant in groundwater in some rural areas where there is a large number of pigs.

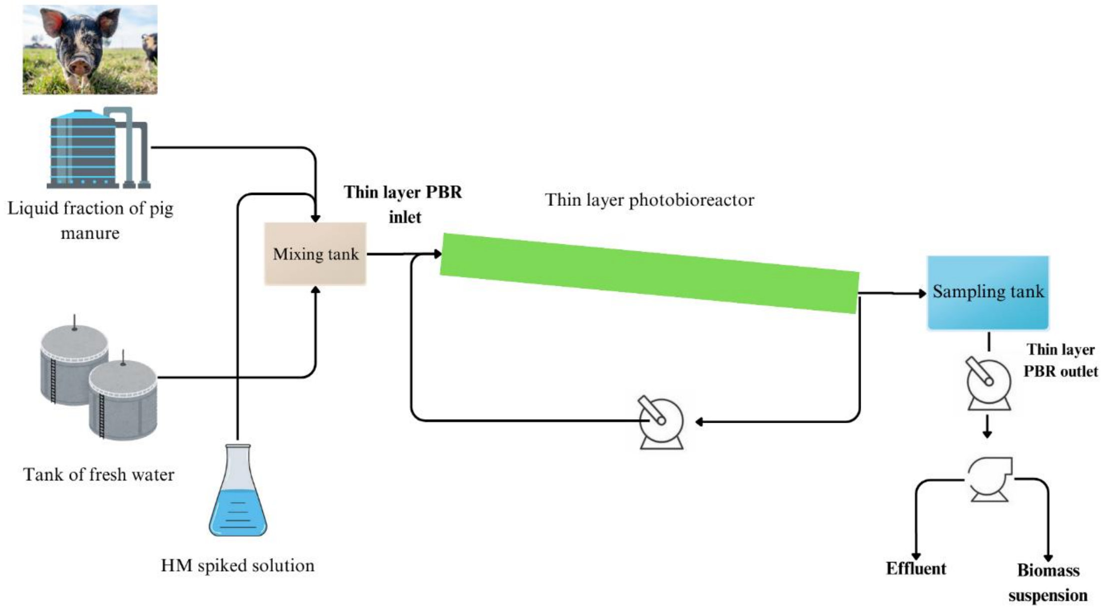


Figure 9.1. Scheme of the microalgae-bacteria treatment of the diluted liquid fraction of swine manure (up) in a pilot scale thin layer photobioreactor in IFAPA facilities at University of Almería (down)

Thus, experiments lasting 35 days, were carried out to study the bioelimination of micropollutants (toxic metals) during the treatment of liquid livestock waste (liquid manure) in photobioreactors (PBR) in which consortia of microalgae and bacteria coexist. Samples of both PBR feed and effluent were collected on days 12, 15, 29, 32 and 35 of PBR operation.

Subsequently, one-fifth of the total reactor volume was extracted in the morning following the homogenization of the reactor. This extracted volume was then transferred to a separate container and centrifugated using an industrial SSD 6-06-007 centrifuge (GEA Westfalia Separator, Oelde, Germany). The resulting harvested biomass was subjected to freezing and lyophilization, while the liquid phase was frozen at -20°C for further analyses in the laboratory of the University of Valladolid laboratory.

Table 9.2. Copper, zinc, arsenic spiking concentrations in thin-layer cascade photobioreactor

	Pollutant	Concentration
HM spiked assay	Copper ($CuCl_2 \cdot 2H_2O$)	5 mg/L
	Zinc ($ZnCl_2$)	15 mg/L
	Arsenic ($Na_2HAsO_4 \cdot 7H_2O$)	20 µg/L

The specific objective in the case will be to study the distribution of heavy metals between the recovered water and the algal biomass obtained. Liquid samples were taken on different days during the 35 days of operation fromboth the influent and the effluent. One fifth of the total reactor volume was harvested in the morning after homogenizing the reactor. This volume was transferred to a separate tank and centrifuged using an industrial SSD 6-06-007 centrifuge (GEA Westfalia Separator, Oelde, Germany). The harvested biomass was frozen and lyophilized, and the liquid phase was frozen at −20 °C until further analysis. From the liquid phase, 1.5 L were used to determine the TOC, IC, TN, NH_4^+, and PO_4^{3-}, and 500 mL was taken to determine dissolved heavy metal /HM) concentrations. From the lyophilized biomass, 1 g was taken to analyze HM concentration in the solid phase. For heavy metals and arsenic analysis and TOC, TN and NH_4^+-N measurement, samples were filtered using a 0.45 mm nylon membrane filter. All experiments were performed in duplicate.

9.2.2 *Analytical methods*

Total organic carbon (TOC), inorganic carbon (IC) and total nitrogen (TN) were measured using a TOC-V CSH analyzer equipped with a TNM-1 chemiluminescence module (Shimadzu, Kyoto, Japan). The ammonia nitrogen ($N - NH_4^+$) concentration was determined using an ammonium selective electrode (Orion 9512 HPWBNWP ammonia, Thermo Scientific, Waltham, MA, USA). and PO_4^{3-} concentrations were determined by high performance liquid chromatography (Waters 515 HPLC pump) coupled to a detector based on ion conductivity (Waters 432, HPLC-IC). Biomass concentration was determined according to standard methods.

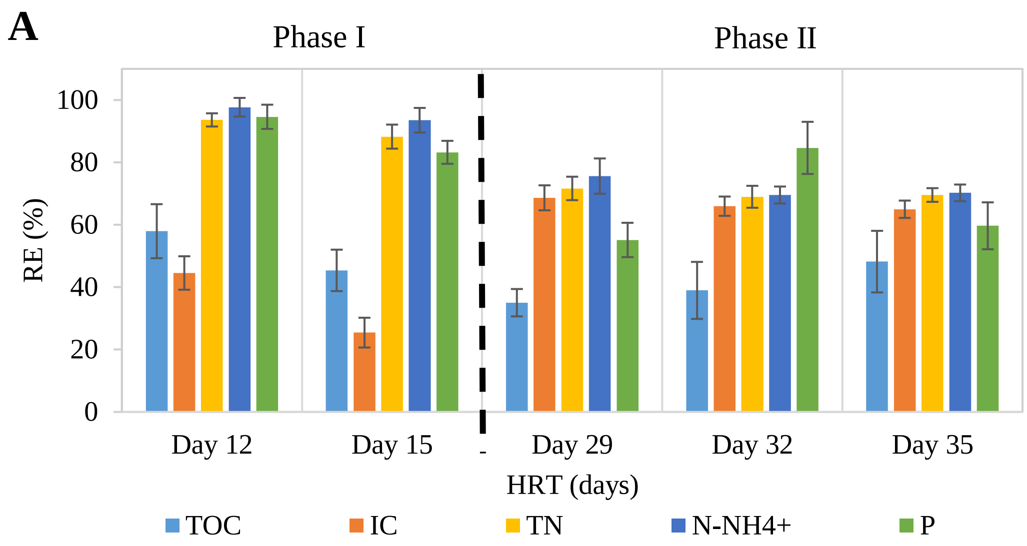
The concentration of Cu(II) and Zn(II) was determined by inductively coupled plasma spectrometry together with an optical emission spectrophotometer (ICP-OES) (Varian 725-ES, Agilent, Santa Clara, CA, USA). Arsenic (As) was measured using an inductively coupled plasma source mass spectrometer (ICP-MS) in an octopolar reaction system (HP 7500c, Agilent, USA). All plastic and glass containers were washed in diluted HNO_3 (10% v/v) for 24 h and rinsed 3 times with Milli-Q water before and after use with HMs and As. The removal efficiencies (RE) of TOC, IC, TN, NH_4^+, PO_4^{3-}, Cu, Zn and As were calculated according to Equation (3.12).

For solid samples of biomass, 0.1 g of lyophilized biomass was subjected to acid digestion through microwave-assisted with 10.0 mL of 69% nitric acid for analysis (Panreac AppliChem, Spain) in a Mileston Ethos Plus microwave oven. Digestion was carried out with a temperature ramp from 25ºC to 180ºC for 20 min followed by 10 min at 180ºC (Bakircioglu et al., 2011). The digestion is controlled with EasyWave 3 software. After the digestion, the resulting liquid is diluted to 30 g with deionised water. Acid digestions were carried out by duplicate. The uptake of Cu(II), Zn(II) and As(V) by the biomass (q) was calculated according to Equation (3.10) from Materials and Methods section.

9.3 Results and discusion

9.3.1 Photobioreactor performance and biomass viability

Results for nutrient removal and biomass viability are shown in **Figure 9.2.**



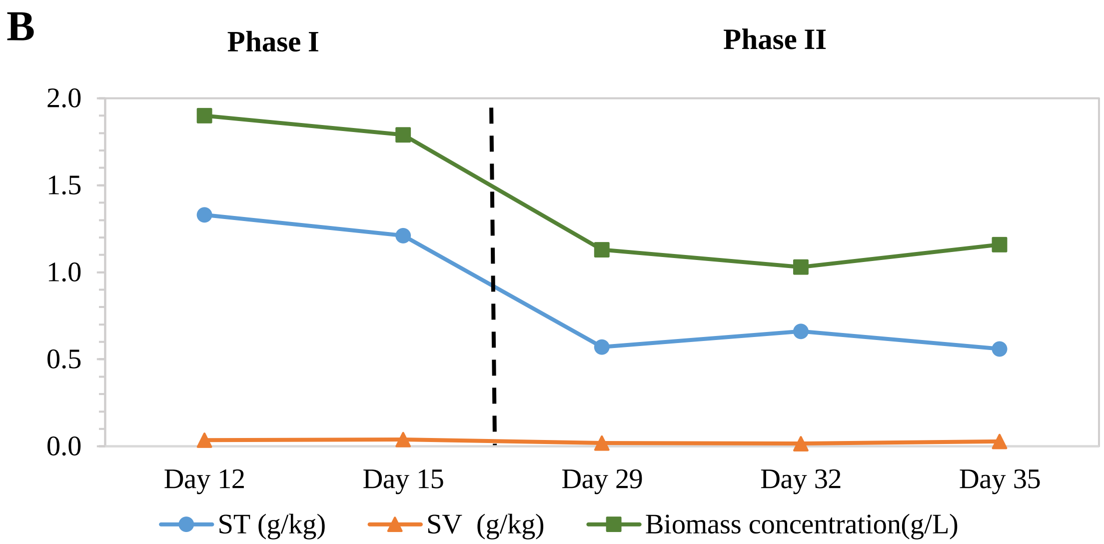


Figure 9.2. Photobioreactor performance results in terms of TOC-RE, IC-RE, TN-RE, NH_4^+-RE and PO_4^{3-}-RE (A) and biomass viability of microalgae-bacteria consortia in terms of ST (g/kg) VS (g/kg)) before and after HM addition to the photobioreactor (B).

TOC, TN, IC, NH_4^+ and PO_4^{3-} were measured in the liquid fraction of the wastewater from photobioreactor feed before and after doping with HM. TOC-RE before addition or doping with heavy metals averaged 52%, while after doping with heavy metals decreased to 35 %, 38.4 % and 38.9 % on day 29, 32 and 35 of contact time. On the other hand, the elimination of nitrogen presented a quite similar behaviour, prior to HM addition, eliminations of this nutrient of 91% were obtained, while after doping this percentage decreased substantially to 71.7%, 68.7% on day 29 and 32 and was maintained until day 35. The ammonium elimination profile was similar to that of nitrogen with eliminations close to 100% in the first phase prior to HM addition, while after doping there was also a drop in the elimination of this nutrient to 75.6%, 69.6% and 70.2% on day 29, 32 and 35. Finally, for the elimination of soluble phosphorus, values close to 90% were reached on days 12 and 15 prior to doping with metals in the photobioreactor. After doping, soluble phosphorus removal percentages of 55.1% were obtained for day 29, 84.7 % for day 32, which is a consistent increase, and 59.7 % for day 35. Phosphate and nitrate ions were measured at the outlet of the photobioreactor and were not detected. The moderately good results may be due to the fact that the experiments in the current study were performed in a thin-layer cascade photobioreactor, which allows good sunlight penetration. On the other hand, it is also important to highlight the low or in this case undetected nitrate concentrations at the outlet of the photobioreactors, so nitrification is not going to be a common process in this type of thin layer PBR, since the shallow depth of their cultures maximizes the availability of sunlight and promotes phototrophic growths of the cultures that develop. (Ciardi et al., 2022)

Results obtained in the present study are in agreement with those obtained in the literature. In the work of Collao et al. (2022), for a *Chlorella vulgaris*-bacteria consortia PBR with an HRT of 22 days and an initial nutrient concentration of 713, 256 and 141 mg/L of TOC, TN and $\mathrm{NH_4^+}$ respectively, achieves nutrient removals of 77, 53 and 24% respectively. On the other hand, in the work of Gao et al. (2018), for PBR treatment of wastewater from pig slurry with an initial concentration of 744, 138 and 88 mg/L of TOC, TN and TP, respectively, nutrient removals of 60 to 80% are achieved. Besides, when the consortium used is that of *Scenedesmus obliquus* with bacteria, nutrient removal removals are maintained. Hernández et al. (2013), treated the liquid fraction from pig slurry in 5L photobioreactors with the microalgae *Chlorella sorokiniana* and activated sludge at a HRT of 10 days. Removal values of 58.1, 82.7 and 58% of $s-COD$, $\mathrm{NH_4^+}$ and PO_4^{3-} were obtained. The high percentage of ammonium elimination in this study stands out, due to the low proportion of ammonium found with respect to other slurries. The initial concentration of ammonium in this PWW was 12 mg/L (Hernández et al., 2013), much lower than in the present work. The study by Zhou et al. (2019), present $s-COD$, NH_4^+ and PO_4^{3-} removals of 55, 46 and 75% respectively. García et al. (2018) studied the dynamics of microalgae population during piggery wastewater (PWW) treatment in four open photobioreactors operated at 27 days of hydraulic retention time, and inoculated with *Chlorella sp*., *Acutodesmus obliquus*, *Oscillatoria sp.* and in the absence of inoculum (R4). Efficient PWW treatment occurred regardless of the microalgae inoculated. Initial concentrations were 459, 452 and 482 for TOC (mg/L) respectively. 285, 242, 227 and 294 mg/L for IC, 174, 166 and 165 mg/L for TN and 2.4, 2.1, 1.9 and 1.8 mg/L for TP. Removal efficiencies accounted for 86, 63, 82 and 90 % for *Chlorella sp*., 87, 69, 83 and 91 for *Acutodesmus obliquus*, 86, 71, 83 and 92% for *Oscillatoria sp.* and, 86, 62, 85 and 92% for control pond in the absence of inoculum for TOC, IC, TN and TP respectively. (García et al., 2017) evaluated the performance of four open algal-bacterial photobioreactors operated at 26 days of hydraulic retention time during the treatment of 10 (×10) and 20 (×20) times diluted piggery wastewater under indoor and outdoor conditions for four months. The highest TOC-RE, TP-RE and Zn-RE were 94, 100 and 83% respectively in indoors PBR in the x10 diluted PWW, while the highest TN-RE (72 %) was recorded outdoors in ×10 PWW. (Guo et al., 2020) evaluated bioremediation performance of microalgae-based treatment technologies for nutrients and heavy metal removal in piggery wastewater. *Chlorella vulgaris, Scenedesmus obliquus* and *Neochloris oleoabundans* were selected for mono-cultivation or co-cultivation with fungi or activated sludge. The highest removal efficiency of TOC, TN and TP in piggery wastewater were 87.29%, 87.26% and 90.17% by co-cultivation of *Sc. obliquus* with activated sludge. In addition, Zeng et al., (2024) for a *C. vulgaris*-consortia with piggery digestate, obtained removal rates of COD, NH_4^+, TN, and TP at a Cu (II) concentration of 0.5 mg/L of 89.0%, 53.7%, 69.6%, and 47.3%, respectively.

The significant differences in the removal efficiency of TOC, IC, TC, TN, NH_4^+ and PO_4^{3-} before and after HM spiking were evaluated using an analysis of variance (ANOVA), as shown in **Table 9.3**. Based on the statistical analysis, significant differences were found for nutrient removal before and after HM addition for IC, TC, TN and NH_4^+($p<0.05$), where the removal efficiency increased significantly for IC and decreased for TN and NH_4^+. On the other hand, the biomass concentration was also significantly negatively affected by the addition of HM to the photobioreactor, as the biomass concentration goes from an average of up to 2 g/L prior to metal spiking to 1.16 g/L on day 35 of the process.

Table 9.3. Statistical analysis (ANOVA) of TOC, IC, nutrient removal and biomass concentration in the photobioreactor before and after HM addition

	ANOVA	Sum of squares	DoF	Root mean square	F	p-value
TOC-RE	Between groups	244,245	1	244,245	8,29	0,064
	Within groups	88,387	3	29,462		
	Total	332,632	4			
IC-RE	Between groups	1195,745	1	1195,745	18,718	0,023
	Within groups	191,647	3	63,882		
	Total	1387,392	4			
TC-RE	Between groups	689,281	1	689,281	28,278	0,013
	Within groups	73,127	3	24,376		
	Total	762,408	4			
TN-RE	Between groups	541,875	1	541,875	81,099	0,003
	Within groups	20,045	3	6,682		
	Total	561,92	4			
NH_4^+-RE	Between groups	679,728	1	679,728	66,51	0,004
	Within groups	30,66	3	10,22		
	Total	710,388	4			
PO_4^{3-}-RE	Between groups	602,112	1	602,112	3,156	0,174
	Within groups	572,42	3	190,807		
	Total	1174,532	4			
Biomass concentration (g/L)	Between groups	0,654	1	0,654	128,128	0,001
	Within groups	0,015	3	0,005		
	Total	0,669	4			

9.3.2 *Removal of Cu, Zn and As from PWW*

Results for removal efficiency of Cu, Zn and As in the phase II of the PBR are shown in **Figure 9.3.**

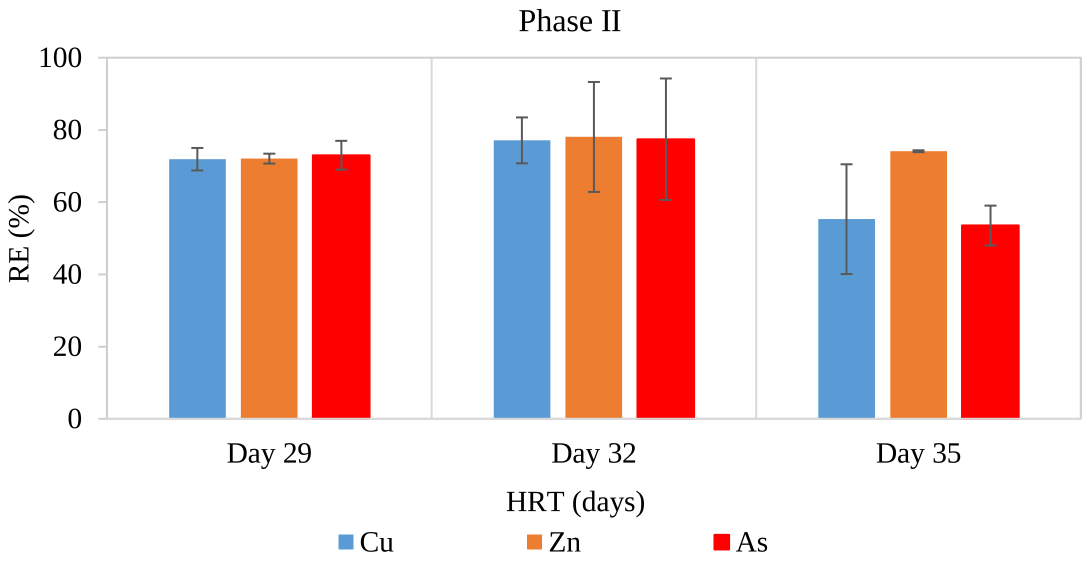


Figure 9.3. Removal efficiency (RE, %) for Cu, Zn and As after photobioreactor doping.

During the first 15 days of operation of the thin layer photobioreactor, liquid samples were taken from the reactor to be analysed to determine if the targeted heavy metals were present in the liquid fraction of the resulting suspension or whether they are absorbed in the microalgae-bacteria based consortium biomass. After the photobioreactor was doped with a mixture of the indicated heavy metals, copper, zinc and arsenic, metal eliminations were obtained for copper of 71.9 %, 77.1 % and 55.3 %, for Zn, 72.1 %, 78.1 % and 74.1 %, and finally for arsenic, removal efficiencies of 73 %, 77.4 % and 53.5 % were obtained for HRT days 29, 32 and 35, respectively. For copper and arsenic, a slight decrease in bioelimination percentages was observed around the last day of contact time, while zinc removal remained constant throughout the entire HRT. Results obtained in the present study agree with those obtained in the literature. Collao et al., (2022) studied bioremediation potential of a *Chlorella vulgaris*-bacteria consortia for an initial concentration for Cu, Zn and As of 100, 100 and 0.5 mg/L at the PBR inlet. RE of 83, 69, and 19%, respectively, were obtained for these metals. García et al. (2018) evaluated different strains for heavy metal removal in PBR; *Chlorella sp*. (R1), *Acutodesmus obliquus* (R2) and *Oscillatoria sp.* (R3). Zn-REs in R1, R2, R3 and R4 (control pond) accounted for 49 ± 6, 37 ± 6 and 26 ± 5 respectively for an initial concentration of 0.9, 1.1 and 1.3 mg/L of Zn, which resulted in average effluent Zn concentrations of 0.9 ± 0.2, 1.1 ± 0.1 and 1.3 ± 0.3 mg/L, respectively, at the end of the operational period. The determination of copper and arsenic removal efficiencies was not possible based on the low concentrations of these metals in the PWW for that work. On the other hand, Gao et al. 2018 obtained bioelimination of 46, 50 and 38% for Cu, Zn and As, respectively for heavy metal removal, at initial concentrations of 0.78 mg/L of Cu 0.92 mg/L of Zn and 690 µg/L of As for a Scenedesmus *obliquus consortia*, while bioeliminations of 42, 45 and 35% respectively are achieved for a consortium of *Chlorella vulgaris* with bacteria from wastewater. Cui et al. (2021), studied how *Myriophyllum aquaticum*, a plant that can be used for animal feed, can treat swine wastewater (SW) that contains high concentrations of nitrogen phosphorus, and heavy metals. The average removal efficiencies of Cu, Zn, and Cd by M. aquaticum were 50.4%, 36.4%, and 47.9%, respectively, compared to the control pond. Yang et al. (2015) studied the removal of Cu, Zn and Cd by *Chlorella minutissima* in a synthetic wastewater matrix. The concentrations tested were for Cu, 12.7, 25.4 and 63.5 mg/L, obtaining bioelimination percentages of 83.60, 82.38 and 30.37%. For Zn, initial metal concentrations of 130.8, 261.6 and 392.4 mg/L were tested, obtaining bioelimination percentages of 62.05, 45.87 and 37.95% and finally for Cd, initial metal concentrations of 22. 48, 44.96 and 67.45 mg/L were tested, obtaining bioelimination percentages of 74.34, 54.86 and 38.76% for initial biomass concentrations between 7 and 8 g/L. In the study by Zhou et al. (2019), removal of Cu, Zn, As and Pb was evaluated, reaching RE% of 93, 70, 11 and 72% respectively.

9.3.3 Heavy metal retention in biomass grown in PBRs

Results for heavy metal retention in biomass are shown in **Figure 9.4,** where the retention of the different toxic elements in the biomass grown throughout the whole process of operation of the PBR is shown, both in the stage where the steady state is reached, as well as during the second stage where the doping with toxic metals of the photobioreactor takes place.

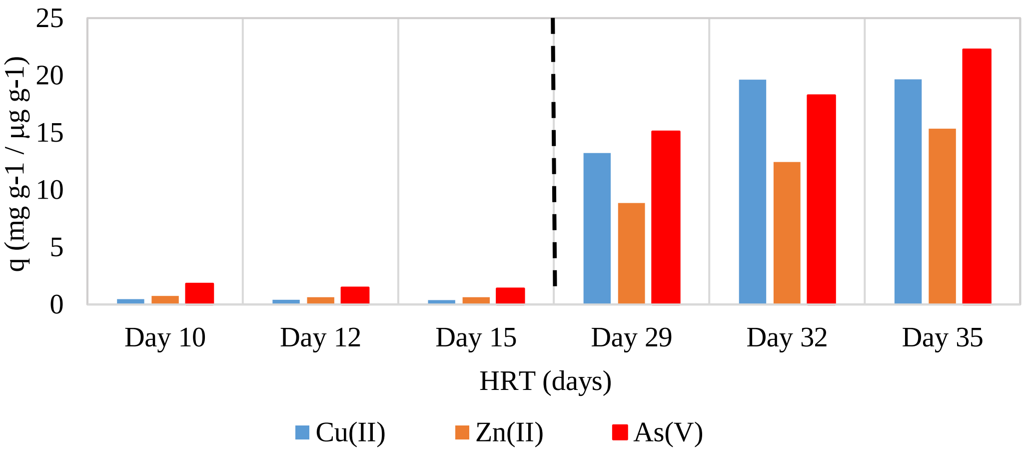


Figure 9.4. Biosorption capacity of *Scenedesmus almeriensis-bacteria* consortia for Cu, Zn and As removal form PWW (biosorption capacity in mg/g for Cu and Zn and in µg/g for As biosorption)

The resulting biomass grown from the PBR was collected in order to determine the amount of heavy metals retained on it. In this case, with the microalgae species *S. almeriensis* as a microalgae-bacteria consortium, the values found in the biomass were 13.65, 20.04 and 20.06 mg/g for copper, 9.53, 13.12 and 16.01 mg/g for zinc. Finally, for arsenic 16.67, 19.81 and 23.82 µg/g were found for days 29, 32 and 35 of the metal-doped test.

Despite the scarcity of recent studies that combine the elimination of both nutrients and heavy metals in wastewater of this type, some similar results were found in the following works. Cui et al., (2021) found that the concentrations of Cu and Cd in *M. aquaticum* at day 75 were in the ranges of 1.92-2.82 and 0.64-1.47 g/kg in dry weight, respectively. On the other hand, Collao et al. (2022) tested a *S. almeriensis-bacteria* consortia in a initial concentration of 0.7 g/L of total suspended solids in photobioreactor for PWW treatment in presence of heavy metals. PBR were doped with concentrations of 100 mg/L for Cu and Zn and 0.5 mg/L for As in separate reactors. Values for these metals in the biomass of 127 and 132 mg/g for Cu and 141 and 209 mg/g for Zn were achieved, while for As, values of 180 and 208 µg/g. Retention capacities depend on several factors, such as the type of microorganisms and their capacity to retain heavy metals, the initial concentration of the metal species as well as the concentration of biomass involved in the process and its viability during treatment. Taking into account these factors and the fact that in the present study lower metal concentrations and inoculum concentrations of around 1 g/L were used, the capacities obtained are comparable.

9.4 Conclusions

Microalgae-bacteria based technologies have proven to be effective in the treatment of wastewater from swine manure. In this work, a thin-layer cascade photobioreactor inoculated with microalgae mainly composed of *S. almeriensis* was tested in order to treat piggery wastewater from swine manure. This treatment has proven to be effective in the simultaneous removal of nutrients and metals. Metals did not have a negative effect in denitrification processes from microalgae consortia. However, a decrease in the removal efficiency of organic matter in terms of TOC was observed once the PBR was spiked with the metal mixture. Maximum removal efficiencies of $77.1 \pm 6.4\%, 78.1 \pm 15.2\%$ and $77.4 \pm 16.8\%$ were achieved for Cu, Zn and As respectively. On the other hand, removal efficiencies averages of $52, 91, 96$ and 89% before doping PBR with toxic elements mixture and, 41, 70, 72 and 67% after doping for TOC, TN, NH_4^+ and PO_4^{3-} were reached, respectively. This technology proved to be effective in the treatment of wastewater from pig manure as an alternative to conventional physico-chemicals treatments.

Acknowledgments

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Chapter 10:

Conclusions

Conclusiones

En la presente tesis doctoral se ha estudiado el empleo de microalgas vivas, bacterias aerobias, así como el consorcio entre microalgas y bacterias con el objetivo de la bioeliminación de elementos tóxicos presentes en la fracción líquida de purín de cerdo, tanto a escala de laboratorio en modo batch como a gran escala en plantas piloto en modo de operación semi-contínuo. Concretamente se evaluó y demostró la eficacia de la especie de microalaga *Scenedesmus almeriensis*, su consorcio con bacterias y bacterias aerobias procedentes de fangos activos frente a la bioeliminación de Cu, Zn, As, Cd, Pb, Hg y U. Se analizó el efecto de los factores de operación más relevantes en los procesos de depuración de este tipo de aguas residuales en fotobiorreactores, isotermas de adsorción, especiación metálica, efecto en la eliminación de nutrientes del medio, etc., con el fin de justificar la viabilidad de esta tecnología basada en consorcios de microalgas-bacterias para la depuración de aguas residuales

En primer lugar, en el Capítulo 4, se mostró el estudio del efecto de diferentes factores, como el tiempo de contacto, la exposición a la luz, la concentración inicial de metal, la presencia de materia orgánica y CO_2 en la biosorción de Cu y Zn en tres biomasas diferentes: microalga *S. almeriensis* cultivada en medio de nutrientes sintético, en la fase líquida de purines de cerdo y una tercera biomasa, fango activo, formada por bacterias aerobias. En términos de capacidades de biosorción, para Cu(II), los valores máximos encontrados fueron; 104.52 mg/g, 81.50 mg/g, y 67.71 mg/g, para cultivo puro de microalga, consorcio, y fango activo respectivamente. Para Zn(II) fueron de 121. 65 mg/g, 96,71 mg/g, y 73,52 mg/g a una concentración inicial de metal de 100 mg/L, tras 72 horas de tiempo de contacto y un pH inicial de 7.5. Los resultados son prometedores ya que se alcanzaron valores de moderados a elevados de bioeliminación, lo que permite considerar el tratamiento de aguas residuales con carga de materia orgánica y altas concentraciones de metales en fotobiorreactores.

En el Capítulo 5, con las condiciones óptimas de tiempo y concentración de metal obtenidas en el capítulo anterior (72 horas y 100 mg/L de cada ión metálico), se realizaron experimentos de biosorción de Cu y Zn para posteriormente tratar de elucidar los mecanismos de retención de estos metales. Se consiguió una bioeliminación de metales del 43% de Cu(II) y del 45% de Zn(II) con la microalga *S. almeriensis* y del 78% de Cu(II) y del 96% de Zn(II) con fango aerobio. Posteriormente, la solubilización selectiva de metales desde la biomasa resultante mostró que los iones Cu(II) y Zn(II) son biosorbidos principalmente por reacciones de intercambio iónico en grupos funcionales protonables y, en menor medida, por complejación e interacción electrostática. La especiación secuencial de metales confirmó también que un alto porcentaje de los metales se encuentra de forma biodisponible en la biomasa, un 69% para el caso del Cu(II) y 94% para el Zn(II) para la microalga y 76% de Cu(II) y 93% de Zn(II) para los fangos aerobios, siendo la biosorción el principal mecanismo de bioeliminación de metales para ambas biomasas, y más notablemente en el caso del Zn(II). El análisis FTIR indica una alta afinidad de unión de estos iones metálicos a los grupos carboxilo, amina, alcohol y amida, y la presencia de Cu(II) y Zn(II) en la superficie celular fue confirmada por ESEM-EDX. En cuanto a la limpieza de la biomasa con fines de aplicación, con ácido acético 0,1 M, se libera >95% de Zn tanto de las microalgas como en el fango activo, mientras que de *S. almeriensis* se extrae >75% de Cu. Así, por ejemplo, la biomasa lavada cumpliría los requisitos más estrictos en el uso de fertilizantes para diferentes aplicaciones.

En el Capítulo 6, se muestra que los fangos aerobios o activos son capaces de bioeliminar hasta un 80% de las especies inorgánicas As(III) y As(V) para concentraciones iniciales de las especies de 0.1 y 0.5 mg/L en las primeras 24 horas de tiempo de contacto, mientras que las microalgas lo hacen en un porcentaje del 80% para la especie DMA en un transcurso de 10 días de tiempo de contacto. A la vista de los resultados obtenidos, se puede afirmar que este tipo de microorganismos tienen un muy buen comportamiento cuanto a la bioeliminación de estas Elementos Traza Tóxicas (ETT), abriendo la posibilidad de utilizar microalgas cultivadas en aguas residuales para comprobar la eficacia de consorcios microalgas-bacterias y su posterior elución para los procesos de valorización de biomasa y las aplicaciones de los bioproductos obtenidos aplicando los conceptos de biorrefinería y economía circular.

En el Capítulo 7, se estudió la eficiencia de eliminación (RE) de 4 elementos traza tóxicos (TTE), cadmio (Cd), plomo (Pb), mercurio (Hg) y uranio (U) por la microalga *S. almeriensis* y fangos activos. *S. almeriensis* presentó altos porcentajes de eliminación, superiores al 70% para todos los ETT en la primera hora de tiempo de contacto. Para el Cd se logró una eficiencia de eliminación máxima del 97% en 24 h de tiempo de contacto, mientras que para el Pb y el Hg se alcanzaron eficiencias de eliminación del 98% y el 99% respectivamente al sexto día de tiempo de contacto. Para el U la eficacia de eliminación fue del 96% a las 8 horas de contacto. Por el contrario, con los fangos activos se obtuvieron porcentajes de eliminación superiores al 95% para todos los TTE durante todo el tiempo de contacto. La eliminación de carbono orgánico total (TOC) en presencia de estos TTE fue significativa tanto en *S. almeriensis* como en los fangos activos, alcanzando valores máximos del 92% y 88% respectivamente en presencia de Pb y el U. Sin embargo, la eliminación de nitrógeno total (TN) fue menor, observándose eliminaciones negativas a partir del día 10 de tiempo de contacto para el fango activo.

En el Capítulo 8, se implementó un Diseño de Experimentos de Taguchi con el objetivo de discutir la mejor combinación de los diferentes parámetros de funcionamiento que se pueden controlar en una planta de tratamiento de aguas residuales y para los cuales se minimiza la fluctuación de los factores de ruido (no controlables). En este caso, se utilizaron fotobiorreactores de consorcios algas-bacterias (A-R1) y biorreactores de bacterias aerobias (B-R2). Por un lado, se estudiaron como factores de control, la concentración inicial de nitrógeno total, el tiempo de contacto y la concentración inicial de biomasa. Por otro lado, como factores de ruido, se incluyeron, el tipo de purín utilizado en cuanto a la relación C/N del mismo, la concentración inicial de metal del alimento y el fotoperiodo luz:oscuridad. Cu(II), Zn(II), As(V) y Cd(II) fueron efectivamente eliminados durante el tratamiento biológico en un fotobiorreactor de tratamiento de PWW, así como los nutrientes (TOC, TN y NH_4^+) presentes en el medio. La eliminación de nutrientes se redujo claramente al aumentar la concentración de metales en la suspensión, mientras que el aumento de la concentración inicial de estos metales no condiciona el aumento de la captación de los mismos, aumentando también la capacidad de biosorción de estos metales. Las eficiencias de eliminación de Cu(II), Zn(II), As(V) y Cd(II) oscilaron entre el 81-98%, 96-97%, 98-72% y 93-99% para A-R1 y B-R2 respectivamente. Además, los porcentajes de eliminación de carbono orgánico total (TOC), nitrógeno total (TN) y NH_4^+ oscilaron entre el 83-88%, 56-63% y 63-89% para A-R1 y B-R2 respectivamente.

Finalmente, en el Capítulo 9, se evaluó el rendimiento de un fotobioreactor formado por un consorcio de *S. almeriensis-bacteria* para la eliminación de Cu, Zn y As, y nutrientes (TOC, TN, NH_4^+ y PO_4^{3-}) procedentes de agua residual de purín de cerdo real. Una vez inoculado el PBR, tras 15 días para alcanzar el estado estacionario se dopó con una mezcla de diferentes concentraciones de Cu, Zn y As. El PBR funcionó durante 20 días adicionales más. Se alcanzaron eficiencias de eliminación máximas de 77, 78 y 77% para Cu, Zn y As respectivamente. Por otro lado, se alcanzaron eficiencias de eliminación máximas de 58, 94, 98 y 95% para TOC, TN, NH_4^+ y PO_4^{3-} respectivamente antes del dopaje con mezcla metálica, mientras que, en presencia de las especies metálicas, se alcanzaron eliminaciones de 48, 72, 76 y 85% de TOC, TN, NH_4^+ y PO_4^{3-} respectivamente.

Una vez demostrada la efectividad de las microalgas y bacterias en la eliminación combinada de metales y nutrientes tanto en laboratorio como a gran escala, así como la posibilidad de recuperar estos metales de la biomasa, se hace necesario en futuros trabajos realizar estudios detallados para aislar y caracterizar nuevas cepas con potencial biotecnológico, evaluar la viabilidad económica y medioambiental de los procesos para garantizar su estabilidad y sostenibilidad a largo plazo, investigar cómo se distribuyen los metales durante el procesamiento de la biomasa para su valorización, explorar el uso de las bacterias púrpuras y, finalmente, investigar la producción de compuestos de alto valor, como la ectoína y los polihidroxialcanoatos (PHA), a partir de estos tratamientos biológicos. Estos estudios contribuirán a optimizar y ampliar las aplicaciones de microalgas y bacterias en la biorremediación y producción de bioproductos seguros.

Conclusions

In this doctoral thesis, the use of live microalgae, aerobic bacteria, as well as the consortium between microalgae and bacteria were studied with the aim of the bioelimination of toxic elements present in the liquid fraction of pig manure on a laboratory scale in batch mode and on a large scale in pilot plants in semi-continuous operation mode. Specifically, the effectiveness of the microalgae species *Scenedesmus almeriensis*, its consortium with bacteria and aerobic bacteria from activated sludge were evaluated and demonstrated for the bioelimination of Cu, Zn, As, Cd, Pb, Hg and U. The effect of the most relevant operating factors in the purification processes of this type of wastewater in photobioreactors, adsorption isotherms, metal speciation, effect on the elimination of nutrients from the medium, etc., was analysed, in order to justify the feasibility of this technology based on microalgae-bacteria consortia for wastewater treatment.

Firstly, in Chapter 4, the study of the effect of different factors, such as contact time, exposure to light, initial metal concentration, presence of organic matter and CO_2 on the biosorption of Cu and Zn in three different biomasses: *S. almeriensis* microalgae grown in synthetic nutrient medium, in the liquid phase of pig manure and a third biomass, activated sludge, consisting of aerobic bacteria. In terms of biosorption capacities, for Cu(II), the maximum values found were; 104.52 mg/g, 81.50 mg/g, and 67.71 mg/g, for pure strain culture, consortium, and activated sludge respectively. For Zn(II) they were 121.65 mg/g, 96.71 mg/g, and 73.52 mg/g at an initial metal concentration of 100 mg/L, after 72 hours of contact time and an initial pH of 7.5. The results are promising as moderate to high bioelimination values were achieved, which allows considering the treatment of wastewater with organic matter load and high metal concentrations in photobioreactors.

In Chapter 5, with the optimal conditions of time and metal concentration obtained in the previous chapter (72 hours and 100 mg/L of each metal ion), Cu and Zn biosorption experiments were carried out, this time in the presence of synthetic wastewater, in order to subsequently try to elucidate the retention mechanisms of these metals. A metal bioelimination of 43% of Cu(II) and 45% of Zn(II) was achieved with the microalga *S. almeriensis* and 78% of Cu(II) and 96% of Zn(II) with aerobic sludge. Subsequently, selective metal solubilisation from the resulting biomass showed that Cu(II) and Zn(II) ions are biosorbed mainly by ion exchange reactions on protonable functional groups and, to a lesser extent, by complexation and electrostatic interaction. Sequential metal speciation also confirmed a high percentage of the metals are bioavailable in the biomass, 69% for Cu(II) and 94% for Zn(II) for the microalgae and 76% of Cu(II) and 93% of Zn(II) for the aerobic sludge, with biosorption being the main mechanism of metal bioelimination for both biomasses, and more notably in the case of Zn(II). FTIR analysis indicates a high binding affinity of these metal ions to carboxyl, amine, alcohol and amide groups, and the presence of Cu(II) and Zn(II) on the cell surface was confirmed by ESEM-EDX. Regarding the cleaning of the biomass for application purposes, with 0.1 M acetic acid, >95% of Zn is released from both microalgae and activated sludge, while >75% of Cu is extracted from *S. almeriensis*. Thus, for example, the washed biomass would meet the most stringent requirements in fertiliser use for different applications.

In Chapter 6, it is shown that aerobic or activated sludge is able to bioeliminate up to 80% of the inorganic As(III) and As(V) species for initial species concentrations of 0.1 and 0.5 mg/L in the first 24 hours of contact time, while microalgae do it in a percentage of 80% for DMA specie within 10 days of contact time. In view of the results obtained, it can be affirmed that this type of biomass perform very well in terms of the bioelimination of these Toxic Trace Elements (TTE), opening up the possibility of using microalgae cultivated in wastewater to test the effectiveness of microalgae-bacteria consortia and their subsequent elution for biomass recovery processes and the applications of the bioproducts obtained by applying the concepts of biorefinery and circular economy.

In Chapter 7, the removal efficiency (RE) of 4 toxic trace elements (TTE), cadmium (Cd), lead (Pb), mercury (Hg) and uranium (U) by the microalgae *S. almeriensis* and activated sludge was studied. *S. almeriensis* showed high removal rates, above 70% for all TTEs in the first hour of contact time. Cd showed a maximum removal efficiency of 97% at 24 h contact time, while Pb and Hg reached removal efficiencies of 98% and 99% respectively at the sixth day of contact time. U showed a removal efficiency of 96% at 8 h contact time. In contrast, activated sludge removal rates above 95% were obtained for all TTEs during the entire contact time. Total organic carbon (TOC) removal in the presence of these TTE was significant in both *S. almeriensis* and activated sludge, reaching maximum values of 92% and 88% respectively in the presence of Pb and U. However, total nitrogen (TN) removal was lower, with negative removals observed from day 10 of contact time for activated sludge with metal treatment.

In Chapter 8, a Taguchi Design of Experiments was implemented with the aim to discuss the best combination of different operating parameters that can be controlled in a wastewater treatment plant and for which the fluctuation of (uncontrollable) noise factors is minimised. In this case, algae-bacteria consortia photobioreactors (A-R1) and aerobic bacteria bioreactors (B-R2) were tested. On the one hand, the initial total nitrogen concentration, the contact time and the initial biomass concentration were studied as control factors. As noise factors, the type of manure used in terms of its C/N ratio, the initial metal concentration of the feed and the light:dark photoperiod were included. Cu(II), Zn(II), As(V) and Cd(II) were effectively removed during biological treatment in a PWW treatment photobioreactor, as well as the nutrients (TOC, TN and NH_4^+) present in the medium. Nutrient removal was clearly reduced with increasing concentration of metals in the suspension, while increasing the initial concentration of these metals does not condition the increase in uptake of these metals, also increasing the biosorption capacity of these metals. The removal efficiencies of Cu(II), Zn(II), As(V) and Cd(II) ranged from 81-98%, 96-97%, 98-72% and 93-99% for A-R1 and B-R2 respectively. In addition, the percentages of total organic carbon (TOC), total nitrogen (TN) and NH_4^+ removal ranged between 83-88%, 56-63% and 63-89% for A-R1 and B-R2 respectively.

Finally, in Chapter 9, the performance of a photobioreactor formed by a *S. almeriensis*-bacteria consortium for the removal of Cu, Zn and As, and nutrients (TOC, TN, NH_4^+ and PO_4^{3-}) from real pig manure wastewater was evaluated. Once the PBR was inoculated, after 15 days to reach the steady state, it was doped with a mixture of different concentrations of Cu, Zn and As. The PBR was run for an additional 20 days. Maximum removal efficiencies of 77.1, 78.1 and 77.4% were achieved for Cu, Zn and As respectively. On the other hand, maximum removal efficiencies of 58, 94, 98 and 95% were achieved for TOC, TN, NH_4^+ and PO_4^{3-}respectively before doping with metal mixture, while, in the presence of the metal species, removals of 48, 72, 76 and 85% were achieved for TOC, TN, NH_4^+ and PO_4^{3-} respectively.

Having demonstrated the effectiveness of microalgae and bacteria in the combined elimination of metals and nutrients on a laboratory scale and on a large scale, as well as the possibility of subsequent recovery of these metals from biomass, it is necessary in future work to study the distribution of metals in biomass recovery processes, study new inoculum such as purple bacteria, and research into new lines such as obtaining products with high added value such as ectoin or PHAs, among others.

Having demonstrated the effectiveness of microalgae and bacteria in the combined removal of both metals and nutrients at laboratory and on a large scale, as well as the possibility of recovering these metals from biomass, it is necessary in future work to carry out detailed studies to isolate and characterise new strains with biotechnological potential, assess the economic and environmental feasibility of the processes to ensure their long-term stability and sustainability, investigate how metals are distributed during biomass processing for valorisation, explore the use of purple bacteria and, finally, investigate the production of high-value compounds, such as ectoin and polyhydroxyalkanoates (PHA), from these biological treatments. These studies will contribute to optimising and expanding the applications of microalgae and bacteria in bioremediation and production of safe bioproducts.

Chapter 11:

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About the Author

Scientific production:

Beatriz Antolín, Milagros Acítores, Juan José Jiménez, Marisol Vega. *"Optimization and validation of GC-MS methods for analysis of VOCs and pesticides in water" Actualidad Analítica, Revista de la Sociedad Española de Química Analítica, SEQA, 68, 21-24, 2019.* https://www.seqa.es/ActualidadAnalitica/AA_68/007.pdf

Antolín, B., Torres, A., García, P.A., Bolado, S., Vega, M., Mechanisms of copper and zinc bioremoval by microalgae and bacteria grown in nutrient rich wastewaters, *Chemosphere* (2024), doi: https://doi.org/10.1016/j.chemosphere.2024.141803.

Antolín, B., Moldes, D., García, P.A., Bolado, S., Vega, M. Factors affecting bioelimination of Cu (II) and Zn (II) by *Scenedesmus almeriensis*, a microalgae-bacteria consortium and activated sludge. *Under writing.*

Antolín, B., Sánchez E., García, P.A., Bolado, S., Vega, M. Bioelimination of As(III), As(V) and DMA by microalgae and activated sludge grown in wastewater treatment plants. *Under writing process.*

Antolín, B., Corchero, Á., García, P.A., Bolado, S., Vega, M. Effect of organic matter in toxic trace metal and nutrient removal by *Scenedesmus almeriensis* and activated sludge. *Under writing process.*

Antolín, B., Irusta-Mata, R., García, P.A., Bolado, S., Vega, M. Effect of operational parameters on photobioreactor performance and metal removal treating piggery wastewater using microalgae-bacteria consortia and activated sludge. *Under writing process.*

Book chapter:

Antolín Puebla, B., Vega Alegre, M., Bolado Rodríguez, S García-Encina, P.A., Algal-based metal recovery. Under revision in Springer in Advances in Biochemical Engineering/Biotechnology. Biological Metal Recovery from Wastewaters

Conference participation

Member of the Organising Committee of "XXII Reunión de la Sociedad Española de Química Analítica" (SEQA 2019) held in Valladolid from 17th to 19th july 2019.

Poster communication: Beatriz Antolín, Milagros Acítores, Juan José Jiménez, Marisol Vega (2019) "Optimization and validation of GC-MS methods for analysis of VOCs and pesticides in water". Congreso SEQA.

SEQA (Spanish society of analytical chemistry) Award for best poster communication, Beatriz Antolín, Milagros Acítores, Juan José Jiménez, Marisol Vega (2019) *"Optimization and validation of GC-MS methods for analysis of VOCs and pesticides in water". Congreso SEQA.*

Poster communication: Beatriz Antolín, David Moldes, Álvaro Corchero, Marisol Vega, Pedro García-Encina (2023). "Bioremoval of Cd, Hg, Pb and U by microalgae and bacteria grown in photobioreactors treating piggery wastewater". 1st Conference of Doctoral Students in Chemistry. Faculty of Science. University of Valladolid.

Poster communication: Beatriz Antolín, David Moldes, Álvaro Corchero, Marisol Vega, Pedro García-Encina (2023). "Bioremoval of Cd, Hg, Pb and U by microalgae and bacteria grown in photobioreactors treating piggery wastewater". 2nd Greenering International Conference. Valladolid, España

Poster communication: Beatriz Antolín, David Moldes, Eduardo Sánchez, Marisol Vega, Pedro García-Encina (2023). "Bioelimination of different arsenic species by microalgae and bacteria grown in wastewater treatment plants". 6th International Conference on eco-Technologies for Wastewater Treatment (Girona) 26-29 June 2023

Fellowships

2024	International Mentor Program 2023-24 of IMFAHE Foundation
2023	Travel and Conference grant for the participation in international conference (UVa fellowship).
2021	Research internship grant to strengthen international cooperation (UVa-Roma-TorVergata fellowship).
2019-2023	JCyL-Predoctoral researcher Fellowship. Spanish Research Council

Research projects

2023-2024	GREENFARM. Project focused on the production of biostimulants and biopesticides from microalgae biomass grown in piggery wastewater. Creation and management of website and social networks dedicated to the dissemination of Greenfarm project results. Organisation of an event to disseminate the results of the Greenfarm project, held at the facilities of the University of Almeria on 17 November 2023.
2021-2024	PROPHACTION**.** The overall objective of PROPHACTION is to develop sustainable resource recovery processes from different biomasses cultivated in wastewater treatment plants through the recovery of proteins, such as amino acids or polypeptides, and the production of PHAs from the N-depleted hydrolysate after protein extraction, guaranteeing the safety and quality of the bioproducts obtained.

	Also, participated in the elaboration and review of the project's mid-term reports.
2019-2021	**PURASOL.** The overall objective of PURASOL is the optimisation of the production of value-added products and water recovery from wastewater treatment with microalgae biomass. Analysis of heavy metals in biomass and resulting liquid fraction. Participation in the preparation and review of the mid-term reports of the project. Attendance at a coordination meeting of the project from 11 to 14 September 2019.

Research stay:

A stay at the Tor Vergata University in Rome (Italy) from September 2021 to December 2021 was carried out. There is a long experience of collaboration with the Algal Biology Laboratory, mainly with Professor Roberta Congestri. In the framework of this project, I carried out a stay at this University to study the assimilation and mechanisms of retention and accumulation of heavy metals by microalgae and bacteria. During this period, I worked with molecular biology techniques and learned to use specific software for the management and identification of nucleotide sequences.

Other activities:

Initiation to writing and publishing scientific articles organised by the Doctoral School, University of Valladolid. December 2019 (4h).

Practical course on basic laboratory techniques in biomedical research. Organised by the IBGM, University of Valladolid. December 2019 (15h).

Online course "Biología molecular. Bases y aplicaciones” organised by the University of Valencia. (24h). December 2019.

Practical workshop on physical-chemical and instrumental analytical techniques. (8h), organised by the Doctoral School, Uva. November and December 2019.

Online course “Biotecnología de Microalgas (35h)” organised by the University of Almeria. November 2019.

Microalgae processes: From fundamentals to industrial scale. Training network courses del CEIA (Centro de Excelencia Internacional Agroalimentario) 2019 given by the University of Almeria. (25h). September 2019.

LC-MS Method validation course. University of Tartu. From 26 November 2019 to 7 February 2020.

Microalgae biorefineries as multi-product integrated biorefineries: A course of combined modelling and experimental approaches (6h). 21-22 February 2020.

Estimation of Measurement Uncertainty in Chemical Analysis. Universidad de Tartu. March-May 2020.

Speaking B2 course. Organised by the Doctoral School of the University of Valladolid. (50h) from 2 March to 8 June 2020.

Writing B2 course (50h). Organised by the Doctoral School of the University of Valladolid from 2nd March to 8th June 2020.

Abstracts y artículos en inglés (ciencias, ciencias de la salud, ingeniería y arquitectura (16h). Celebrado del 8 al 22 de junio de 2020.

"Agilent 2021 Virtual Food Safety Analysis Symposium". From 01/03/2021 to 30/04/2021.

Course of languages for PDI of Uva (60h). Organised by the ESE Malta Academy from 23/08/2021 to 03/09/2021.

Course " Diseño de presentaciones innovadoras" (30h). Organised by VirtUVa del 25 de octubre al 2 de diciembre de 2021.

Course "Introducción a los planes de gestión de datos de investigación" (14h). Organised by VirtUVa del 6 al 26 de octubre de 2021.

Course "Protección del conocimiento: Patentes, protección intelectual y derechos de autor" (8h). Organizado por EsDUVa y realizado los días 13 y 14 de enero de 2022.

Course "Introducción a TEAMS para estudiantes" (3h). Organizado por VirtUVa y realizado entre los días 4 y 11 de febrero de 2022.

Course "Ciencia abierta y su efecto en los datos de investigación" (2h). Organizado por EsDUVa el día 4 de febrero de 2022.

Course "Introducción al Diseño de Experimentos" (8h). Organised by the PhD programme in Chemical and Environmental Engineering, held on 7, 8, 9 and 10 November 2022.

Course "Pon en valor tu investigación en Ciencias Experimentales" (6h). Organised by EsDUVa and held on 22 and 23 November 2022.

Course "Excel para investigadores", organised by the PhD programme in Chemical and Environmental Engineering, 24 November 2022 with a total duration of 2 hours..

Course "Elaboración de una propuesta proyecto" (2h). Organised by the PhD programme in Chemical and Environmental Engineering, 24 November 2022.

BIP Course: "Biological Carbon Capture Technologies". Organised by the Institute for Sustainable Processes. University of Valladolid from 12/06/2023 to 22/06/2023.

Other activities:

I participated in the annual meeting of the project in which this research is framed; "Optimisation of the obtaining of value-added products and water recovery from manure treatment (PURASOL)", funded within the call Retos 2017 by the Ministry of Economy and Competitiveness, held from 11 to 14 September 2019 in Almeria (Spain). I gave an oral presentation; "Behaviour of heavy metals in manure valorisation processes using microalgae" at the Conference on Circular Economy in agricultural environments, held on 12 September 2019 at the Cajamar Experimental Station (Las Palmerillas, Almeria), which aimed to raise awareness of the state of the art of the problems that exist with heavy metals in relation to value-added products generated from microalgae.

Teaching and student mentoring

Collaboration in the teaching of the course “Control y Gestión de Calidad”, taught by the Department of Analytical Chemistry in the Bachelor's Degree in Chemistry during the academic years 2020-2021, 2021-2022 and 2022-2023.

Collaboration in the teaching of the course “Química Experimental III”, taught by the Department of Analytical Chemistry in the Degree in Chemistry during the academic year 2021-2022 and 2022-2023.

Co-supervisor of the master's thesis entitled “Factors influencing the bioremoval of copper and zinc from wastewater using microalgae, bacteria, and their consortia”, presented by Mr. David Moldes Plaza for the Master's Degree in Advanced Techniques in Chemistry. Chemical Analysis and Quality Control (University of Valladolid, academic year 2019/2020). This work received a grade of 10.0.

Co-supervisor of the Master's Thesis entitled “Influencing factors in the bioelimination of arsenic species in wastewater using microalgae and bacteria”, presented by Mr Eduardo Sánchez Iglesias for the Master's Degree in Advanced Techniques in Chemistry. Chemical Analysis and Quality Control (University of Valladolid, academic year 2020/2021). This work received a grade of 9.5.

Co-supervisor of an undergraduate student Final Degree Project entitled “Estudio comparativo de la bioeliminación de iones de Cu(II) y Zn(II) con biomasas de microalga *Scenedesmus almeriensis* y de fangos activos para el tratamiento de aguas residuales”, submitted by Ms. Alba Torres to apply for the degree of Graduate in Chemistry (University of Valladolid, academic year 2020/2021). This work received a grade of 8.8.

Co-supervisor of a Master Thesis entitled "Bioelimination of toxic trace elements with microorganisms grown in wastewater treatment plants", presented by Mr. Álvaro Corchero Quirce for the title of Master in Advanced Techniques in Chemistry. Chemical Analysis and Quality Control (University of Valladolid, academic year 2021/2022). It received a grade of 9.3.

Co-supervisor of an undergraduate student Final Degree Project entitled “Bioeliminación y biotransformación de arsénico en fotobiorreactores de tratamiento de aguas residuales”, presented by Mr. Jaime Alcalde Mento for the degree of Graduate in Chemistry (University of Valladolid, academic year 2021/2022). This work received a grade of 8.9.