Universidad de Valladolid

Facultad de Ciencias

Trabajo Fin de Máster

Máster en Técnicas Avanzadas en Química. Análisis y Control de Calidad Químicos

FACTORS INFLUENCING THE BIOREMOVAL OF COPPER AND ZINC FROM WASTEWATER USING MICROALGAE, BACTERIA, AND THEIR CONSORTIA

Autor: *David Moldes Plaza*

Tutoras: *Marisol Vega Alegre y Beatriz Antolín Puebla*

"Hay que reírse cuando dicen que la ciencia fracasa. Tontería: Lo que fracasa es la mentira; la ciencia marcha adelante, arrollándolo todo"

Pío Baroja, *El árbol de la ciencia.*

AGRADECIMIENTOS

Aunque el trabajo científico esté redactado en inglés, quisiera hacer los agradecimientos en español.

Este trabajo ha sido realizado en el Instituto de Procesos Sostenibles de la Universidad de Valladolid, ISP, dentro del GIR QAMAQ (Química Analítica, Medio Ambiente y Quimiometría). Agradezco a ambas estructuras de investigación que me hayan permitido utilizar sus medios e instalaciones para llevar a cabo este trabajo.

Lo primero de todo, quiero dar las gracias a Beatriz, a quien agradezco el constante acompañamiento y asesoramiento durante la realización de este trabajo (tanto en el laboratorio, como fuera de él), y por su permanente disponibilidad a resolver cualquier duda que me surgía, ya que este campo era totalmente novedoso para mí.

Agradezco también al personal del Laboratorio de Técnicas Instrumentales de la Uva (LTI), y muy especialmente a Begoña Ribón, por su paciencia para enseñarme a utilizar el ICP-OES y ayudarme con la determinación de metales en cientos de disoluciones empleando dicha técnica analítica.

De forma muy especial, quiero expresar mi agradecimiento a mi tutora Marisol Vega por su apoyo incondicional, confianza y ayuda prestados en el desarrollo de este trabajo, y a la que debo una gran parte del químico que soy hoy en día. Querría también mencionar a Silvia Bolado, Catedrática de Ingeniería Química de la Universidad de Valladolid, gracias a la cual he podido entrar a este apasionante mundo de la bioremediación de metales, y en cuyos laboratorios he desarrollado la mayor parte del trabajo experimental. Gracias también a Paco y Charo, personal técnico del Departamento de Química Analítica, que siempre están cuando se les necesita, solucionan cada problema que tenemos y de los que he aprendido mucho.

Me gustaría también dar las gracias a todos los buenos profesores que he tenido, a lo largo del grado, pero también en la secundaria y en primaria, he sido muy afortunado y de cada uno he aprendido una barbaridad. También agradecer a mis amigos, los cuales me han acompañado en los buenos y malos momentos, y particularmente a "los químicos", por estar ahí durante estos cinco años, la química no hubiera sido tan apasionante sin vosotros.

Por último, quiero dar gracias a mi familia, que siempre me apoya y me da ánimos para seguir, y en particular a mis padres por toda su ayuda pasiva o activa durante todos estos años, y a mi hermana, que siempre está ahí y me saca una sonrisa en cualquier situación.

ABSTRACT

Water pollution by toxic heavy metals is a severe socio-sanitary problem that requires efficient, environmentally friendly, and economically viable solutions.

Typical pig diets have a high content of phytates, which reduces the availability of Zn and Cu. Thus, to ensure animal health, welfare and productivity, pig diets are supplemented with these elements that are partially released to the ambient through the urine and feces. These residues represent an alarming problem nowadays, due to their high concentrations of carbon, nitrogen, phosphorous, and, of course, heavy metals and pharmaceutical products. Therefore, it is essential to develop an effective treatment of slurry generated in livestock facilities, which not only prevents contamination but also allows the recovery of organic matter and nutrients present in them, applying the concept of circular bioeconomy.

This work aims to provide a sustainable solution to this issue, using microorganisms to treat and valorize wastewater from the food industry, in general, and livestock, in particular. The study focuses on the elimination of two metals (Cu and Zn) that, although essential at low concentrations, can be toxic when prolonged exposure to concentrations higher than required takes place.

A bibliographic review has been carried out to evaluate the retention capacity of these elements by microalgae, bacteria, and their consortia, the experimental conditions in which metal retention takes place, and the predominant bioaccumulation mechanisms in each type of biomass.

In the experimental part, a complete factorial design of 144 experiments has been applied to evaluate the effect of six factors on the bioelimination capacity of copper and zinc and the growth of the biomass. Three types of biomass were used: a pure *Scenedesmus Almeriensis* strain, a bacterial sludge, and a consortium of *Scenedesmus Almeriensis* and bacteria grown in slurry water. Furthermore, we selected other factors intending to study whether they influenced the retention process or not, such as organic matter, the CO_2, the initial concentration of metals, the light, and the contact time.

After the statistical analysis of the results, it was determined that the most important factors are the type of biomass (the pure microalgae showed the highest metal retention capacities), the initial metal concentration (the higher the concentration, the higher the retention), and stirring time (short times resulted in higher retention). For pure microalgae and slurry-grown biomass, significant biomass growth was observed.

The results obtained from the retention capacities are promising since very high values were reached for copper and zinc metals, which makes it possible to consider the treatment of wastewater with high organic load and metals in photobioreactors as a promising method for the elimination heavy metal.

RESUMEN

La contaminación de las aguas por metales pesados tóxicos es un grave problema sociosanitario que requiere soluciones eficientes, respetuosas con el medio ambiente y económicamente viables.

Típicamente, las dietas porcinas tienen un alto contenido en fitatos, que reducen la disponibilidad de Zn y Cu. Con el fin de asegurar su correcto desarrollo, los piensos que se dan a los cerdos se suplementan con estos dos elementos. La mayor parte de esos metales se expulsa con las heces, que suponen actualmente un problema medioambiental muy importante debido a sus elevadas concentraciones de carbono, nitrógeno y fósforo y, por supuesto de metales pesados y productos farmacéuticos. Resulta imprescindible, en la actualidad, un tratamiento efectivo de los purines generados en las instalaciones ganaderas, que no sólo evite la contaminación, sino que permita la recuperación de la materia orgánica y nutrientes presentes en los mismos, aplicando el concepto de bioeconomía circular.

Este trabajo pretende dar una solución sostenible a este problema, usando microorganismos para tratar y valorizar aguas residuales de la industria alimentaria, en general, y ganadera, en particular. El estudio se centra en la eliminación de dos metales (Cu y Zn) que, aunque esenciales a bajas concentraciones, pueden resultar tóxicos cuando tiene lugar una exposición prolongada a concentraciones superiores a la requerida.

Se ha llevado a cabo una revisión bibliográfica para evaluar la capacidad de retención de estos elementos por microalgas, bacterias y sus consorcios, las condiciones experimentales en las que la retención de metales tiene lugar y los mecanismos de bioacumulación predominantes en cada tipo de biomasa.

En este trabajo se ha aplicado un diseño factorial completo de 144 experimentos para evaluar el efecto de seis factores sobre la capacidad de bioeliminación de cobre y cinc y sobre el crecimiento de la biomasa. Se hizo uso de tres tipos de biomasa: una cepa *Scenedesmus Almeriensis* pura, un fango de bacterias, y un consorcio de *Scenedesmus Almeriensis* y bacterias crecido en aguas de purín. Además, seleccionamos otros factores con la intención de estudiar si influían en el proceso de retención, como la materia orgánica, el CO_2, la concentración inicial de metales, la luz, y el tiempo de contacto con la disolución.

Tras el análisis estadístico de los resultados, se determinó que los factores más importantes son el tipo de biomasa (la microalga pura mostró las capacidades de retención de metales más altas), la concentración inicial de metal (a mayor concentración, mayores retenciones), y el tiempo de agitación (tiempos cortos resultaron en mayores retenciones). Para la microalga pura y biomasa crecida en purín, se observó un crecimiento de biomasa significativo.

Los resultados que se obtuvieron de las capacidades de retención son prometedores, pues se alcanzaron valores muy altos para los metales cobre y cinc, que hacen que podamos considerar el tratamiento de aguas residuales con elevada carga orgánica y metales en fotobioreactores un método prometedor para la eliminación de metales pesados.

CONTENT

1 INTRODUCTION 1

1.1 WATER POLLUTION AS AN ENVIRONMENTAL PROBLEM 1
1.1.1 POLLUTION BY HEAVY METALS 2
1.1.2 METHODS TO IMPROVE WATER QUALITY: AN OVERVIEW 12
1.2 BIOREMEDIATION AS A SOLUTION TO WATER POLLUTION ISSUES 14
1.2.1 BIOACCUMULATION 15
1.2.2 BIOSORPTION 20
1.2.3 THERMODYNAMICS AND KINETICS OF BIOSORPTION [54], [60]–[68] 30

2 AIM OF THIS WORK 43

3 BIOSORPTION OF HEAVY METALS 45

3.1 BACTERIA AS BIOSORBENTS 45
3.1.1 WHAT ARE BACTERIA? 45
3.1.2 HEAVY METAL RETENTION MECHANISMS 48
3.1.3 BIOSORBENT CAPACITIES 49
3.1.4 FACTORS AFFECTING BACTERIA BIOSORPTION 51
3.1.5 REVIEW OF THE BACTERIA USED FOR HEAVY METAL REMOVAL 53
3.2 MICROALGAE AS BIOSORBENTS 57
3.2.1 WHAT ARE MICROALGAE? 57
3.2.2 USES AND APPLICATIONS OF MICROALGAE 58
3.2.3 BIOSORBENT CAPACITIES 59
3.2.4 HEAVY METAL RETENTION MECHANISMS 60
3.2.5 FACTORS AFFECTING MICROALGAE BIOSORPTION 61
3.2.6 REVIEW OF MICROALGAE USED FOR HEAVY METAL REMOVAL 63
3.3 MICROALGAE-BACTERIA CONSORTIA AS BIOSORBENTS 67
3.3.1 WHAT ARE MICROALGAE-BACTERIA CONSORTIA? 67
3.3.2 WHY MICROALGAE-BACTERIA CONSORTIUMS? 68
3.3.3 MICROALGAE-BACTERIA INTERACTIONS 69
3.3.4 APPLICATIONS TO WASTEWATER TREATMENT AND LIMITATIONS 70
3.3.5 REVIEW OF MICROALGAE-BACTERIA CONSORTIA USED FOR HEAVY METAL REMOVAL 71

4 METHODOLOGY AND MATERIALS 73

4.1 EXPERIMENTAL DESIGN 73
4.2 BIOMASS CHARACTERIZATION 75
4.2.1 MOISTURE AND VOLATILE SOLIDS ANALYSIS 76
4.2.2 ANALYSIS OF LIPIDS 76
4.2.3 ANALYSIS OF PROTEINS 77
4.3 REAGENTS 77
4.4 ANALYTICAL PROCEDURE FOR MULTIMETALLIC BIOSORPTION EXPERIMENTS 78

5 RESULTS AND DISCUSSION 81

5.1 BIOMASS COMPOSITION 81
5.2 MULTIMETALLIC BIOSORPTION EXPERIMENTS 81
5.2.1 BIOMASS GROWTH EXPERIMENTS 82
5.2.2 METAL RETENTION CAPACITY 87
5.3 ISOTHERMS 99

6 CONCLUSIONS 103

APPENDIX 105

REFERENCES 115

1 INTRODUCTION

1.1 Water pollution as an environmental problem

When it comes to human progress and advancement, everyone agrees that industrialization has had an unprecedented impact on our lives. However, we must not ignore the fact that these improvements have taken place at the cost of environmental quality [1]. In general terms, pollution refers to the occurrence of troublesome substances (pollutants) in the environment above an acceptable limit, which can harmfully affect life. Sources of pollution can be both natural (such as geothermal, comets, or volcanic activities) and anthropogenic, which have arisen on account of fast industrialization and extensive use of hydrocarbons, pesticides, chlorinated hydrocarbons, and heavy metals [2].

According to the World Health Organization (WHO), "Safe and readily available water is important for public health, whether it is used for drinking, domestic use, food production or recreational purposes. Everyone has the right to sufficient, continuous, safe, acceptable, physically accessible, and affordable water for personal and domestic use" [3]. However, in 10 or 20 years ahead, the world will have a 40% deficit of water, because of the increasing demand and contamination of the resources available [4]. Moreover, the influence of climate change (higher temperatures and changes to the water cycle), will provoke water issues and will result in increased flooding, more severe droughts, and enhanced toxicity of chemical contaminants [5]. At the same time, large amounts of wastewater are being generated with a high concentration of several nutrients (eutrophication could occur), together with a heavy load of toxic trace elements and toxic organic compounds (antibiotics, dyes). Hence, sustainable ways to treat wastewater must be found.

Some primary sources of water contamination include the textile industry, mining, pharmaceuticals, municipal wastewater, agriculture, farming, and food processing [6]. Focusing on the food industry, as reported by the Institute of Mechanical Engineers, the production of 1 kg of meat needs between 5000 and 15000 L of water [7], which means that part of that clean water backup available is employed to feed the animals, mostly pigs and cows. Additionally, most of the meat industry wastewater returns to the environment as a high contaminant residue rich in ammonia (urine), metals, antibiotics, and steroidal hormones.

Besides, this wastewater does not only contain toxic molecules, but it also restrains various pathogenic organisms such as protozoan bacteria, helminths, viruses, which can cause several diseases. Consequently, all those sources of contamination will release pollutants to the environment (as a solid, liquid, or gaseous), which will enter the food chain, and thus, they will have an effect on living organisms.

Thus, efficient approaches to clean up wastewater are an urgent need, because the surrounding media could be affected, leading to contamination and pollution of water bodies, eutrophication, reduction in dissolved oxygen, alterations in the ecosystem, and last but not least, it entails human health risks [8], [9]. Moreover, as stated earlier, our reservoirs of water are limited, so we should assess whether the massive consumption of water in meat production is worth it.

One straightforward solution would be to reduce our meat consumption (fewer animals to feed will imply less water consumed). Nevertheless, this is not a realistic future in the short run, as meat consumption is a pillar of our cultural background.

The ideal solution would be to treat the wastewater with the appropriate technology and give a brand-new use for that water since some activities do not require a high-water quality standard. Taking advantage of all the nutrients that are within the wastewater, new fuels, or fertilizers could be synthesized. The problem comes in deciding what the best way to face the issue successfully is. In the incoming sections, we will look in more detail the strategies available nowadays.

1.1.1 Pollution by heavy metals

There is a wide range of different pollutants that can be found in wastewaters, some organic compounds such as dyes, antibiotics, phenols, and hormones, but also metals and metalloids.

Figure 1.1 exemplifies the different pollutants discharged by three primary sources: agricultural, municipal, and industrial [10].

Organic compounds can undergo degradation processes in the environment, forming less toxic species. In contrast, metals and metalloids are not bio-degradable, so they persist in the ecosystem leading to bioaccumulation in the food chain, causing harsh environmental and health issues. Among the effects of metal accumulation, they may cause DNA damage, renal abnormalities, organ failure, allergy, fertility reduction, etcetera. Hence, it is vital to reduce the concentration of these toxic trace elements (TTEs) in wastewater effluents [11]–[13].

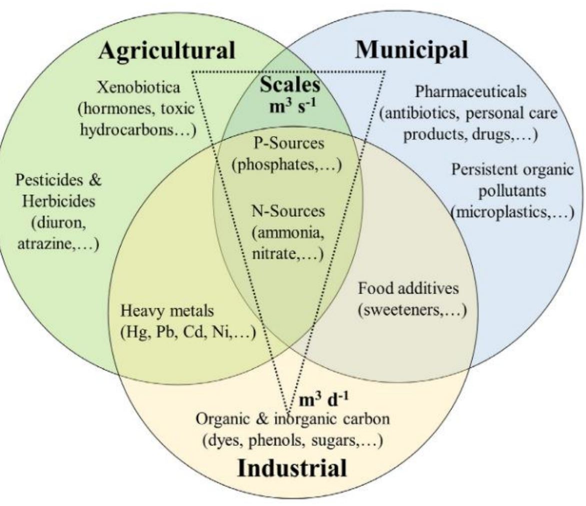


Figure 1.1

Main sources of different pollutants.

There is a bit of controversy and misunderstanding when it comes to defining what is (or not) a metal. In agreement with inorganic chemistry, a metal is a species that present lower electronegativities and non-directional interactions. Metals are good reducing agents; they typically form basic oxides, and they have low ionization energies (metals are commonly found in their oxidized states in nature). The non-metals have higher electronegativities and tend to gain electrons (except for the noble gases). In between those two, a small group called "metalloids" appears, which possess properties of both metals and non-metals. Figure 1.2 shows the periodic table divided into the three groups mentioned [14], [15].

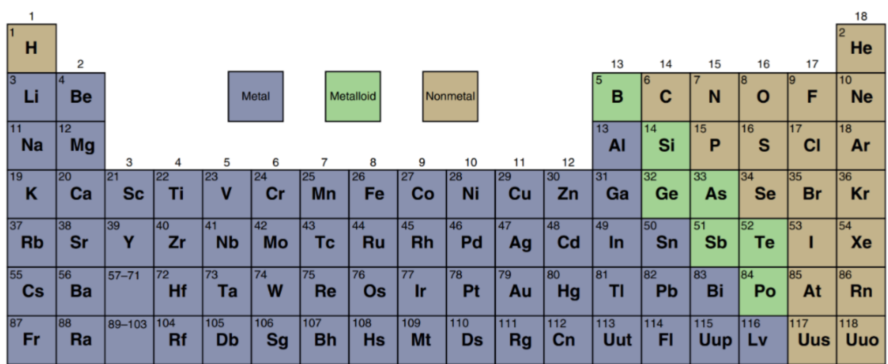


Figure 1.2

The periodic table of the elements, is divided into three regions: metals, metalloids and non-metals.

In the environmental argot, a heavy metal is defined as an element with metallic properties having an atomic number greater than 20, and it usually includes metals and metalloids with an atomic density five times greater than water [16], [17]. Thus, following this criterium, some of the heavy metals (HMs) which are usually found in nature are Zn, Cu, Mn, Ni, Co, Hg, Pb, Cd, Cr, Fe, Ag, V, As, Sb, Sn (these three last ones are formerly metalloids). Some of these HMs, such as V, Co, Fe, Ni, Mn, Mo, Cr, Cu, and Zn, have functional roles that are crucial for diverse physiological and biochemical activities in the body (they are essential parts of enzymes, proteins, or have homeostasis roles). However, high doses can be harmful to the body. Others, such as Cd, Hg, Pb, Ag, or As, in minimal quantities have tremendous effects in the body, leading to acute and chronic toxicity in humans [18].

Even though some heavy metals are typical components of the Earth's crust, due to the industrial revolution, their concentration in different environmental compartments has increased. HMs are released into the environment through natural and anthropogenic activities (rapid urbanization, wastewater residues, industrial and mining activities, and agricultural activities). Figure 1.3 displays the primary sources of the most relevant HMs [15].

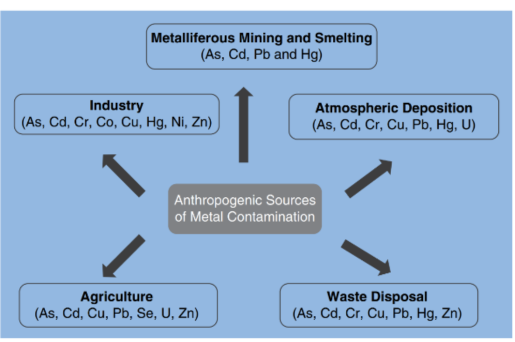


Figure 1.3

Main anthropogenic sources along with the more common metals they release.

As a result of these activities, soil, surface water, and groundwater can quickly become contaminated by heavy metals. Due to their polarity, HMs may be adsorbed, dissolved, or absorbed in many different ways [19].

The chemistry between the metals, the aquatic system, and the organic matter is rather complicated. There is an equilibrium among free ions, organic/inorganic complexes, and metal bound to organic/inorganic particles, which is shown in Figure 1.4 [20].

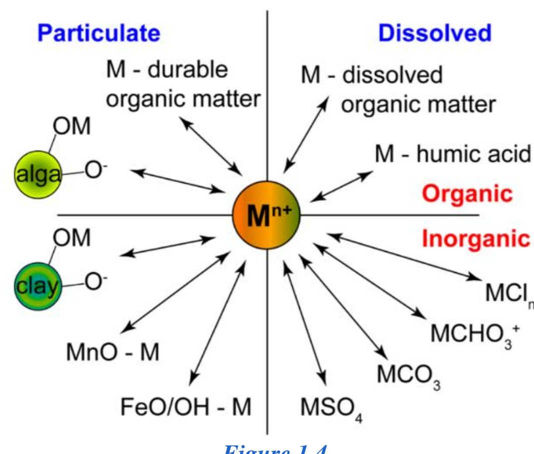


Figure 1.4

Interactions a metal cation can undergo when is surrounded by organic and inorganic matter

As heavy metals are hazardous species, the Environmental Protection Agency of the United States (USEPA) sets a maximum contamination limit (MCL) for each HM depending on its toxicity in different matrices. Table 1.1 shows the MCLG and MCL for some metals in water. MCLG represents the level of a contaminant in drinking water below which there is no known risk to health; MCL is the highest level of a contaminant that is allowed in drinking water. In Spain, the maximum concentration levels of water pollutants are compiled in RD 140/2003 of 7 of February, BOE of 21 of February, the values are similar to the ones of USEPA.

Table 1.1

MCLG and MCL values established by USEPA for heavy metals in water.

Contaminant	MCLG / ppm	MCL / ppm
Sb	0.006	0.006
Cd	0.005	0.005
Cr (Total)	0.1	0.1
Pb	0	0.015
Hg	0.002	0.002
Cu	1.3	1.3
Zn	5	5
As	0	0.01

Thus, heavy metals present a significant threat to the environment and public health. Due to numerous activities, they are discharged into water bodies through wastes. If we aim to reduce the impact on the ecosystem, it is crucial to remove them before their release, which is a challenge as they cannot be chemically or biologically degraded [21].

Figure 1.5 exemplifies the different pathways and mechanisms heavy metals can follow, to finally reach humans (or living organisms, in general) [18]:

Figure 1.5

Pathways through which metals can reach humans.

To illustrate how HM lead to harmful effects in the organism, Figure 1.6 shows a good scheme [18]:

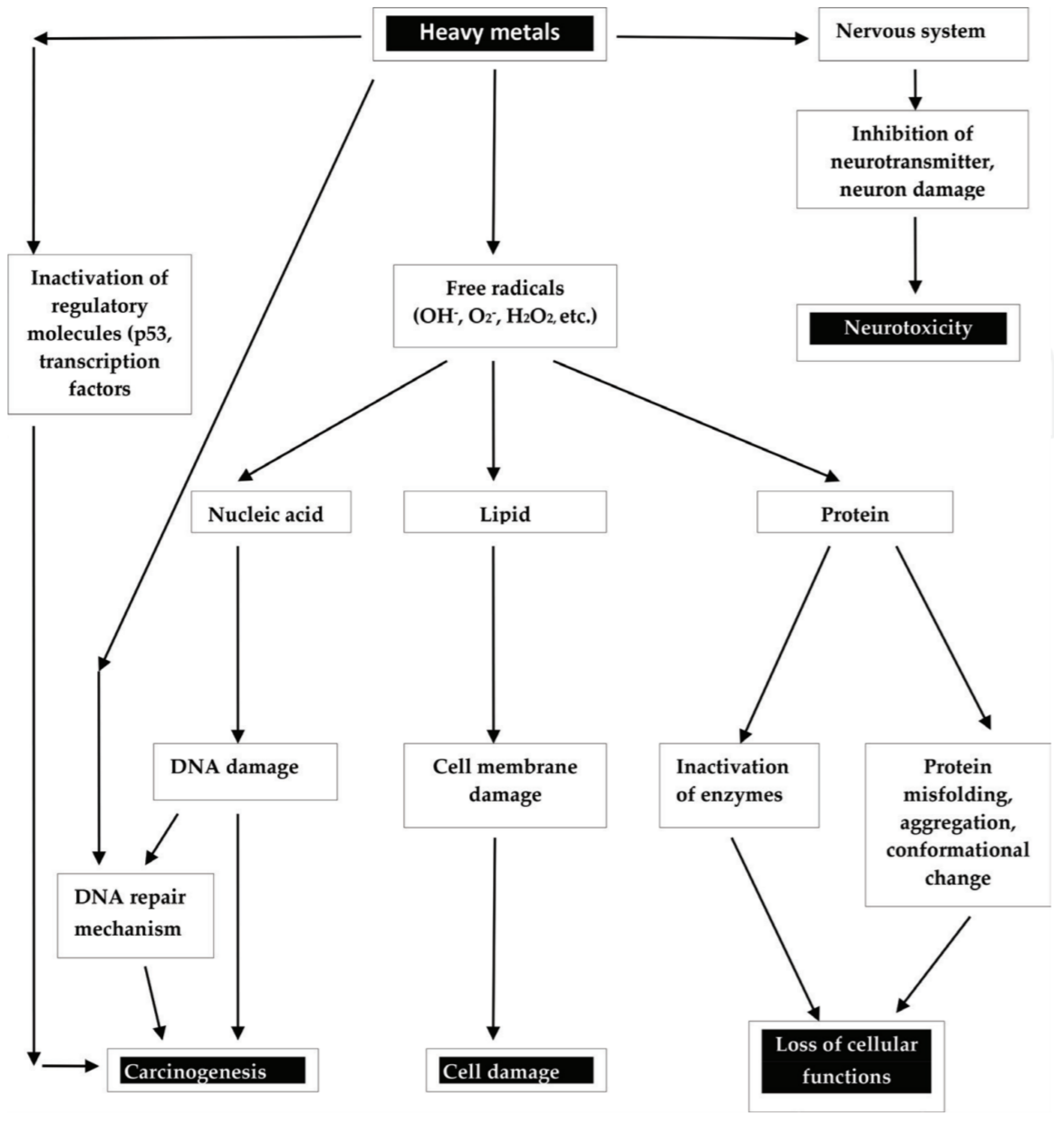


Figure 1.6

Different courses and mechanisms heavy metals can follow to damage living organisms metabolism.

Heavy metals produce stress and oxidation of biological molecules. They also are known to generate free radicals, which may drive oxidative stress and cause other cellular damages. The mechanism of free radical generation depends on the type of heavy metal. Heavy metals can also induce carcinogenic effects, as they can target signaling proteins that participate in apoptosis, cell cycle regulation, DNA repair, or DNA methylation.

As this work is going to focus only on copper and zinc, we will study a bit deeper about the chemistry of these elements in the following sections.

1.1.1.1 Copper (Cu)

Copper is the last transition metal of the first row. Its most common oxidation states are 0, +I, and +II, although +III, +IV, and +V have also been detected. In biological systems, it usually presents +I, +II, or +III [14]. In Earth, copper is found mainly as copper pyrite (chalcopyrite), $CuFeS_2$, which accounts for about 50% of all Cu deposits. Other forms of copper are Cu_2S (chalcocite), Cu_2O (cuprite), and, $Cu_2CO_3(OH)_2$ (malachite). The mean concentration of Cu on the Earth's crust is around 68 mg/kg, and it has two stable isotopes, ^{63}Cu, and ^{65}Cu, with a relative abundance of 69.09 and 30.91%, respectively [22].

Copper is used in industries to produce cables, wires, etc., due to its excellent conductive properties. Copper is also used in a variety of biocidal products for water disinfection, wood protection, or preventing fouling. Thus, copper can enter the environment through waste dumps, domestic wastewater, timber treatment, phosphate fertilizer production, wastewater from animal farms (as the feed is often enriched with copper), and natural sources. Copper is found in plants and animals because it is essential at low-level intakes for healthy development, but high dosages of copper are toxic to living organisms [23], [24].

Cu works as a micronutrient. A few examples will exemplify this fact. For instance, some invertebrate animals have proteins called hemocyanins, whose function is equivalent to that of hemoglobin (but instead of iron, the metallic center to which oxygen is bound is copper). It is less efficient than hemoglobin. Figure 1.7 shows the equilibrium reaction of oxygen coordination, in which two copper units in the protein start with an oxidation state of +I. They are close to each other, and when a molecule of oxygen is around, a peroxide bridge is formed, and the copper atoms are oxidized.

Deoxyhemocyanin $\underset{-\,O_2}{\overset{+\,O_2}{\rightleftharpoons}}$ Oxyhemocyanin

Figure 1.7

Equilibrium reaction of how oxygen interacts with the metal centres.

Copper is essential for all eukaryotes because it forms the cytochrome c oxidase, a transmembrane protein localized in the mitochondria (the number of subunits changes depending on the organism). It is the enzyme that closes the respiratory electron transport chain (it transfers 8 electrons from the cytochrome c to the oxygen, reducing it to water). Figure 1.8 shows how cytochrome c looks like [25].

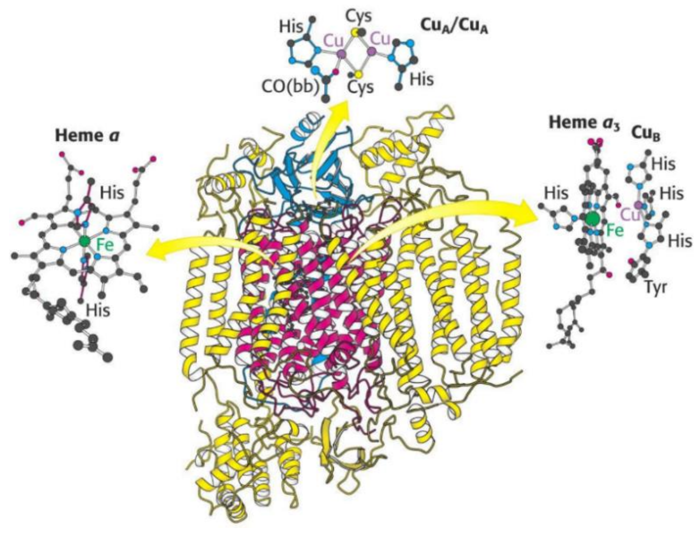
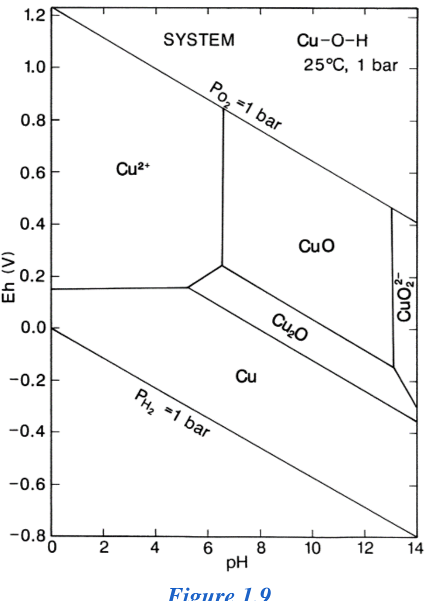


Figure 1.8

Complex IV (Citochrome c oxidase). It has 13 subunits (in humans).

Cu is also found as part of the superoxide dismutase protein, which is responsible for the elimination of the O_2^- anion, by converting it to O_2. Other enzymes in which copper is located are amine oxidases, ferroxidases, hephaestin, or tyrosinases.

An average adult usually contains between 50-120 mg of copper [26]. Therefore, copper is an essential micronutrient. Our metabolism is not capable of synthesized it, so copper must be taken up from the diet. The absence of Cu in people can lead to anemia, bone, and cardiovascular issues, weakening in mental and sensory systems, defective keratinization of hair, a decrease in levels of synapses, dopamine, and norephedrine, among others [24]. Although copper excess usually does not occur (because absorption/excretion mechanisms prevent that situation to happen), some people have the Wilson disease (copper accumulation in specific tissues of the body), which is related to aggravated symptoms of Alzheimer's disease. However, the cause is a genetic disorder [27].



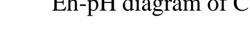


Figure 1.9

Eh-pH diagram of Cu

In acid media, copper is found as the divalent cation Cu^{2+} or as the acuo-complex, as can be seen in the Eh-pH diagram of the Cu-O-H system (Figure 1.9) [28].

When the pH rises, then its base-conjugates may appear. However, in the water, they will not be very favorable because parallel reactions will take place between the ligands of the proteins and sugars of the organic matter and the Cu^{2+} and Cu^{+}, leading to protein-metal interactions [29].

1.1.1.2 Zinc (Zn)

Zinc is the second most abundant trace element in the human body. An average adult has about 3 g of Zn, so it is not difficult to infer that zinc is vital to growth and development, not only for humans but for all forms of life [30]. The mean concentration of zinc in the Earth's crust is 76 ppm (a bit more abundant than copper). Zinc occurs as carbonate, silicate, or phosphate. The significant ores of zinc are ZnS (known as zinc blende) and $ZnCO_3$ (calamine). The most abundant isotopes of zinc are ^{64}Zn, ^{66}Zn, and ^{68}Zn, although it has others less abundant. Zinc has plenty of uses, e.g., anti-corrosion coating (immersion in molten zinc, electrolytic deposition, or spraying with liquid metal). Also, some alloys are built from Zn and sometimes Cu or Al [22], [24].

Zinc is much more reactive than copper (the redox potential is so low that it is easily oxidized). It is not strictly considered a transition metal, because the electronic configuration is d^{10} (only elements with partially filled d-orbitals are transition metals). Like for copper, when going to the right in the first transition element row, the *d* orbitals are stabilized, so the d-electrons are not likely to be put into play. Thus, chemistries with a low oxidation number will prevail. The chemistry of Zn is confined to Zn^{2+} (in aqueous solution). Its chemistry is very similar to that of Mg^{2+}. However, the electronegativity is a bit higher, and the energy gap between s and p orbitals is more significant in Zn, which favors the formation of more covalent compounds [14], [22].

Since the d^{10} configuration does not have any crystal field stabilization, Zn is not going to have any structural preferences. The geometry will depend on the Zn^{2+} cation and the steric requirements of the ligands. In most cases, 4-coordinate tetrahedral complexes will be preferred, along with the octahedral configuration. The energetic cost of changing the geometry is not going to be high either, so dynamic exchanges and different Zn coordination may take place. Zn^{2+} is not an active redox center, so it will not take part in electron-transfer processes (as for copper). However, it is a hard metal center -highly concentrated charge in comparison to its relatively small ionic radius- so coordination by nitrogen and oxygen donors is favored. As a hard metal center, it is going to be highly polarizing, so the activity of Zn^{2+} enzymes will depend on its Lewis acidity.

The predominant species in water will be $Zn(OH_2)_6^{2+}$ (up to a pH around 9). However, in a media with organic matter, Zn^{2+} will be coordinated with carboxyl, amino, and thiol groups of proteins, lipids, and sugars. As a Lewis center, Zn will play a role in increasing the acidity of water when it is coordinated to Zn, and also to enhance de electrophile of the carbonyl groups coordinated to Zn (+I effect). Zinc is vital for virtually all cellular functions, as it is present in an estimated 3000 human proteins. Moreover, it has additional functions in regulation. Thus, Zn is not the only key to the correct function of a wide range of enzymes, but it also has an essential structural and signaling functions.

Figure 1.10 shows some Zn enzyme complexes found in essential enzymes, as it has very similar functionalities; they look very similar (only differ on the ligands).

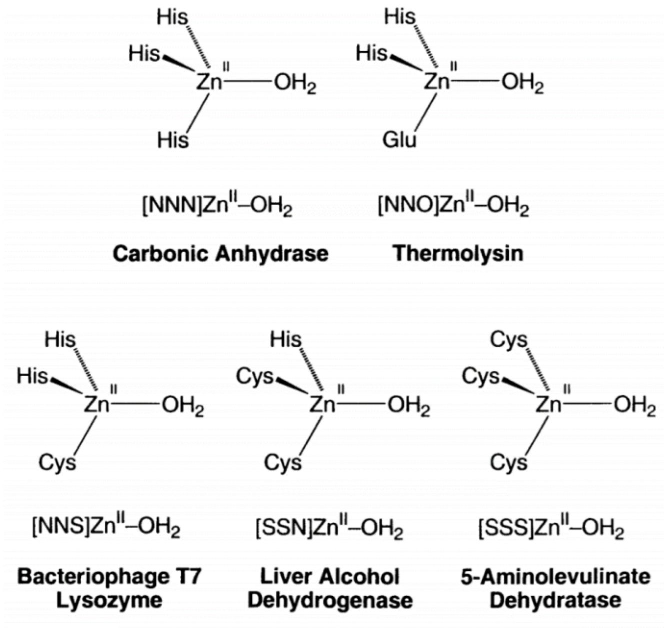


Figure 1.10

Active sites coordination motifs in representative zinc enzymes

For example, human carbonic anhydrase II (CAII) is present in red blood cells and catalyzes the reversible hydration of CO_2 (Reaction 1).

$$2\,H_2O + CO_2 \rightleftarrows H_3O^+ + HCO_3^-$$

Reaction 1

It is a way to liberate the CO_2 generated in our cells as a result of the aerobic respiration process. A water molecule coordinates with the metal center and is deprotonated by a molecule of histidine. The nucleophile HO^- attacks the carboxylic center of CO_2, and finally, HCO_3^- is obtained. A scheme is shown in Figure 1.11.

Figure 1.11

Mechanism of the anhydrase, and how the Lewis character of zinc is crucial for it.

As was mentioned before, Zn is a vital constituent of a multitude of enzymes: carboxypeptidases, cytidine deaminase, 5-Aminolevulinate dehydratase, thermolysin, or neutral protease [31].

Moreover, Zn holds proteins together; for example, For instance, insulin is stored in a hexameric form held together via Zn^{2+} binding, and it is a critical component in many transcription factors such as the so-called "zinc fingers" [32].

Like for copper, it is fundamental to know the zinc chemistry in water, and for that porpoise, we need the Eh-pH diagram, which is shown in Figure 1.12, [28].

In brief, Zn is a fundamental micronutrient and a less dangerous metal (compared to Cu and As, because it is present at higher concentrations in the organism). A deficiency in Zn can lead to several dysfunctions such as growth disorders, lousy wound healing, or immunological disorder. However, a high level of Zn consumption, (breathing Zn vapors and ingesting Zn-defiled foods and water) is also dangerous, as it may cause liver failure, pancreatic harm, bloody urine, lower levels of high-density lipoprotein cholesterol, nausea and vomiting. However, this situation is unlikely to happen because the tolerance limits for Zn are quite high. Zinc is discharged into the environment mainly due to the industrial operations, [24].

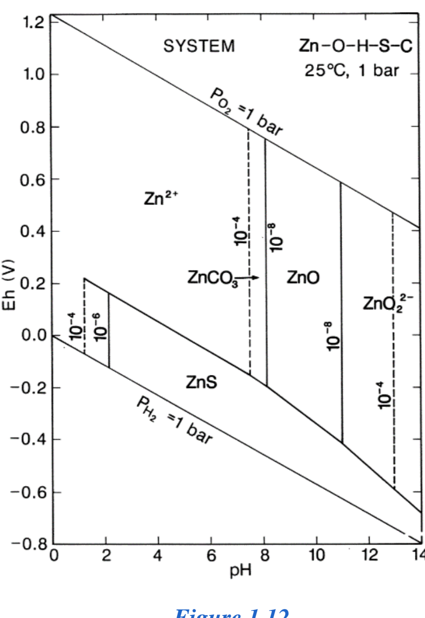


Figure 1.12

Eh-pH diagram for part of the system Zn-O-H-S-C.

To end this section, for essential metals such as copper and zinc, there are dose-response curves, which indicates the range within the dose of each metal is not harmful: there is normal homeostasis (Figure 1.13) [33].

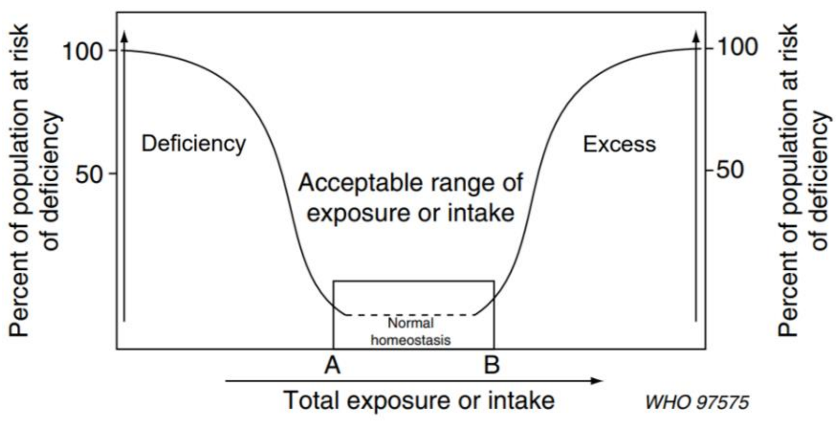


Figure 1.13

Dose-response curve for a certain (unknown) compound.

1.1.2 Methods to improve water quality: an overview

Many techniques have been tried for the treatment of contaminated water, and in particular, for metal removal. They can be split into two big categories:

- ***Abiotic or Physicochemical (no organisms involved):*** precipitation, ion exchange, adsorption, membrane filters, or electrochemical technologies.

- ***Biotic or Bioremediation (are based on biological materials):*** phytoremediation, biotransformation, biomineralization, bioacummulation, and biosorption.

Physicochemical techniques usually have low efficiencies (notably when metal concentration is in the range of 1–100 mg/L), demand high costs of operation, and generally produce contaminant byproducts [6], [24], [34], [35].

In the group of physicochemical methods for HM removal, we found:

- Chemical precipitation: hydroxide precipitation, carbonate precipitation, and sulfide precipitation.
- Chemical oxidation or reduction (use of electrochemical techniques), which include electrodeposition, and electro-dialysis.
- Lime coagulation.
- Ion exchange (using resins, starch xanthate).
- Reverse osmosis.
- Solvent extraction.
- Evaporation recovery.
- Adsorption-based technologies, which either employ inorganic adsorbents (natural minerals, ores, clay, and waste materials from industries or involve organic adsorbents (like waste materials derived from plants or animals).

Most physicochemical water treatment technologies (chemical precipitation, evaporation, electrodeposition, ion-exchange, membrane separation) suffer from ineffectiveness and high cost in the treatment of wastewater when the concentration of heavy metals is low (incomplete metal removal) [13], [36], [37].

However, adsorption and inorganic exchange have shown to be a practical approach, and nowadays, it is the most used technology in water treatment processes.

An overview of the physical methods is found in Figure 1.14 [38]. It exemplifies the processes for the removal of chromate ions, although similar mechanisms will take place for cationic species.

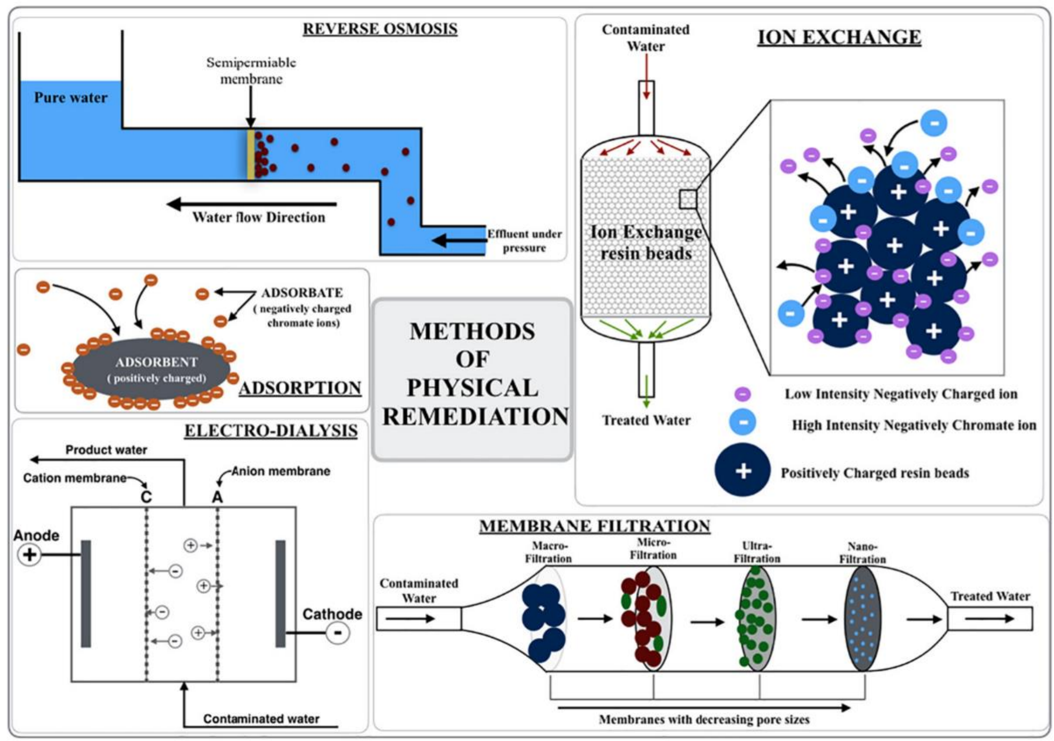


Figure 1.14

Physical methods for HMs removal from wastewate.

New adsorbents for the removal of the organic and inorganic contaminants from the wastewater have been investigated, such as nanocarbon, metals oxides, polymers, clays, or waste materials (industrial and agricultural) [11], [17].

An entirely new branch of treatments arose a few years ago in the field of magnetic materials (Figure 1.15). They can be used as adsorbents and, in contrast to traditional adsorbents that are difficult to separate from the water after the process, magnetic materials can be quickly and efficiently separated using a magnet. Nevertheless, magnetism is not the only characteristic of their use. The extraordinary surface charge and redox activity characteristics are prominent reasons for their qualification when considering other materials [39], [40].

In recent years, researchers have focused on waste biomass for the removal of dyes and heavy metals for water treatment, due to their availability, renewable nature, and low cost. Indeed, it is nothing else but applying the principles of adsorption but using biological materials as adsorbents [5], [39], [41].

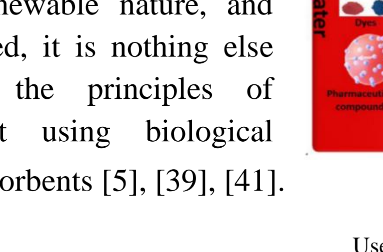


Figure 1.15

Use of different magnetic adsorbent materials in wastewater treatment application.

This new category of treatment methods is the so-called "biotic", and they will be studied in-depth in the next section.

1.2 Bioremediation as a solution to water pollution issues

Generally, efficient solutions can be found by attending to how nature behaves. Bioremediation is the use of biological materials for the removal of pollutants. The technique utilizes innate biological mechanisms to eliminate contaminants using microorganisms and plants (or their products) to heal polluted environments. Chemical and physical techniques are often more expensive and ineffective, especially for low metal concentrations than bioremediation techniques. Also, conventional methods generate significant amounts of toxic sludge [12], [42]–[44].

Five main processes are considered as bioremediation techniques [17], [39]:

- ***Phytoremediation:*** Use of natural organisms (algae and plants) to clean up pollutants from the environment *in situ*. They have high uptake capacities.

- ***Biotransformation:*** Converting toxic compounds into less toxic species using biological systems. It is an excellent method for organic pollutants, but as we mentioned before, it is not suitable for heavy metals as they are not biodegradable and are accumulated into the organisms.

- ***Biomineralization:*** Different organisms transform toxic compounds into their less toxic mineral forms.

- ***Bioaccumulation:*** It is an active metabolic process, requires respiration to remove pollutants, which break through the cell wall.

- ***Biosorption:*** It is a metabolically passive process in which pollutants are retained on the cell surface.

Bioremediation has a promising potential in removing heavy metals from the environment in an eco-friendly manner because they are genuinely low-cost, have high efficiencies in HM removal from dilute solutions. Moreover, both living and non-living organisms can be used, so the options available are very wide [17].

Nowadays, although phytoremediation is gaining some attention, it is an "in-situ" method, so the scaling up of the processes seems rather tricky. However, bioaccumulation or biosorption can be carried out ex-situ in a treatment plant.

Therefore, in the following sections, we will study bioaccumulation and biosorption profoundly.

1.2.1 Bioaccumulation

Bioaccumulation occurs when the absorption rate of the pollutant is higher than the rate of losing it. Thus, the contaminant remains inside the organism. Organisms can usually resist concentrations of chemicals up to certain levels, beyond which these chemicals become toxic and endanger the organism. Therefore, the candidate organisms should have a wide tolerance of one or more contaminants, and they must demonstrate exceptional properties when transforming a toxic element into a non-toxic form, which will allow them to keep the pollutants inside (thus, reducing the toxicity in the outside).

Bioaccumulation is defined as the intracellular accumulation of the pollutant, which occurs in two stages (Figure 1.16):

1. Fast stage (molecule/element is adsorbed). This stage matches the biosorption process, which will be explained later.

2. The slow step that includes transport of sorbate into the inside of cells by the active transport system.

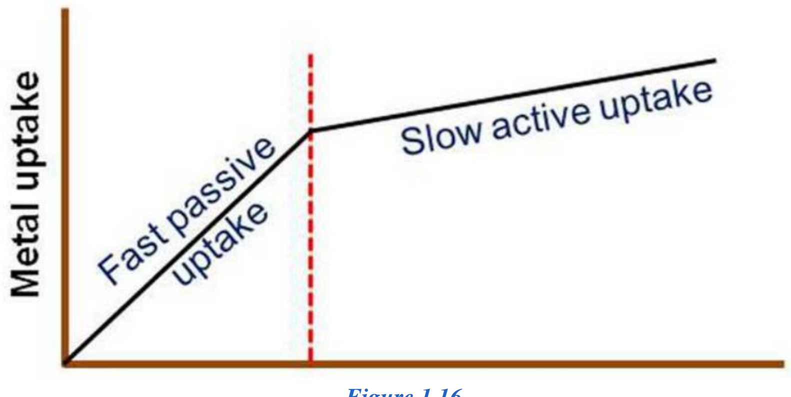


Figure 1.16

Stages for metal bioaccumulation.

Bioaccumulation is a non-equilibrium process. Figure 1.17 illustrates a simple siderophore mechanism through which the heavy metal crosses the cell membrane and reaches the cytoplasm, where is going to be kept in vacuoles [44].

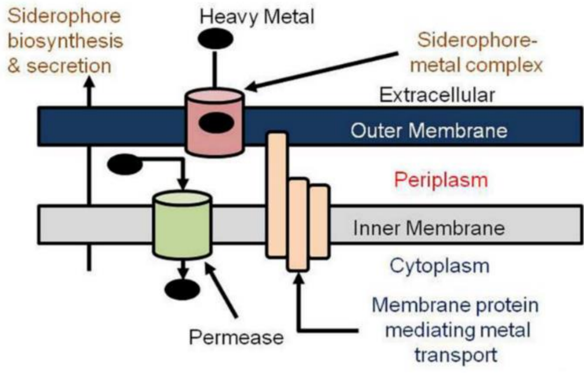


Figure 1.17

Heavy metal removal by a siderophore mechanism.

The whole process is more complex, as can be seen in Figure 1.18, as there are plenty of possible mechanisms of biosorption, and only when the heavy metal is sorbed, it is going to be absorbed into the cell [45].

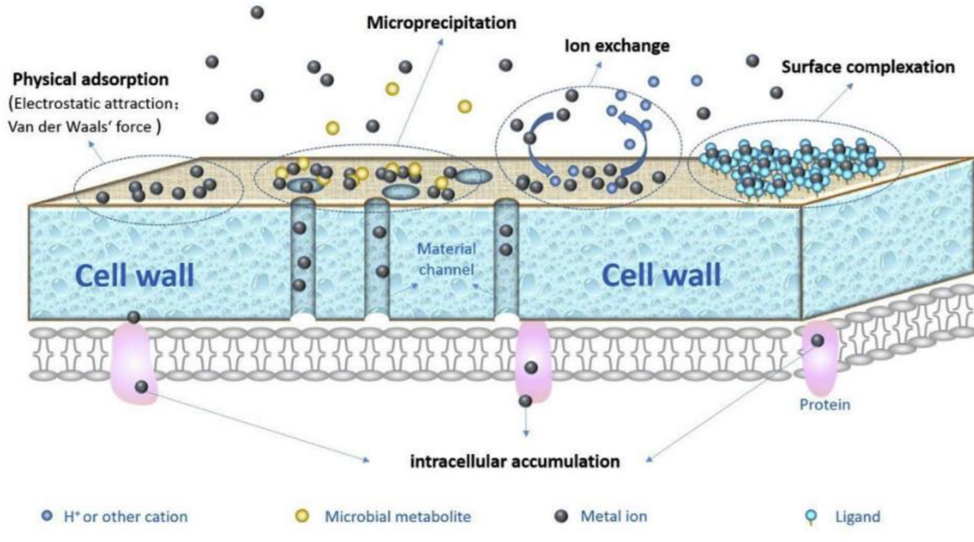


Figure 1.18

Different biosorption mechanisms previous to the metal uptake.

The uptake of elements across the bilayer can be divided into three different mechanisms: passive diffusion, facilitated transport, and active uptake [46].

The intracellular accumulation phase is produced when the extracellular concentration of HM is higher than its intracellular concentration, which is going to be valid until the saturation limit is reached [17].

In bioaccumulation, pollutants are transported across cell walls and membranes. Inside the cells, they are bound to intracellular structures. Different organisms have been used for bioaccumulation studies such as plants, fungi, fish, algae, microalgae, bacteria, and microalgae-bacteria consortia, among others. To select the best organisms, we must investigate the mechanisms and genes associated with bioaccumulation as well as the genes that account for the tolerance towards the concentrations of metals [47].

To that end, molecular biology has been used in such investigations. Techniques such as mass spectrometry-based proteomics or genome sequencing of microorganisms with potential bioaccumulation capacities have shed light on investigating candidate genes to be targeted for improving the bioaccumulation efficiency of the organism. Furthermore, bioinformatics and mathematical modeling have gained importance in the investigation of the properties and potential candidate organisms to predict the concentration of chemicals that can be tolerated by them [44], [47].

1.2.1.1 Bioaccumulation mechanisms

Scientists often describe three different mechanisms for bioaccumulation of metal ions (Figure 1.19) [17], [46]:

A. Metal ions compete for binding to ion carriers, or they either enter the cell after binding low-molecular-weight molecules (such as amino acids), via endocytosis.

B. They could also penetrate by using ionic channels or intermembrane proteins.

C. Via active transport, which can be either primary or secondary (then, it will require the aid of another molecule).

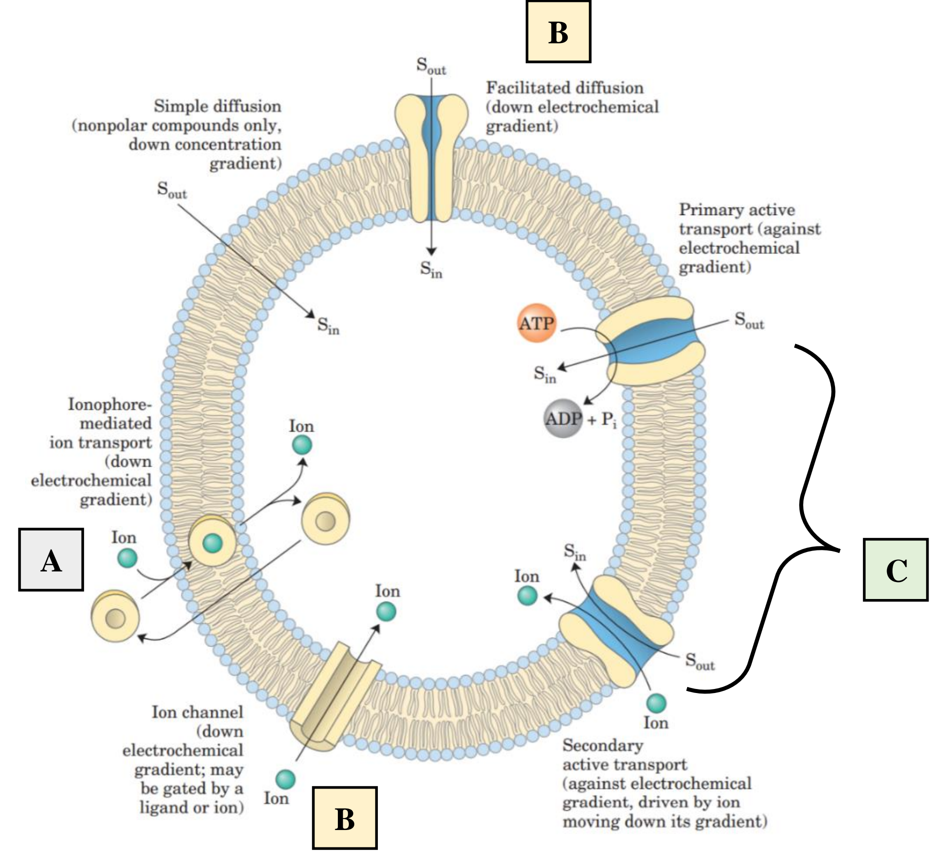


Figure 1.19

Summary of active and passive transport types across the cell membrane.

We assume that a charged ion could never cross a cell membrane via simple diffusion (many repulsive forces to evade). Nevertheless, indeed, some of them will go through the membrane by facilitated diffusion mechanisms (ion channels, siderophores). However, it is unlikely that the microorganism we choose has the appropriate channels for the ions we would like to remove (as they should be concrete in size). Hence, the primary bioaccumulation mechanism will be active transport. In physicochemical terms, in order to the bioaccumulation to be produced, we need to overcome an electrochemical gradient.

The transport across the membrane obeys the laws of thermodynamics. Therefore, it will only take place if there is a release of Gibbs free energy:

$$\Delta G < 0$$

Besides, many ions will be inside and outside the cell (electroneutrality must be kept), but this charge separation creates a transmembrane electrical gradient, in short, a membrane potential. The flux of ions can be down or against the gradient, and if it is against the gradient, then energy is consumed.

The Gibbs free energy for a metal ion crossing the membrane is shown below:

$$\Delta G = \Delta G_{Chemical} + \Delta G_{Electrical} = RT \cdot ln \frac{c_{Inside}}{c_{Outside}} + z \cdot F \cdot \Delta V_{Membrane}$$

The electrical output of the Gibbs free energy takes into account the electric potential energy (how much energy we must spend in transporting 1 mol of a substance of charge z to a solution of a specific potential). F is Faraday constant.

The membrane potential ($\Delta V_{Membrane}$) produces a force opposing ion movements that increase the electric potential, so it drives ion movement that reduces the electric potential. Thus, a charged solute (a metal ion) tends to move spontaneously depending on both the chemical gradient (the difference in solute concentration) and the electrical gradient across the cell membrane. This agrees with the second law of thermodynamics: molecules tend to spontaneously assume the distribution of greatest randomness and lowest energy (because it is the one most probable, as it has more microstates that lead to the same macrostate).

The process will go like this:

1. Water-ion interactions must disappear, and protein-ion or ion-channel interactions may show off. The process needs energy in order to disrupt dipole-ion interactions.

2. Then, the ion must diffuse about 3 nm (the usual thickness of a cell membrane), repulsive interactions must be overcome.

3. The ion finally arrives at the inside of the cell and gets rehydrated. Finally, it is stored in the corresponding organelle.

The activation energy for the translocation of a polar solute across the bilayer is considerable (they are virtually impermeable). However, these "vehicles or channels" reduce the activation energy for transportation, as they open an alternative route for transportation and thus, facilitating the process [25].

Even though practically all heavy metal accumulation follows an active transport mechanism, some metals can form neutral complexes that can go through the cell membrane via passive diffusion, although for metals, the partitioning coefficient (K_{ow}) is rather low. K_{ow} is "the ratio of the concentration of a chemical in n-octanol and water at equilibrium at a specified temperature" [48]. Examples are shown in Table 1.2 [46].

Table 1.2

Partitioning coefficient values for different metal species.

Metal	Inorganic Complexes	K_{ow}	Ref.	Organic Complexes	K_{ow}	Ref.
Hg	$HgCl_2$	3.3	[1]	Hg(cysteine)$_2$	3.7	[2]
	HgOHCl	1.2	[1]	Hg(thiourea)$_2$	4.6	[2]
	$Hg(OH)_2$	0.05	[1]			
	HgSHOH, $Hg(SH)_2$	26	[3]			
	$Hg(S)_6$	26	[3]			
	Hg^0	~10^3	[7]			
		4.2	[1]			
CH_3Hg	CH_3HgCl	1.7	[1]	CH_3Hg(cysteine)	50	[2]
	CH_3HgOH	0.07	[1]	CH_3Hg(thiourea)	630	[2]
	CH_3HgSH	28	[2]			
	$(CH_3)_2Hg$	180	[1]			
Cd	$CdCl_2$	0.002	[8]	Cd(dithiocarbamate)$_2$	1000	[4]
Ag	AgCl	0.09	[5]	–		
Cu	—			Cu(oxine)$_2$	400	[4]
				Cu(oxine)$_2$	70	[6]
				Cu(chloroxine)$_2$	325	[6]
				Cu(dichloroxine)$_2$	690	[6]
				Cu(dibromoxine)$_2$	3715	[6]
				Cu(dithiocarbamate)$_2$	630	[4]
Pb	–			Pb(dithiocarbamate)$_2$	10^4	[4]

References: 1: Mason et al. [9] and Morel and Hering [10] and references therein; 2: Lawson and Mason [8]; 3: Benoit et al. [11]; 4: Phinney and Bruland [12]; 5: Reinfelder and Chang [13]; 6: Kaiser and Escher [14]; 7: Jay et al. [15]; 8: Gutknecht [16].

Both facilitated transport and passive diffusion do not require any energy consumption. However, the cell (and thus, the organism) must be alive in order to build and maintain the different proteins and channels that allow metal ion transportation. For active transport, the situation is even worse, as it needs energy before the transport, so it is never going to work. Hence, we can conclude that when the cell dies, there is not going to be any bioaccumulation of the metal. Nevertheless, that does not imply that the biomass is no longer useful for bioremediation because other processes can take place even if the cells are dead.

Additionally, it has been demonstrated that bioaccumulation promotes the synthesis of proteins like metallothioneins (with thiol groups) that are meant to coordinate with metal ions, preventing them from disrupting the metabolic activity of the cell. They are usually generated in response to the presence of metal ions in the growth medium, so it could be said that bioaccumulation is, at least in some cases, a defense mechanism for the microorganism.

The main advantage derived from bioaccumulation is unit processes are reduced: harvesting, drying, processing, and storage [47], [49]. The disadvantage of this method is that a very high concentration of toxic metals may cause the death of the cells, and thus, no more bioaccumulation will be performed.

1.2.1.2 Factors influencing bioaccumulation

The process is complicated and depends on several factors:

- The growth medium used (in our case, the wastewater).
- The pH of the medium.
- The working temperature.
- The presence of other contaminants that, at the same time, can be bioaccumulated, or can act as inhibitors for the growth of the organism, which will affect the absorption of our pollutant/s of interest.

The last factor is the most problematic, as it makes it impossible to treat a solution with a high load of pollutants. Moreover, it is necessary to supply external source of energy to growing cells, although it is possible to keep alive the organism used if the minimum nutrient requirements are supplied by the medium in which they are living.

1.2.2 Biosorption

Before going deep into what biosorption is, it is good to compare it with bioaccumulation (at the end, biosorption is just the first step of any bioaccumulation process). Table 1.3 presents the comparison [47], [50].

Table 1.3

Comparison between bioaccumulation and biosorption.

Property	Bioaccumulation	Biosorption
Cost	The process is costly because Keep the suitable conditions for live cell is very difficult.	Low cost, most of the biosorbents are made from waste from the industry, and the costs of transportation and operating costs are low.
Storage	In order to maintain the culture medium, it needs extra metabolic energy.	Easy storage.
Selectivity	Better than biosorption.	Weak and improved with chemical modification.
Adaptation	It does not have much flexibility and is affected by high concentrations of metal or salt.	Suitable linking sites are compatible with various ions.
The desire to establish a link	Depending on the toxicity of the pollutant.	In a favorable condition, the likelihood is high
Recovery and reuse of adsorbent	Since most toxic substances are accumulated inside the cell, the possibility of regeneration and use is very limited.	There is a possibility of proper absorption and reuse of it in repeated cycles.
Collect toxic substance	Given the feasibility, there is no possibility of using cells in the next cycle.	With appropriate selection, it is possible to collect toxic material. In many cases, acid or alkaline solutions are used to collect toxic substances.

Biosorption differs from bioaccumulation in the way pollutants are bound, they get only attached to the surface of the cell, but they do not undergo any accumulation. Thus, no energy is depleted. It is a simple physicochemical process, analog to traditional adsorption, but now the sorbent is biological-based, and the possible binding sites are going to be more heterogeneous. Simply, bioaccumulation is what traditionally is known as absorption, while biosorption is analog to adsorption (Figure 1.20).

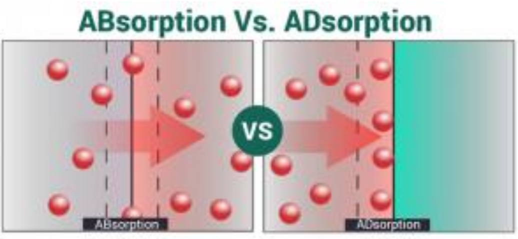


Figure 1.20

Visual difference between an absorption process versus an adsorption process.

Biosorption does not depend on metabolism, and it is an equilibrium process. Thus, when an equilibrium situation is reached, it can be shifted towards metal desorption with the appropriate eluent and conditions.

The history of biosorption began in the 1990s, when Prof. Bohumil Volesky (University in Canada) provided the first theoretical basis of the process, and described some metal cations retained by the biomass, which was able to concentrate the metal up to 1000 times. Moreover, the process showed to be specific for certain metals. Since then, many metals have been tried successfully to be sorbed by biological matrixes. Some biosorbents are bacteria, algae, fungi, the skin of animals and fruits (and their residues), or biopolymers (chitosan, calcium alginate) [51].

A diagram about how biosorption is applied in wastewater remediation is shown in Figure 1.21 [47]:

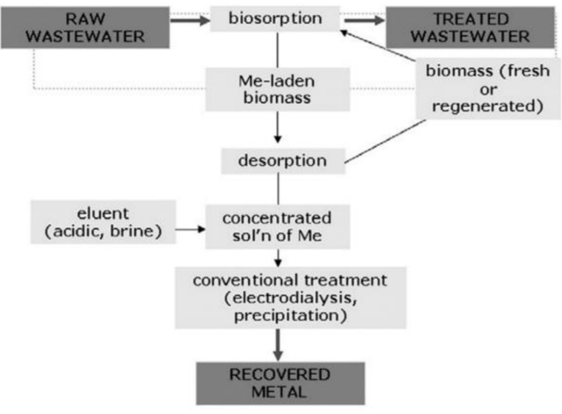


Figure 1.21

Scheme of a biosorption-based metal recovery process.

A summary of how the biosorption process works is in Figure 1.22, we have contaminated wastewater, and by using a biosorbent and a post-filtration step, we get water cleaner (without the yellow pollutant) [39].

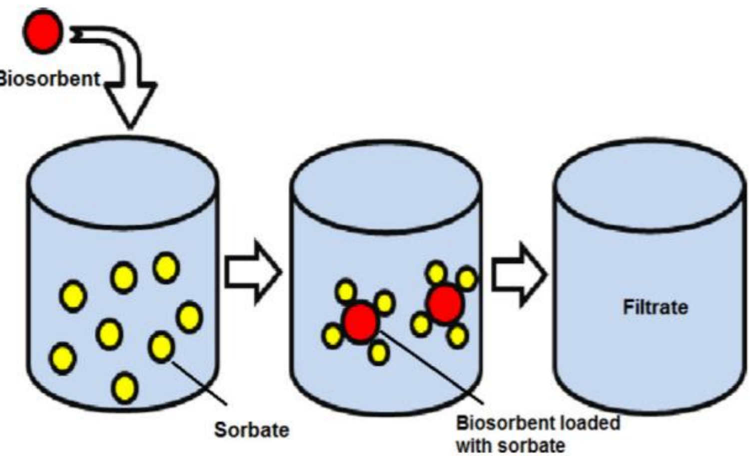


Figure 1.22

A simplified version of the biosorption process.

Once the biosorbent is loaded with the contaminants, it can be regenerated and reused, and the medium that had been in contact with it is now free of toxic elements. It is crucial to correctly choose the desorbing agent because we aim to desorb all the pollutants attached to the sorbent with the lowest amount of volume possible. Moreover, treatment with the wrong eluent may damage the sorbent (and, therefore, its sorptive capacities). The eluted solution loaded with toxic elements must be treated. Ultimately, if there are some useful metals (such as Cu or Ag), they could be recovered. Another approach could be to burn the solid residue to transform it into a compact solid or recover the metal from the ashes.

The advantages of this methodology are the low operational cost (as there are plenty of biological sorbents that in theory will be reused) and the low quantity of sewage sludge produced (conventional methods generate a more significant amount of residues). The process has shown to be particularly useful in the treatment of dilute effluents, such as wastewater. The disadvantage, when compared with traditional sorbents, is that the lifetime of biosorbents is generally shorter [47], [52].

There are many different types of biosorbents, but they can be split into two categories:

I. ***Low-Cost:*** Can be gathered directly from the environment (renewable sources), and it includes waste or byproducts produced by the industry.

II. ***High-Cost:*** They are unique materials based on biological organisms, but with some modification in order to enhance their sorption properties.

Biosorbents applied in wastewater treatment should possess the following properties [50]:

a. High biosorption capacity and proper kinetics.

b. The right size, shape, and physical properties.

c. The separation step from the solutions must be cheap, rapid, and complete.

d. Strong mechanical strength, thermal stability, and excellent chemical resistance are required.

e. Available, and the preparation must be cost-effective.

f. Regenerable and reusable to decrease operation costs.

In Figure 1.23, a diagram of the whole process, from biosorbent selection to its application to a real wastewater matrix, is shown [50]:

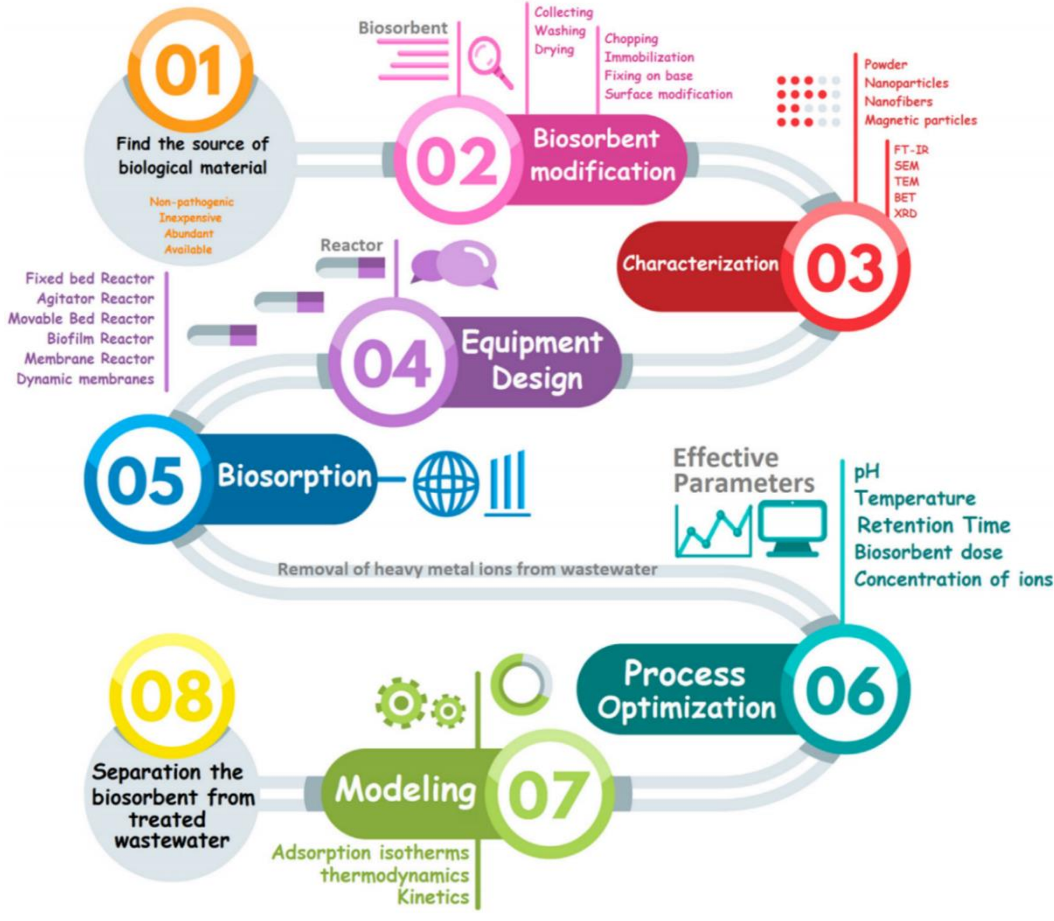


Figure 1.23

Steps of a biosorption process for heavy metal removal.

1.2.2.1 Biosorption mechanisms

The mechanisms of biosorption are generally based on physicochemical interactions between metal ions and the functional groups present on the cell surface, which can trap several heavy metal ions. The most common organic functions involved are carboxylate, hydroxyl, amino, and phosphoryl. They usually are negatively charged and are part of sphingolipids, phospholipids, sugars, and proteins, which are the essential components of all cell membranes.

In contrast with bioaccumulation, biosorption is usually fast and reversible; the equilibrium can be reached within a few minutes, although it depends on the type of biosorbent and matrix used. The activation energy required is up to three times lower than for bioaccumulation (21 kJ/mol versus 63 kJ/mol). As a reversible process, the metals can be desorbed, so regeneration and reuse of the biosorbent are usually straightforward [51], [53].

The mechanism involves several processes, including electrostatic interaction, ion exchange, precipitation, redox process, or surface complexation. An overview of all possible processes is in Figure 1.24 [9]:

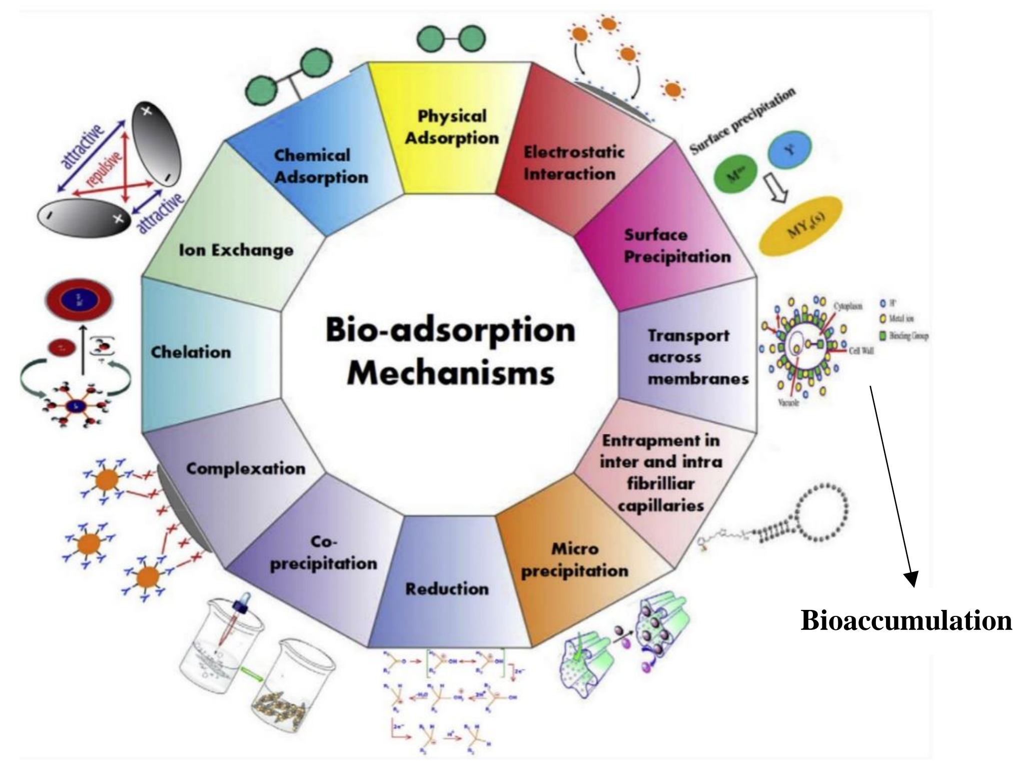


Figure 1.24

Mechanisms of biosorption and bioaccumulation that can happen on a cell.

The actual mechanism involved in metal biosorption is not entirely understood. Additionally, one metal can be sorbed due to more than one mechanism, so in complex mixtures with more metals involved, it is impossible to know what is happening in the solution accurately.

Before introducing the two main types of biosorption, we have to set the typical terminology used in this field. The adsorptive in our problem would be the metal ions present in the wastewater, the adsorbent would be the alga, bacteria of their consortia, and the adsorbate is the complex formed through the interaction of the adsorptive with the adsorbent (depending on the nature of the interaction, we will have chemical or physical adsorption) [54]. Sometimes the adsorptive is called the adsorbate in order to simplify the terminology, although it is not the most rigorous and the IUPAC recommends the use of adsorptive [55].

We can divide all the mechanisms showed above into two categories, depending on the nature of the interaction:

A. **Chemisorption:** Strong interaction, similar to the covalent bonding. It is a selective process which requires relatively high activation energy. Complexation, ion exchange or chemical adsorption, are within this category. The general reaction that describes this process is the following:

$$M^{n+} + n\,(AB) \rightleftarrows \; n\,A^{+} + MB_n$$

Where M^{n+} is the metal ion, AB is the chemical form of the sites available, and n is the number of available sites along the cell membrane.

As this is an equilibrium process, it will have an equilibrium constant. Considering molar concentrations:

$$K = \frac{[A^{+}]^{n} \cdot [MB_n]}{[M^{n+}] \cdot [AB]^{n}}$$

According to the reports, the ion-exchange mechanism could lead the biosorption process in some microorganisms.

Chemisorption increases with temperature up to a specific value, from which it begins to decrease (Figure 1.25).

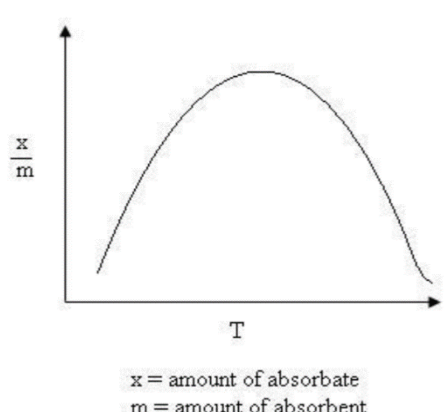


Figure 1.25

Dependence de chemisorption capacity with temperature.

B. Physisorption: It is a labile interaction mostly due to Van Der Waals forces. The activation energy is shallow (or it even does not exist), and the process is not specific. Physisorption decreases with temperature (Van Der Waals interactions are disrupted), as is shown in Figure 1.26.

The equation that describes this process is much simpler, as it only depends on the metal ion and the sorbent Van Der Waals interactions:

$$x\, M^{n+} + B \rightleftarrows (M_x B)^{n+}$$

B stands for the unoccupied cell membrane sites of the biosorbent.

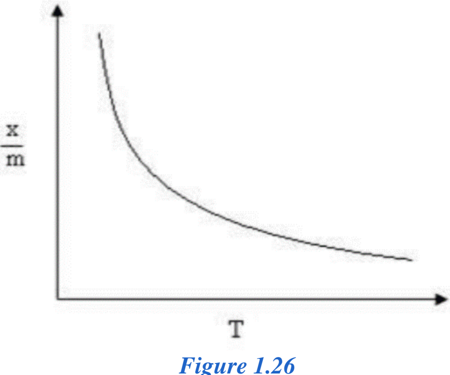


Figure 1.26

Dependence of physisorption with temperature.

However, not all the possible mechanisms are equally probable. Some of them, such as ion exchange, complexation/chelation, or chemical/physical adsorption, have a more significant contribution to the amount of heavy metal adsorbed. Thus, the more influential factors of the process must be utterly related to those mechanisms. As cell membranes usually have a net negative charge, removal of metal cations is going to be favored, although anion can also be removed by other functional groups [47].

In order to study more deeply this process, there are three different stages in which biosorption takes place (whether it is chemisorption or physisorption):

1. The metal ion must be transported from the aqueous phase to the interface solution-cell membrane. Fick's diffusion law governs this step.

2. Then, the ion must go from the interface to the actual active sites of the cell membrane.

3. Finally, fixation of the ion is done via physisorption or chemisorption.

Therefore, the biosorption process takes place in the liquid-solid interface.

For specific pressure and temperature conditions, when the adsorption process is progressing, the concentration of the ion in the aqueous phase decreases, while its concentration on the surface will increase.

When all the active sites are occupied, or there is no more metal ion in the solution, the process stops (saturation point). In these circumstances, it exists a relationship between the amount of ion retained in the solid phase and its concentration in the aqueous phase, which is commonly known as isotherms (more details and information in *Section 1.2.3*).

Figure 1.27 shows the isotherms of two different biosorbents, both reaching the saturation point, but one is better than the other because its maximum metal uptake is higher [56].

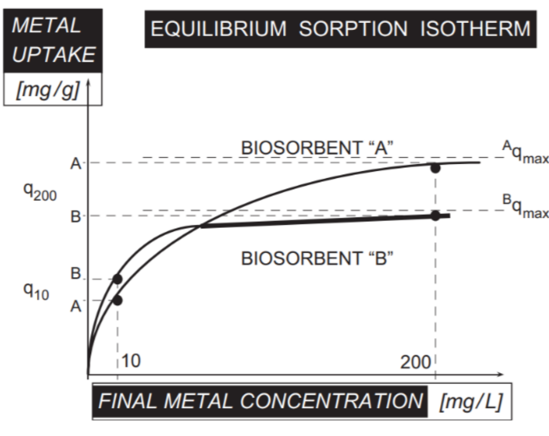


Figure 1.27

Two isotherms for two different biosorbents, with different q_{max}.

1.2.2.2 Factors influencing biosorption

It is not hard to find that the factors conditioning the process are going to be firmly related to the cell membrane structures and aqueous chemistry. Thus, the six most important factors are temperature, pH, reaction time, functional groups available, presence of other competing ions, and biosorbent dose. However, the agitation rate and the biosorbent size also contribute, but in a lesser way [50], [57].

Since ion exchange is one of the primary mechanisms, protons present in the media are going to compete with metal cations for the binding sites. Moreover, the pH affects the form the metal cations are in the solution; if it is too high, they may appear forming hydroxides, and if it is too low, then the metal can be kicked off the cell membrane while protons occupy its place instead. Thus, pH profoundly influences the process. This pH behavior is crucial when we want to recover the metal cations and subsequent regeneration of the biosorbent.

In the same way, the functional groups and biosorbent dose are intimately related to each other: the higher the dose, the larger the number of functional groups available, and depending on the nature of those groups, the binding is going to be more or less effective. Concerning the presence of more than one ion, depending on the different charge to volume ratio, they will have a particular affinity towards the functional groups. Besides, the number of active sites is linked to how the biosorbent is found; if it is immobilized, then the active surface is reduced (although the following separation step is going to be more comfortable).

These factors are common for practically all types of organisms, as the chemical composition of their cell wall is relatively similar. However, there are differences, and for example, the fungi have a cell surface that can bind cations and anions easily at the same time (typically, there is a preference for cation binding). We will study the morphology of the bacteria and alga cells in their corresponding sections.

As there is a wide variety of functional groups capable of performing ionic exchange reactions, such as carboxyl, amido, sulfonate, imidazole phosphoryl, or amino, the process of biosorption is highly favorable in the cell surface. Each group has a specific acidity constant, which determines its binding capacity ($pH > pKa$, then the groups are deprotonated and ready for binding metal ions). Therefore, if we want to find a new sorbent with as high uptake as possible before performing a set of experiments, it is a good idea to do a potentiometric titration to determine the concentration of each group in the cell membrane. In Table 1.4, some of the most common functional groups present in cells are related to its pKa, the biomolecules where they can be found, and also the preference of the metal nature (hard or soft) [56].

Table 1.4

pKa values of the binding groups that can be found in the cell surface. Hard/Soft indicates the charge to radius ratio the functional groups prefer. The acronyms stand for: PS = polysaccharides; UA = uronic acids; SPS = sulfated PS; Cto = chitosan; PG = peptidoglycan; AA = amino acids; TA = teichoic acid; PL = phospholipids; LPS = lipoPS.

Binding group	Structural formula	pK_a	HSAB classif.	Ligand atom	Occurrence in selected biomolecules
Hydroxyl	–OH	9.5–13	Hard	O	PS, UA, SPS, AA
Carbonyl (ketone)	>C=O	–	Hard	O	Peptide bond
Carboxyl	–C=O, OH	1.7–4.7	Hard	O	UA, AA
Sulfhydryl (thiol)	–SH	8.3-10.8	Soft	S	AA
Sulfonate	O, –S=O, O	1.3	Hard	O	SPS
Thioether	>S	–	Soft	S	AA
Amine	$–NH_2$	8–11	Int.	N	Cto, AA
Secondary amine	>NH	13	Int	N	Cti, PG, peptide bond
Amide	–C=O, NH_2	–	Int	N	AA

At the same time, the characterization of the biosorption sites can be done using techniques such as IR/FTIR, Raman spectroscopy, X-Ray Photoelectron Spectroscopy (XPS) or Electron Microscopy (SEM or TEM) [47], [50].

Also, affinity constants between a group and a sorbate can be determined, which enables a better description of the selectivity of the biomass for different sorbates.

The adsorption process can be endothermic or exothermic. Usually, it is exothermic because of the new bonds created to reduce the enthalpy of the products. However, temperature affects the solubility and stability of metal ion species, the functional groups, and their future complex. Temperature influences the kinetics of the process lowering its activation energy, but it also can have a down-side effect on the biosorbent. However, some researchers have suggested that temperature affects less than the biosorbent dose or the pH.

In the case of live biosorbent, the organism used will have a specific range of temperatures within it is going to develop better, so this factor directly affects the amount of heavy metal sorbed. Moreover, higher temperature increases the mass transfer coefficient (more collision rate between heavy metal ions and active groups on the biosorbent surface). If the biosorption process is exothermic, increasing the temperature will damage the biosorbent active sites and decreases biosorption, the Gibbs free energy of adsorption will acquire a higher value so that the process will be less favorable or not even spontaneous.

These factors are always present, but some novel approaches try to externally modify the process, increasing the metal uptake as much as possible through chemical or physical tools [58]:

- ✓ Biosorbent pretreatment, in order to increase the active sites. It can be physical (heat or fragmentation), or chemical (acid-base, oxidant, organic solvents).

- ✓ Introduction of nanoparticles (similar to the example in *Section 1.1.2*), Molecular Organic Frameworks (MOFs), and some biopolymers have also been tried in combination with the biological material.

To sum up, there is yet a lot to know about the temperature effect in the whole process, and studies have shown different results. What we can say with relative confidence is that temperature does not play a determinant role in biosorption, although it has some influence [50], [59].

1.2.3 Thermodynamics and kinetics of Biosorption [54], [60]–[68]

We will try to tackle the problem of biosorption from a physicochemical point of view. Such as any other problem, thermodynamics rules all the processes in nature, and making use of it, we will obtain equations for final equilibrium, interfacial energy, and isotherms. Also, kinetics plays a crucial role, as it is going to inform about the rate at which the process takes place.

1.2.3.1 Thermodynamic equilibrium of Biosorption

The interface or phase boundary is the inhomogeneous region between the homogeneous light or α phase (the aqueous solution were the pollutants are) and the homogeneous dense or β phase (the biosorbent) (Figure 1.28 I, the real situation). To simplify the mathematical formulation of the adsorption process, Gibbs proposed an idealized model (Figure 1.28 II), where the interphase volume is equal to zero, but its thermodynamical properties (n, U, S) remain intact.

Guggenheim came up with an intermediate model, assigning an arbitrary but specific thickness to the interface (Figure 1.28 III) [62].

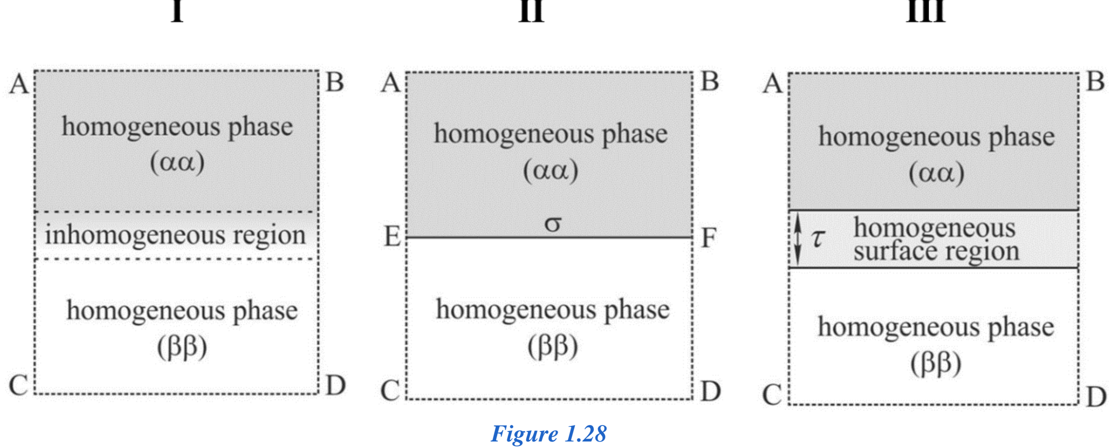


Figure 1.28

Schematic conceptualization of the interface region in adsorption processes: I, real situation; II, Gibbs model; III, Guggenheim model. The authors used ther terminology αα and ββ when refering to the phases, but α and β will be used here instead.

The Gibb´s approximation will be adopted here to explain the adsorption thermodynamics and to deduce the isotherms and equations used in most articles dealing with this topic.

To describe the process, we need to use a state function. As the adsorption problem is generally conditioned by pressure and temperature, we will use the general expression for Gibbs free energy of a particular process with i number of components (that represents the maximum non-expansive work the system can perform at certain p and T). We must add an additional term that accounts for the extra work needed to modify the surface $(\gamma \cdot dA)$.

$$\mathrm{d}G = V \cdot dp - S \cdot dT + \sum_{i=1} \mu_i \cdot dn_i + \gamma \cdot dA$$

Where γ is the surface tension coefficient, that can be measured via the Wilhelmy or the Langmuir methods.

Thus, the different system properties of each phase and the interphase are set. Properties without any sub or top mark account for the value of the system globally, they can be calculated as the sum of the properties of each phase

$$n_i = n_i{}^{\alpha} + n_i{}^{\beta} + n_i{}^{\sigma} \quad \forall \ i$$

Where n, symbolize the number of moles, and α, β and σ represent the upper phase, the lower phase, and the interphase, respectively. At equilibrium conditions, the chemical potential of a species *i* is the same in all the phases:

$$\mu_i^{\alpha} = \mu_i^{\beta} = \mu_i^{\sigma} \quad \forall \ i$$

As we are assuming Gibbs model, the number of moles (n_i) in phases α and β can be defined considering its concentration, c, in each phase and its volume, and the moles of the interphase can be defined concerning those of α and β.

$$n_i{}^{\sigma} = n_i - n_i{}^{\alpha} - n_i{}^{\beta} = n_i - c_i^{\alpha} \cdot V^{\alpha} - c_i^{\beta} \cdot V^{\beta}$$

Dividing $n_i{}^{\sigma}$ by the surface area, a new magnitude called surface concentration or interfacial excess, $\Gamma_i{}^{\sigma}$, is obtained.

$$\Gamma_i{}^{\sigma} = \frac{n_i{}^{\sigma}}{A}$$

At constant p and T, the Gibbs free energy of the interphase is defined as:

$$G^{\sigma} = \gamma \cdot A + \sum_{i=1} \mu_i \cdot n_i$$

Differentiating the above equation:

$$dG^{\sigma} = (\gamma \cdot dA + A \cdot d\gamma) + \sum_{i=1} (\mu_i \cdot dn_i + n_i{}^{\sigma} \cdot d\mu_i)$$

However, although the chemical potentials depend on the number of moles, at constant pressure and temperature, the equation must be equal to the one at the beginning:

$$A \cdot d\gamma + \sum_{i=1} n_i{}^{\sigma} \cdot d\mu_i = 0$$

This is known as the Gibbs-Duhem condition equation, widely used in thermodynamics. It is a need to give coherence to the macroscopic and microscopic (differential) treatment, which must converge.

Reordering the equation:

$$A \cdot d\gamma = -\sum_{i=1} n_i{}^{\sigma} \cdot d\mu_i$$

As we are interested in the surface concentration (how many moles of *i* are retained in the σ phase):

$$d\gamma = -\sum_{i=1} \Gamma_i{}^{\sigma} \cdot d\mu_i$$

The chemical potential of a component in a liquid phase follows the equation:

$$\mu_i = \mu_i{}^0 + RT \cdot ln a_i$$

As we want to solve the differential equation, we must differentiate it:

$$d\mu_i = 0 + RT \cdot dln a_i = RT \cdot \frac{da_i}{a_i} \cong RT \cdot \frac{dc_i}{c_i}$$

In the case of a binary mixture and considering that *i=1* is the solvent (thus, its surface concentration will be zero), after integration we obtain:

$$\Gamma_2{}^{\sigma} = -\frac{1}{RT} \cdot c_2 \cdot \left(\frac{\partial \gamma}{\partial c_2}\right)$$

$\Gamma_2{}^{\sigma} > 0$ would mean that there is a concentration of the solute 2 in the interface (adsorption occurs). However, $\Gamma_2{}^{\sigma} < 0$ means that the solute avoids the surface.

The above equation is the Gibbs isotherm equation for an adsorption process of a solute *i=2* with a specific solvent *i=1*. Considering the adsorption of two metals (in this work Cu and Zn) the equation will look like:

$$d\gamma = -\Gamma_{Cu}{}^{\sigma} \cdot d\mu_{Cu} - \Gamma_{Zn}{}^{\sigma} \cdot d\mu_{Zn}$$

$$d\gamma = -\frac{1}{RT}(\Gamma_{Cu}{}^{\sigma} \cdot dlnc_{Cu} + \Gamma_{Zn}{}^{\sigma} \cdot dlnc_{Zn})$$

However, there are several issues to be taken into account before applying these equations to an adsorption problem:

1. In a liquid, the surface tension coefficient is easily measured. However, when it comes to solids (biosorbents), it is a magnitude not easy to account for. A straightforward solution is measuring the amount of metal that remains in the solution, and the amount adsorbed.

2. The Gibbs equation derived is only valid for non-electrolyte adsorption, so a different version of the equation, taking into account the electrochemical potential, is required. Nevertheless, this is not simple because we do not know exactly all the forms in which the metal may be present in the solution.

3. The equation is only valid for liquid surfaces (reversible deformation); in solids, elastic tensions have to be considered, so an additional term $(\gamma_{plas} - \gamma)dA$ must be added, which difficult the differential equation solving.

4. It has not been considered that the adsorbent it charged (as it has multiple functional groups, and it generally is considered to have a net negative charge). Thus, the problem becomes even more complicated as now an electric field has to be introduced, and different models are proposed to describe its decay (Helmholtz, Gouy-Chapman, Stern).

Langmuir and Fowler were some of the first scientists to encounter this experimental sorption problem. They follow a different approach than the one of Gibbs, and they came out with a model that works quite well for a multitude of different cases. Although they proposed it for a Gas-Solid system, its formulation is analog to a Liquid-Solid problem and can be generalized. Several assumptions are made:

- The adsorbent has a certain amount of active sites, and one solute species can only occupy each active site.

- The energy of all the active sites is equal, i.e., the probability of a site being occupied is the same.

- There is no interaction between the adsorbed species.

- A monolayer is formed (not multi-layer possibilities).

It may seem that the approximations are exceedingly rough (it neglects quantum mechanics, and we have atomic particles). However, the Langmuir-Fowler isotherm surprisingly provides good fits and results for many adsorption problems, and together with other isotherms (BET, Freundlich) is one of the most used.

In the end, we are looking for an expression that relates the amount of solute adsorbed (in other words, the retention capacity q), with the concentration of the solute in the solution (c) at a constant temperature.

$$q_i = f(c_i) \qquad T = constant$$

The retention capacity and the concentration follow these equations:

$$q_i = \frac{m_i^{Adsorbed}}{m_{Adsorbent}} [=] \frac{mg}{kg}$$

$$c_i = \frac{m_i^{Solution}}{V^{Solution}} [=] \frac{mg}{L}$$

When equilibrium is reached, q_{eq} and c_{eq} can be measured, which is much simpler than measuring the surface tension coefficient.

We are going to use that equilibrium condition to derive the Langmuir isotherm equation.

The general equilibrium reaction for an adsorption process of a species A, regardless of whether it is physi or chemisorption, is the following:

$$A\,(free) \rightleftarrows A\,(adsorbed)$$

The rate of A to get adsorbed will have a specific rate constant k_{ads}, while the desorption process of A from the adsorbent will have a desorption rate constant, k_{des}. Moreover, as the surface is finite, there must be a limit surface concentration of A, which receives the name of saturated surface concentration, $\Gamma_A{}^s$. Langmuir defined a typical parameter called θ as the fraction of occupied sites (Figure 1.29):

$$\theta = \frac{\Gamma_A{}^{Adsorbed}}{\Gamma_A{}^s}$$

Figure 1.29

Two situations, where θ<1 (there are still free active sites), and when θ = 1 (all active sites are occupied).

The rate of each process can be expressed as the product of kinetic rate constant times the concentration of the species involved. For adsorption, we need the concentration of sites available (1-θ) and the concentration that remains in the solution. For the desorption process, we only need the occupied sites:

$$Adsorption: \quad v_{Ads} = k_{Ads} \cdot (1-\theta) \cdot c_A^{Solution}$$

$$Desorption: \quad v_{Des} = k_{Des} \cdot \theta$$

At the equilibrium, both rates must be equal, thus:

$$k_{Ads} \cdot (1-\theta) \cdot c_A^{Solution} = k_{Des} \cdot \theta$$

The equilibrium constant (K_c) is calculated as the quotient of adsorption and desorption constant rates:

$$K = K_c = \frac{k_{Ads}}{k_{Des}}$$

Clearing θ, we will get an equation that relates to the concentration of the solute in the adsorbent, with the concentration in the solution.

$$\theta = \frac{\Gamma_A^{Adsorbed}}{\Gamma_A{}^S} = \frac{K \cdot c_A^{Solution}}{1 + K \cdot c_A^{Solution}}$$

That is the Langmuir-Fowler isotherm equation derived from a kinetic point of view. The thermodynamic approach is more complicated for Liquid-Solid systems (although for Gas-Solid systems, it is quite easy to demonstrate).

We can rewrite the Langmuir equation with the amounts defined at the beginning (q_{eq} and c_{eq}):

$$\theta = \frac{q_{eq}}{q_{Saturation}} = \frac{K \cdot c_{eq}}{1 + K \cdot c_{eq}} \Rightarrow q_{eq}(c_{eq}) = \frac{q_{Saturation} \cdot K \cdot c_{eq}}{1 + K \cdot c_{eq}}$$

Graphically, plotting θ (or q_{eq}) against the concentration (Figure 1.30), it can be seen that, at a particular concentration, the limit ($\theta=1$), which means that all the active points of the adsorbent have been occupied, is reached. Furthermore, there are two limit cases:

$$K \cdot c_{eq} \ll 1 \Rightarrow \theta \cong K \cdot c_{eq} \quad (Weak\ adsorbate)$$

$$K \cdot c_{eq} \gg 1 \Rightarrow \theta \cong 1 \quad (Strong\ adsorbate)$$

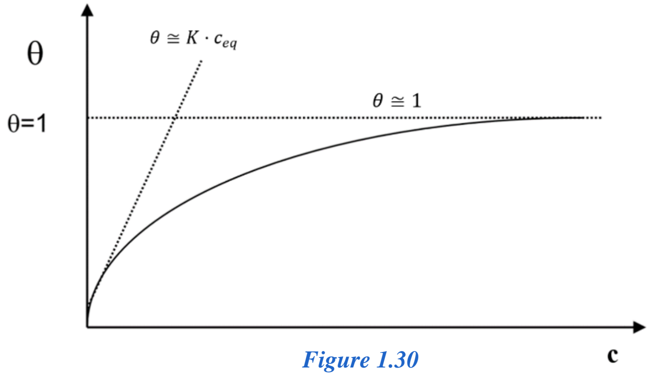


Figure 1.30

Biosorption plot.

Mathematically, this function has a horizontal asymptote, which coincides with the saturation retention capacity:

$$\lim_{c_{eq}\to\infty} \frac{q_{Saturation} \cdot K \cdot c_{eq}}{1 + K \cdot c_{eq}} = IND \left(\frac{\infty}{\infty}\right) \Longrightarrow \lim_{c_{eq}\to\infty} \frac{q_{Saturation} \cdot K \cdot c_{eq}}{K \cdot c_{eq}} = q_{Saturation}$$

Thus, when we obtain q_{sat} value, we will know that our graph has an asymptote in $y = q_{sat}$ (when $q_{eq} = q_{sat}$, so when $\theta = 1$).

The equation is most commonly used in its linear form, in order to obtain an estimate of the true values of the parameters q_{sat} and *K*.

$$\frac{c_{eq}}{q_{eq}} = \frac{1}{K \cdot q_{Saturation}} + \frac{1}{q_{Saturation}} \cdot c_{eq}$$

From the linear regression analysis, K and the saturated retention capacity for that species can be estimated. If K results in a large number, then adsorption is very favorable, and the adsorbate will be highly organized. Both parameters should be positive.

There are other isotherms such as Freundlich, BET, Dubinin–Radushkevich, Elovich, Jovanovic, or Temkin, among others. The most used for metallic liquid-solid adsorptions are the Langmuir and Freundlich isotherm, together with a combination of them. We will briefly describe those two:

- ***Freundlich isotherm:*** It still is a biparametric equation, and it has no physicochemical derivation, it is only a mathematical fit, so the parameters are not going to have any physical meaning. It also has a linear form:

$$q_{eq} = K_F \cdot c_{eq}^{\frac{1}{n}} \quad \Rightarrow \quad lnq_{eq} = lnK_F + \frac{1}{n} lnc_{eq}$$

K_F and n are the new parameters. K_F characterizes the adsorption (the larger the K_F, the better the adsorption), while n is related to the energetic homogeneity of the surface; low n values indicate that the adsorption is favorable because at low c_{eq} values the q_{eq} increases. It works better at lower concentrations than the Langmuir isotherms, although it fails at higher concentrations of adsorbed species.

- ***Langmuir-Freundlich isotherm: Langmuir-Freundlich isotherm:*** It is a mixture of both isotherms, and now it has three parameters. It assumes that the surface is homogeneous (like Langmuir model), but admitting the existence of some interactions between the adsorbed species; it can be seen as a cooperative process, which reflects more accurately the real situation [69]

$$\frac{q_{eq}}{q_s} = \frac{K_{LF} \cdot c_{eq}^{\gamma}}{1 + \sum_{i=1}^{n} a_{LF_i} \cdot {c_{eq}}_i^{\gamma_i}}$$

Now K_{LF} a_{LF_i}, and γ are the new parameters. γ vary from 0 to 1, and when it is equal to 1, this model converges to the Langmuir model.

All the isotherms explained are meant to be for only one solute adsorbed. For multispecies adsorption processes, we have to generalize them, and the equations get more complicated, as shown in Table 1.5:

Table 1.5

Isotherms for single and multi-adsorption

Isotherm	Single adsorption	Multi adsorption
Langmuir-Fowler	$\frac{q_{eq}}{q_{Saturation}} = \frac{K \cdot c_{eq}}{1 + K \cdot c_{eq}}$	$\frac{q_{eq}}{q_{Sat}} = \frac{K \cdot c_{eq}}{1 + \sum_{i=1}^{N} K_i \cdot {c_{eq}}_i}$
Freundlich	$q_{eq} = K_F \cdot c_{eq}^{\frac{1}{n}}$	${q_{eq}}_I = K_{Fi} \cdot \sum_{i=1}^{N} {c_{eq}}_i^{\frac{1}{n_i}}$

1.2.3.2 Thermodynamic data

In the previous section, the biosorption equilibrium has been studied from a thermodynamic point of view. However, any thermodynamic property that could inform us about the nature of the biosorption itself has been mentioned.

To study the thermodynamics of the biosorption process, the Gibbs free energy, enthalpy, and entropy of the system must be determined, making use of the Van´t Hoff equation.

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = -RT \cdot lnK_c$$

$$\ln K_c = \frac{\Delta S^0}{R} - \frac{\Delta H^0}{R} \cdot \frac{1}{T}$$

To determine if the biosorption of a heavy metal occurs spontaneously, we have to consider the enthalpy and the entropy together. The negative value of the Gibbs free energy (ΔG^0) will indicate that the process is spontaneous at a specific temperature.

A variation of ΔG^0 with temperature means that the entropy contribution is playing an important role (as we mentioned, in some biosorption process, the temperature did not affect too much).

To calculate entropy and enthalpy, the equilibrium constant is plotted versus the inverse of the temperature. If it turns out that entropy is positive, that will indicate that randomness between solids and solutions increased during the biosorption on biosorbent active sites.

Moreover, depending on the value of the enthalpy obtained, it is possible to know if an increase in temperature will favor the process. According to the Le Chatelier principle, when the temperature of a system increases, it shifts to the endothermic direction (in order to counter that temperature increase).

- $\Delta H^0 < 0$, if T increases, the adsorption process is going to be unfavorable (adsorption will prefer to be conducted at lower T).

- $\Delta H^0 > 0$, an augmentation in temperature will lead to a better adsorption performance.

1.2.3.3 Kinetics of Biosorption [70]

Indeed, the thermodynamics of biosorption is vital to the process of taking place. However, another critical aspect must be taken into account: the kinetics. In short, thermodynamics will tell us how much metal is sorbed, while the kinetics will inform us about how long is it going to take [12].

In traditional kinetic problems, the rate of the process is related to the production or consumption of a specie. In adsorption processes, now we will have the solute uptake rate.

To establish a kinetic model, attention must be paid to the the critical steps of the process. In general, four different stages can be distinguished in an adsorption process (Figure 1.31):

1. Transport of the solute (in this work, the metal ions) to the beginning of the interphase, the boundary layer.

2. Transport of the solute through that interphase to the surface of the adsorbent (mass transfer diffusion step).

3. Transport in the surface to the active sites (pore-like diffusion).

4. Energetic interaction between the adsorbate and the adsorbent.

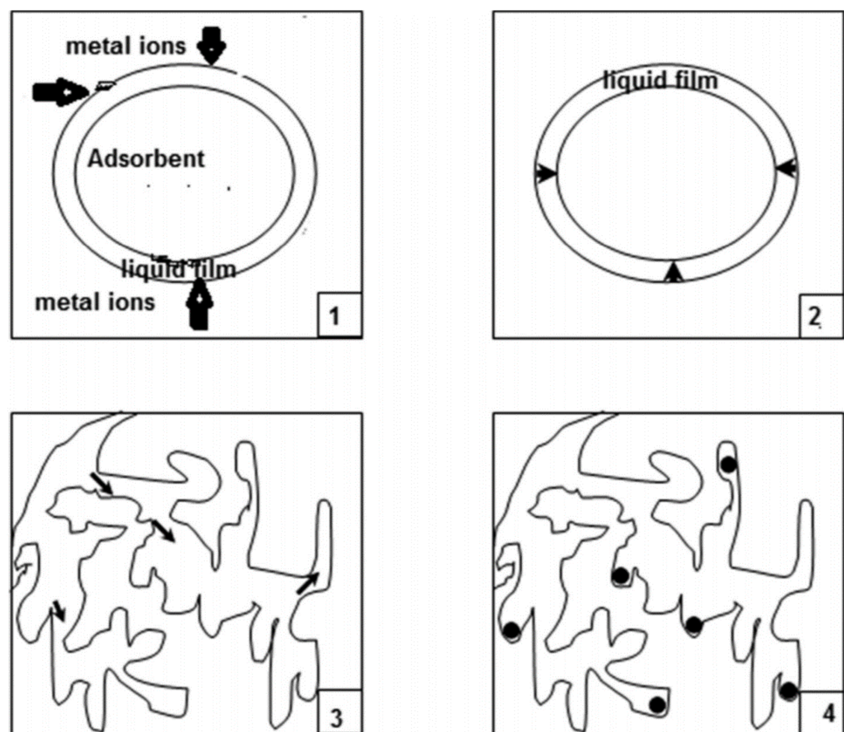


Figure 1.31

The four stages of biosorption mechanism

By and large, the first and last steps are not the rate-controlling steps as the adsorbent is not involved, and the energetic interaction is usually fast. Therefore, one of the two intermediate stages are going to condition the overall kinetics of the process, and both are related to diffusion-like steps [71]. However, this may change depending on the biosorbent and the conditions used.

For a kinetic model to be built, the information of the concentrations of the solute species is needed and can be obtained from the isotherms, so a proper scheme of how this model is constructed can be the one showed in Figure 1.32:

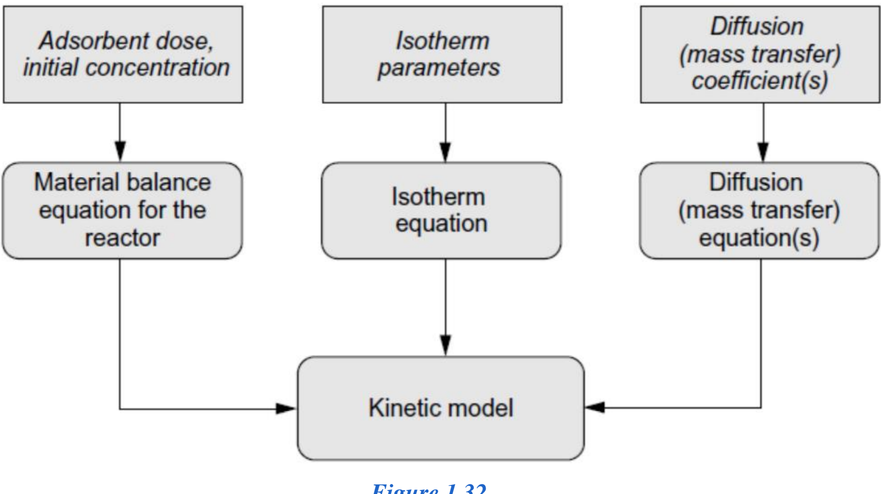


Figure 1.32

Information needed to build an adsorption kinetic model.

There is a multitude of different kinetic models. Nevertheless, for biosorption kinetics, two are preferred above others: the pseudo-first and second-order models since adsorption of metal ions generally agree with reversible first-order kinetics, which considers that the occupation of sorption sites is proportional to the number of unoccupied sites

Both pseudo-first and second-order equations are similar. It is strange to assign a specific reaction order (as it is always determined experimentally unless the mechanism is perfectly known). There is no logical way by which biosorption can be assigned either pseudo-first or pseudo-second order.

We can start by trying to set an expression for the effective concentration of the number of sites available at a specific time t (c_t/c_0). That must be equal to the total amount available (1) minus the total amount of adsorbed at that time divided by the theoretically amount of species adsorbed at equilibrium (q_t/q_{eq}).

$$\frac{c_t}{c_0} = 1 - \frac{q_t}{q_{eq}}$$

When $q_t = q_{eq}$ then $c_t = 0$ (the equilibrium is reached, no more active sites available).

Now that we have a mathematical expression for the concentration of available sites, we can get an expression for the rate of disappearance of those active sites:

$$v = -\frac{dc_t}{dt} = k_x \cdot {c_t}^x$$

Where x states for the unknown reaction order coefficient, k_x is the rate constant.

- ***Pseudo-First order model:*** Assuming that x equals 1, and mixing the rate equation with the previous one:

$$v = -\frac{dc_t}{dt} = k_1 \cdot c_t = k_1 \cdot c_0 \cdot \frac{(q_{eq} - q_t)}{q_{eq}}$$

It is a differential equation, and we can change c_t for q_t. The variable change is done below:

$$dc_t = d\left(1 - \frac{q_t}{q_{eq}}\right) = 0 - \frac{dq_t}{q_{eq}} = \frac{-1}{q_{eq}} dq_t$$

$$v = -\frac{dc_t}{dt} = \frac{-1}{dt}\left(\frac{-1}{q_{eq}} dq_t\right) = \frac{1}{q_{eq}} \cdot \frac{dq_t}{dt}$$

Equalling that new expression with the old one:

$$v = \frac{1}{q_{eq}} \cdot \frac{dq_t}{dt} = k_1 \cdot c_0 \cdot \frac{(q_{eq} - q_t)}{q_{eq}} \Rightarrow \frac{dq_t}{dt} = k_1^{'} \cdot (q_{eq} - q_t)$$

The reason why it is called "pseudo-first-order" is the original rate constant (*k_1*) times the initial concentration (*c_0*):

$$\frac{1}{q_{eq} - q_t} \cdot dq_t = k_1^{'} \cdot dt$$

The reason why it is called "pseudo-first-order" is that the rate constant is included q_{eq} (which is a constant). Taking integrals in both sides, and considering the integral limits from time and concentration cero, to a time t and a concentration q_t:

$$\int_0^{q_t} \frac{1}{q_{eq} - q_t} \cdot dq_t = \int_0^t k_1^{'} \cdot dt$$

$$\ln\left(\frac{q_{eq}}{q_{eq} - q_t}\right) = k_1^{'} \cdot t$$

Reordering the equation, the exponential expression is obtained:

$$\frac{q_{eq}}{q_{eq} - q_t} = e^{k_1^{'} \cdot t} \Rightarrow q_t = q_{eq} \cdot \left(1 - e^{-k_1^{'} \cdot t}\right)$$

By converting the equation into its linear form, q_{eq} and $k_1^{'}$ can be found.

$$\ln(q_{eq} - q_t) = ln\, q_{eq} - k_1^{'} \cdot t$$

- ***Pseudo-Second-order model:*** It is applied when the adsorption mechanism is the rate-controlling step, so it begins with low rates and suddenly higher ones. We must pose the same equation, but with the second-order (x=2).

$$v = -\frac{dc_t}{dt} = k_2 \cdot {c_t}^2 = k_2 \cdot {c_0}^2 \cdot \frac{\left(q_{eq} - q_t\right)^2}{\cdot {q_{eq}}^2} = {k_2}' \cdot \left(q_{eq} - q_t\right)^2$$

$${k_2}' = \frac{k_2 \cdot {c_0}^2}{q_{eq}}$$

Solving the differential equation, the integrated linear equation can be obtained:

$$\int_0^{q_t} \frac{1}{\left(q_{eq} - q_t\right)^2} \cdot dq_t = \int_0^t - {k_2}' \cdot dt$$

$$\frac{1}{q_{eq} - q_t} = \frac{1}{q_{eq}} + {k_2}' \cdot t$$

In the experimental work, we will get q_t, and t, so we will check which model fits best according to the results. Other models describe the kinetics of the biosorption (Table 1.6), although they are used to a lesser extent as they fit the data worse than the previous. The two principal are:

- ***Elovich model:*** it is applied when biosorption is carried out at a heterogeneous surface.
- ***Intraparticle diffusion model:*** it defines a diffusion rate constant, and it describes better the diffusion process. The two equations depend on how vital the diffusion is in the process.

Table 1.6

A summary of the different equations required for each kinetic model [50].

Model	Parameters	Equation
Pseudo-First	q_{eq} *and* ${k_1}'$	$\ln\left(q_{eq} - q_t\right) = ln\, q_{eq} - {k_1}' t$
Pseudo-Second	q_{eq} *and* ${k_2}'$	$\frac{1}{q_{eq} - q_t} = \frac{1}{q_{eq}} + {k_2}' \cdot t$
Elovich	α *(initial value of biosorption rate)* β *(desorption coefficient)*	$q_t = \beta \ln(\alpha\beta) + \beta \ln t$
Intraparticle Diffusion	K_i *(diffusion rate constatnt)* C *(intraparticle accumulation)* K_{ID} *(biosorption constant)*	$q_t = K_i t^{0.5} + C$ $\ln(1 - \frac{q_t}{q_{eq}}) = -K_{ID} \cdot t$

2 AIM OF THIS WORK

The general goal of the project in which this research is framed is the treatment of pig manure wastewater with microalgae in photobioreactors to remove nutrients (N, C, and P), heavy metals and antibiotics, and the valorization of the harvested biomass. More precisely, it would be a fractional valorization, which consists of tracking the concentration of heavy metals in the products obtained, in order to remove them during the process.

Concerning the recovery of heavy metals, the goal is to retain the metal ions on the biomass to obtain water free of heavy metals that can be used for irrigation, and later to desorb the metals from the biomass before the valorization treatments to extract valuable and non-toxic byproducts. Figure 2.1 shows the steps of the global process schematically:

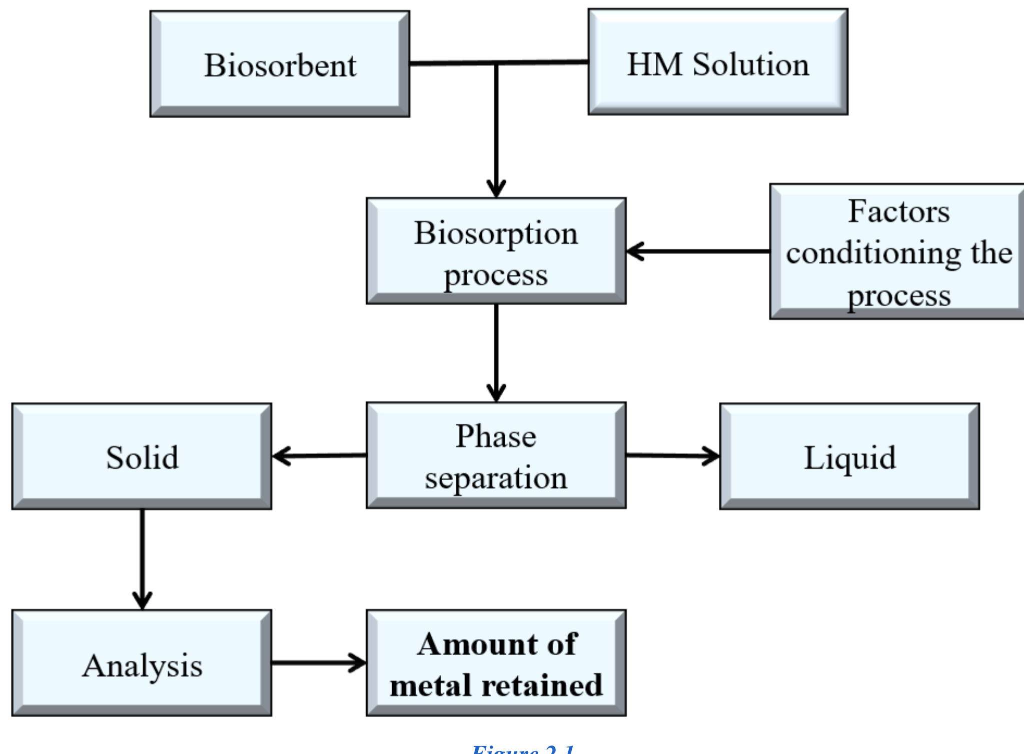


Figure 2.1

Overall view of the whole bioremediation process to remove heavy metals from a HM solution.

The main objective of the research presented here is to investigate the factors influencing the bioremediation of the most common and abundant toxic trace elements in piggery wastewater, Cu and Zn, as well as their retention mechanisms on microalgae, bacteria and their consortia. Due to COVID-19 pandemic, some experimental work was not performed (for example, arsenic addition or mechanistic experiments were not conducted).

In order to give a broad view of the actual state-of-the-art of the three biomasses used, a review including theoretical insights of each type of biomass, along with a collection of several biosorption experiments on the removal of Cu and Zn has been carried out.

Regarding the experimental section, three different types of biomass are assayed with the purpose to study their retention capabilities: (a) microalgal-bacterial biomass consortia grown in a photobioreactor for treatment of pig slurry, (b) pure microalgae grown in a photoreactor with a synthetic nutrient medium, and (c) sludge from biological wastewater treatment (only bacteria).

The effect of several factors likely influencing the biomass growth and the metal removal efficiency are investigated: metal concentration in the wastewater, stirring time, with or without light intensity, and availability of inorganic and organic carbon. The tested levels of the factors are combined in a full factorial experimental design with 144 experiments to investigate the significant main effects and factor interactions, and to identify the factor levels yielding the maximum retention capacity for both copper and zinc.

Due to de COVID-19 pandemic, only the Design of Experiments was completed, and no further investigations in the retention mechanisms or lixiviation were done.

3 BIOSORPTION OF HEAVY METALS

In this section, we will study three of the most used biomasses when leading with bioremediation of heavy metals, and especially in biosorption techniques. This biomasses are bacteria, microalgae, and a consortia bacteria-microalgae.

Finally, a review of several experiments using those biomasses will be displayed, in order to see which factors usually provide the highest metal removals (which is our experimental aim).

3.1 Bacteria as biosorbents

Bacteria are found everywhere (water, organic matter, living bodies of plants and animals, or soil). They are essential for the degradation of chemical species and the recycling of nutrients, such as nitrogen, sulfur, and phosphorus. Among the microscopic organisms, the bacteria are the most important in wastewater treatment plants because they grow using the nutrients available in the matrix [72], [73].

3.1.1 What are bacteria?

According to Carl Woese, terrestrial living organisms can be classified into three domains:

I. Archaea *II. Bacteria* *III. Eukarya*

For many years archaea and bacteria have been included in the same group, the prokaryotes (not defined nucleus). However, due to ARN sequencing, and genomic and proteomic studies, Woese found that archaea are as different from bacteria as they are from eukaryotes. In the bacteria domain, there are only unicellular organisms with simple morphologies, but with a great variety of metabolisms. All complex living forms, together with other more simple (such as microalgae or protozoans), belong to the Eukarya domain [74].

Bacteria are formed by 80% water and 20% dry material. Inside that 20%, approximately 90% is organic, and only 10% corresponds to inorganic compounds. A simple organic formula for a bacterial cell can be $C_{60}H_{87}O_{23}N_{12}P$ [75]. Bacteria have no defined nucleus and rarely present organelles with an envelope; their genetic material is found dispersed within the cell. Most bacteria are 0.3–3.0 µm in size and exist with four sorts of shapes determined by the cell wall, which regulates the ability of bacteria to compete for substrate and nutrients, along with its ability to swim and flocculate. The shapes are the following:

Rod (*bacillus*)
Sphere (*coccus*)
Spiral (*spirillum*)
Comma (*vibrio*)

The other structural features of bacteria (apart from the nucleoide) are plasmids, inclusions, and ribosomes, although some of them may also present vesicles that may contain gas or enzymes (Figure 3.1) [76].

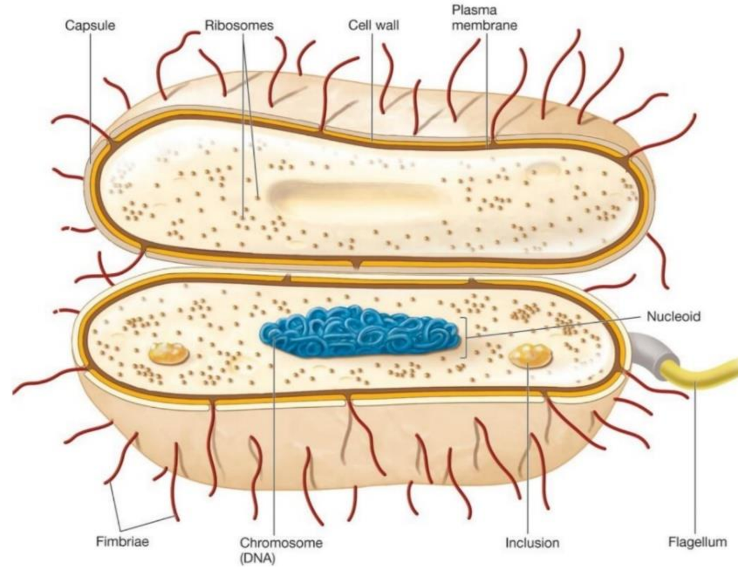


Figure 3.1

Morphology of a typical bacteria cell.

Their reproduction is asexual, usually by binary fission, and it highly depends on temperature. The genetic material is duplicated by dividing the DNA into two equal parts when enough nutrients and size have been reached. The new two cells formed are identical to the previous cell as the genetic material has not changed; nevertheless, there can be genetic recombination between different bacteria strains. The process is fast, and, as a result, colonies are formed [73].

The bacteria envelope is the structure involved in the biosorption/bioaccumulation process. Unlike eukaryote cells, the bacteria envelope can be divided into three different parts:

1) ***Bacteria capsule:*** It is the outer envelope, formed by glycoproteins and polysaccharides. It confers mechanical resistance to the cell. It can also have pilis, which are small protein prolongations that help the bacteria in the sticking process to a substrate.

2) ***Bacteria wall:*** It is similar to one of the vegetal cells but more porous. It is constituted by murine (peptidoglycan) and protects the cell from hypotonic or hypertonic media.

3) ***Plasmatic membrane:*** It is comparable to those of the eukaryotes. It has a lipid bilayer in which proteins are inserted in an irregular array. Here is where biosorption/bioaccumulation is going to be produced.

Based on the composition of the cell wall, there are two different types of bacteria: gram-negative and gram-positive (the negative/positive refers to Gram´s stain test). Gram-positive bacteria have a thick cell wall that contains many layers of peptidoglycan and teichoic acids, which provide rigidity to the cell wall by attracting and bonding to cations. Examples of gram-positive are *Corynebacterium, Bacillus, Clostridium,* or *Listeria*. Gram-negative bacteria have a thin cell wall that contains only a few layers of peptidoglycan surrounded by a membrane of lipopolysaccharides and lipoproteins (examples are *Escherichia, Klebsiella, Pseudomonas* or *Salmonella*). Figure 3.2 shows the two different cell walls:

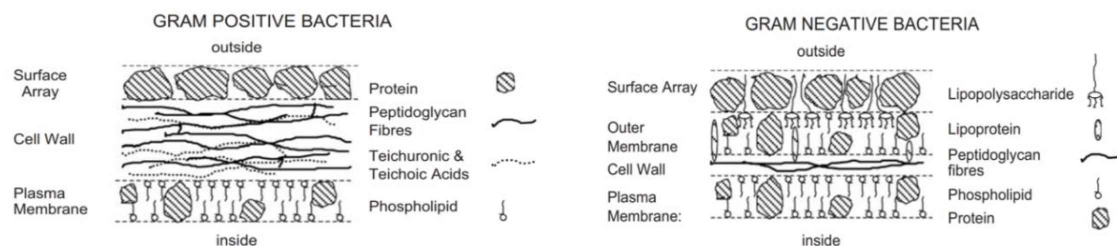


Figure 3.2

Cell wall structure and composition of Gram positive and Gram negative bacteria.

Their cell composition is going to be crucial because it determines which species are better adsorbed, as the process highly depends on the surface involved.

Bacteria can be heterotrophic or autotrophic, although bacteria living in wastewater media tend to be heterotrophs (that is, 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). Heterotrophic activity declines rapidly below 4°C and stops at 1°C [73].

Depending on how they use oxygen, bacteria are divided into three groups:

- ***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.

- ***Anaerobes:*** They cannot use oxygen.

Bacteria are famous for producing unique cellular structures that protect them from external danger, such as the presence of chemicals, desiccation, heat, and other environmental factors -like pH-. The defense mechanisms are the formation of endospores and capsules [72].

3.1.2 Heavy metal retention mechanisms

Heavy metals may be attached to the functional groups that surround the cell membrane and cell wall of the bacteria, or they can enter the cell by crossing the lipid bilayer. As mentioned in *Section 1.2.1*, the layer is impermeable to charged species, so transport mechanisms must exist.

All the biosorption and bioaccumulation mechanisms studied previously are valid for bacteria, although now there are three primary mechanisms involved: precipitation, complexation with nitrogen and oxygen ligands, and ion exchange reactions with teichoic acids and peptidoglycan. While for bioaccumulation, the mechanisms are: diffusion, facilitated diffusion, and active transport [77].

A scheme that summarizes the mechanisms through which heavy metals can be retained by the bacteria (whether it is living or not) is shown in Figure 3.3. The primary mechanism is adsorption, as it does not depend on energy metabolism (and the activation barrier is much lower than for absorption) [12], [78].

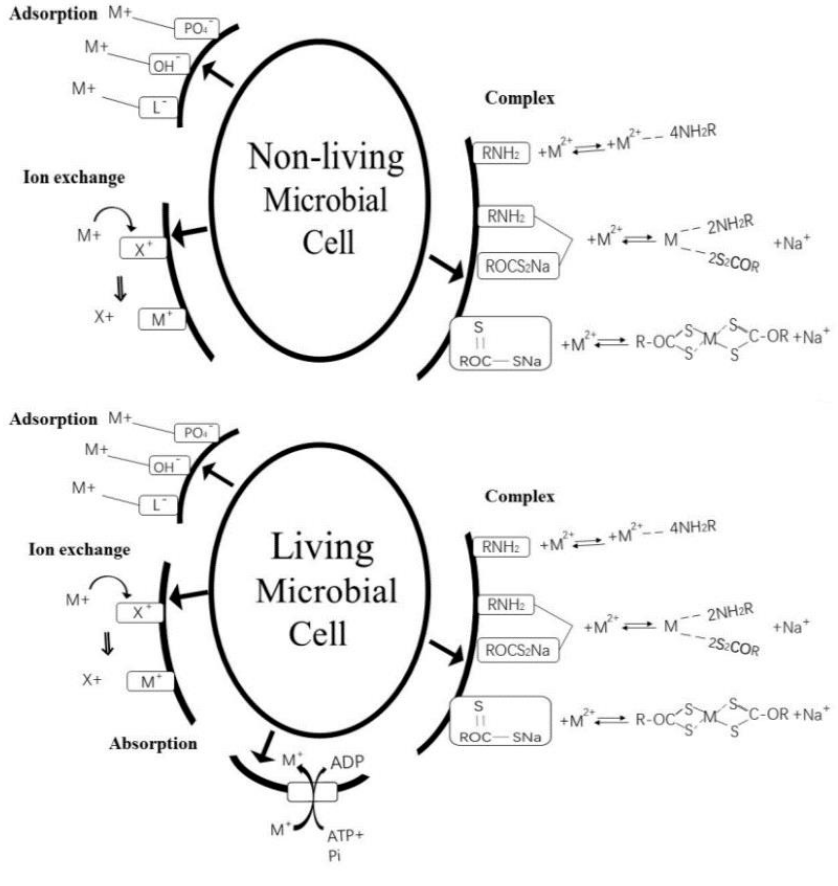


Figure 3.3

Mechanistic overview to understand what may happen on a living cell, or on a dead cell.

It is worth mentioning that metal removal capacity varies depending on the type of bacteria (if it is gram-positive or gram-negative). Gram-positive bacteria have a high content in teichoic acid polymers, alanine, glutamic acid, and glycerol. All the latter compounds are good ligand donors, and together with many phosphate groups, they will confer a strong negative charge on the surface of the cell wall. On the other hand, the cell wall of gram-negative bacteria have lower amounts of teichoic acid and contains more enzymes, glycoproteins, and phospholipids, resulting in a lessened net negative charge.

Therefore, the interaction between the cell surface of gram-positive bacteria and the metal ion is going to be stronger than that with gram-negative bacteria. Consequently, it is reasonable to think that the heavy metal accumulation load by gram-positive bacteria is more significant than that of gram-negative organisms [79]

3.1.3 Biosorbent capacities

Most bacteria are 0.3–3.0 μm in size. In comparison to eukaryote cells, they are considerably smaller. Due to their tiny size, they possess a huge surface-to-volume ratio and, therefore, a large surface area available for biosorption and bioaccumulation processes, which is traduced in fastest removal rates and higher metabolic rate and growth [75].

However, not only alive bacteria can act as biosorbents. Dead bacteria have even higher biosorption capacities, and they usually outperform living cells of the same strains. Additionally, using biotechnology techniques and genetic engineering, a new world of opportunities is opened for the design of the perfect organism. For example, new functional groups can be placed on the surface, or specific metal-binding peptides can be added. These improvements will enhance the affinity and selectivity for target compounds, such as metals [44].

The knowledge of the cell wall composition and its differences among bacteria strains are vital to understanding their biosorption power. Peptidoglycan (in both, although thicker layer in gram +) and teichoic acid (only in gram +) are essential as they provide ion-exchange active groups in their structures [56].

Furthermore, the pili filaments coating the bacteria capsule, approximately 2–5 nm long, also play adsorption roles retaining some metal ions from the bulk solution as they have a net negatively charged surface that attracts and removes positively charged soluble metals (Figure 3.4). From the pili, ions can be transported inside the cell [73].

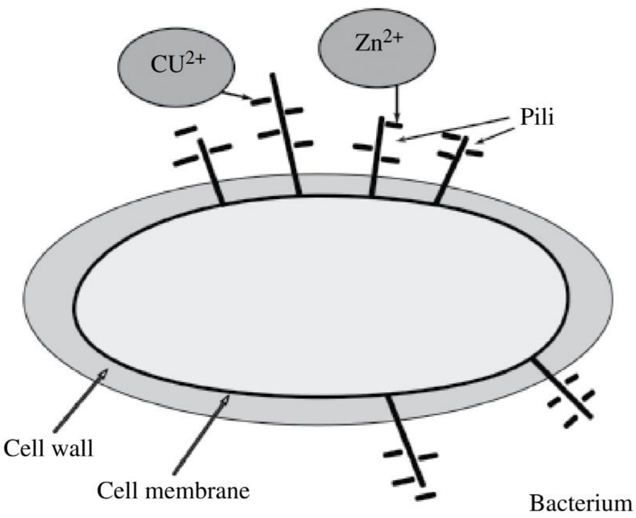


Figure 3.4

Scheme of metal adsorption on bacteria pilis.

When dealing with heavy metals, as they are highly toxic for the cells, it is necessary to make sure that metal concentration does not exceed the bacteria tolerance, as an excess of metal will disrupt and kill the biomass. An exceptional feature of bacteria as biosorbent is that most of them have a wide tolerance towards one or more metals, even at high concentrations. For example, some bacteria are capable of tunning their cell wall composition depending on the metals that surround them [80].

Moreover, bacteria can grow almost everywhere, and some of them can develop defensive mechanisms towards heavy metals intoxication. It is said that bacteria are either resistant or tolerant toward a pollutant. Tolerance is defined as the ability to survive in a polluted environment by itself, while resistance is the capacity to survive in a highly concentrated medium via detoxication mechanisms. The resistance of a bacteria is encoded in the genetic material of its plasmids (possibly due to mutations), and the genes will only be expressed in the presence of certain metals. In the case of tolerant bacteria, they have developed several mechanisms, for instance, an efflux system that can pump toxic elements out if the concentration starts to increase. Due to all of the above, bacteria are meant to have an excellent sorption capacity [12], [77].

However, one of the main disadvantages of employing bacteria as biosorbent (although this can be extended to the use of other microorganisms for bioremediation) is their low mechanical strength -as they "break" in centrifugation processes, and they cannot be separated via decantation either- and low density -as the bacteria solution has a similar density to the one of water-. These two drawbacks keep bioremediation from being a real alternative for an industrial-scale application. The solution lies in adopting immobilization techniques onto an inert matrix. This approach will enhance the biosorbent renewability, and it will aid in the separation of the "clean" water from the sorbent that retains the metals [13].

3.1.4 Factors affecting bacteria biosorption

Notwithstanding, the efficiency of the removal technique will not only depend on the type of bacteria used. There are other factors that have an influence when using bacteria [13].

These factors can be grouped into two categories: *biotic* (if they come from a response of the microorganism itself) or *abiotic* (if it is an external parameter), and as they are crucial in this research, we will take a more in-depth look to them [1].

3.1.4.1 Biotic factors [1], [72]

- ***Cell size and composition:*** As a response to the heavy metal presence, the microorganism can change its cell size and composition, which plays a defense mechanism role. That implies having less active sites available for metal ion binding.

- ***Biomass concentration:*** Is is a crucial factor. Generally, an increase in the biomass concentration is related to the rise in the amount of solute sorbed (as the number of active sites is boosted). The total amount of solute biosorbed per unit of biosorbent tends to decrease when increasing biosorbent dosage because there are more active sites, and they are not going to be occupied (Langmuir isotherm). Additionally, higher biomass concentration is meant to lead to cell aggregation, which will reduce the active points availability. Thus, even though some results show an increase in the removal efficiency by increasing the sorbent dosage, chiefly, when the biomass concentration is reduced, the highest amounts of metal are reported (per unit of biomass).

3.1.4.2 Abiotic factors [1], [71], [78]

- ***pH:*** Biosorption strongly depends on the pH. Firstly, because it conditions microbial growth and enzyme activity. Secondly, as it is one of the parameters that control the number of active sites (if they are protonated, at low pH, biosorption is not going to be produced). Finally, because it conditions the chemical form of dissolved heavy metals, as the pH affects the hydration outer sphere of the metal ions and their mobility. As the cell-membrane composition differs among species, the optimum pH value must be found for each one, although the range within is usually located between 5.5-7.0, with some exceptions.

- ***Ionic Strength:*** If many other ions are present in the medium, they may disrupt the electrostatic interactions among the organic functional groups and the heavy metal ions we aim to retain, leading to lower removal capacities. The activity coefficient will also be altered.

- ***Initial metal concentration:*** If the maximum tolerated concentration is surpassed, the bacteria metabolism is altered, thus decreasing metal uptake. Nonetheless, using low concentrations may difficult the mass transfer step (which is going to be more easily overcome with higher concentrations). Thus, when dealing with non-living organisms, large initial metal concentrations can be used, as the removal efficiency increases. However, when using living microorganisms, an intermediate concentration must be found in order not to disturb its metabolic functions.

- ***Effects of nutrients:*** Similarly to the initial metal concentration, depending on whether we use living or non-living microorganisms, this effect will have higher or minor importance. Each species will have an optimum value of nutrients (if there is a lack of them, then vital metabolic functions are not going to be fulfilled). Moreover, a good carbon source can lead to a growth of biomass, thereby more active sites will be available.

- ***Contact Time:*** Commonly, biosorption increases with an enhancement of the contact time, as the metal has more time to reach the active sites. In practice, the saturation limit usually is achieved within the first 1-3 hours so that more time will imply more energy consumption but no significant increase in removal efficiency.

- ***Stirring rate:*** Like in electrochemistry, a reasonable stirring rate is needed to overcome external mass transfer resistance (because the diffusion layer will narrow). Hence, an optimum stirring rate must be found, as it is presumed to be different among species.

- ***Temperature:*** The medium temperature directly affects both the rate of heavy metal sorption and the growth of the microorganism, as the metabolic activity relies on temperature. High temperatures can trigger enzyme and protein denaturation so that the microorganism can reduce its activity. On the other hand, low temperature may affect the membrane fluidity, interfering with the transport systems and functional groups involved with metal binding. We must find a compromise solution. As in the case of pH, there is a range within 25-35ºC that usually provides the best removal efficiencies, although the specific temperature value must be studied and will vary with the microorganism used. Nevertheless, compared with the above factors, the temperature is meant to affect the process to a lesser extent.

3.1.5 Review of the bacteria used for heavy metal removal

An intensive revision of the literature has been carried out to demonstrate how useful and important bacteria have become as a biosorbent, and to understand better which strains and conditions provide the best performances (only concerning Cu and Zn removal). As presenting all the conditions reviewed for each experiment could be a bit messy, firstly, only retention capacities (expressed in mg of the metal per g of biosorbent or either in % of removal) are going to be shown. Later on, the best experiments will be selected, and their requirements will be broken down. The number of metal ions retained per weight unit of biosorbent (q_{eq}) and the removal percentage is described by these equations:

$$q_{eq} = \frac{V \cdot (c_0 - c_{eq})}{m_{Sorbent}} = \frac{(c_0 - c_{eq})}{c_{Sorbent}}$$

$$\%Removal = \%R = \frac{c_0 - c_{eq}}{c_0} \cdot 100 = \frac{q_{eq} \cdot m_{Sorbent}}{c_0 \cdot V} \cdot 100$$

$* \, c_{eq}$ *is the equilibrium concentration of the metal in the solution*

The q_{eq} varies as a function of the final metal concentration in the solution. However, most articles will give the q_{sat} directly after the isotherm experiments (that is, the maximum retention capacity for specific biomass).

The tables regarding zinc and copper are presented:

Table 3.1

Table with bacteria species, adsorption capacities, and percentages of removal for zinc.

Bacteria strain	Ret Capacity (mg/g)	Removal (%)	Reference number
Pseudomonas putida	6.90	80.00	[81]
Pseudomonas aeruginosa AT 18	77.50	87.70	[82]
Pseudomonas jessenii	4.39	---	[83]
Pseudomonas putida	3.66	---	[84]
Rhodobium marinum NW16	---	24.05	[85]
SRB, Desulfovibrio	---	94.60	[86]
Bacillus firmus MS-102	722.00	61.80	[87]
Thiobacillus ferrooxidans	172.40	---	[88]
Pseudomonas putida CZ1	24.40	---	[89]
Pectobacterium sp ND2	34.27	68.54	[90]
Streptomyces rimosus	30.00 - 80.00	---	[91]

Table 3.2

Table with bacteria species, adsorption capacities, and percentages of removal for copper.

Bacteria strain	Ret Capacity (mg/g)	Removal (%)	Reference number
Micrococcus luteus	408.00	---	[92]
Desulfovibrio desulfuricans-zeolite carrier	---	98.20	[84]
Pseudomonas jessenii	10.22	---	[83]
Pseudomonas putida	5.52	---	[93]
Bacillus cereus FIT10	---	87.16	[94]
Rhodobium marinum NW16	---	46.99	[85]
Rhodobacter sphaeroides HY01	---	> 96.00	[95]
SRB, *Desulfovibrio*	---	98.90	[86]
Bacillus sp	---	88.60	[96]
Bacillus firmus MS-102	860.00	74.90	[87]
Geobacillus thermodenitrificans CCM 2566	63.70	6.38	[97]
Pseudomonas putida CZ1	27.60	---	[89]
Enterobacter sp	32.50	---	[98]
Pectobacterium sp ND2	38.63	77.26	[90]
Eichhornia spp and SRB	---	90.00	[99]
Pseudomonas aeruginosa AT 18	86.95	95.00	[82]

Attending to the best values of the removal percentage and retention capacity, three experiments for each heavy metal are selected in order to describe their experimental conditions, such as pH, initial metal concentration, biosorbent dose, contact time, temperature, and stirring rate.

Bacteria have been widely used in wastewater treatment due to its high accessibility, low cost, high abundance in the environment, and due to its excellent efficiency for organic matter oxidation.

On the next page, Table 3.3 shows the best experiments with their set up conditions.

Table 3.3

Experimental conditions of Cu(II) and Zn(II) removal by different bacteria strains

a: 2 g/L was found as the optimum biosorption dose.

b: Result after treatment with NaOH (1M).

c: Values refers to an experiment with a mix of four metals simultaneously: Cu, Zn, Mn and Cr. Individual experiments gave higher %Removal.

Heavy Metal	Bacteria strain	pH	Initial Concentration mg/L	Biomass concentration g/L	Agitation rate rpm	Temperature °C	Time h	Max Uptake mg/g	Removal %	Reference
Cu (II)	*Rhodobacter sphaeroides HY01*	---	10.00	---	150	35	48	---	> 96.00	[95]
Cu (II)	*Eichhornia spp* and SRB	5.0/ 5.5	100.00	0.8 - 2.0[a]	150	30 ± 2	24	33.40	85.00	[99]
Zn (II)	*Streptomyces rimosus*	7.5	100.00	3.0	250	20	4	30.00 - 80.00[b]	---	[91]
Zn (II)	*Bacillus firmus MS-102*	6.0	1000.0	0.85	---	25	0.17	722.0	61.80	[87]
Cu (II)		4.0	1000.0	---	100	23	---	860.0	74.90	
Zn (II)	SRB, *Desulfovibrio*	---	5.00	---	---	---	---	---	94.60	[86]
Cu (II)		5.5	25.00					---	98.90	
Zn (II)	*Pseudomonas aeruginosa AT 18*	7.0	80.00	0.5 - 1.0	150	---	72	77.50[c]	87.70[c]	[82]
Cu (II)		6.3	50.00					86.95[c]	95.00[c]	

3.2 Microalgae as biosorbents

3.2.1 What are Microalgae?

Algae belong to the eukaryotic group of organisms. They are photosynthetic organisms (can convert CO_2 into biomass), and they mainly live in aquatic environments. Attending to their size, they can be divided into two groups [100]:

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

Microalgae are the first producers of O_2 (they allowed the emergence of plant and animal life on Earth). Furthermore, microalgae still play a crucial role in the food chain as an elementary supply of biomass. They adapt themselves to several environments and can be found in acidic media or saline waters. They either live alone or via symbiotic interactions with other microorganisms. The difference between plants and algae is that the later is more like “primitive” plantlike organisms that contain chlorophyll *a*, and perform photosynthesis, but they are not as complicated as traditional plants (embryophytes) [100], [101].

Microalgae are a very diverse group (the number of species ranges from 22,000 to 26,000). One typical classification is done based on their pigment composition, which results in nine classes. The six more essential groups are [102]:

- *Chlorophyceae* (green algae).
- *Phaeophyceae* (brown algae).
- *Pyrrophyceae* (dinoflagellates).
- *Chrysophyceae* (golden-brown algae).
- *Bacillariophyceae* (diatoms).
- *Rhodophyceae* (red algae).

Depending on their pigments, the O_2 and CO_2 balance will vary; for example, green algae are meant to produce more O_2 than the oxygen consumed, while red algae behave the opposite.

Microalgae have a defined nucleus, a plasma membrane, and its cytosol contains chloroplasts, amyloplasts, elaioplasts, and mitochondria. Along with chlorophylls, they may contain other pigments such as carotenoids and phycobiliproteins. Microalgae are predominantly photoautotrophic (inorganic carbon as a carbon source), although they can be heterotrophic (organic carbon as a carbon source) or mixotrophic (they can use both types of carbon). The molecular formula of microalgae is approximately $C_{106}H_{263}O_{110}N_{16}P$ [4], [100].

Some of the properties related to the metal biosorption are going to be similar to those studied for bacteria so that they are described more briefly.

3.2.2 Uses and applications of microalgae

Microalgae are not only a handy tool in wastewater remediation. Their biomass can be transformed in biofuels due to their lipidic content (Figure 3.5) [8].

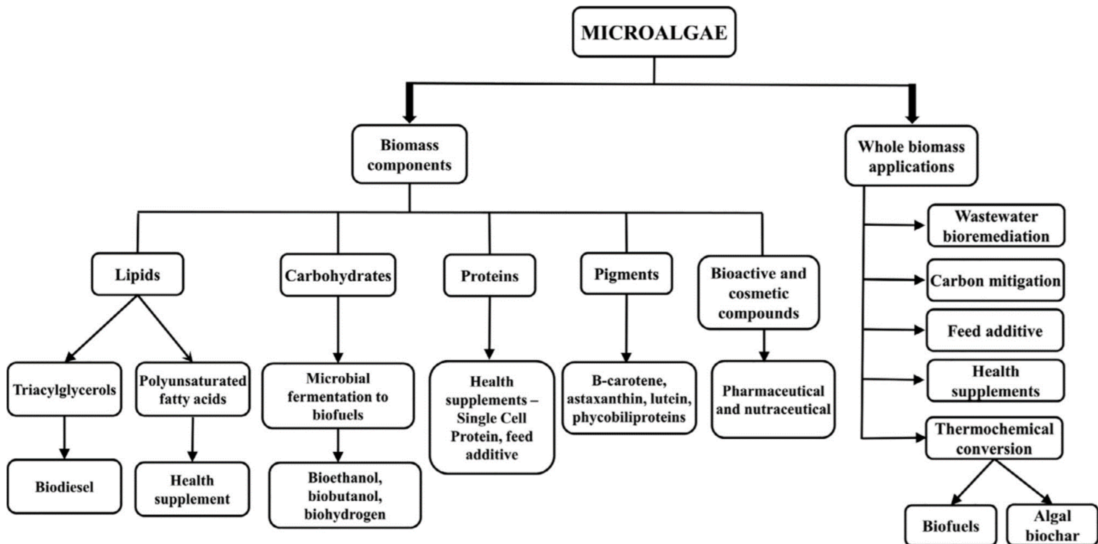


Figure 3.5

Applications of whole biomass and by-products derived from microalgae.

Unlike bacteria, live microalgae produce polysaccharides and lipids. Thus, not only they remove pollutants from the medium in which they grow, but their biomass can be used later in other applications such as pigment, or protein production, in the synthesis of bioactive compounds (such as biodiesel, bioethanol), or even their biomass can be employed as a fertilizer (as it has almost all nutrients needed).

As a bioremediation tool, microalgae have numerous benefits: they present simple requirements for their development (water, light, CO_2, and nutrients), and they can live in many habitats, even at extreme conditions. Microalgae are an essential alternative for contaminant removal, as they can effectively remove a wide variety of pollutants while assimilating inorganic nitrogen and phosphorus due to their metabolism (thus, reducing eutrophication). Employing microalgae to wastewater remediation is a "green" solution, as microalgae may be benefited from the nutrients present in the wastewater matrix, while they can remove heavy metal, pharmaceuticals, or dyes. This association can be grouped as a life cycle assessment (LCA) tool [103]. Once the wastewater has been cleaned, we can reuse the biomass, or employ it in the production of the several valued products shown in Figure 3.6, which will indeed reduce the overall operational cost of the process.

The whole process for wastewater treatment using microalgae is condensed below (Figure 3.6) [104]. Sometimes the microalgae can replace aerobic treatment.

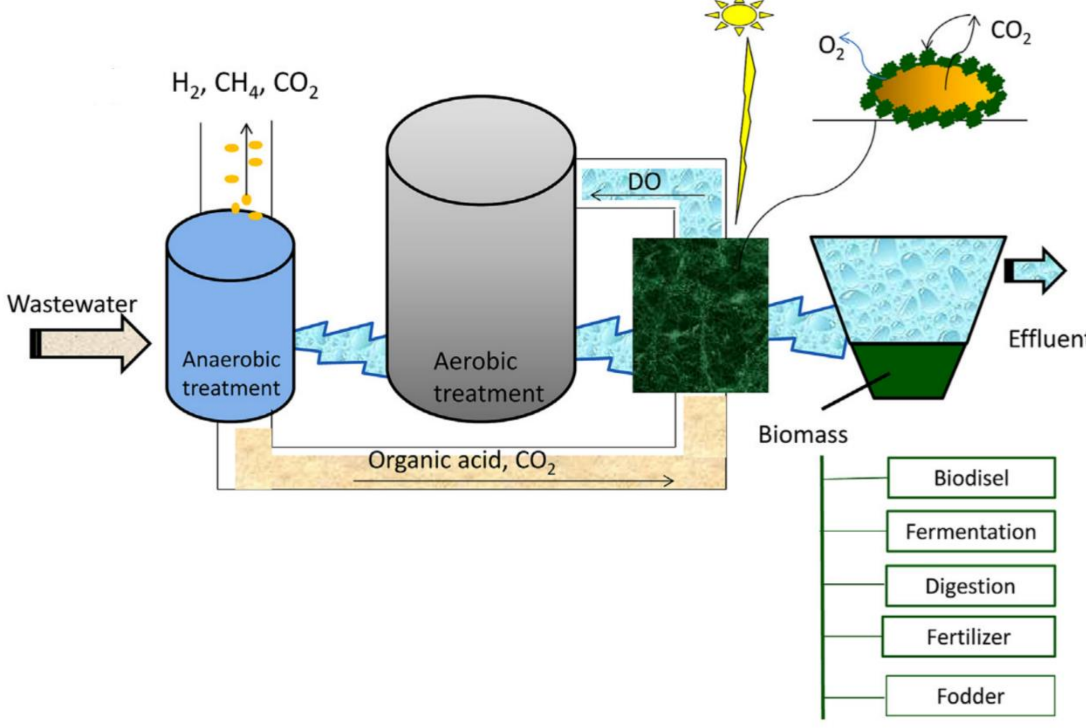


Figure 3.6

Example of a wastewater treatment process using microalgae

The most used species of microalgae for wastewater treatment are *Chlorella sp.*, *Arthrospira sp.*, *Scenedesmus sp.*, and *Nannochloropsis sp.*, their resistance to growth in bacteria consortia at the stressing conditions existing in a wastewater treatment photobioreactor [10].

3.2.3 Biosorbent capacities

Analog to bacteria, the biosorbent potential of microalgae is due to its membrane and cell wall, as there will be a multitude of functional groups that will interact with heavy metals.

Although the exact composition of the wall and the bilayer may vary among microalgae (brown, green, red), an example of a brown type is shown in Figure 3.7, in order to see the differences concerning bacteria [56].

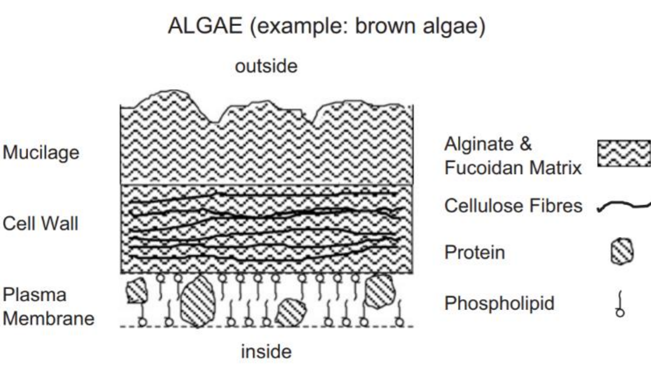


Figure 3.7

Composition of the cell wall of brown algae.

As stated earlier for bacteria, both living and non-living microalgae can be used. However, if additional products such as proteins, lipids, or starch are wanted to be obtained, then the non-living option gets depleted (as the process will be less effective).

They do not have a high surface-to-volume ratio as bacteria. Nevertheless, it is large enough to allow high removal efficiencies, together with its selectivity towards a wide range of heavy metals. Moreover, living microalgae can tolerate heavy metals easily [105].

Besides their outstanding removal capacities and their eco-friendly behavior, the use of microalgae is a robust and straightforward process, which has no toxicity hinders, has a fast growth rate, and can produce value-added products [11].

3.2.4 Heavy metal retention mechanisms

Like every living organism, microalgae need metals such as B, Co, Cu, Fe, or Zn, which play a role in many enzymatic and metabolic processes. Nevertheless, at high concentrations, they can be harmful. Microalgae have strategies of self-protection against their toxicity, such as gene regulation, metal immobilization, redox enzymes, or chelation/precipitation of metals. The cell wall of microalgae is negatively charged, and along its surface, many reactive groups are placed, with active binding sites that can interact with the metals present in the medium.

Ion-exchange is meant to be the dominant mechanism, followed by complexation and microprecipitation. One of the differences with respect to HMs retention by bacteria is that heavy metals get encapsulated into vacuoles once they entered the inside of the cell. Figure 3.8 shows the mechanisms that apply to microalgae bioremediation [11], [57].

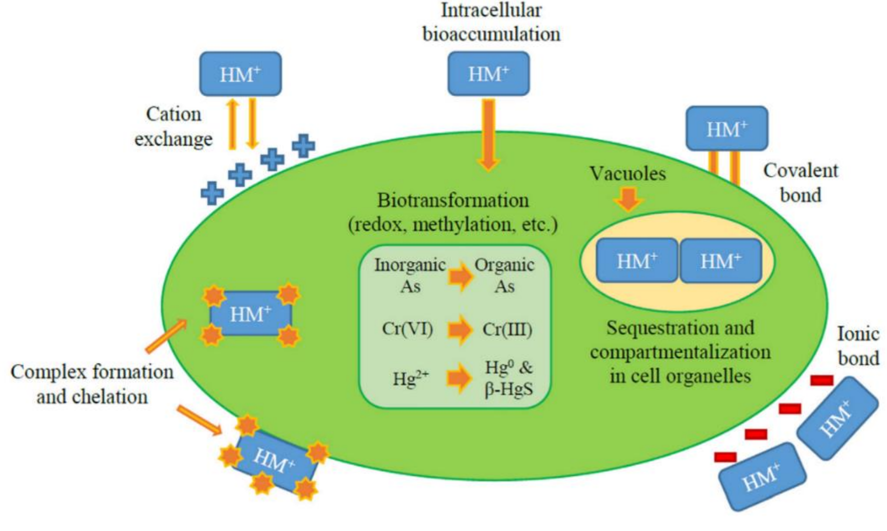


Figure 3.8

Metal retention mechanisms occurring in the microalgae cell.

3.2.5 Factors affecting microalgae biosorption

Besides metal-microalgal interaction, biotic and abiotic parameters directly affect the removal of heavy metals by microalgae biomass [17], [105].

3.2.5.1 Biotic factors

- ***Size and life stages:*** The size strongly affects the biochemical composition, the metabolism, and the growth of the microalgae. For instance, smaller microalgae performed photosynthesis with a higher yield, together with fast growth rates. If the population size of the microalgae increases, there will be better removal capacities. As mentioned before, a smaller size also provides a higher surface-to-volume ratio, which benefits the process of metal biosorption.

- ***Species:*** Each microalgae species has a slightly different cell wall composition, and having more content of some proteins may be crucial to more efficient removal of certain heavy metals. Related to this is the tolerance of the microalgae to the presence of toxic elements, which will vary amongst the species and the genus.

- ***Biomass concentration:*** Like in bacteria, a moderate increase in biomass concentration can lead to a higher removal capacity (more active sites), but if higher biomass concentrations are used, lower retention capacities could be obtained.

3.2.5.2 Abiotic factors

- ***pH:*** It is one of the most critical parameters affecting metal biosorption because it affects both the metal chemistry in water, and the binding sites of the microalgae. When pH is low, functional groups remain protonated (hindering positively charged cations from binding due to repulsive forces). As pH increases, more ligands (carboxyl, phosphate, amino, or imidazole groups) will become deprotonated, acquiring a negative charge, and allowing metal cation to bind. Studies [106], [107] suggest that zero-point charge was found at pH 3.0 for the algal biomass, so above 3.0, the algal cells would have a net negative charge. Depending on the functional group, the pH range within which it is deprotonated changes. For example, carboxyl groups predominate at pH 2-5. Thus, higher pH results in the facilitation of metal uptake since the cell surface are more negatively charged, while at higher pH levels, precipitation of most metals tends to occur. Hence, it is necessary to determine the optimal pH for algae–metal interactions. The influence of pH on metal accumulation by algae is quite species-specific, and it also highly depends on the metal species involved.

- ***Ionic strength:*** When ionic strength increases, the active sites for the heavy metals are reduced, as there will be competition with other cations.

- ***Salinity and hardness:*** To some extent, it is related to ionic strength and pH. Generally, higher salinity and hardness values are traduced in a reduction of heavy metal toxicity (so lower removal capacities are reported), because there are more competing ions for the active sites.

- ***Contact time:*** Generally, an increase in contact time enhance the biosorption efficiency. At 180 min, equilibrium is usually reached, although the optimum value may vary among species.

- ***Temperature:*** Higher temperatures promote the solubility of metal ions, decreasing the biosorption. Nonetheless, the binding can be endothermic or exothermic, so there will be an optimal temperature for each metal and species. However, studies available about the effect of temperature upon metal biosorption by microalgae are not entirely consistent. Usually, a temperature range between 25-35ºC is recommended.

- ***Multimetal interaction:*** Real wastewaters usually contain a mixture of metals: Cr, Ni, Cd, Hg, Na, K, and many others. Biosorption of metals is usually inhibited by the presence of other metals in solution, due to competitive interactions. Moreover, as we could be dealing with living biomass, it is not the same for them to consider a special harmful effect or combined toxic effects owed to several heavy metals. There are few reports on combined effects, although it could be said that the preferential binding is related to the relative strength of the interaction between types of metal ions and the types of biomass (a metal cation with higher charge-to-radius ratio will be more prone to bind than other with lower ratio).

- ***Initial metal concentration:*** Like in bacteria, the total amount of metal removed will depend on the initial concentration added. According to the adsorption isotherms, there will also be a maximum sorption level, so further increases in initial metal concentrations will not provide higher removal capacities if that limit is surpassed.

- ***Biomass pretreatment:*** Although biomass itself can bind metals, we can enhance the metal biosorption by using reactants that change the biorsorbent surface significantly. Hence, the goal is to facilitate contact between metal and active sites or to create new binding points. The most frequent pretreatment agents are:

  (i) $CaCl_2$, it is the most economical method.
  (ii) NaOH, which increases electrostatic attraction to metal cations.
  (iii) HCl, it displaces light metals by protons, and can also dissolve polysaccharide compounds of the wall, creating new binding sites.

3.2.6 Review of microalgae used for heavy metal removal

Similarly, as we did for bacteria, a revision of the literature on Cu and Zn removal using microalgae has been carried out.

Table 3.4

Table with microalgae species, adsorption capacities, and percentages of removal for zinc.

a: Strange results, with all the data, the %R results in a value higher than 100, which is not possible.

Microalgae strain	Ret Capacity (mg/g)	Removal (%)	Reference number
Chlorophyceae spp	---	88.00	[108]
Oscillatoria princeps	---	90.00	[109]
Chlorella vulgaris[a]	714.94	---	[110]
Chlorella pyrenoidosa	---	78.00	[111]
Chlamydomonas reinhardtii	---	55.00	[112]
Tetraselmis marina AC16-MESO	---	92.00	[113]
Heterochlorella sp. MAS3	---	43.00	[114]
Sargassum sp	1.14	≈ 90	[115]
Cladophora fascicularis	94.05	---	[116]
Dead Spirulina sp	389.0	---	[117]
Chlorella minutissima UTEX2341 (freeze-dried)	16.16	≈ 98	[118]
Chlorella vulgaris	---	64.70	[119]
Scenedesmus spinosus	---	55.00	
Chlorella pyrenoidosa	3.25	83.14	[120]

On the next page, the table for zinc results is shown (Table 3.5).

Table 3.5

Table with microalgae species, adsorption capacities, and percentages of removal for zinc.

Microalgae strain	Ret Capacity (mg/g)	Removal (%)	Reference number
Chlorophyceae spp	---	91.90	[108]
Scenedesmus obliquus	836.50	---	[121]
Scenedesmus obliqus	---	80.00	[111]
Desmodesmus sp. MAS1	---	68.00	[114]
Sargassum sp	0.81	≈ 90	[115]
Desmodesmus pleiomorphus	360.00	---	[122]
Chlorella minutissima UTEX2341	123.46	≈ 96	[118]
Scenedesmus obliquus	6.67	---	[123]
Scenedesmus quadricauda	5.03	---	
Chlorella vulgaris	105.29	---	[124]
Chlorella vulgaris	11.90	72.60	[125]

Nevertheless, for large-scale applications, pure microalgae, as described above, are not usually used. Nowadays, there is an increasing interest in using swine or wastewater (with some nutrients and metal) in order to remove the pollutants, providing at the same nutrients for growth. The occurrence of bacteria is such substrates that lead to the formation of microalgae- bacteria consortia.

The next table shows the most useful experimental conditions for the biosorption of Cu(II) and Zn(II) with microalgae (Table 3.6).

Compared to bacteria, sometimes microalgae are more interesting because it has higher growth rates, and low nutrient content is needed. Additionally, in contrast to bacteria or fungi, it does not generate toxic substances.

Table 3.6

Best experiments (with their conditions) found in the literature, for the removal of Cu and Zn, using microalgal biosorbent.

Heavy Metal	Microalgae strain	pH	Initial Concentration mg/L	Biomass concentration g/L	Agitation rate rpm	Temperature ºC	Time h	Max Uptake mg/g	Removal %	Reference
Cu (II)	*Oscillatoria princeps*	4.0	10.00	10.0	200	25	4	0.10	90.00	[109]
Cu (II)	*Chlorella pyrenoidosa*	2.0	---	---	---	---	96	---	78	[111]
Cu (II)	*Tetraselmis marina AC16-MESO*	---	5.00	---	---	20.0	72	---	92.00	[113]
Cu (II	*Chlorella pyrenoidosa*	6.3	5.00	1.28	250	28	12	3.25	83.14	[120]
Zn (II)	*Scenedesmus obliqus*	6.0/ 7.0	75.00	0.02	---	25	24	836.50	---	[121]
Zn (II)	*Scenedesmus obliqus*	---	---	---	---	---	48	---	80	[111]
Zn (II)	*Chlorella vulgaris*	6.0	30-300	0.4	150	---	5	105.29	---	[124]
Zn (II)	*Desmodesmus sp. MAS1*	3.5	20.00	---	100	23	---	---	68.00	[114]
Zn (II)	*Sargassum sp*	5.0	---	---	---	---	1	1.14	≈ 90	[115]
Cu (II)		5.5	---	---	---	---		0.81	≈ 90	
Zn (II)	*Chlorella minutissima UTEX2341*	6.0	---	4.0	140	28	3	123.46	≈ 96	[118]
Cu (II)		4.0	---					16.16	≈ 98	

3.3 Microalgae-Bacteria consortia as biosorbents

Algae form a large group of eukaryotes, which is one of the primary producers in the food web. However, in nature, they are not found alone; in most of the cases, they enlist with other microorganisms in a symbiotic relationship. Symbiosis is defined as "living together", and it can be harmful or beneficial [126].

In the following sections, the microalgae-bacteria consortia will be defined, along with their inner interactions, mechanisms, and advantages concerning pure cultures.

3.3.1 What are microalgae-bacteria consortia?

It is well known that algae emerged from the association of a prokaryote (cyanobacteria) with a eukaryote. Via endocytosis, the photosystems the cyanobacteria possessed were retained by the new cell, although it followed different evolutionary lineages, leading to the actual red, green algae, and glaucophytes. Hence, photosynthetic eukaryotes born [127].

As microalgae are cells bigger than bacteria, it is usually considered that bacteria get attached to microalgae via extracellular polymeric substances (a mixture of polymers) that the microalgae produced, which is responsible for keeping the microorganisms together. This process of consortia formation takes place on a solid substrate, and when the growth reaches a stationary state (no more biomass is created), then it gets detached spontaneously, obtaining the consortia biomass free to use. Figure 3.9 exemplifies the process accurately [128].

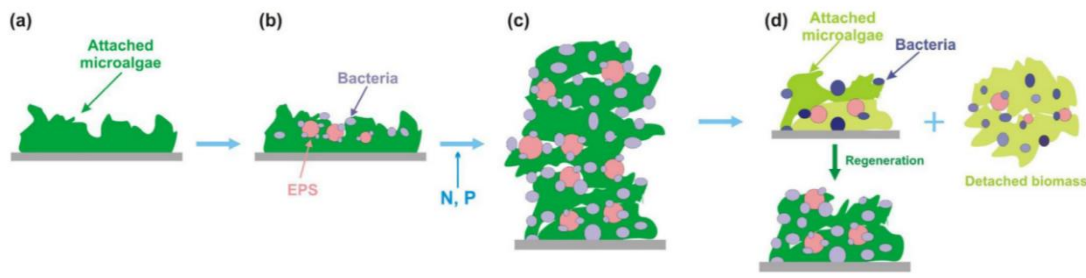


Figure 3.9

Microalgae-bacteria consortia formation:

a) Microalgae is attached to the solid substrate.
b) Bacteria adhere to microalgae.
c) Growth of the consortia with nutrients (N and P).
d) Detached biomass from the substrate.

As we have seen, there is incredibly high biodiversity within the microalgae and the bacteria, so their consortia will be even more complicated, and their interactions will not be easy to study and understand.

3.3.2 Why microalgae-bacteria consortiums?

Microalgae and bacteria both remove nutrients and pollutants from the medium in which they are. However, they have several disadvantages: expensive biomass harvesting, insufficient biomass productivity, or energy-intensive extraction. The consortiums aid to overcome these issues by using symbiotic interactions between microalgae and bacteria, which may spur the growth and will enhance the removal efficiencies [129].

Polycultures are based in a mix of different microorganisms, and they can be advantageous for nutrient and pollutant removal, as they combine different metabolic activities. Additionally, they adapt themselves more easily to environmental conditions, allowing them to create a robust system. Generally, cooperative interactions are established between the microorganisms, which facilitates this process, together with better removal efficiency [130]. Furthermore, microalgae are more sensitive than bacteria towards specific compounds, which include heavy metals that can be found in all environmental compartments. However, when being part of a consortium, the strength, and tolerance of the system increases [131].

Despite the advantages consortiums offer, most studies have focused on pure cultures, so there is little information about the interaction mechanisms, physiology, or diversity, which in part explains why this methodology has not been employed at an industrial level. Nevertheless, the use of pure cultures is not practical for large-scale applications yet, as it is challenging to maintain and keep the optimal sorption capacity, given the different environmental conditions they may encounter. Alternatively, consortiums emerge as an ideal solution [132].

Needless to say, the capacity of microalgae-bacteria as biosorbent is given by the capacities of its constituents. Bacteria and microalgae have their retention mechanisms, so the mixture of them will also have those properties, and the mechanisms will be the same we have talked about previously, mainly biosorption and bioaccumulation. Under the interactions between them, the system will have more chances to prosper (Figure 3.10) [133].

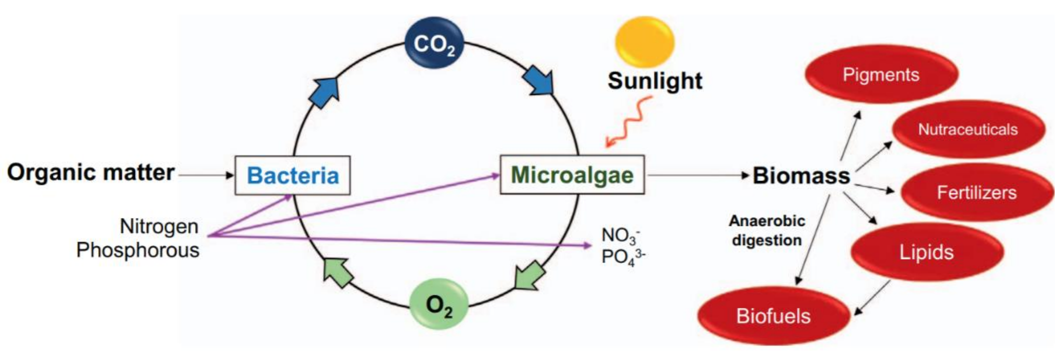


Figure 3.10

A simplified view of how bacteria and microalgae interact and the applications of the resulting biomass.

3.3.3 Microalgae-bacteria interactions

The high biodiversity in the consortium leads to complex interactions between and within the microalgae and bacteria. Depending on how the relationship is, there can be three different types of associations from an ecological point of view. Moreover, evidence suggests that the interactions are species-specific [127]–[129], [134]:

A. ***Mutualism:*** Each microorganism provides some essential molecules or stimulus to the other, so they get a net benefit from the interaction. Microalgae provide O_2 and organic nutrients, while bacteria give inorganic N, P, vitamins…For example, bacteria are known to supply Vitamin B_{12} to algae, in exchange for fixed carbon. Additionally, microalgae provide organic carbon to bacteria and use the CO_2 for the photosynthesis, which triggers an enhanced growth.

B. ***Commensalism:*** One microorganism benefits from the other, but without damaging nor helping the other. It is very similar to mutualism; in fact, studies relate one or the other depending on the environmental conditions in which the consortium is.

C. ***Parasitism:*** In this case, one microorganism harms the other by releasing harmful substances. Many bacteria have an algicidal effect, as well as some microalgae, excrete some bactericide substances.

The main interactions are mutualism and parasitism, and commensalism is not as well study as it is far less common. A summary with the primary interactions an modes of interactions is shown in Figure 3.11:

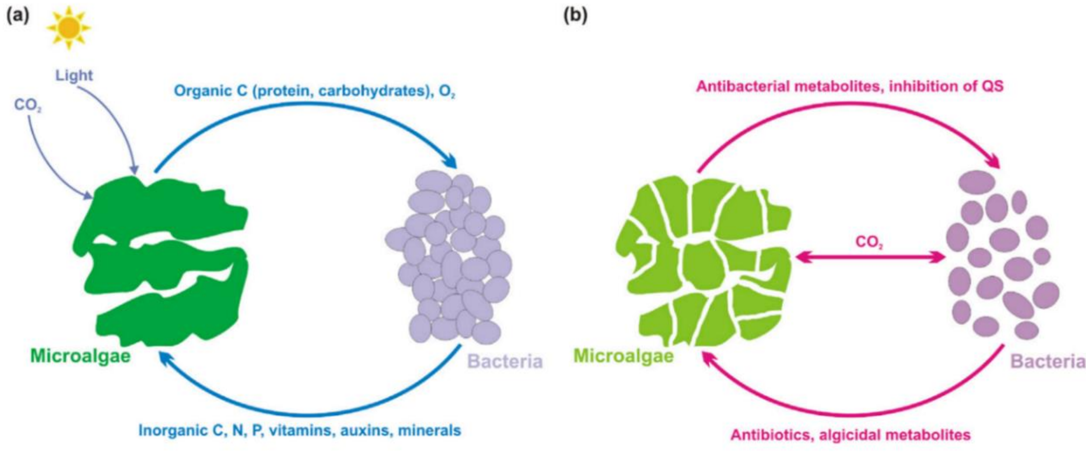


Figure 3.11

Representation of mutualistic or cooperative (a) and parasitic or competitive (b) interactions between bacteria and microalgae.

Together with nutrients exchange, there are more complex interactions, such as gene transfer, or cell to cell communication via specific substances and receptors such as phytohormones, or the indole acetic acid (which in the case of a marine algae, is sent by bacteria in exchange for organosulfur compounds). Despite being ecologically significant, antagonistic interactions are hidden by the overall beneficial effect of the microalgae-bacteria relationship.

Nevertheless, there are not only interactions between the microorganisms, but also between the consortium and the environment. Solar irradiance, temperature, pH, salinity, or nutrient disposal greatly influences the microalgae-bacteria consortia relationship.

3.3.4 Applications to wastewater treatment and limitations

Microalgae-bacteria consortiums are effective in nutrient and pollutants removal. They inherently have other associated advantages for wastewater treatment, such as the reduction of the cost related to oxygenation or the reduction of the greenhouse gases, as the CO_2 or CH_4 can be metabolized.

There has been little research in microalgal-bacterial consortia for heavy metals removal. The first step must be to choose appropriately the microorganisms that are going to form the consortia, as the interaction between them will condition the uptake capacities. Then, it could be interesting to carry out studies involving biotechnology or genetic engineering, in order to improve the characteristics of the initial system, such as keeping homeostasis in the long run, or the maintenance of the effectiveness of the consortia in time.

Most of the studies related to microalgae-bacteria consortiums have been performed in laboratory scale units, so they may not be representative of real situations. Hence, further advances must be carried out, such as:

- Study of the effect of environmental conditions.
- Outdoor, or in situ experiments.
- Deep understanding of the microalga-bacteria interactions.
- Design of mathematical models describing the behavior of these consortia, which will help in the scale-up of the process.

In brief, consortiums combine microorganisms with different metabolic capacities, distinct metal-binding ability, and different affinities towards nutrients. In order to apply them successfully, a profound understanding of their whole complexity must be reached. If finally, this approach overcomes the drawbacks nowadays has, it can be the best treatment for bioremediation.

3.3.5 Review of microalgae-bacteria consortia used for heavy metal removal

The literature regarding microalgae-bacteria consortia for heavy metal removal is scarce. Nevertheless, there is more information about consortiums applied to nutrient removal [135]. Table 3.7 compile the experiments found:

Table 3.7

Best experiments (with their conditions) found in the literature, for the removal of Cu and Zn, using microalgal-bacteria consortia as biosorbent.

<table>
<tr><th>Heavy Metal</th><th>Microalgae and Bacteria strains</th><th>pH</th><th>Initial Concentration mg/L</th><th>Biomass concentration g/L</th><th>Agitation rate rpm</th><th>Temperature ºC</th><th>Time h</th><th>Max Uptake mg/g</th><th>Removal %</th><th>Reference</th></tr>
<tr><td>Cu (II)</td><td>Algae and bacteria from wastewater treatment plant (biomass)</td><td>4.0</td><td>< 100.0</td><td>0.4</td><td>---</td><td>---</td><td>0.33</td><td>18.36</td><td>≈ 80.0</td><td>[136]</td></tr>
<tr><td>Cu (II)</td><td>Chlorella sorokiniana and Ralstonia basilensis</td><td>5.0</td><td>20.0</td><td>1.2</td><td>150</td><td>26</td><td>40</td><td>8.5</td><td>51.0</td><td>[137]</td></tr>
<tr><td>Cu (II)</td><td>Sulfate-reducing bacteria-microalgae (many types of microalgae and bacteria)</td><td>5.5</td><td>100.0</td><td>---</td><td>---</td><td>---</td><td>24</td><td>45.28</td><td>> 98</td><td>[138]</td></tr>
<tr><td>Cu (II</td><td>Enterobacter sp. AMD01 and Chlorella sp (sequential process)</td><td>---</td><td>84.6</td><td>---</td><td>---</td><td>---</td><td>24</td><td>---</td><td>98.9</td><td>[139]</td></tr>
<tr><td>Zn (II)</td><td>PRB-AB system (mixture of several bacteria with Chollera Vulgaris)</td><td>8.5</td><td>---</td><td>---</td><td>---</td><td>---</td><td>---</td><td>---</td><td>98.0</td><td>[140]</td></tr>
<tr><td>Zn (II)</td><td rowspan="2">Stichococcus and blue-green Phormidium algae</td><td>---</td><td>0.01</td><td rowspan="2">---</td><td rowspan="2">---</td><td rowspan="2">---</td><td rowspan="2">---</td><td>---</td><td>90.0</td><td rowspan="2">[141]</td></tr>
<tr><td>Cu (II)</td><td>---</td><td>0.04</td><td>---</td><td>62.0</td></tr>
</table>

4 METHODOLOGY AND MATERIALS

4.1 Experimental design

The design of experiments (DoE) is a statistical approach that allows us to determine if specific factors have a significant influence on a process response (Figure 4.1). A factor can be changed independently from the other factors, and every factor is assayed at different values (factor levels) to investigate their effect on the response. The ultimate purpose of an experimental design is to find the suitable combination of levels of the control factors that maximize or minimize the response, or that keep it at a given (nominal) value, using the fewer number of experiments to get the maximum information about the process.

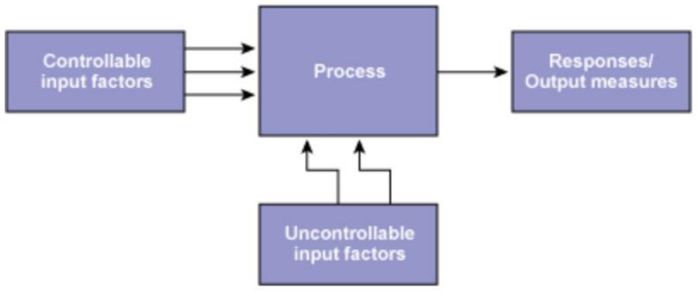


Figure 4.1

A simplified diagram of how factors influence the process and the response.

The fundamentals of DoE rely on the analysis of variance (ANOVA), which in short is a hypothesis test based on the F-test (comparison of variances). With ANOVA, we study how controllable factors influence the variability of the response, if this variability is higher than the random variability inherent to the experimentation caused by uncontrollable factors affecting the process, then we will say that the factor affects the response [142]–[144].

The stages of DoE are:

1. Select the output variables to be optimized. In this study, the retention capacity of the biomass towards metal ions Cu(II) and Zn (II) (mg metal per g of biosorbent) is the parameter to be maximized, as the objective is to remove these elements from wastewater.

2. Once the output variable is chosen, we must determine the potential influencing factors -that can be controlled and tuned- along with their levels and establish the combinations among factors to be assayed. According to the literature review made, six different factors were considered, two factors at three levels (the type of biomass, and the initial metal concentration in the solution to be treated), and four factors at two levels (organic carbon, included as pentone; inorganic carbon, added as CO_2; light, and contact time).

In real wastewater samples, the metal concentration would be a noise factor, as it is impossible to know precisely its amount. However, in this design, the initial concentration of each metal is known. Control factors are displayed in Table 4.1:

Table 4.1

Control factors and their levels assayed. In biomass factor, **A** represents pure microalgae, **S** the activated sludge, and **P** microalgae-bacteria consortium grown in piggery wastewater (pig slurry).

		Level		
Control Factors	***Symbol***	**-1**	**0**	**1**
Biomass	*B*	A	S	P
Organic Matter / mg $_{peptone}$ per L $_{solution}$	*OM*	0	---	80
Light	*L*	No	---	Yes
Time / h	*T*	1	---	72
CO_2 / mL	*IC*	0	---	20
Concentration Cu^{2+} - Zn^{2+} /mg·L^{-1}	*C*	15 - 40	60 - 70	100 - 100

The election of the three biomasses is supported by the information given in Chapter 3: they are the most common biomasses used for metal remediation.

Organic matter is very frequently present in wastewater. The levels chosen were absence and presence. The absence of OM could negatively influence the capacity of microorganisms to survive and grow, and therefore to cope with heavy metals. Organic matter was added as pectone, a water-soluble mixture of polypeptides and amino acids formed by the partial hydrolysis of protein, which can be used by microorganisms as a source of carbon to grow. The value was selected according to literature [145].

Microalgae need light to subsist. The levels used for this factor were: ***No*** (ambient light in the laboratory), and ***Yes*** (high-intensity light supplied with LED lamps in cycles on-off of 12 h).

The contact time affects the capacity of the microorganism to uptake metals. Terse times can be insufficient to remove the metals effectively, whereas too long times can lead to toxic effects on the organisms. Levels of 1 hour are usually sufficient for significant uptake of heavy metals. The level of 3 days was selected as it is the average stay time of microalgae in a photobioreactor for piggery wastewater treatment.

Microalgae use carbon dioxide as a source of carbon. The availability of abundant CO_2 may improve the growth and resistance of microalgae against high concentrations of toxic elements. The two levels assayed were no supply or supply of extra CO_2.

Copper and zinc are the major heavy metals always present in pig manure, and therefore the factor metal was assayed mixing both elements.

The concentration range was selected based on the composition of the liquid fraction of pig manure reported by the American Society of Agricultural Engineers [146].

Taking all the factors into account, a full factorial design was selected in order to have sufficient degrees of freedom to investigate not only the main factors but also their possible interactions. Only first-order interactions will be considered as higher-order interactions are not very likely to happen, and thus they will be neglected in this work. The total number of experiments required in a full factorial design of two factors at three levels and four factors at two levels is $3^2 \times 2^4 = 144$ experiments. Due to the high number of experiments, no replications were made. All the combinations of the factor levels are displayed in Table A.1, Table A.2, and Table A.3, in the Appendix.

3. Select in which order the experimentation is going to be performed. By using a random design, the influence of uncontrolled factors will be reduced. However, this approach is more time-consuming. An intermediate technique called random-blocks is used in order to optimize laboratory time. In our case, we made blocks by biomass type in order to use homogeneous material from the same batch, and sub-blocks by the factor OM.

4. Finally, the results of the experiments are interpreted using Analysis of Variance, ANOVA, employing the Yates´s algorithm to compute the estimates of main effects and the interactions [142]. A significance level of 5% was selected, so significant effects were considered those with a p-value below 0.05. Statistical calculations were performed using Statgraphics Centurion 18 (Statgraphics Technologies, USA).

4.2 Biomass characterization

Biomass A (pure microalgae strain of *Scenedesmus Almeriensis*) was grown in a photoreactor fed with a synthetic solution of nutrients (fertilizer). Biomass P (microalgae-bacteria consortium with *Scenedesmus Almeriensis* as major microalgae specie) was harvested from a photobioreactor for pig manure wastewater treatment. Both microalgae biomasses were kindly provided by the University of Almeria (Spain). Biomass S was the activated sludge collected from a biological wastewater treatment plant, mainly formed by bacteria. The biomasses were stored in the dark at 4°C for quality assurance purposes, mainly to ensure a constant composition for every 48 experiments.

It is essential to know how much water the biomass contains to calculate the retention capacity accurately. Other parameters of the biomass such as volatile solids (a measurement of the total organic matter), and the lipid and protein contents were determined. All measurements were done in duplicate

4.2.1 Moisture and volatile solids analysis

The moisture content (or the dry weight) is calculated by drying a weighed amount of fresh biomass of 1 g at 50 °C until constant weight:

$$\boldsymbol{Moisture}\ (\%) = 100 - \%Dry\ Weight = 100 \cdot \left(1 - \frac{m_{Dry\ Biomass}}{m_{Wet\ biomass}}\right)$$

For the calculations, it is more straightforward to use and calculate the %Dry Weight (or total solids), as it directly gives the dry biomass used.

The dried mass (around 0.5g) was transferred to a porcelain crucible and calcinated in a muffle furnace at 550 ºC for 24 h, and the ashes cooled and weighed. The mass loss, expressed as volatile solids, VS, is a measure of the organic matter in the sample.

$$\boldsymbol{VS}\ (\%) = \frac{m_{Dry\ biomass} - m_{ashes}}{m_{Dry\ biomass}} \cdot 100$$

4.2.2 Analysis of lipids

The Bligh and Dyer method was employed (as it was the one that was optimized in the research group).

100 mg of a lyophilized sample is weighed in a glass tube and mixed with Al_2O_3 in a 1:1 ratio. The mixture is ground manually for 15 minutes.

Then 2 mL of chloroform:methanol 2:1 are added, the tube was shaken to mix and centrifuged 3 min at 7800 rpm. The liquid phase is transferred to a Falcon tube. The extraction step is repeated up to five times with 1 mL of chloroform:methanol solution, collecting the liquid fractions until the supernatant is clear, and the precipitate turns whitish (pigments are also extracted).

Next, 3 mL of 0.1 M HCl and 0.3 mL of 0.5 % $MgCl_2$ are added to the tube containing the extract to separate proteins. After mixing and centrifugation in the same conditions as referred before, three phases are obtained: an aqueous phase on top, the precipitated proteins at the bottom and a lipidic intermediate layer that is recovered with a Pasteur glass pipette and transferred to a dry pre-weighed glass tube.

The lipidic layer is then dried by evaporating the solvents at room temperature, or applying gentle heat, in a fume hood, until constant weight.

The content of total lipids is calculated as follows:

$$\boldsymbol{Lipids}\ (\%) = \frac{m_{Lipids}}{m_{Lyophilized\ biomass}} \cdot 100$$

4.2.3 Analysis of proteins

The content of proteins was related to the organic N content of the samples, using a conversion factor of $f = 6.25$. Organic N was determined by the Kjeldahl method as it is detailed below:

An aliquot of 0.1-0.2 of dry biomass is accurately weighed on a filter paper. The paper is wrapped like a little ball, and placed inside a digestion tube, together with a dielectric piece, 6 mL of 96% H_2SO_4 and one tablet of the Kjeldahl catalyst. The tubes are placed in the digestor, assembling the fume collector, and establishing a heating program with three stages: 20 min ramp-up to 150ºC, other 20 min up to 270ºC, and finally 1 h at 370º. Once finished, solutions are allowed to cool down to room temperature.

The digests are then distilled with 6 M NaOH. The distilled ammonia is collected into an Erlenmeyer flask containing an excess of the orthoboric acid solution and an indicator (a mixture of methyl red and methylene blue), where ammonia reacts to give a stoichiometric amount of borate ions (the solution turns green) that are titrated with a standard solution of sulphuric acid (the solution turns purple at the endpoint). The equations relating the percentage of nitrogen in the sample with the mol of titrating solution spent are shown below:

$$mmol\ NH_3(distilled) = mmol\ N = mmol\ H_30^+ = 2\ mmol\ H_2SO_4$$

$$\boldsymbol{Proteins}\ (\%) = 6.25 \cdot \frac{2 \cdot mmol\ H_2SO_4\ \cdot 14.007}{1000 \cdot m_{Dry\ sample}(g)} \cdot 100$$

4.3 Reagents

All the chemicals employen in this study were analytical grade (Sigma Aldrich, Germany). Plastic and glass containers were washed in dilute HNO3 (10% v/v) for 24 hours and rinsed three times with Mili-Q water (R>18 MΩ cm) before use.

The metal ions studied were introduced as $CuCl_2 \cdot 2H_2O$ and $ZnCl_2$.

The culture growth medium used was Bristol, whose composition is displayed in Table 4.2:

Table 4.2

Different reagents and concentrations of the Bristol medium.

Compound	Concentration / M
$NaNO_3$	0.30
$CaCl_2·2H_2O$	0.02
$MgSO_4·7H_2O$	0.03
K_2HPO_4	0.04
KH_2PO_4	0.13
NaCl	0.04

The Bristol medium was not a single bottle, as there could be precipitation issues, six different bottles for each salt were prepared in that specific concentration.

4.4 Analytical procedure for multimetallic biosorption experiments

The 144 experiments consisting of all possible combinations of the factor levels were carried out as follows:

Around 1 g of A, S, or P fresh biomass is weighed and placed into a glass bottle of 500 mL, the actual weight depending on the moisture percentage, to achieve a final biomass concentration between 0.5 and 1 g of dry biomass/L.

200 mL of the bimetallic solution containing the desired concentration of Cu(II) Zn(II), and the Bristol medium (the final concentration of each salt must be 10 mg/L) is introduced into the bottle together with a magnetic stir bar. For the experiments containing organic matter, 16 mg of peptone are added to obtain a concentration of 80 mg/L.

The pH is one of the most critical parameters in the biosorption experiments, it must be kept between 5.5 and 8.0, for the reasons studied in *Sections 1.2.2, 3.1.4, and 3.2.5* [147]. Due to ionic-exchange reactions, the pH could vary during the experiments, so we must ensure that it is within that interval. The initial pH for the three biomasses was below 7.0 at the three metal concentration levels. The microalgae-bacteria biomass (P) was the most acidic one. The pH was adjusted to 6-7 with 0.1 M NaOH. When inorganic carbon is required, 20 mL of CO_2 is injected with a syringe through the septum, the addition of CO_2 did not show a significant decrease in the pH. Bottles are covered with a septum. The suspensions are then stirred with or without LED light (at 1000 $\mu E·m^{-2}·s^{-1}$, and 12:12 h photoperiod) during the desired time (1 or 72 hours).

The suspensions pH stayed stable after 72 h (pH 6-7) for biomasses P, S, but it rose to 8-9 for the pure microalgae biomass. This pH increase could be due to photosynthetic reactions, as CO_2 is consumed and there is not bacterial activity (and CO2 production) in experiments with biomass A.

When the contact time is completed, the stirring is stopped, and the bottles are left to stand and settle. The supernatant and the solid phase are then separated through a decantation process, and the solid transferred to a 50 mL Falcon tube, which is centrifuged at 4500 rpm for 7 min.

The supernatant is removed, and the biomass washed 2-3 times with Milli-Q water in order to remove the remaining solution containing metals. The washing liquid is removed after centrifugation as before (we check that metals did not remain in that washing liquid by analyzing several samples via ICP-OES).

Then, the Falcon tube with the biomass is taken to an oven at 50ºC for two days, to eliminate the water embedded in the solid. Dry biomass was weighed to know the biomass growth during the 72 h experiments . Biomass growth does not affect the uptake capacity as it is expressed in mg metal per g of biomas, but this information is necessary in order to estimate the percentage of metal removal from solutions.

Once the residual biomass is dry, a portion of around 0.1 g is weighed into a PTFE vessel, together with 5 mL of 68% HNO_3. The mixture is heated in a microwave oven at a constant rate from room temperature up to 180 ºC for 10 min, and then for 20 minutes at 180 ºC.

After digestion, the resulting solution is transferred to a volumetric flask (50-100 mL, depending on the concentration level) and filled with Milli-Q water. The concentration of the resulting solutions containing the biosorbed metals copper and zinc are finally determined by inductively coupled plasma – optical emission spectrometry using a Varian 725-ES ICP-OES instrument. The instrument was calibrated with copper and zinc standards prepared by dilution in 3% nitric acid. Plasma gas and auxiliary gas flows of 15.0 L Ar/min and 1.5 L Ar/min were used, respectively, and the plasma potency was 1.3 kW. Nebulizer pressure was 180 kPa and washing time, 10 s.

The characteristic wavelengths for copper and zinc, according to UNE-EN ISO 11885, are shown in Table 4.3. For this work, we have used the 202.548 nm wavelength for Zn, while for Cu was the 324.754 nm wavelength. Despite the small spectral interference that copper may originate in the line of Zn, this wavelength was chosen due to its high intensity, compared to the one at 206.200 nm.

Table 4.3

Most used lines (and their metal interferences) for Cu, and Zn.

Element	Wavelength / nm	Interferent elements
Cu	324.754	*Cr, Fe, Mo, Ti*
	327.396	*Co, Ti*
Zn	202.548	*Cr, Cu, Co, Ni*
	206.200	*Cr*
	213.857	*Cu, Fe, Ni*

With the concentration obtained in the ICP-OES, the volume of the flask used, and the dry biomass weighed the retention capacity (q) is calculated following the equation below:

$$\boldsymbol{q_i}\left(\frac{\boldsymbol{mg}\ metal\ i}{\boldsymbol{g}\ biomass}\right) = \frac{c_i\left(\frac{mg}{L}\right) \cdot V_{Flask}\ (mL)}{m_{Dry\ Biomass} \cdot 1000}$$

Figure 4.2 presents the typical set up used for the experimentation method explained above:

Figure 4.2

Microalgae biomass (pure *Scenedesmus almeriensis*) subjected to light conditions.

5 RESULTS AND DISCUSSION

5.1 Biomass composition

Table 5.1 displays the average results ($n = 2$) of the compositional parameters measured in the three types of biomass investigated in this work.

Table 5.1

Average results (n=2) of compositional parameters of the three different biomasses. The uncertainty of each value is given by ± the standard deviation (s).

A is the pure microalgae, **S** the activated sludge, and **P** the microalgae-bacteria consortia cultivated in pig slurry.

	A	*S*	*P*
Moisture / %	85.17 ± 0.23	98.03 ± 0.01	84.85 ± 0.14
Volatile Solids / %	75.0 ± 0.9	56.26 ± 0.26	65.1 ± 1.7
Lipids / %	9.1*	4.6 ± 0.1	8.5 ± 1.4
Proteins / %	38.9 ± 2.8	33.2 ± 0.7	42.1 ± 1.1

The water content of fresh biomasses ranged from 84.8 % (biomass from pig slurry treatment) to 98.0 % (active sludge). The value of moisture for pure microalgae is very similar to the one found in biomass grown in pig slurry.

The volatile solids results are referred to the dry biomass. From the results of total and volatile solids, the organic matter contents can be derived. As volatile solids and organic matter content are closely related, the activated sludge is the biomass with the lowest amount of organic matter.

The results of lipids' contents show that P and A biomasses have a very similar lipidic composition. For the pure microalgae (*) there is no uncertainty estimation as only one replicate measurement could be made. The activated sludge has a significantly lower lipid content, which is reasonable as the bilayer composition of microalgae is richer in lipids.

Concerning the protein content, it was found that the activated sludge has the lowest amount, which is feasible as eukaryotic cells usually have more proteins.

5.2 Multimetallic biosorption experiments

The combination of factor levels of the 144 experiments, and the results of the retention capacity of copper and zinc, are displayed in Table A.1, Table A.2, and Table A.3 of the Appendix. Furthermore, the results of biomass growth (for the 72-hour experiments) are also shown in Table A.4 of the Appendix.

In the following sections, the statistical analysis of the results will be performed.

5.2.1 Biomass growth experiments

It is expected that metal concentration affects to different extent the growth capacity of bacteria and microalgae as copper and zinc are essential elements. However, they can become toxic if the concentration exceeds a specific limit, which depends on the organism.

Growth percentages above 100% indicate an increase in the amount of biomass in the bottle and, therefore, the growth of the microorganisms during the 72 h experiments. On the contrary, results below 100% are due to loss of biomass, related to an insufficient or excessive amount of metals in solution. Other factors (pH, nutrients availability, light) could also be influencing biomass development and survival. These effects were investigated using analysis of variance.

5.2.1.1 One-Way ANOVA

For the three biomasses, one-way ANOVA was carried out in order to determine whether the metal concentration significantly affects the growth of the microorganisms and to identify the concentration yielding maximum biomass production.

The factor will be significant if the p-value obtained is lower than the significant level chosen, in our case, 0.05. Table 5.2 collects the p-values for the effect of metal concentration factor on the growth of the three biomasses investigated. Full ANOVA tables are displayed in the Appendix, Tables A.6-A.8.

Table 5.2

p-values for the effect of metal concentration on each biomass growth.. The values in red indicate that the factor is significant.

	A	S	P
p-value	0.0000	0.0000	0.0000

The factor metal concentration significantly affected the growth of the three types of biomass (p-values < 0.05), which means that at least one concentration level yields a significantly different growth.

The least significant difference (LSD) test (see Tables A.9-A.11 in the Appendix) in combination with the box-and-whisker plot allowed to identify the factor level providing optimal biomass growth.

The LSD test compares the absolute difference between the mean responses of two factor levels with a critical value calculated as:

$$LSD = t_{crit} \cdot \sqrt{MS_R \cdot \left(\frac{2}{n_j}\right)}$$

Where the MS_R is the mean square of the residuals, and n_j is the number of replicates. If the experimental difference exceeds the critical value, then the responses at those factor levels are significantly different.

Box-and-whisker plots show the median (blue line) and mean (red dot) values at each metal concentration level; the length of the box and the whiskers provide information on the shape (dispersion) of the distribution of the results. Overlapping boxes indicate that there are no significant differences in the variable at those factor levels.

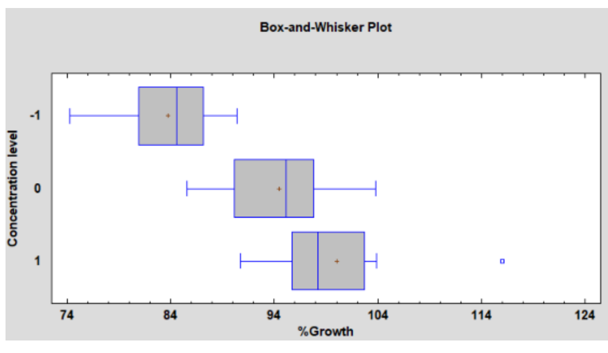


Figure 5.1

Box-and-Whisker plot for the pure microalgae (A).

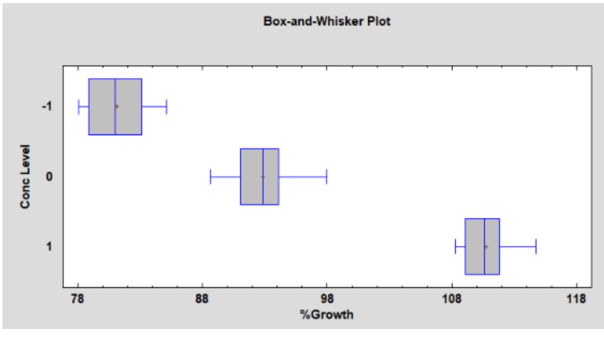


Figure 5.2

Box-and-Whisker plot for the activated sludge (S).

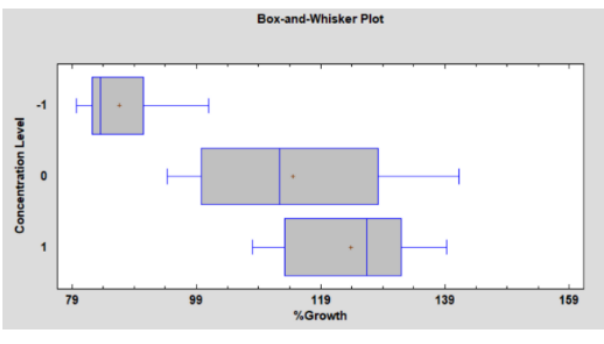


Figure 5.3

Box-and-Whisker plot for the biomass cultivated in pig slurry (P).

Both statistical tools demonstrated that:

- Biomass A (cultivated with inorganic fertilizers in the absence of bacteria), showed a relevant decrease of biomass amount in the solution after three days for the lowest metal concentration (level -1), and only a slight growth at the highest metal level (+1). However, the difference between the higher two concentrations was no significant.
- In the case of biomass S (activated sludge), all the metal concentration levels resulted in significant differences in biomass amount in solution after three days. Growth was only observed at the higher metal concentration used, level 1, whereas biomass loss was observed at the lower concentrations.
- Biomass P (harvested from a photoreactor treating pig slurry), behaved similarly to biomass A, the biomass amount in the solution decreased at the lower metal concentration, level -1, and increased at the higher metal concentrations. Only significant differences were found between the lowest concentration, level -1, and levels 0 and 1.
- Biomass growth was obtained in all cases at the maximum metal concentration assayed. The highest increase was observed for biomass P, probably due to the symbiotic interaction.

5.2.1.2 Multifactor ANOVA

Multifactor ANOVA was performed to evaluate if other experimental factors assayed (light intensity, presence of organic matter, OM) exert additional influence on biomass growth and to verify if they interact with the metal concentration factor.

The p-values derived from ANOVA calculations are gathered in Table 5.3. The original tables are in the Appendix (Table A.12-A.14).

Table 5.3

p-values of experimental factors and their interactions for the three biomasses.

	p-value		
Factors	***A***	***S***	***P***
Concentration	**0.0006**	**0.0000**	**0.0000**
OM	0.4068	0.0958	0.4565
Light	0.7331	0.1336	**0.0000**
Concentration-OM	0.9559	0.2812	0.2395
Concentration-Light	0.1641	0.7099	**0.0095**
OM-Light	0.2259	0.7922	0.4199

Besides metal concentration, light intensity and its interaction with concentration are significant, but only for biomass P (microalgae and bacteria consortia cultivated in pig manure). The interaction is not very significant, but it indicates that the biomass P grows better when only ambient light is provided, and the concentration level is the highest. To demonstrate this, Figure 5.4 shows the interaction plot between concentration and light:

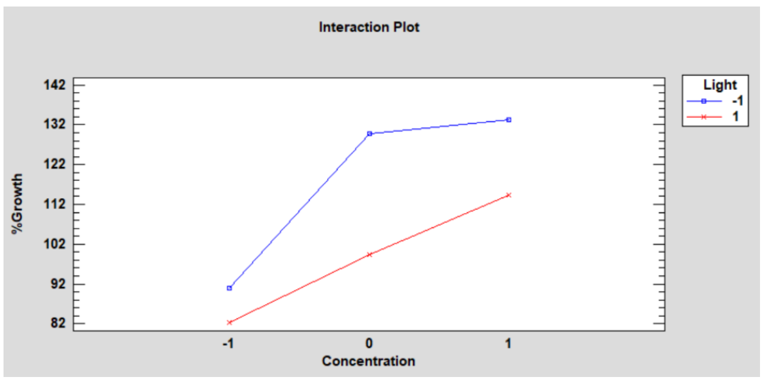


Figure 5.4

Interaction plot between the factors light and concentration.

The blue line (ambient light) is always above the red line, which means that the %Growth is always higher when no light is applied. However, growth increases steadily with increasing concentration at the higher level of light, whereas at low light exposure the average growth is similar at medium and maximum levels of metal concentration. We expected that light was a significant factor for A and P biomasses, as they both contain photosynthetic microalgae. Nonetheless, it appears only to affect the growth of the pig slurry biomass. The activated sludge is not affected by light, as it does not contain photosynthetic bacteria.

Finally, the ANOVA of the results of biomass growth percentage was repeated, including the biomass type factor in the analysis to verify if the increase or loss of biomass differs significantly between the three types of biosorbents when metal concentration and other experimental factors vary.

Table 5.4

ANOVA table for multifactor analysis results.

Analysis of Variance for %Growth - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Biomass	3436,04	2	1718,02	48,95	0,0000
B:Concentration	9312,38	2	4656,19	132,67	0,0000
C:OM	8,75014	1	8,75014	0,25	0,6197
D:Light	951,643	1	951,643	27,11	0,0000
INTERACTIONS					
AB	1304,5	4	326,125	9,29	0,0000
AC	60,4628	2	30,2314	0,86	0,4285
AD	1315,25	2	657,623	18,74	0,0000
BC	54,6511	2	27,3256	0,78	0,4643
BD	148,448	2	74,224	2,11	0,1309
CD	4,66142	1	4,66142	0,13	0,7170
RESIDUAL	1825,03	52	35,0967		
TOTAL (CORRECTED)	18421,8	71			

All F-ratios are based on the residual mean square error.

To shed light on which factors and levels produce more growth, the Graphical ANOVA for %Growth will help us (Figure 5.5). To the right, levels increasing %Growth (positive residuals), and in the left levels that reduce %Growth(negative residuals).

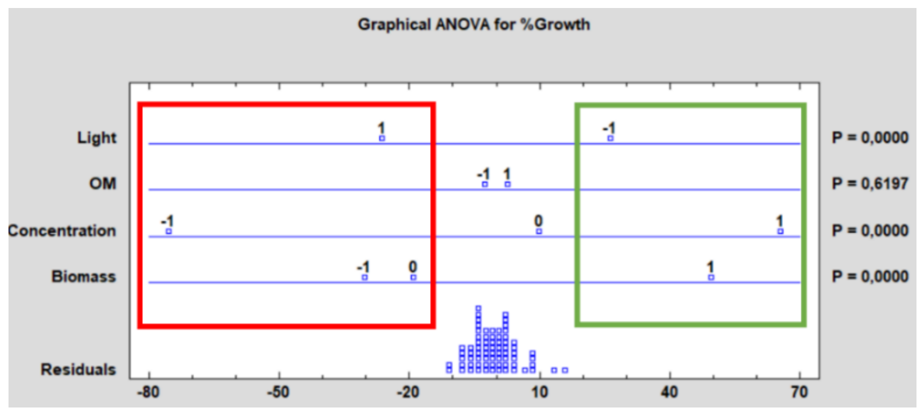


Figure 5.5

Graphical ANOVA for %Growth.

The additional significant effect of biomass type was observed (p<<0.05), indicating a different response of the three biosorbents to copper and zinc concentration.

The ANOVA table, the LSD analysis for the biomass levels, and the Graphical ANOVA for %Growth, demonstrated that:

- Metal concentration, biomass type, light intensity, and the interactions Biomass-Light and Biomass-Concentration are significant.

- The LSD test results (Table A.15 of the Appendix) show that the activated sludge and the pure microalgae have a comparable %Growth, while they significantly differ from the pig slurry biomass, which shows higher %Growth.

- Having no intense light, using the pig slurry biomass, and the highest level concentration of metals, lead the maximum growth of biomass. Both levels of the OM, along with the intermediate metal concentration level, do not produce growth nor decay of the microorganisms

5.2.2 Metal retention capacity

In the following section, we will study how the factors influence heavy metal retention capacity. Table 4.1 shows the different factor levels that are going to be used from now on. It is worth to note that the retention capacity, expressed in mg metal per g of dry biomass, does not depend on the growth of the biomass.
For the analysis, Pareto charts will be used. Pareto charts are a visual way to express an ANOVA table, from which the effects that have a significant influence will be derived. Pareto charts also indicate if the influence is positive or negative towards the dependent variable (in our problem, the retention capacity).

An approach to start is with the global design of experiments, taking into account all the factors (6 factors, 144 experiments). Tables A.1-A.3 in the Appendix show all the combinations of control factors levels and the retention capacity values obtained for each experiment. There will always be two Pareto charts, as we have two response variables, the retention capacity of Cu and the retention capacity of Zn. In the Pareto charts, the vertical blue lines account for the significance level, $\alpha=0.05$; if a factor surpasses that barrier is because its p-value is lower than 0.05 (thus, it is significant).

For the 144 experiments, the Pareto charts are shown in Figure 5.6 and Figure 5.7:

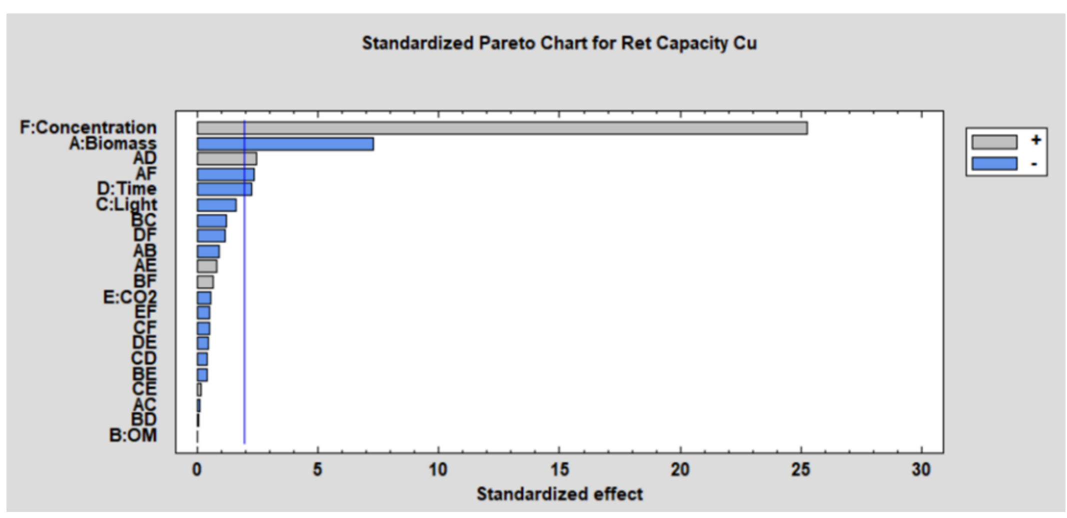


Figure 5.6

Pareto chart for the retention capacity of copper.

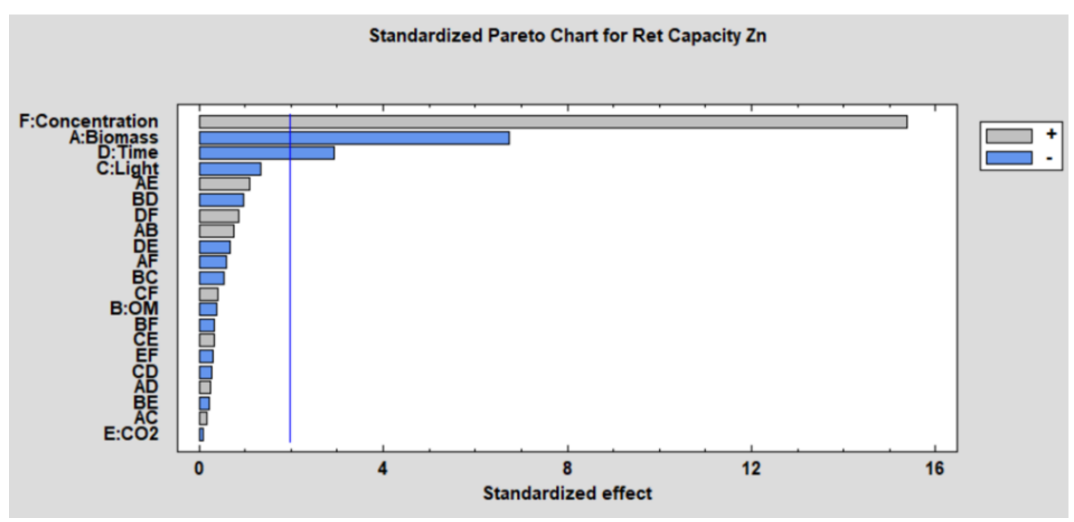


Figure 5.7

Pareto chart for the retention capacity of zinc.

In order to simplify, the p-values of each factor (and the possible interactions) will be displayed in tables. The cells in green will indicate that the effect has a significant influence, and the sign inside the parenthesis will mark whether the effect reduces (negative) or increases (positive) the retention capacity.

The p-values for the five factors assayed and the most relevant interactions are collected in Table 5.5:

Table 5.5

Table of effects for both metals. The negative and positive, refers the kind of influence the effect has on the response.

		Cu	Zn
EFFECTS	Biomass	0.0000 (-)	0.0000 (-)
	OM	No	No
	Light	No	No
	Time	0.0247 (-)	0.0040 (-)
	CO_2	No	No
	Concentration	0.0000 (+)	0.0000(+)
	Biomass-Time	0.0147 (+)	No
	Biomass-Conc	0.0199 (-)	No

No: Factor no significant (p-value > 0.05).

Attending to the results, it is clear that both copper and zinc retention capacities are affected by the type of biomass used, the contact time with the metal solution, and the initial concentration of the metal. Moreover, for copper interactions of some factors with the biomass have resulted also slightly significant.

The combination of factors that gives the optimum retention capacity is displayed in Table 5.6, along with the maximum values of retention capacity for copper and zinc obtained in those conditions:

Table 5.6

Optimum values for Cu^{2+} and Zn^{2+} removal.

Factors	Level	Retention capacity Cu / mg·g^{-1}	Retention capacity Zn / mg·g^{-1}
Concentration	-1	**93.95**	**103.99**
OM	1		
Light	-1		
Time	-1		
CO_2	-1		
Concentration	1		

The polynomial functions that best fits the experimental values for copper and zinc retention capacity were obtained by multivariate linear regression. Therefore, coefficients for each factor will be provided, so if we tune the factor levels, we can predict the retention capacity (q_i) to be obtained.

$$q_{Cu} = 42.5 - 8.1 \cdot B + 0.028 \cdot OM - 1.46 \cdot L - 2.04 \cdot T - 0.49 \cdot IC + 27.8 \cdot C$$

$$q_{Zn} = 60.4 - 11.1 \cdot B + 0.52 \cdot OM - 1.79 \cdot L - 3.94 \cdot T - 0.11 \cdot IC + 25.3 \cdot C$$

In these equations B, OM, L, T, IC and C symbolize, respectively, the factors biomass type, organic matter supplementation, light intensity, contact time, inorganic carbon supplementation as CO_2 and Cu-Zn concentration. The magnitude of the coefficient is related to the contribution of the factor to the response, i.e., with the importance of the effect.

5.2.2.1 Effects of factors by time

As was seen in *Section 5.2.1*, the biomass grows during the 72-hour experiments, at least for the high concentration experiments. Thus, the factor time could be hiding other factor effects. For instance, OM or CO_2 can be depleted before the 72 hours. Therefore, to separate the effect of time from the others, we split the full design into a smaller one, formed by the 72 experiments of one-hour duration (assuming there is no significant biomass growth within that period).

The p-values of the ANOVA are shown below (Table 5.7), and the full table with all the factor level combinations at 1-hour time is shown in the Appendix (Table A.5):

Table 5.7

ANOVA p-values for the factors effects of the 72 experiments of one hour.

	EFFECTS					
	Biomass	OM	Light	CO_2	Conc	Biomass-Conc
Cu	0.0000 (-)	No	No	No	0.0000 (+)	0.0013 (-)
Zn	0.0000 (-)	No	No	No	0.0000 (+)	No

From these results, we conclude that the biomass and the initial concentration of copper and zinc have a significant influence on the retention capacity, and their effect is not conditioned by the time.

To perform a full analysis, we made another six sub-designs, keeping the concentration, and then the biomass constant, in order to see if the factors biomass type or initial metal concentration are also "hiding" the remaining effects. Keeping the concentration constant, the following p-values were obtained using ANOVA (Table 5.8):

Table 5.8

ANOVA p-values of factors at the three different concentrations, and at 1-hour time.

	EFFECTS					
-1	Biomass	OM	Light	CO_2	Biomass-OM	Biomass-Biomass
Cu	0.0000 (-)	No	0.0382 (-)	No	0.0039 (+)	0.0001 (+)
Zn	0.0033 (-)	No	No	No	0.0187 (+)	0.0000 (+)

	EFFECTS					
0	Biomass	OM	Light	CO_2	Biomass-CO2	Biomass-Biomass
Cu	0.0000 (-)	No	No	No	No	0.0000 (+)
Zn	0.0000 (-)	No	No	No	0.0348 (+)	0.0000 (+)

	EFFECTS				
1	Biomass	OM	Light	CO_2	Biomass-Biomass
Cu	0.0000 (-)	No	No	No	0.0001 (+)
Zn	0.0004 (-)	No	No	No	0.0000 (+)

The biomass, and the interaction with itself (something that has not many sense), is the most significant effect when considering only the one-our experiments. This means that no matter what other parameters we tune, the biomass (when the time is kept constant) is going to condition our retention capacity output.

Considering the biomass constant, ANOVA was applied again obtaining the p-values shown in Table 5.9:

Table 5.9

ANOVA p-values of factors at the three different biomasses, and at 1-hour time.

A (-1)	EFFECTS				
	OM	Light	CO_2	Conc	Conc-Conc
Cu	No	No	No	0.0000 (+)	0.0001 (-)
Zn	No	No	No	0.0000 (+)	0.0004 (-)

S (0)	EFFECTS					
	OM	Light	CO_2	Conc	Conc-Conc	OM-Conc
Cu	No	No	No	0.0000 (+)	No	No
Zn	No	No	No	0.0000 (+)	0.0099 (-)	No

P (1)	EFFECTS			
	OM	Light	CO_2	Conc
Cu	No	No	No	0.0000 (+)
Zn	No	No	No	0.0000 (+)

As before, the concentration appears again as the unique effect that influences the response.

5.2.2.2 Effects of factors by concentration level

Now, we are interested in how the factors behave when the concentration is kept constant. That gives three sub-designs of 48 experiments that were interpreted with ANOVA (Table 5.10):

Table 5.10

ANOVA p-values of factors at the three different concentrations.

-1	EFFECTS					
	Biomass	OM	Light	Time	CO_2	Biomass-Light
Cu	0.0000 (-)	No	0.0131 (-)	No	No	0.0207 (+)
Zn	0.0005 (-)	No	No	0.0111 (-)	No	No

0	EFFECTS								
	Biomass	OM	Light	Time	CO_2	Biomass-Time	Biomass-Biomass	Biomass-CO_2	OM-Time
Cu	0.0000 (-)	No	No	0.0011 (-)	No	0.0017 (+)	0.0000 (+)	No	No
Zn	0.0000 (-)	No	No	0.0000 (-)	No	0.0008 (+)	0.0000 (+)	0.0251 (+)	0.0075 (-)

1	EFFECTS							
	Biomass	OM	Light	Time	CO_2	Biomass-OM	Biomass-Time	Biomass-Biomass
Cu	0.0000 (-)	No	No	0.0301 (-)	No	0.0443 (-)	0.0032 (+)	0.0000 (+)
Zn	0.0000 (-)	No	No	No	No	No	No	0.0000 (+)

At all concentration levels, the biomass has a significant and negative influence, which means that the lowest biomass level, labeled A, is providing the highest retention capacity values. Then, time seems to be also a significant factor, although depending on the concentration level, it affects one metal or both. There are plenty of interactions that are not explained easily.

5.2.2.3 Effects of factors by biomass type

Analog reasoning can be done when considering the biomass. To evaluate the influence of the other assayed factors on the retention capacity of each biomass, evaluated individually, new sub-designs were implemented, leaving out the biomass as a factor, so that we will have three identical setups: for pure microalgae, activated sludge, and microalgae-bacteria consortia from the photobioreactor treating pig slurry.

Following the same reasoning, the concentration could be the next factor that influences the most on the response so that we could remove this effect from each of the biomass (we will have eight different Pareto charts for each biomass, four concerning copper, and other four concerning zinc).

With the same data, we can build another type of plot: the mean´s plot, which consists of representing the mean results obtained at each factor level to visualize the variation produced in the Retention Capacity by each factor. As we want to see differences in concentration, we will construct two plots (for the two metal) at the three concentration levels. We calculate the global mean of each biomass, and the corresponding means for the effects at their levels (e.g., the mean of all experiments with no organic matter, at -1 initial concentration of copper). Each graph will have three different horizontal lines (corresponding to each biomass studied), and they will have several lines with a particular slope, the higher the slope, the better the influence of the factor on the retention capacity at that concentration level, and in that biomass.

Thus, we will first see which factors influence the retention capacity, and then with the ANOVA results, we will determine which factors are statistically significant.

However, first of all, the full DoE analysis for each type of biomass will be displayed (Table 5.11):

Table 5.11

ANOVA p-values of the factors influencing the retention capacity in each type of biomass.

	EFFECTS				
A	**OM**	**Light**	**Time**	**CO_2**	**Concentration**
Cu	No	No	0.0870 (-)	No	0.0000 (+)
Zn	No	No	0.0181 (-)	No	0.0000(+)

	EFFECTS								
S	**OM**	**Light**	**Time**	**CO_2**	**Concentration**	**OM-Conc**	**OM-Time**	**Conc-Conc**	**Time-Conc**
Cu	No	No	0.0000 (-)	No	0.0000 (+)	0.0088 (+)	0.0068 (+)	No	0.0000 (-)
Zn	No	No	0.000 0(-)	No	0.0000 (+)	No	No	0.0008 (-)	No

	EFFECTS								
P	**OM**	**Light**	**Time**	**CO_2**	**Conc**	**Conc-Conc**	**Light-Conc**	**OM-Time**	**Time-Conc**
Cu	No	0.0050 (-)	No	No	0.0000 (+)	0.0277 (-)	No	No	0.0096 (+)
Zn	No	No	0.0110 (-)	No	0.0000 (+)	No	No	0.0105 (-)	0.0199 (+)

From these results, some conclusions can be derived:

- For **A**, the contact time and the initial metal concentration are the main parameters conditioning the removal of metal for both zinc and copper.
- For **S**, light does not influence at all (bacteria in the activated sludge do not perform photosynthesis). Both copper and zinc retention capacities are influenced by contact time and OM, which could be related to the metabolism of the biomass. Some interactions appear, but they are not easy to interpret.
- For **P**, we have a bit of controversy. On the one hand, it seems that light influences only the retention capacity of copper, while contact time behaves the same for the retention capacity of zinc. On the other hand, the initial concentration significantly affects the retention capacity of both metals. It is a bit weird that one factor influences exclusively to one metal; more experiments should be done to clarify this situation.
- In all cases, longer contact time shows a negative influence on the retention capacity. This could be due to the living microorganisms retain metals faster at the beginning, but they can become resistant, thus expelling the metals (as they could be harmful at higher concentrations).

Once we have studied the overall factors that affect the retention capacity in each biomass, we can exclude the concentration factor (as it is the only one that is statistically significant in all biomasses), to determine whether that great influence could be hiding other possible factors.

In the Mean´s plots, the thinner lines indicate the global mean of each biomass, while the thicker ones (and darker in their respective color) show how the retention capacity changes when the level of the effect shifts. We will analyze the Graphs (Figure 5.8 and Figure 5.9) and the p-value tables (Table 5.12):

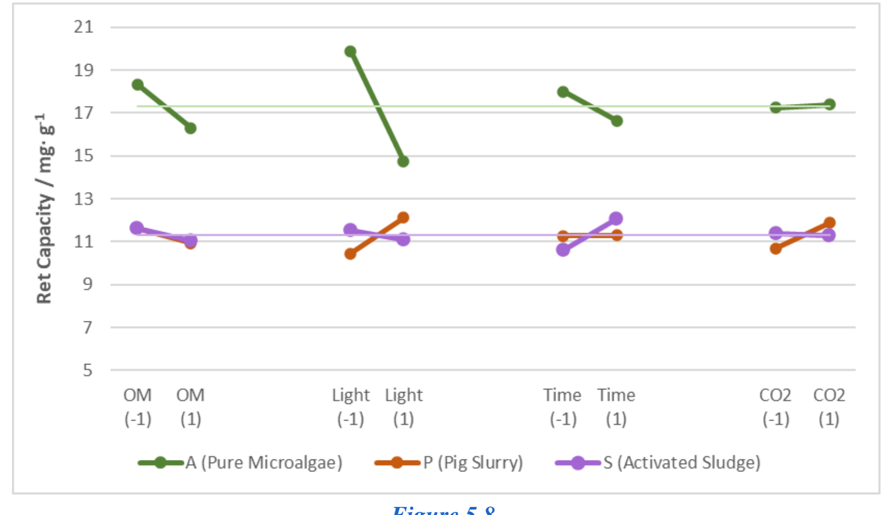


Figure 5.8

Mean´s plot for copper at -1 concentration level.

For copper, the light shows an influence on the retention capacity of pure microalgae, while the activated sludge is unaffected, and the pig slurry biomass shows an opposite behavior than the pure microalgae regarding the light, although it has no much slope. The rest of the factors present almost horizontal lines (that means, no effects on the retention capacity due to the level change). The pure microalgae shows the highest retention capacities.

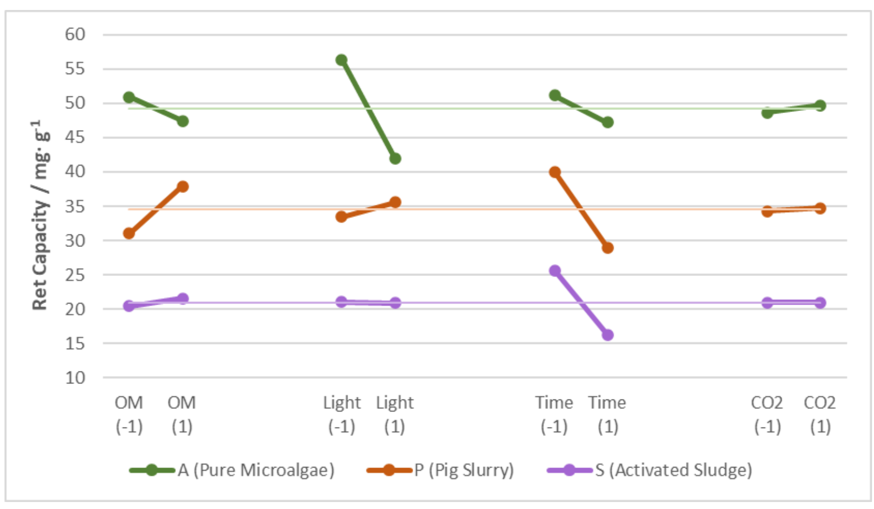


Figure 5.9

Mean´s plot for zinc at -1 concentration level.

For Zn, light is again the most critical parameter for pure microalgae, but it seems that also time is influencing the activated sludge and the pig slurry. The activated sludge gives the lowest values for the retention capacity of Zn, and it seems to be influenced by the OM as well.

To check these observations, we attend to the p-values (Table 5.12):

Table 5.12

p-values for the effects at concentration -1, for each biomass.

	EFFECTS				
A	**OM**	**Light**	**Time**	**CO_2**	**Light-Time**
Cu	No	0.0064 (-)	No	No	No
Zn	No	0.0038 (-)	No	No	0.0381 (-)

	EFFECTS				
S	**OM**	**Light**	**Time**	**CO_2**	**OM-Time**
Cu	No	No	0.0019 (+)	No	No
Zn	0.0207 (+)	No	0.0000 (-)	No	0.0205 (-)

	EFFECTS			
P	**OM**	**Light**	**Time**	**CO_2**
Cu	No	No	No	No
Zn	No	No	0.0170 (-)	No

Attending both Mean´s plot and p-value table, we can conclude that:

- For biomass **A** (microalgae), light has a significant effect on the retention capacity of both copper and zinc, together with an interaction light-time. Moreover, all those effects lead to lower retention capacities for both metals. In other words, the light affects negatively to the removal of metal ions and the interaction light-time (longer times with light, seems to inhibit some retention mechanisms) also has an adverse effect. Despite all of the latter, biomass A presents the highest retention capacity values.

- For biomass **S** (bacteria), time affects both metals (although with distinct sign), while the OM only influences the retention capacity of zinc (as was seen before). Zinc also has a significant OM-Time interaction.

- For biomass **P** (microalgae-bacteria consortium), only time seems to have a significant effect on the retention capacity of zinc, according to its p-value. Moreover, while for the retention capacity of copper, P gives almost identical results as S, in the case of zinc removal, it provides the highest retention capacity values.

We move to the intermediate concentration level, 0, so below are displayed the Mean´s plot (Figure 5.10 and Figure 5.11):

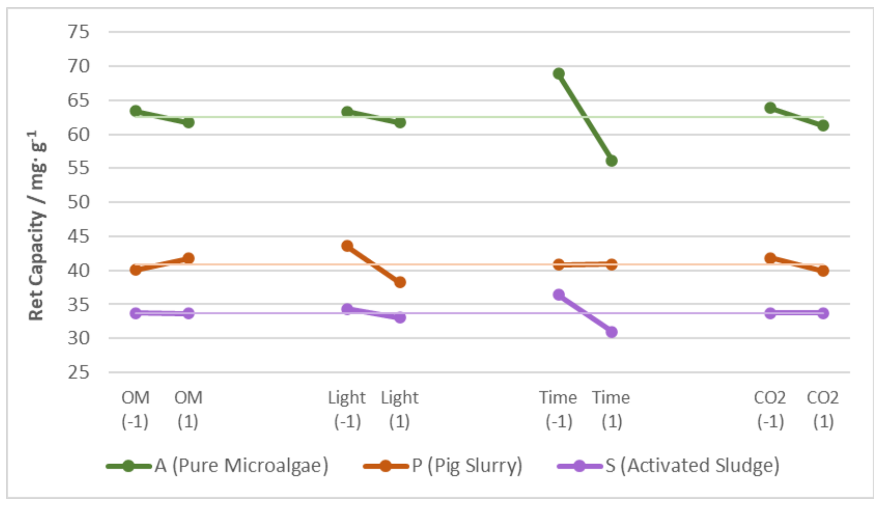


Figure 5.10

Mean´s plot for copper at 0 concentration level.

When the concentration level rises, for copper, the time starts to have a more acute effect in pure microalgae, which remains as the more efficient biomass retaining metals. The light and the remaining effects do not have a significant effect on the retention capacity when changing their concentration levels.

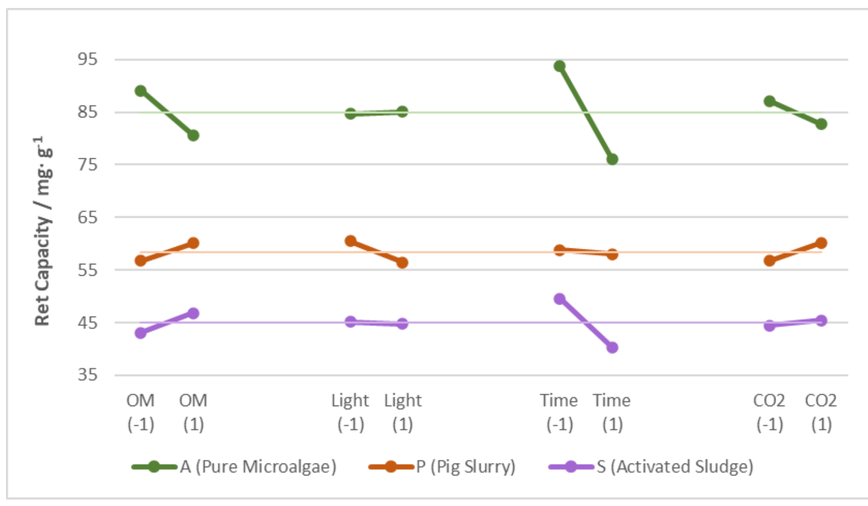


Figure 5.11

Mean´s plot for zinc at 0 concentration level.

For zinc, OM and time are the most important factors for pure microalgae. Light is no longer significant (horizontal line). Maybe Zn^{2+} can be complexed better by the organic matter than Cu^{2+}, and that is the reason why the shift in the OM level influences the retention capacity.

To statistically validate these observations, we attend to the p-value table (Table 5.13):

Table 5.13

p-values for the effects at concentration 0, for each biomass.

	EFFECTS					
A	**OM**	**Light**	**Time**	**CO_2**	**OM-Time**	**OM-Light**
Cu	No	No	0.0030 (-)	No	0.0088 (-)	0.0204 (-)
Zn	0.0421 (-)	No	0.0024 (-)	No	Yes (-)	0.0476 (-)

	EFFECTS					
S	**OM**	**Light**	**Time**	**CO_2**	**Light-CO_2**	**OM-Time**
Cu	No	No	0.0005 (-)	No	0.0401 (-)	0.0008 (+)
Zn	0.0392 (+)	No	0.0010 (-)	No	No	No

	EFFECTS					
P	**OM**	**Light**	**Time**	**CO_2**	**OM-Time**	**OM-Light**
Cu	0.0063 (-)	0.0029 (-)	No	No	0.0062 (-)	No
Zn	No	No	No	0.0254 (+)	0.0052 (-)	No

Attending both Mean´s plot and p-value table, we can conclude that:

- For **A**, the time has a significant effect on the retention capacity of both copper and zinc, and light becomes a negligible effect. The OM significantly influences the retention capacity of zinc, and there is an interaction between OM-Light that affects both retention capacities (although it is rather weak, as it is close to 0.05, our significance level). As for the -1 concentration level, A is the biomass with the highest retention capacity values (both for copper and zinc).

- For **S**, time persists as a significant factor for the retention capacities of copper and zinc. The OM only influences zinc, like in A. Light-CO_2 is another weak interaction. Again, it is the biomass providing the lowest retention capacity values.

- For **P**, we do not have a factor that influences both metals. In contrast, we have that for the removal of copper, the OM, the light, and the interaction OM-Time affects significantly (and negatively), while for zinc only the CO_2 and the OM-Time interaction have an influence.

Finally, we get to the highest concentration level, 1. The Mean´s plots are shown in Figure 5.12 and Figure 5.13, and the p-value tables in Table 5.14:

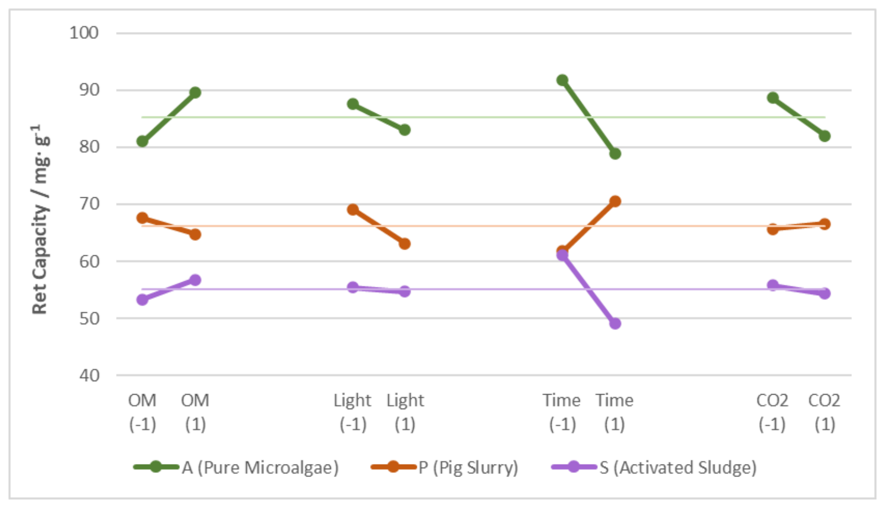


Figure 5.12

Mean´s plot for copper at 1 concentration level.

For copper, contact time seems to be a significant factor for all the biomasses, although its effect is beneficial for the pig slurry biomass (more retention capacity), but detrimental to the other biomasses. For pure microalgae, it seems that OM has an influence not observed until now.

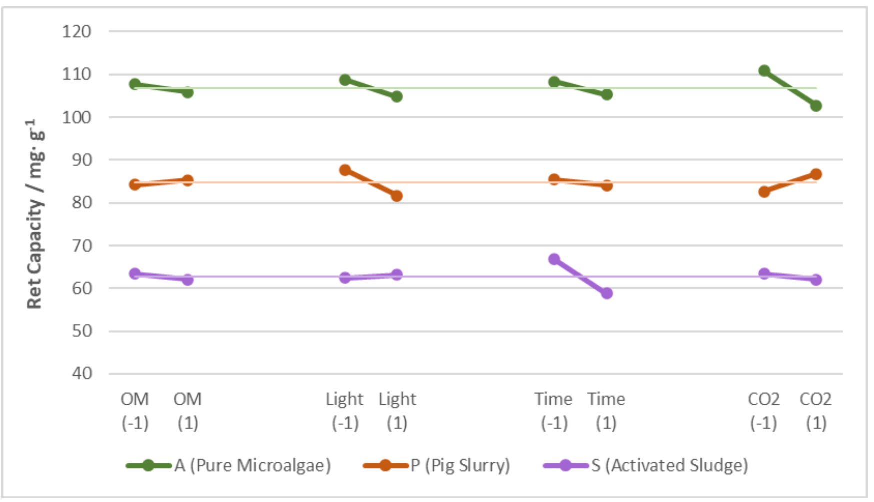


Figure 5.13

Mean´s plot for zinc at 1 concentration level.

Unlike for copper, now it seems that no factor influences the retention capacity towards zinc, all the effect lines are entirely horizontal (except the time for S, and maybe CO_2 for A and P). Nonetheless, the ranking of the biomasses according to their retention capacity remains the same.

Turning to the p-value table (Table 5.14), we obtain:

Table 5.14

p-values for the effects at concentration -1, 0 and 1.

	EFFECTS				
A	**OM**	**Light**	**Time**	**CO_2**	**OM-Time**
Cu	No	No	No	No	No
Zn	No	No	No	No	No

	EFFECTS			
S	**OM**	**Light**	**Time**	**CO_2**
Cu	0.0306 (+)	No	0.0001 (-)	No
Zn	No	No	0.0028 (-)	No

	EFFECTS			
P	**OM**	**Light**	**Time**	**CO_2**
Cu	No	No	No	No
Zn	No	No	No	No

Attending both Mean´s plot and p-value table, we can resolve that:

- For **A**, although the slope for the CO2 had a certain slope, there is no significant factor (at α=0.05).
- For **S**, time persists as a significant factor for the retention capacities of copper and zinc, as well as the OM, that only influences zinc
- For **P**, despite the slopes saw in the light and the CO_2 factors, like for A, there is no significant effect (p-values below 0.05).

Additional conclusions that can be extracted from the Mean´s plots is that for the retention capacity, the factors do not seem to have a considerable influence on it. In fact, most of the time, the lines have no slope (they are entirely horizontal), which means that the biomass is conditioning the retention capacity.

5.3 Isotherms

Paying attention to the data obtained, we realized that there were no enormous variations in values of metal retention capacity of the investigated biomasses despite the different conditions the samples were subjected to. Hence, we tried to fit the retention capacity data to an isotherm. First of all, we need to select an isotherm model (Langmuir is usually the most common), and then we should linearize it, in order to make a quadratic regression model, and thus obtain the critical parameters, the maximum retention capacity (or saturation capacity, $q_{Saturation}$) and the Langmuir constant, K.

$$\frac{c_{eq}}{q_{eq}} = \frac{1}{K \cdot q_{Sat}} + \frac{1}{q_{Sat}} \cdot c_{eq}$$

Plotting $\frac{c_{eq}}{q_{eq}}$ vs c_{eq}, and after the regression analysis, the slope and the intercept are obtained, which allow to estimate the retention capacity of saturation (the maximum amount of metal the biosorbent can take, according to the Langmuir model), and the Langmuir constant K, which informs about the strength of the interaction.

The data used to try to make the isotherms were the experiments of 72-hours. We assumed their retention capacity was q_{eq}. To calculate the concentration of metals in the solution, we applied a mass balance, in which we take into account the initial amount introduced, that must be either in the biosorbent ($q_{eq} \cdot c_{Biosorbent}$) or in the solution ($c_{eq} \cdot V$), the equation we have is shown below:

$$c_o \cdot V = q_{eq} \cdot c_{Biosorbent} + c_{eq} \cdot V$$

Where c_o is the initial concentration of the metal added, V is the solution volume, q_{eq} the experimental value of the metal retention capacity of biomass, and $c_{Biosorbent}$ is the concentration of biomass in the solution after the 72-hour experiment. The calculated c_{eq} values are shown in Tables A.1-A.3 from the Appendix, so the plot building is straightforward.

The isotherm is built as a function of different concentrations, so takings into account the results obtained for the 48 experiments at the same time (72h) that could lead us to an isotherm. Because of the graphs, the linear fit to the experimental data is not satisfactory (R^2 obtained are below 0.8), however, giving the fact that those results are not meant to be applied for an isotherm study, and all data points are different, as they have a distinct combination of factor levels, this lack of fit could be explained to the vast amount of noise the data introduces. Nevertheless, the signs of the Langmuir parameters found are coherent (except for the copper in the pig slurry.

The results are shown in Table 5.15:

Table 5.15

Regression line parameters, and the Langmuir isotherm values for each metal for each biomass.

		Regression parameters		Isotherm Parameters	
		Slope	Intercept	*q sat (mg/g)*	*K (L/mg)*
Pure Microalgae	*Copper*	0,02	0,04	60,24	0,45
	Zinc	0,01	0,06	79,37	0,23
Activated Sludge	*Copper*	0,01	0,62	100,00	0,02
	Zinc	0,02	0,44	48,78	0,05
Pig Slurry	*Copper*	**-0,0088**	**0,90**	**-113,64**	**-0,01**
	Zinc	0,0066	0,41	151,52	0,02

As an example, Figure 5.14 and Figure 5.15 present the plot for copper and zinc for the pure microalgae. The other graphs are displayed in the Appendix (Figures A.1-A.5):

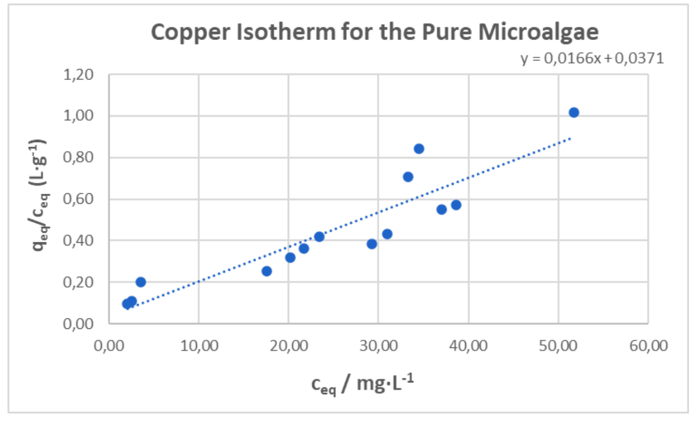


Figure 5.14

Copper isotherm (q_{eq}/c_{eq} versus c_{eq}).

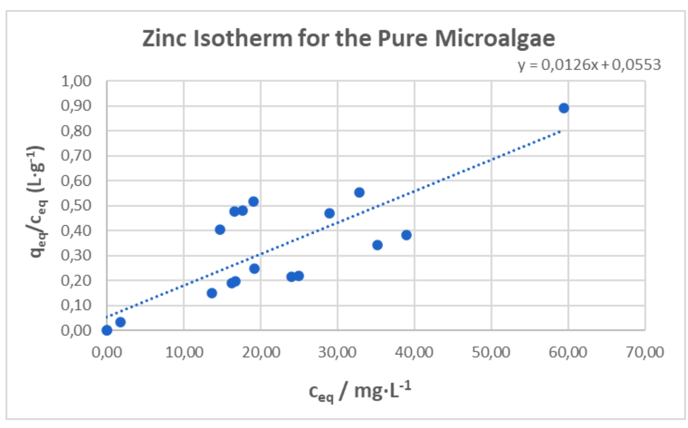


Figure 5.15

Zinc isotherm (q_{eq}/c_{eq} versus c_{eq}).

The red values appearing in the pig slurry that may point biosorption is not the primary mechanism, additional mechanisms are taking place, and thus it does not follow the isotherm properly. To visualize why the Langmuir parameters should be both positive, Desmos (https://www.desmos.com/calculator/r66ffma58p) will shed some light. When K has a positive value, we have a function with a positive image, while when it acquires negative values, we obtain a more complex function. To build up these plots, the value for the tables above, q_{sat} = 113.64 and K=0.01 were used, and then we construct the plot with those values but negative (Figure 5.16 and Figure 5.17, respectively):

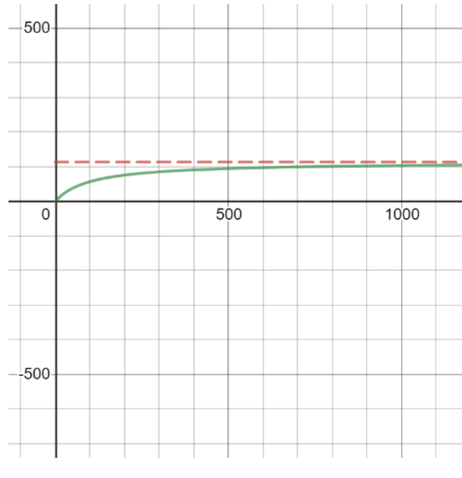


Figure 5.16

Representation of the function (q_{eq} vs c_{eq}) when q_{sat} = 113.64 and K = 0.01. For Cu in pig slurry.

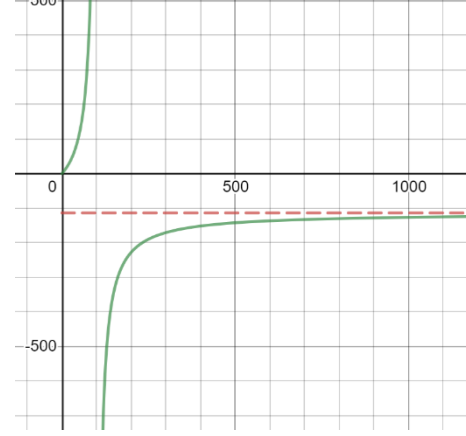


Figure 5.17

Representation of the function (q_{eq} vs c_{eq}) when q_{sat} = -113.64 and K = -0.01. For Cu in pig slurry.

The dotted red line is the horizontal asymptote whose value is $y = q_{sat}$. When increasing the q_{sat}, the asymptote will increase, which means that more adsorbate will be adsorbed; therefore, it makes no physical sense, it acquires a negative value. The K parameter tune how much concentration is needed to reach that limit (and a negative value will mean that there is no interaction).

6 CONCLUSIONS

Taking all the above into account, we can conclude several facts:

- ✓ First of all, an extensive review of biosorption experiments on zinc and copper removal was done. Some of the most used parameters are the same chosen for the design of experiments, such as the pH range between 4-6, or biomass concentration of 1 g/L. A couple of the most promising experiments was highlighted, giving values for the removal percentage of the ions between 80 and almost 100 percent.

- ✓ Turning to the experimental section, the effect of six factors on the retention capacity of biomass towards copper and zinc has been investigated: biomass type (pure microalgae Scenedesmus *Almeriensis*, activated sludge containing bacteria and microalgae-bacteria consortium cultivated in a photoreactor treating pig sludge), the concentration of bimetallic solutions of copper and zinc (three levels), contact time (1 and 72 h), light intensity (ambient and LED lamps) and supplementation of organic matter (0 and 80 mg/L) and CO_2 (no or yes) have been assayed using a complete factorial experimental design with 144 experiments.

- ✓ The type of biomass and the metal concentration level are the factors that most influence the retention capacity of both metals. According to the ANOVA results and the mean´s plot, the pure microalgae is the biomass that provides the highest retention capacity values, while maximum retention capacity was also achieved when using the highest metal concentration levels. As in real wastewater samples, the concentration would be a noise factor, the most critical parameter to select for treating wastewater polluted with copper and zinc is the biomass; in our case, the one that provided the best results was the pure microalgae (a *Scenedesmus Almeriensis* strain).

- ✓ The biomass grows better in the presence of high metal concentration. In other words, the range of concentrations used does not have harmful effects on the biomasses, or at least it does not reduce their retention capacities.

- ✓ It is highly probable that some retention will take place in the inorganic part of the biomass or that some metal precipitation occurs, as due to the biomass characterization, the content of organic matter was rather low so to explain those high values for the retention capacity the inorganic contribution can help. Thus, as we are not sure if the process is exclusively biosorption, it would be more appropriate to call the process "retention" or "removal" of metals. This is only a hypothesis that should be checked in future research with new experiments, such as metal speciation, to uncover the chemical forms in which copper and zinc are present after the contact time.

- ✓ The Langmuir isotherm did not provide very conclusive results for the pig slurry data of retention capacity, although it seems to fit well with the pure microalgae and the activated sludge data.

- ✓ From the mean´s plot, we can also conclude that the pig slurry is more similar (in terms of retention capacity) to the activated sludge than to the pure microalgae. This may be incongruent from a biological point of view, as the purine has more percentage of microalgae than of bacteria. However, the results point in that direction. Maybe an explanation could be that the pure microalgae uses some retention mechanisms when it is alone, but in consortia with bacteria, the interactions between the two microorganisms modify their behavior towards heavy metals.

Therefore, promising results are hoped to be obtained by using the pure microalgae biomass, with the combination of factor levels as follows: 1h contact time, OM supplement, and preventing intense light nor CO_2 addition. That combination should be first assayed in real wastewaters, in order to see whether it works or not, and then another design must be planned, in order to optimize the process in wastewaters.

APPENDIX

Table A.1

Design of experiments for the pure microalgae (A). All the combination levels along with the retention capacity results of copper and zinc.

Label	Biomass	OM	Light	Time	CO_2	Concentration	Ret Cap Cu / $mg \cdot g^{-1}$	Ret Cap Zn / $mg \cdot g^{-1}$
A1	-1	-1	-1	-1	-1	-1	18,60	52,71
A2	-1	-1	-1	-1	-1	0	69,53	99,65
A3	-1	-1	-1	-1	-1	1	86,83	103,29
A4	-1	-1	-1	-1	1	-1	24,61	67,01
A5	-1	-1	-1	-1	1	0	55,36	79,65
A6	-1	-1	-1	-1	1	1	90,58	104,21
A7	-1	-1	1	-1	-1	-1	19,02	52,57
A8	-1	-1	1	-1	-1	0	65,44	98,68
A9	-1	-1	1	-1	-1	1	94,39	113,65
A10	-1	-1	1	-1	1	-1	18,25	49,65
A11	-1	-1	1	-1	1	0	68,84	97,31
A12	-1	-1	1	-1	1	1	93,88	110,31
A13	-1	1	-1	-1	-1	-1	16,52	46,16
A14	-1	1	-1	-1	-1	0	79,70	102,11
A15	-1	1	-1	-1	-1	1	94,22	110,89
A16	-1	1	-1	-1	1	-1	16,91	51,69
A17	-1	1	-1	-1	1	0	78,32	99,24
A18	-1	1	-1	-1	1	1	96,89	113,22
A19	-1	1	1	-1	-1	-1	13,06	39,19
A20	-1	1	1	-1	-1	0	65,05	84,06
A21	-1	1	1	-1	-1	1	90,44	108,19
A22	-1	1	1	-1	1	-1	17,07	50,35
A23	-1	1	1	-1	1	0	68,91	88,61
A24	-1	1	1	-1	1	1	86,27	102,46
A25	-1	-1	-1	1	-1	-1	21,27	60,92
A26	-1	-1	-1	1	-1	0	60,21	83,90
A27	-1	-1	-1	1	-1	1	71,55	112,14
A28	-1	-1	-1	1	1	-1	17,96	53,57
A29	-1	-1	-1	1	1	0	55,67	77,41
A30	-1	-1	-1	1	1	1	67,09	102,67
A31	-1	-1	1	1	-1	-1	13,95	34,77
A32	-1	-1	1	1	-1	0	68,90	91,16
A33	-1	-1	1	1	-1	1	67,66	101,28
A34	-1	-1	1	1	1	-1	13,03	36,19
A35	-1	-1	1	1	1	0	63,09	85,09
A36	-1	-1	1	1	1	1	76,16	113,80
A37	-1	1	-1	1	-1	-1	23,73	66,23
A38	-1	1	-1	1	-1	0	60,84	75,90
A39	-1	1	-1	1	-1	1	99,18	115,47
A40	-1	1	-1	1	1	-1	19,56	52,57
A41	-1	1	-1	1	1	0	46,94	59,50
A42	-1	1	-1	1	1	1	94,09	108,15
A43	-1	1	1	1	-1	-1	11,83	36,63
A44	-1	1	1	1	-1	0	40,96	61,38
A45	-1	1	1	1	-1	1	104,52	121,65
A46	-1	1	1	1	1	-1	11,85	36,71
A47	-1	1	1	1	1	0	52,81	74,47
A48	-1	1	1	1	1	1	50,88	66,58

Table A.2

Design of experiments for the activated sludge (S). All the combination levels along with the retention capacity results of copper and zinc.

The values in **red** were experiments that went wrong, so their values were not correct. In order to perform the DoE analysis, we average experiments with similar conditions, as due to COVID-19 pandemic, we could not repeat those experiments.

Label	Biomass	OM	Light	Time	CO_2	Concentration	Ret Cap Cu / $mg \cdot g^{-1}$	Ret Cap Zn / $mg \cdot g^{-1}$
S1	0	-1	-1	-1	-1	-1	11,02	24,67
S2	0	-1	-1	-1	-1	0	39,62	49,08
S3	0	-1	-1	-1	-1	1	59,97	69,24
S4	0	-1	-1	-1	1	-1	11,86	25,21
S5	0	-1	-1	-1	1	0	39,71	49,74
S6	0	-1	-1	-1	1	1	60,37	68,27
S7	0	-1	1	-1	-1	-1	11,38	24,32
S8	0	-1	1	-1	-1	0	38,12	47,57
S9	0	-1	1	-1	-1	1	57,77	67,28
S10	0	-1	1	-1	1	-1	10,36	23,90
S11	0	-1	1	-1	1	0	37,76	47,58
S12	0	-1	1	-1	1	1	58,56	68,43
S13	0	1	-1	-1	-1	-1	9,88	26,19
S14	0	1	-1	-1	-1	0	34,22	50,32
S15	0	1	-1	-1	-1	1	62,07	60,87
S16	0	1	-1	-1	1	-1	10,22	27,05
S17	0	1	-1	-1	1	0	35,77	52,14
S18	0	1	-1	-1	1	1	61,80	61,27
S19	0	1	1	-1	-1	-1	10,42	27,95
S20	0	1	1	-1	-1	0	35,66	53,73
S21	0	1	1	-1	-1	1	67,71	73,52
S22	0	1	1	-1	1	-1	9,62	26,11
S23	0	1	1	-1	1	0	30,31	46,19
S24	0	1	1	-1	1	1	59,92	64,99
S25	0	-1	-1	1	-1	-1	12,52	16,52
S26	0	-1	-1	1	-1	0	27,51	36,39
S27	0	-1	-1	1	-1	1	47,79	58,49
S28	0	-1	-1	1	1	-1	12,40	16,42
S29	0	-1	-1	1	1	0	30,04	38,78
S30	0	-1	-1	1	1	1	48,00	60,94
S31	0	-1	1	1	-1	-1	11,59	15,73
S32	0	-1	1	1	-1	0	29,65	38,92
S33	0	-1	1	1	-1	1	47,51	57,47
S34	0	-1	1	1	1	-1	11,76	16,58
S35	0	-1	1	1	1	0	27,40	36,55
S36	0	-1	1	1	1	1	46,87	57,17
S37	0	1	-1	1	-1	-1	12,46	16,47
S38	0	1	-1	1	-1	0	32,40	39,06
S39	0	1	-1	1	-1	1	53,74	62,03
S40	0	1	-1	1	1	-1	11,99	16,08
S41	0	1	-1	1	1	0	35,65	45,51
S42	0	1	-1	1	1	1	49,40	57,90
S43	0	1	1	1	-1	-1	11,68	16,16
S44	0	1	1	1	-1	0	32,49	40,64
S45	0	1	1	1	-1	1	49,77	58,37
S46	0	1	1	1	1	-1	12,11	16,53
S47	0	1	1	1	1	0	33,07	46,72
S48	0	1	1	1	1	1	49,61	57,99

Table A.3

Design of experiments for the pig slurry (P). All the combination levels along with the retention capacity results of copper and zinc.

Label	Biomass	OM	Light	Time	CO_2	Concentration	Ret Cap Cu / $mg \cdot g^{-1}$	Ret Cap Zn / $mg \cdot g^{-1}$
P1	1	-1	-1	-1	-1	-1	**10,82**	**33,38**
P2	1	-1	-1	-1	-1	0	**42,85**	**56,31**
P3	1	-1	-1	-1	-1	1	**72,15**	**95,14**
P4	1	-1	-1	-1	1	-1	**13,75**	**52,67**
P5	1	-1	-1	-1	1	0	**46,90**	**65,71**
P6	1	-1	-1	-1	1	1	**52,54**	**70,39**
P7	1	-1	1	-1	-1	-1	**8,18**	**24,87**
P8	1	-1	1	-1	-1	0	**36,08**	**47,00**
P9	1	-1	1	-1	-1	1	**57,64**	**68,80**
P10	1	-1	1	-1	1	-1	**9,45**	**33,37**
P11	1	-1	1	-1	1	0	**37,46**	**51,29**
P12	1	-1	1	-1	1	1	**72,81**	**96,45**
P13	1	1	-1	-1	-1	-1	**13,95**	**49,93**
P14	1	1	-1	-1	-1	0	**41,67**	**58,19**
P15	1	1	-1	-1	-1	1	**68,79**	**94,80**
P16	1	1	-1	-1	1	-1	**10,49**	**39,50**
P17	1	1	-1	-1	1	0	**43,22**	**69,84**
P18	1	1	-1	-1	1	1	**59,10**	**89,07**
P19	1	1	1	-1	-1	-1	**13,15**	**49,40**
P20	1	1	1	-1	-1	0	**37,83**	**53,22**
P21	1	1	1	-1	-1	1	**51,23**	**71,84**
P22	1	1	1	-1	1	-1	**10,45**	**37,11**
P23	1	1	1	-1	1	0	**40,63**	**69,06**
P24	1	1	1	-1	1	1	**60,00**	**96,71**
P25	1	-1	-1	1	-1	-1	**9,23**	**26,13**
P26	1	-1	-1	1	-1	0	**46,34**	**60,01**
P27	1	-1	-1	1	-1	1	**71,52**	**85,35**
P28	1	-1	-1	1	1	-1	**5,10**	**14,45**
P29	1	-1	-1	1	1	0	**48,03**	**65,58**
P30	1	-1	-1	1	1	1	**81,50**	**96,33**
P31	1	-1	1	1	-1	-1	**10,02**	**26,10**
P32	1	-1	1	1	-1	0	**44,44**	**66,23**
P33	1	-1	1	1	-1	1	**70,34**	**87,03**
P34	1	-1	1	1	1	-1	**9,41**	**23,59**
P35	1	-1	1	1	1	0	**43,30**	**65,69**
P36	1	-1	1	1	1	1	**63,27**	**79,45**
P37	1	1	-1	1	-1	-1	**10,16**	**25,07**
P38	1	1	-1	1	-1	0	**38,73**	**52,08**
P39	1	1	-1	1	-1	1	**72,24**	**84,34**
P40	1	1	-1	1	1	-1	**10,15**	**26,58**
P41	1	1	-1	1	1	0	**40,74**	**55,74**
P42	1	1	-1	1	1	1	**75,32**	**86,47**
P43	1	1	1	1	-1	-1	**8,59**	**19,51**
P44	1	1	1	1	-1	0	**33,81**	**50,49**
P45	1	1	1	1	-1	1	**57,56**	**69,81**
P46	1	1	1	1	1	-1	**10,17**	**22,45**
P47	1	1	1	1	1	0	**37,54**	**51,94**
P48	1	1	1	1	1	1	**60,58**	**75,34**

Table A.4

Combination of factor and their levels for the 72-hour experiments (to study the growth of the biomass).

Label	Biomass	OM	Light	CO_2	Concentration	Ret Cap Cu / mg·g^{-1}	Ret Cap Zn / mg·g^{-1}	%Growth
A25	-1	-1	-1	-1	-1	21,27	60,92	**84,8**
A26	-1	-1	-1	-1	0	60,21	83,90	**74,3**
A27	-1	-1	-1	-1	1	71,55	112,14	**84,9**
A28	-1	-1	-1	1	-1	17,96	53,57	**89,3**
A29	-1	-1	-1	1	0	55,67	77,41	**85,5**
A30	-1	-1	-1	1	1	67,09	102,67	**94,3**
A31	-1	-1	1	-1	-1	13,95	34,77	**96,0**
A32	-1	-1	1	-1	0	68,90	91,16	**92,8**
A33	-1	-1	1	-1	1	67,66	101,28	**99,6**
A34	-1	-1	1	1	-1	13,03	36,19	**103,9**
A35	-1	-1	1	1	0	63,09	85,09	**116,0**
A36	-1	-1	1	1	1	76,16	113,80	**96,6**
A37	-1	1	-1	-1	-1	23,73	66,23	**90,4**
A38	-1	1	-1	-1	0	60,84	75,90	**79,2**
A39	-1	1	-1	-1	1	99,18	115,47	**82,5**
A40	-1	1	-1	1	-1	19,56	52,57	**84,3**
A41	-1	1	-1	1	0	46,94	59,50	**96,2**
A42	-1	1	-1	1	1	94,09	108,15	**99,4**
A43	-1	1	1	-1	-1	11,83	36,63	**103,8**
A44	-1	1	1	-1	0	40,96	61,38	**87,5**
A45	-1	1	1	-1	1	104,52	121,65	**94,9**
A46	-1	1	1	1	-1	11,85	36,71	**96,8**
A47	-1	1	1	1	0	52,81	74,47	**90,8**
A48	-1	1	1	1	1	50,88	66,58	**101,5**
S25	0	-1	-1	-1	-1	12,52	16,52	**84,3**
S26	0	-1	-1	-1	0	27,51	36,39	**82,0**
S27	0	-1	-1	-1	1	47,79	58,49	**81,5**
S28	0	-1	-1	1	-1	12,40	16,42	**80,6**
S29	0	-1	-1	1	0	30,04	38,78	**88,6**
S30	0	-1	-1	1	1	48,00	60,94	**91,2**
S31	0	-1	1	-1	-1	11,59	15,73	**94,5**
S32	0	-1	1	-1	0	29,65	38,92	**98,0**
S33	0	-1	1	-1	1	47,51	57,47	**112,7**
S34	0	-1	1	1	-1	11,76	16,58	**109,4**
S35	0	-1	1	1	0	27,40	36,55	**114,7**
S36	0	-1	1	1	1	46,87	57,17	**110,9**
S37	0	1	-1	-1	-1	12,46	16,47	**78,7**
S38	0	1	-1	-1	0	32,40	39,06	**79,0**
S39	0	1	-1	-1	1	53,74	62,03	**85,2**
S40	0	1	-1	1	-1	11,99	16,08	**78,1**
S41	0	1	-1	1	0	35,65	45,51	**92,6**
S42	0	1	-1	1	1	49,40	57,90	**91,0**
S43	0	1	1	-1	-1	11,68	16,16	**93,8**
S44	0	1	1	-1	0	32,49	40,64	**93,2**
S45	0	1	1	-1	1	49,77	58,37	**110,9**
S46	0	1	1	1	-1	12,11	16,53	**108,3**
S47	0	1	1	1	0	33,07	46,72	**108,8**
S48	0	1	1	1	1	49,61	57,99	**110,3**
P25	1	-1	-1	-1	-1	9,23	26,13	**90,2**
P26	1	-1	-1	-1	0	46,34	60,01	**101,0**
P27	1	-1	-1	-1	1	71,52	85,35	**90,8**
P28	1	-1	-1	1	-1	5,10	14,45	**82,0**
P29	1	-1	-1	1	0	48,03	65,58	**133,1**
P30	1	-1	-1	1	1	81,50	96,33	**123,3**
P31	1	-1	1	-1	-1	10,02	26,10	**141,3**
P32	1	-1	1	-1	0	44,44	66,23	**121,5**
P33	1	-1	1	-1	1	70,34	87,03	**133,0**
P34	1	-1	1	1	-1	9,41	23,59	**139,3**
P35	1	-1	1	1	0	43,30	65,69	**130,9**
P36	1	-1	1	1	1	63,27	79,45	**129,8**
P37	1	1	-1	-1	-1	10,16	25,07	**82,8**
P38	1	1	-1	-1	0	38,73	52,08	**84,4**
P39	1	1	-1	-1	1	72,24	84,34	**82,5**
P40	1	1	-1	1	-1	10,15	26,58	**79,7**
P41	1	1	-1	1	0	40,74	55,74	**94,3**
P42	1	1	-1	1	1	75,32	86,47	**99,0**
P43	1	1	1	-1	-1	8,59	19,51	**103,3**
P44	1	1	1	-1	0	33,81	50,49	**100,5**
P45	1	1	1	-1	1	57,56	69,81	**122,9**
P46	1	1	1	1	-1	10,17	22,45	**108,1**
P47	1	1	1	1	0	37,54	51,94	**111,3**
P48	1	1	1	1	1	60,58	75,34	**115,3**

Table A.5

Combination of factor and their levels for the 1-hour experiments.

Label	Biomass	OM	Light	CO_2	Concentration	Cap Ret Cu	Cap Ret Zn
A1	-1	-1	-1	-1	-1	18,60	52,71
A2	-1	-1	-1	-1	0	69,53	99,65
A3	-1	-1	-1	-1	1	86,83	103,29
A4	-1	-1	-1	1	-1	24,61	67,01
A5	-1	-1	-1	1	0	55,36	79,65
A6	-1	-1	-1	1	1	90,58	104,21
A7	-1	-1	1	-1	-1	19,02	52,57
A8	-1	-1	1	-1	0	65,44	98,68
A9	-1	-1	1	-1	1	94,39	113,65
A10	-1	-1	1	1	-1	18,25	49,65
A11	-1	-1	1	1	0	68,84	97,31
A12	-1	-1	1	1	1	93,88	110,31
A13	-1	1	-1	-1	-1	16,52	46,16
A14	-1	1	-1	-1	0	79,70	102,11
A15	-1	1	-1	-1	1	94,22	110,89
A16	-1	1	-1	1	-1	16,91	51,69
A17	-1	1	-1	1	0	78,32	99,24
A18	-1	1	-1	1	1	96,89	113,22
A19	-1	1	1	-1	-1	13,06	39,19
A20	-1	1	1	-1	0	65,05	84,06
A21	-1	1	1	-1	1	90,44	108,19
A22	-1	1	1	1	-1	17,07	50,35
A23	-1	1	1	1	0	68,91	88,61
A24	-1	1	1	1	1	86,27	102,46
S1	0	-1	-1	-1	-1	11,02	24,67
S2	0	-1	-1	-1	0	39,62	49,08
S3	0	-1	-1	-1	1	59,97	69,24
S4	0	-1	-1	1	-1	11,86	25,21
S5	0	-1	-1	1	0	39,71	49,74
S6	0	-1	-1	1	1	60,37	68,27
S7	0	-1	1	-1	-1	11,38	24,32
S8	0	-1	1	-1	0	38,12	47,57
S9	0	-1	1	-1	1	57,77	67,28
S10	0	-1	1	1	-1	10,36	23,90
S11	0	-1	1	1	0	37,76	47,58
S12	0	-1	1	1	1	58,56	68,43
S13	0	1	-1	-1	-1	9,88	26,19
S14	0	1	-1	-1	0	34,22	50,32
S15	0	1	-1	-1	1	62,07	60,87
S16	0	1	-1	1	-1	10,22	27,05
S17	0	1	-1	1	0	35,77	52,14
S18	0	1	-1	1	1	61,80	61,27
S19	0	1	1	-1	-1	10,42	27,95
S20	0	1	1	-1	0	35,66	53,73
S21	0	1	1	-1	1	67,71	73,52
S22	0	1	1	1	-1	9,62	26,11
S23	0	1	1	1	0	30,31	46,19
S24	0	1	1	1	1	59,92	64,99
P1	1	-1	-1	-1	-1	10,82	33,38
P2	1	-1	-1	-1	0	42,85	56,31
P3	1	-1	-1	-1	1	72,15	95,14
P4	1	-1	-1	1	-1	13,75	52,67
P5	1	-1	-1	1	0	46,90	65,71
P6	1	-1	-1	1	1	52,54	70,39
P7	1	-1	1	-1	-1	8,18	24,87
P8	1	-1	1	-1	0	36,08	47,00
P9	1	-1	1	-1	1	57,64	68,80
P10	1	-1	1	1	-1	9,45	33,37
P11	1	-1	1	1	0	37,46	51,29
P12	1	-1	1	1	1	72,81	96,45
P13	1	1	-1	-1	-1	13,95	49,93
P14	1	1	-1	-1	0	41,67	58,19
P15	1	1	-1	-1	1	68,79	94,80
P16	1	1	-1	1	-1	10,49	39,50
P17	1	1	-1	1	0	43,22	69,84
P18	1	1	-1	1	1	59,10	89,07
P19	1	1	1	-1	-1	13,15	49,40
P20	1	1	1	-1	0	37,83	53,22
P21	1	1	1	-1	1	51,23	71,84
P22	1	1	1	1	-1	10,45	37,11
P23	1	1	1	1	0	40,63	69,06
P24	1	1	1	1	1	60,00	96,71

Statistical tables from the Growth experiments

One-way ANOVA tables are shown for each type of biomass:

Table A.6

One-way ANOVA for pure microalgae.

One-Way ANOVA for concentration (Pure)						
Source of variations	**SS**	**DoF**	**MS**	**F_{calc}**	**p-value**	**F_{crit}**
Concentration	1092,21	2	546,10	**13,59**	**1,63E-04**	**3,47**
Residual	843,78	21	40,18			

Table A.7

One-way ANOVA for the activated sludge.

One-Way ANOVA for concentration (Sludge)						
Source of variations	**SS**	**DoF**	**MS**	**F_{calc}**	**p-value**	**F_{crit}**
Concentration	3547,39	2	1773,70	**281,26**	**6,91E-16**	**3,47**
Residual	132,43	21	6,31			

Table A.8

One-way ANOVA for the pig slurry.

One-Way ANOVA for concentration (Pig slurry)						
Source of variations	**SS**	**DoF**	**MS**	**F_{calc}**	**p-value**	**F_{crit}**
Concentration	5978,18	2	2989,09	**18,50**	**2,33E-05**	**3,47**
Residual	3392,37	21	161,54			

LSD values are displayed in the following tables:

Table A.9

LSD values for the concentration in the pure microalgae.

Contrast	Sig.	Difference	+/- Limits
-1 - 0	*	-10,695	6,59111
-1 - 1	*	-16,255	6,59111
0 - 1		-5,56	6,59111

* denotes a statistically significant difference.

Table A.10

LSD values for the concentration in the activated sludge

Contrast	Sig.	Difference	+/- Limits
-1 - 0	*	-11,68	2,61298
-1 - 1	*	-29,565	2,61298
0 - 1	*	-17,885	2,61298

* denotes a statistically significant difference.

Table A.11

LSD values for the concentration in the pig slurry

Contrast	Sig.	Difference	+/- Limits
-1 - 0	*	-27,8589	13,2159
-1 - 1	*	-37,1419	13,2159
0 - 1		-9,28301	13,2159

* denotes a statistically significant difference.

Multifactor ANOVA tables for each type of biomass (considering the remaining parameters: concentration, OM, and light):

Table A.12

Multifactor ANOVA table for the pure microalgae.

Analysis of Variance for %Growth_1 - Type III Sums of Squares

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
MAIN EFFECTS					
A:Concentration	1092,06	2	546,029	13,38	0,0006
B:OM	29,8597	1	29,8597	0,73	0,4068
C:Light	4,94134	1	4,94134	0,12	0,7331
INTERACTIONS					
AB	3,69003	2	1,84502	0,05	0,9559
AC	168,338	2	84,169	2,06	0,1641
BC	65,5051	1	65,5051	1,60	0,2259
RESIDUAL	571,446	14	40,8176		
TOTAL (CORRECTED)	1935,84	23			

All F-ratios are based on the residual mean square error.

Table A.13

Multifactor ANOVA table for the activated sludge.

Analysis of Variance for %Growth_1 - Type III Sums of Squares

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
MAIN EFFECTS					
A:Concentration	3547,69	2	1773,85	311,42	0,0000
B:OM	18,1656	1	18,1656	3,19	0,0958
C:Light	14,446	1	14,446	2,54	0,1336
INTERACTIONS					
AB	15,8461	2	7,92305	1,39	0,2812
AC	4,00093	2	2,00047	0,35	0,7099
BC	0,410817	1	0,410817	0,07	0,7922
RESIDUAL	79,7427	14	5,69591		
TOTAL (CORRECTED)	3680,31	23			

All F-ratios are based on the residual mean square error.

Table A.14

Multifactor ANOVA table for the pig slurry.

Analysis of Variance for %Growth - Type III Sums of Squares

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
MAIN EFFECTS					
A:Concentration	5977,13	2	2988,56	82,72	0,0000
B:OM	21,1876	1	21,1876	0,59	0,4565
C:Light	2247,5	1	2247,5	62,21	0,0000
INTERACTIONS					
AB	114,574	2	57,2868	1,59	0,2395
AC	478,483	2	239,241	6,62	0,0095
BC	24,9492	1	24,9492	0,69	0,4199
RESIDUAL	505,805	14	36,1289		
TOTAL (CORRECTED)	9369,63	23			

All F-ratios are based on the residual mean square error.

Finally, the LSD test for the biomass levels is shown in the following table:

Table A.15

LSD of the different levels considering the biomass factor.

Multiple Range Tests for %Growth by Biomass

Method: 95,0 percent LSD

Biomass	Count	LS Mean	LS Sigma	Homogeneous Groups
-1	24	92,7271	1,20928	X
0	24	94,9183	1,20928	X
1	24	108,354	1,20928	X

Contrast	Sig.	Difference	+/- Limits
-1 - 0		-2,19125	3,43174
-1 - 1	*	-15,6267	3,43174
0 - 1	*	-13,4354	3,43174

* denotes a statistically significant difference.

Isotherms graphs

To end the Appendix, the remaining graphs plotting q_{eq}/c_{eq} versus c_{eq} are displayed (Figures A.1-A.4):

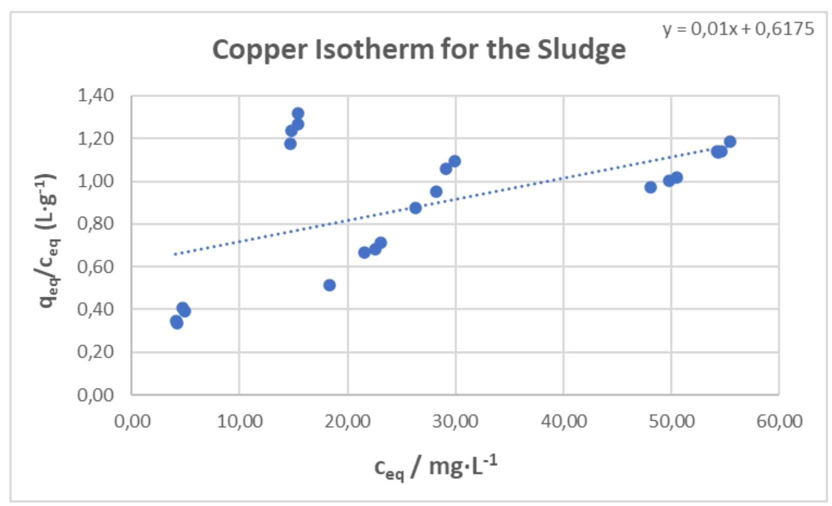


Figure A.1

Copper isotherm (q_{eq}/c_{eq} versus c_{eq}).

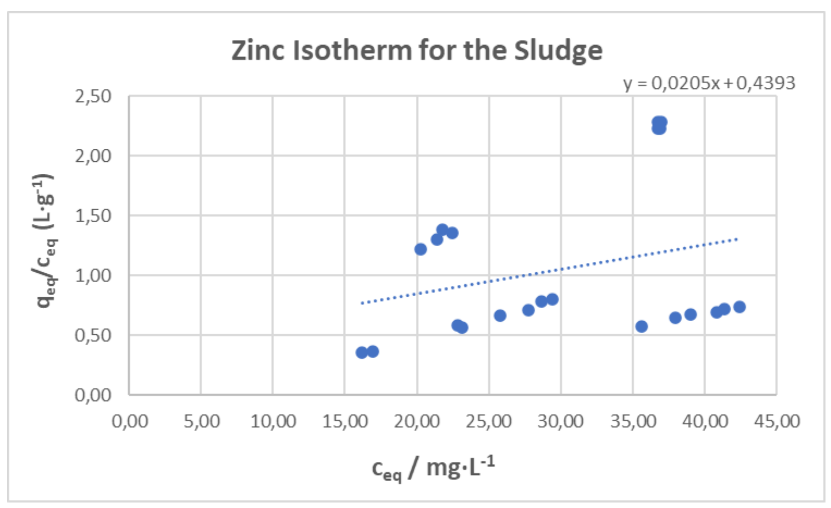


Figure A.2

Zinc isotherm (q_{eq}/c_{eq} versus c_{eq}).

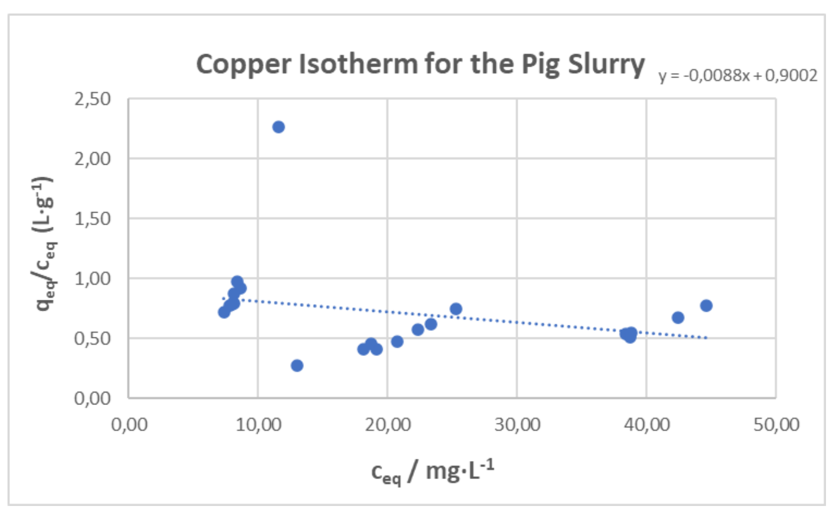


Figure A.3

Copper isotherm (q_{eq}/c_{eq} versus c_{eq}).

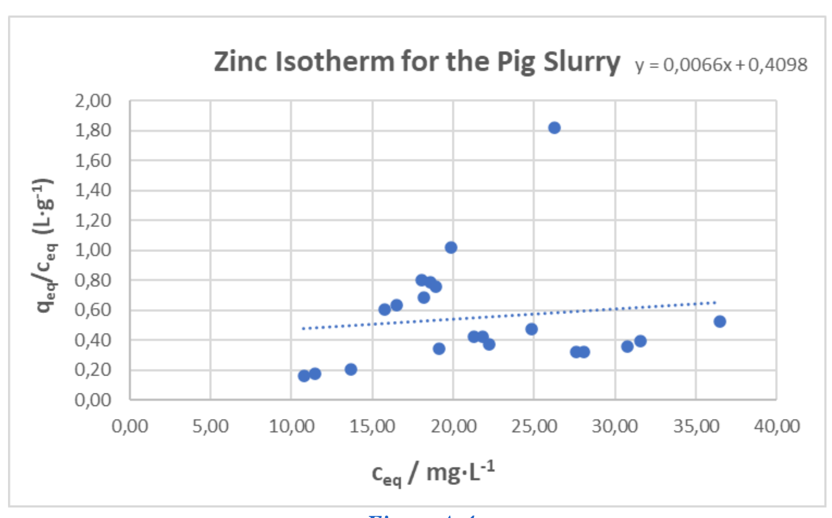


Figure A.4

Zinc isotherm (q_{eq}/c_{eq} versus c_{eq}).

REFERENCES

[1] J. M. Jacob *et al.*, “Biological approaches to tackle heavy metal pollution: A survey of literature,” *J. Environ. Manage.*, 2018, doi: 10.1016/j.jenvman.2018.03.077.

[2] N. Gaur, G. Flora, M. Yadav, and A. Tiwari, “A review with recent advancements on bioremediation-based abolition of heavy metals,” *Environ. Sci. Process. Impacts*, 2014, doi: 10.1039/c3em00491k.

[3] W. H. Organization, “‘Drinking Water,’” [Online]. Available: www.who.int/news-room/fact-sheets/detail/drinking-water (accessed May 01, 2020).

[4] K. Li *et al.*, “Microalgae-based wastewater treatment for nutrients recovery: A review,” *Bioresour. Technol.*, 2019, doi: 10.1016/j.biortech.2019.121934.

[5] L. Joseph, B. M. Jun, J. R. V. Flora, C. M. Park, and Y. Yoon, “Removal of heavy metals from water sources in the developing world using low-cost materials: A review,” *Chemosphere*, 2019, doi: 10.1016/j.chemosphere.2019.04.198.

[6] S. K. Bhagat, T. M. Tung, and Z. M. Yaseen, “Development of artificial intelligence for modeling wastewater heavy metal removal: State of the art, application assessment and possible future research,” *J. Clean. Prod.*, 2020, doi: 10.1016/j.jclepro.2019.119473.

[7] M. M. Mekonnen and A. Y. Hoekstra, “The green, blue and grey water footprint of farm animals and animal products,” *Value of Water Research Report Series*. 2010.

[8] D. Nagarajan, A. Kusmayadi, H. W. Yen, C. Di Dong, D. J. Lee, and J. S. Chang, “Current advances in biological swine wastewater treatment using microalgae-based processes,” *Bioresour. Technol.*, 2019, doi: 10.1016/j.biortech.2019.121718.

[9] S. Singh *et al.*, “Current advancement and future prospect of biosorbents for bioremediation,” *Sci. Total Environ.*, 2020, doi: 10.1016/j.scitotenv.2019.135895.

[10] F. Wollmann *et al.*, “Microalgae wastewater treatment: Biological and technological approaches,” *Eng. Life Sci.*, 2019, doi: 10.1002/elsc.201900071.

[11] Y. K. Leong and J. S. Chang, “Bioremediation of heavy metals using microalgae: Recent advances and mechanisms,” *Bioresour. Technol.*, 2020, doi: 10.1016/j.biortech.2020.122886.

[12] A. S. Ayangbenro and O. O. Babalola, “A new strategy for heavy metal polluted environments: A review of microbial biosorbents,” *Int. J. Environ. Res. Public Health*, 2017, doi: 10.3390/ijerph14010094.

[13] M. A. Laskar, R. Kumar, and M. A. Barakat, “Immobilized Microbial Biosorbents for Wastewater Remediation,” *Adv. Mater. Waste Water Treat.*, 2017, doi: 10.1002/9781119407805.ch4.

[14] C. E. Housecroft and A. G. Sharpe, *Inorganic chemistry*, vol. 1. 2005.

[15] R. Deshmukh, D. K. Tripathi, and G. Guerriero, *Metalloids in Plants. Advances and future prospects*. 2020.

[16] Y. de Jing, Z. li He, and X. e. Yang, “Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils.,” *J. Zhejiang Univ. Sci. B.*, 2007, doi: 10.1631/jzus.2007.B0192.

[17] K. Suresh Kumar, H. U. Dahms, E. J. Won, J. S. Lee, and K. H. Shin, “Microalgae - A promising tool for heavy metal remediation,” *Ecotoxicol. Environ. Saf.*, 2015, doi: 10.1016/j.ecoenv.2014.12.019.

[18] G. Azeh Engwa, P. Udoka Ferdinand, F. Nweke Nwalo, and M. N. Unachukwu, *Mechanism and Health Effects of Heavy Metal Toxicity in Humans*. 2019.

[19] B. Basu, A; Saha, D; Saha, R; Ghosh, T; Saha, "A review on sources, toxicity and remediation technologies for removing arsenic from drinking water," *Res. Chem. Intermed.*, 2014.
[20] K. S. Egorova and V. P. Ananikov, "Toxicity of Metal Compounds: Knowledge and Myths," *Organometallics*, 2017, doi: 10.1021/acs.organomet.7b00605.
[21] M. A. Khan, R. Ali, K. Rao, and M. Ajmal, "Heavy Metal pollution and its control through Non-conventional adsorbents (1998-2007): a review," *J. Int. Environ. Appl. Sci.*, 2016.
[22] N. N. G. and A.Earnshaw, *Chemistry of the Elements*, Scond. 1998.
[23] A. for T. S. and D. Registry and D. of T. I. Branch, *Toxicological profile for copper*. 2004.
[24] K. H. Vardhan, P. S. Kumar, and R. C. Panda, "A review on heavy metal pollution, toxicity and remedial measures: Current trends and future perspectives," *J. Mol. Liq.*, 2019, doi: 10.1016/j.molliq.2019.111197.
[25] M. M. C. Albert L. Lehninger, David L. Nelson, *Principles of Biochemistry*. 2005.
[26] T. R. Ross, A.C., Caballero, B., Cousins, R.J., Tucker, K.L., Ziegler, *Modern nutrition in health and disease*. 2014.
[27] G. J. Brewer, "Copper excess, zinc deficiency, and cognition loss in Alzheimer's disease," *BioFactors*, 2012.
[28] D. G. Brookins, *Eh-pH for Geochemistry*. 2012.
[29] V. Krstić, T. Urošević, and B. Pešovski, "A review on adsorbents for treatment of water and wastewaters containing copper ions," *Chem. Eng. Sci.*, 2018, doi: 10.1016/j.ces.2018.07.022.
[30] R. Crichton, "Zinc – Lewis Acid and Gene Regulator," in *Biological Inorganic Chemistry*, Third edit., 2017.
[31] G. Parkin, "The bioinorganic chemistry of zinc: Synthetic analogues of zinc enzymes that feature tripodal ligands," *Chem. Commun.*, 2000, doi: 10.1039/b004816j.
[32] L. Zhu, Z. Yuan, J. T. Simmons, and K. Sreenath, "Zn(ii)-coordination modulated ligand photophysical processes-the development of fluorescent indicators for imaging biological Zn(ii) ions," *RSC Adv.*, 2014, doi: 10.1039/c4ra00354c.
[33] M. N. Gunnar F. Nordberg, Bruce A. Fowler, *Handbook of the toxicology of metals*. 2007.
[34] K. Vijayaraghavan and Y. S. Yun, "Bacterial biosorbents and biosorption," *Biotechnol. Adv.*, 2008, doi: 10.1016/j.biotechadv.2008.02.002.
[35] P. Giovanella, G. A. L. Vieira, I. V. Ramos Otero, E. Pais Pellizzer, B. de Jesus Fontes, and L. D. Sette, "Metal and organic pollutants bioremediation by extremophile microorganisms," *J. Hazard. Mater.*, 2020, doi: 10.1016/j.jhazmat.2019.121024.
[36] N. Haq, K. Rajeev, and M. A. Khan, "Application of Carbon Nanotubes in Heavy Metals Remediation," *Crit. Rev. Environ. Sci. Technol.*, 2014.
[37] S. Deng and P. Y. Ting, "Fungal Biomass with Grafted Poly(acrylic acid) for Enhancement of Cu(II) and Cd(II) Biosorption," *Langmuir*, 2005.
[38] R. Jobby, P. Jha, A. K. Yadav, and N. Desai, "Biosorption and biotransformation of hexavalent chromium [Cr(VI)]: A comprehensive review," *Chemosphere*, 2018, doi: 10.1016/j.chemosphere.2018.05.050.
[39] M. I. A. Abdel Maksoud, A. M. Elgarahy, C. Farrell, A. H. Al-Muhtaseb, D. W. Rooney, and A. I. Osman, "Insight on water remediation application using magnetic nanomaterials and biosorbents," *Coord. Chem. Rev.*, 2020, doi: 10.1016/j.ccr.2019.213096.
[40] C. and S. K. Martinez-Boubeta, "Chapter 20 - Magnetic Nanoparticles for Water Purification," in *Nanoscale Materials in Water Purification*, 2019, pp. 521–552.

[41] X. X. G. B. J. B. Y. Q, “Removal of anionic pollutants from liquids by biomass materials: A review,” *J. Mol. Liq.*, 2016.
[42] C. Mani, D., Kumar, “Biotechnological advances in bioremediation of heavy metals contaminated ecosystems: an overview with special reference to phytoremediation,” *Int. J. Environ. Sci. Technol.*, 2014.
[43] F. B. Ata Akcil, Ceren Erust, Sevda Ozdemiroglu, Viviana Fonti, “A review of approaches and techniques used in aquatic contaminated sediments: metal removal and stabilization by chemical and biotechnological processes,” *J. Clean. Prod.*, 2015.
[44] K. A. Mosa, I. Saadoun, K. Kumar, M. Helmy, and O. P. Dhankher, “Potential biotechnological strategies for the cleanup of heavy metals and metalloids,” *Front. Plant Sci.*, 2016, doi: 10.3389/fpls.2016.00303.
[45] H. Qin, T. Hu, Y. Zhai, N. Lu, and J. Aliyeva, “The improved methods of heavy metals removal by biosorbents: A review,” *Environ. Pollut.*, 2020, doi: 10.1016/j.envpol.2019.113777.
[46] R. P. Mason, “Trace Metals and Organisms: Essential and Toxic Metals, Organometallics, Microbial Processes, and Metal Bioaccumulation,” in *Trace Metals in Aquatic Systems*, 2013, pp. 370–421.
[47] K. Chojnacka, “Biosorption and bioaccumulation - the prospects for practical applications,” *Environ. Int.*, 2010, doi: 10.1016/j.envint.2009.12.001.
[48] P. . Schwarzenbach, R.P. . Gschwend and D. M. Imoden, *Environmental Organic Chemistry*, Second Edi. 2004.
[49] Z. Aksu and G. Dönmez, “Combined Effects of Molasses Sucrose and Reactive Dye on the Growth and Dye Bioaccumulation Properties of Candida tropicalis,” *Process Biochem.*, 2005.
[50] A. A. Beni and A. Esmaeili, “Biosorption, an efficient method for removing heavy metals from industrial effluents: A Review,” *Environ. Technol. Innov.*, 2020, doi: 10.1016/j.eti.2019.100503.
[51] C. Mack, B. Wilhelmi, J. R. Duncan, and J. E. Burgess, “Biosorption of precious metals,” *Biotechnol. Adv.*, 2007, doi: 10.1016/j.biotechadv.2007.01.003.
[52] Y. Orhan, J. Hrenovič, and H. Büyükgüngör, “Biosorption of heavy metals from wastewater by biosolids,” *Eng. Life Sci.*, 2006, doi: 10.1002/elsc.200520135.
[53] J. Kaduková and E. Virčíková, “Comparison of differences between copper bioaccumulation and biosorption,” *Environ. Int.*, 2005, doi: 10.1016/j.envint.2004.09.020.
[54] H. Butt, K. Graf, and M. Kappl, *Physics and Chemistry of Interfaces*. 2003.
[55] M. Thommes *et al.*, “Physisorption of gases, with special reference to the evaluation of surface area and pore size distribution (IUPAC Technical Report),” *Pure Appl. Chem.*, 2015, doi: 10.1515/pac-2014-1117.
[56] B. Volesky, “Biosorption and me,” *Water Res.*, 2007, doi: 10.1016/j.watres.2007.05.062.
[57] L. Bulgariu and M. Gavrilescu, *Bioremediation of Heavy Metals by Microalgae*, no. 3. Elsevier Inc., 2015.
[58] D. Huang *et al.*, “Megamerger of biosorbents and catalytic technologies for the removal of heavy metals from wastewater: Preparation, final disposal, mechanism and influencing factors,” *J. Environ. Manage.*, 2020, doi: 10.1016/j.jenvman.2019.109879.
[59] R. Gomez-Gonzalez, F. J. Cerino-Córdova, A. M. Garcia-León, E. Soto-Regalado, N. E. Davila-Guzman, and J. J. Salazar-Rabago, “Lead biosorption onto coffee grounds: Comparative analysis of several optimization techniques using equilibrium adsorption models and ANN,” *J. Taiwan Inst. Chem. Eng.*, 2016, doi: 10.1016/j.jtice.2016.08.038.
[60] P. Atkins and J. de Paula, *Atkins' Physical Chemistry*, Eighth edi. 2006.
[61] A. W. Adamson and A. P. Gast, *Physical Chemistry of Surfaces*, Sixth edit. 1997.

[62] G. G. Láng, “Basic interfacial thermodynamics and related mathematical background,” *ChemTexts*, 2015, doi: 10.1007/s40828-015-0015-z.
[63] J. C. Berg, “Thermodynamics of Interfacial Systems,” in *An Introduction to Interfaces and Colloids. The bridge to Nanoscience*, 2009, pp. 107–213.
[64] X. F. Wu, Y. L. Hu, F. Zhao, Z. Z. Huang, and D. Lei, “Ion adsorption components in liquid/solid systems,” *J. Environ. Sci. (China)*, 2006, doi: 10.1016/S1001-0742(06)60057-0.
[65] M. D. LeVan and G. Carta, “Adsorption and Ion Exchange,” in *Perry's Chemical Engineers' Handbook*, Eighth Edi., 2008, pp. 1–66.
[66] M. A. Khosa and A. Ullah, “Mechanistic insight into protein supported biosorption complemented by kinetic and thermodynamics perspectives,” *Adv. Colloid Interface Sci.*, 2018, doi: 10.1016/j.cis.2018.09.004.
[67] Y. Liu and Y. J. Liu, “Biosorption isotherms, kinetics and thermodynamics,” *Sep. Purif. Technol.*, 2008, doi: 10.1016/j.seppur.2007.10.002.
[68] J. López, “Ecuación de Langmuir en líquidos,” *Educ. Química*, 2015.
[69] Z. Hajahmadi, H. Younesi, N. Bahramifar, H. Khakpour, and K. Pirzadeh, “Multicomponent isotherm for biosorption of Zn(II), CO(II) and Cd(II) from ternary mixture onto pretreated dried Aspergillus niger biomass,” *Water Resour. Ind.*, 2015, doi: 10.1016/j.wri.2015.07.003.
[70] R. Sudha, K. Kalpana, T. Rajachandrasekar, and S. Arivoli, “Comparative Study on the Adsorption Kinetics and Thermodynamics of Metal Ions onto Acid Activated Low Cost Pandanus Carbon,” *J. Chem.*, 2007.
[71] M. N. Sahmoune, “Performance of Streptomyces rimosus biomass in biosorption of heavy metals from aqueous solutions,” *Microchem. J.*, 2018, doi: 10.1016/j.microc.2018.05.009.
[72] M. H. Gerardi and M. C. Zimmerman, *Wastewater Pathogens*. 2005.
[73] M. H. Gerardi, “Lower Life forms. Bacterias,” in *The Biology and Troubleshooting of Facultative Lagoons*, 2015, pp. 45–57.
[74] C. R. Woese and G. E. Fox, “Phylogenetic structure of the prokaryotic domain: The primary kingdoms (archaebacteria/eubacteria/urkaryote/16S ribosomal RNA/molecular phylogeny),” *Proc. Natl. Acad. Sci. U. S. A.*, 1977.
[75] M. H. Gerardi, *Wastewater Bacteria*. 2006.
[76] J. W. Woolverton, L. Sherwood, and C. J., *Prescott's Microbiology*, Tenth edit. 2017.
[77] P. Prabhakaran, M. A. Ashraf, and W. S. Aqma, “Microbial stress response to heavy metals in the environment,” *RSC Adv.*, 2016, doi: 10.1039/c6ra10966g.
[78] Y. Jin, Y. Luan, Y. Ning, and L. Wang, “Effects and mechanisms of microbial remediation of heavy metals in soil: A critical review,” *Appl. Sci.*, 2018, doi: 10.3390/app8081336.
[79] T. Tsuruta, “Selective accumulation of light or heavy rare earth elements using gram-positive bacteria,” *Colloids Surfaces B Biointerfaces*, 2006, doi: 10.1016/j.colsurfb.2006.04.014.
[80] M. Nanda, V. Kumar, and D. K. Sharma, “Multimetal tolerance mechanisms in bacteria: The resistance strategies acquired by bacteria that can be exploited to ‘clean-up’ heavy metal contaminants from water,” *Aquat. Toxicol.*, 2019, doi: 10.1016/j.aquatox.2019.04.011.
[81] R. Pardo, M. Herguedas, E. Barrado, and M. Vega, “Biosorption of cadmium, copper, lead and zinc by inactive biomass of Pseudomonas Putida,” *Anal. Bioanal. Chem.*, 2003.

[82] R. M. Pérez Silva, A. Ábalos Rodríguez, J. M. Gómez Montes De Oca, and D. Cantero Moreno, “Biosorption of chromium, copper, manganese and zinc by Pseudomonas aeruginosa AT18 isolated from a site contaminated with petroleum,” *Bioresour. Technol.*, 2009, doi: 10.1016/j.biortech.2008.06.057.

[83] M. Rajkumar and H. Freitas, “Influence of metal resistant-plant growth-promoting bacteria on the growth of Ricinus communis in soil contaminated with heavy metals,” *Chemosphere*, 2008, doi: 10.1016/j.chemosphere.2007.11.038.

[84] H. K. In, C. Jin-Ha, O. J. Jeong, K. Young-Kee, and O. Jeong-Woo, Choi Byung-Keun, “Development of a Microbe-Zeolite Carrier for the Effective Elimination of Heavy Metals from Seawater.,” *J. Microbiol. Biotechnol.*, 2015.

[85] S. Panwichian, D. Kantachote, B. Wittayaweerasak, and M. Mallavarapu, “Removal of heavy metals by exopolymeric substances produced by resistant purple nonsulfur bacteria isolated from contaminated shrimp ponds,” *Electron. J. Biotechnol.*, Jul. 2011, doi: 10.2225/vol14-issue4-fulltext-2.

[86] M. G. Kiran, K. Pakshirajan, and G. Das, “Heavy metal removal from multicomponent system by sulfate reducing bacteria: Mechanism and cell surface characterization,” *J. Hazard. Mater.*, 2017, doi: 10.1016/j.jhazmat.2015.12.042.

[87] H. Salehizadeh and S. A. Shojaosadati, “Removal of metal ions from aqueous solution by polysaccharide produced from Bacillus firmus,” *Water Res.*, 2003, doi: 10.1016/S0043-1354(03)00418-4.

[88] H. L. Liu, B. Y. Chen, Y. W. Lan, and Y. C. Cheng, “Biosorption of Zn(II) and Cu(II) by the indigenous Thiobacillus thiooxidans,” *Chem. Eng. J.*, 2004, doi: 10.1016/S1385-8947(03)00210-9.

[89] X. C. Chen, Y. P. Wang, Q. Lin, J. Y. Shi, W. X. Wu, and Y. X. Chen, “Biosorption of copper(II) and zinc(II) from aqueous solution by Pseudomonas putida CZ1,” *Colloids Surfaces B Biointerfaces*, 2005, doi: 10.1016/j.colsurfb.2005.10.003.

[90] T. Liu, J. H. Hou, J. B. Wang, W. Wang, X. Y. Wang, and J. L. Wu, “Biosorption of heavy metals from aqueous solution by the novel biosorbent Pectobacterium sp. ND2,” *Environ. Prog. Sustain. Energy*, 2018, doi: 10.1002/ep.12757.

[91] N. Mameri *et al.*, “Batch zinc biosorption by a bacterial nonliving Streptomyces rimosus biomass,” *Water Res.*, 1999, doi: 10.1016/S0043-1354(98)00349-2.

[92] Z. M. Puyen, E. Villagrasa, J. Maldonado, E. Diestra, I. Esteve, and A. Solé, “Biosorption of lead and copper by heavy-metal tolerant Micrococcus luteus DE2008,” *Bioresour. Technol.*, 2012, doi: 10.1016/j.biortech.2012.09.036.

[93] G. Uslu and M. Tanyol, “Equilibrium and thermodynamic parameters of single and binary mixture biosorption of lead (II) and copper (II) ions onto Pseudomonas putida: Effect of temperature,” *J. Hazard. Mater.*, 2006, doi: 10.1016/j.jhazmat.2005.11.029.

[94] P. Dhanwal *et al.*, “Biosorption of Heavy Metals from Aqueous Solution by Bacteria Isolated from Contaminated Soil,” *Water Environ. Res.*, 2018, doi: 10.2175/106143017x15131012152979.

[95] H. Yang, H. Ma, B. Shi, L. Li, and W. Yan, “Experimental study of the effects of heavy metal ions on the hydrogen production performance of Rhodobacter sphaeroides HY01,” *Int. J. Hydrogen Energy*, 2016, doi: 10.1016/j.ijhydene.2016.04.199.

[96] H. Nõlvak *et al.*, “Microbial community changes in TNT spiked soil bioremediation trial using biostimulation, phytoremediation and bioaugmentation,” *J. Environ. Eng. Landsc. Manag.*, 2013, doi: 10.3846/16486897.2012.721784.

[97] L. Babák, P. Šupinová, M. Zichová, R. Burdychová, and E. Vítová, “Biosorption of Cu, Zn and Pb by thermophilic bacteria - Effect of biomass concentration on biosorption capacity,” *Acta Univ. Agric. Silvic. Mendelianae Brun.*, 2012, doi: 10.11118/actaun201260050009.

[98] W. Bin Lu, J. J. Shi, C. H. Wang, and J. S. Chang, “Biosorption of lead, copper and cadmium by an indigenous isolate Enterobacter sp. J1 possessing high heavy-metal resistance,” *J. Hazard. Mater.*, 2006, doi: 10.1016/j.jhazmat.2005.10.036.
[99] S. Dave, M. Damani, and D. Tipre, “Copper remediation by Eichhornia spp. and sulphate-reducing bacteria,” *J. Hazard. Mater.*, 2010, doi: 10.1016/j.jhazmat.2009.08.073.
[100] S. Tebbani, R. Filali, F. Lopes, D. Dumur, and D. Pareau, “Chapter 1: Microalgae,” in *CO2 Biofixation by Microalgae: Modeling, Estimation and Control*, First edit., 2014, pp. 1–22.
[101] M. A. Borowitzka, “Chapter 3: Biology of microalgae,” in *Microalgae in Health and Disease Prevention*, no. 1998, 2018, pp. 23–72.
[102] S. Hemaiswarya, R. Raja, R. Ravikumar, and I. S. Carvalho, “Microalgae taxonomy and breeding,” *Biofuel Crop. Prod. Physiol. Genet.*, 2013, doi: 10.1079/9781845938857.0044.
[103] M. P. de Souza, M. Hoeltz, L. Brittes Benitez, Ê. L. Machado, and R. de C. de Souza Schneider, “Microalgae and Clean Technologies: A Review,” *Clean - Soil, Air, Water*, 2019, doi: 10.1002/clen.201800380.
[104] Y. Wang *et al.*, “Perspectives on the feasibility of using microalgae for industrial wastewater treatment,” *Bioresour. Technol.*, 2016, doi: 10.1016/j.biortech.2016.09.106.
[105] C. M. Monteiro, P. M. L. Castro, and F. X. Malcata, “Metal uptake by microalgae: Underlying mechanisms and practical applications,” *Biotechnol. Prog.*, 2012, doi: 10.1002/btpr.1504.
[106] Z. Aksu, “Equilibrium and kinetic modelling of iron adsorption by eggshells in a batch system: effect of temperature,” *Sep. Purif. Technol.*, 2001, doi: 10.1016/j.desal.2006.04.052.
[107] R. H. Crist, K. Oberholser, N. Shank, and M. Nguyen, “Nature of Bonding between Metallic Ions and Algal Cell Walls,” *Environ. Sci. Technol.*, 1981, doi: 10.1021/es00092a010.
[108] R. Saavedra, R. Muñoz, M. E. Taboada, M. Vega, and S. Bolado, “Comparative uptake study of arsenic, boron, copper, manganese and zinc from water by different green microalgae,” *Bioresour. Technol.*, 2018, doi: 10.1016/j.biortech.2018.04.101.
[109] A. H. Sulaymon, A. A. Mohammed, and T. J. Al-Musawi, “Removal of lead, cadmium, copper, and arsenic ions using biosorption: Equilibrium and kinetic studies,” *Desalin. Water Treat.*, 2013, doi: 10.1080/19443994.2013.769695.
[110] B. N. T. and J. P. G. S.K. Mehta, “Enhanced sorption of Cu2+ and Ni2+ by acid-pretreated Chlorella vulgaris from single and binary metal solutions,” *J. Appl. Phycol.*, 2002, doi: 10.1023/A.
[111] G. J. Zhou, F. Q. Peng, L. J. Zhang, and G. G. Ying, “Biosorption of zinc and copper from aqueous solutions by two freshwater green microalgae Chlorella pyrenoidosa and Scenedesmus obliquus,” *Environ. Sci. Pollut. Res.*, 2012, doi: 10.1007/s11356-012-0800-9.
[112] R. Flouty and G. Estephane, “Bioaccumulation and biosorption of copper and lead by a unicellular algae Chlamydomonas reinhardtii in single and binary metal systems: A comparative study,” *J. Environ. Manage.*, 2012, doi: 10.1016/j.jenvman.2012.06.042.
[113] H. Cameron, M. T. Mata, and C. Riquelme, “The effect of heavy metals on the viability of Tetraselmis marina AC16- MESO and an evaluation of the potential use of this microalga in bioremediation,” *PeerJ*, 2018, doi: 10.7717/peerj.5295.

[114] S. Abinandan, S. R. Subashchandrabose, L. Pannerselvan, K. Venkateswarlu, and M. Megharaj, "Potential of acid-tolerant microalgae, Desmodesmus sp. MAS1 and Heterochlorella sp. MAS3, in heavy metal removal and biodiesel production at acidic pH," *Bioresour. Technol.*, 2019, doi: 10.1016/j.biortech.2019.01.053.
[115] P. X. Sheng, Y. P. Ting, J. P. Chen, and L. Hong, "Sorption of lead, copper, cadmium, zinc, and nickel by marine algal biomass: Characterization of biosorptive capacity and investigation of mechanisms," *J. Colloid Interface Sci.*, 2004, doi: 10.1016/j.jcis.2004.01.036.
[116] L. Deng, X. Zhu, X. Wang, Y. Su, and H. Su, "Biosorption of copper(II) from aqueous solutions by green alga Cladophora fascicularis," *Biodegradation*, 2007, doi: 10.1007/s10532-006-9074-6.
[117] D. Hiren, R. Arabinda, and K. I.L., "Bioremediation Potential of Live and Dead Spirulina: Spectroscopic, Kinetics and SEM Studies," *Biotechnol. Bioeng.*, 2006, doi: DOI 10.1002/bit.21190.
[118] J. S. Yang, J. Cao, G. L. Xing, and H. L. Yuan, "Lipid production combined with biosorption and bioaccumulation of cadmium, copper, manganese and zinc by oleaginous microalgae Chlorella minutissima UTEX2341," *Bioresour. Technol.*, 2015, doi: 10.1016/j.biortech.2014.10.124.
[119] C. Urrutia, E. Yañez-Mansilla, and D. Jeison, "Bioremoval of heavy metals from metal mine tailings water using microalgae biomass," *Algal Res.*, 2019, doi: 10.1016/j.algal.2019.101659.
[120] V. R. Moreira, Y. A. R. Lebron, S. J. Freire, L. V. S. Santos, F. Palladino, and R. S. Jacob, "Biosorption of copper ions from aqueous solution using Chlorella pyrenoidosa: Optimization, equilibrium and kinetics studies," *Microchem. J.*, 2019, doi: 10.1016/j.microc.2018.10.027.
[121] C. M. Monteiro, P. M. L. Castro, and F. X. Malcata, "Biosorption of zinc ions from aqueous solution by the microalga Scenedesmus obliquus," *Environ. Chem. Lett.*, 2011, doi: 10.1007/s10311-009-0258-2.
[122] C. M. Monteiro, A. P. G. C. Marques, P. M. L. Castro, and F. X. Malcata, "Characterization of Desmodesmus pleiomorphus isolated from a heavy metal-contaminated site: Biosorption of zinc," *Biodegradation*, 2009, doi: 10.1007/s10532-009-9250-6.
[123] H. H. Omar, "Bioremoval of zinc ions by Scenedesmus obliquus and Scenedesmus quadricauda and its effect on growth and metabolism," *Int. Biodeterior. Biodegrad.*, 2002, doi: 10.1016/S0964-8305(02)00048-3.
[124] A. Ahmad, A. Bhat, and A. Buang, "Biosorption of transition metals by freely suspended and Ca-alginate immobilised with Chlorella vulgaris: kinetic and equilibrium modeling," *J. Clean. Prod.*, 2018.
[125] L. S. Ferreira *et al.*, "Adsorption of Ni2+, Zn2+ and Pb2+ onto dry biomass of Arthrospira (Spirulina) platensis and Chlorella vulgaris. I. Single metal systems," *Chem. Eng. J.*, 2011, doi: 10.1016/j.cej.2011.07.039.
[126] M. B. Cooper and A. G. Smith, "Exploring mutualistic interactions between microalgae and bacteria in the omics age," *Curr. Opin. Plant Biol.*, 2015, doi: 10.1016/j.pbi.2015.07.003.
[127] R. Ramanan, B. H. Kim, D. H. Cho, H. M. Oh, and H. S. Kim, "Algae-bacteria interactions: Evolution, ecology and emerging applications," *Biotechnol. Adv.*, 2016, doi: 10.1016/j.biotechadv.2015.12.003.
[128] J. Liu *et al.*, "Advanced nutrient removal from surface water by a consortium of attached microalgae and bacteria: A review," *Bioresour. Technol.*, 2017, doi: 10.1016/j.biortech.2017.06.054.

[129] B. Zhang *et al.*, “Microalgal-bacterial consortia: From interspecies interactions to biotechnological applications,” *Renew. Sustain. Energy Rev.*, 2020, doi: 10.1016/j.rser.2019.109563.
[130] A. L. Gonçalves, J. C. M. Pires, and M. Simões, “A review on the use of microalgal consortia for wastewater treatment,” *Algal Res.*, 2017, doi: 10.1016/j.algal.2016.11.008.
[131] R. Muñoz and B. Guieysse, “Algal-bacterial processes for the treatment of hazardous contaminants: A review,” *Water Res.*, 2006, doi: 10.1016/j.watres.2006.06.011.
[132] I. E. Mejias Carpio, A. Ansari, and D. F. Rodrigues, “Relationship of Biodiversity with Heavy Metal Tolerance and Sorption Capacity: A Meta-Analysis Approach,” *Environ. Sci. Technol.*, 2018, doi: 10.1021/acs.est.7b04131.
[133] G. Quijano, J. S. Arcila, and G. Buitrón, “Microalgal-bacterial aggregates: Applications and perspectives for wastewater treatment,” *Biotechnol. Adv.*, 2017, doi: 10.1016/j.biotechadv.2017.07.003.
[134] S. R. Subashchandrabose, B. Ramakrishnan, M. Megharaj, K. Venkateswarlu, and R. Naidu, “Consortia of cyanobacteria/microalgae and bacteria: Biotechnological potential,” *Biotechnol. Adv.*, 2011, doi: 10.1016/j.biotechadv.2011.07.009.
[135] Y. Shen, J. Gao, and L. Li, “Municipal wastewater treatment via co-immobilized microalgal-bacterial symbiosis: Microorganism growth and nutrients removal,” *Bioresour. Technol.*, 2017, doi: 10.1016/j.biortech.2017.07.041.
[136] S. Loutseti, D. B. Danielidis, A. Economou-Amilli, C. Katsaros, R. Santas, and P. Santas, “The application of a micro-algal/bacterial biofilter for the detoxification of copper and cadmium metal wastes,” *Bioresour. Technol.*, 2009, doi: 10.1016/j.biortech.2008.11.019.
[137] R. Muñoz, M. T. Alvarez, A. Muñoz, E. Terrazas, B. Guieysse, and B. Mattiasson, “Sequential removal of heavy metals ions and organic pollutants using an algal-bacterial consortium,” *Chemosphere*, 2006, doi: 10.1016/j.chemosphere.2005.09.062.
[138] Y. Li, X. Yang, B. Geng, and X. Liu, “Effective bioremediation of Cu(II) contaminated waters with immobilized sulfate-reducing bacteria-microalgae beads in a continuous treatment system and mechanism analysis,” *J. Chem. Technol. Biotechnol.*, 2018, doi: 10.1002/jctb.5513.
[139] H. Sahoo, D. Senapati, I. S. Thakur, and U. C. Naik, “Integrated bacteria-algal bioreactor for removal of toxic metals in acid mine drainage from iron ore mines,” *Bioresour. Technol. Reports*, 2020, doi: 10.1016/j.biteb.2020.100422.
[140] D. García, I. de Godos, C. Domínguez, S. Turiel, S. Bolado, and R. Muñoz, “A systematic comparison of the potential of microalgae-bacteria and purple phototrophic bacteria consortia for the treatment of piggery wastewater,” *Bioresour. Technol.*, 2019, doi: 10.1016/j.biortech.2018.12.095.
[141] E. Safonova, K. V. Kvitko, M. I. Iankevitch, L. F. Surgko, I. A. Afti, and W. Reisser, “Biotreatment of industrial wastewater by selected algal-bacterial consortia,” *Eng. Life Sci.*, 2004, doi: 10.1002/elsc.200420039.
[142] G. E. P. Box, W. G. Hunter, and J. S. Hunter, *Statistics for Experiments: An Introduction to Design, Data Analysis, and Model Building*. 1978.
[143] W. G. Cochran and G. M. Cox, *Diseños experimentales*. 1990.
[144] S. N. Deming and S. L. Morgan, *Experimental design: A chemometric approach*. 1987.
[145] R. Saavedra, R. Muñoz, M. E. Taboada, and S. Bolado, “Influence of organic matter and CO2 supply on bioremediation of heavy metals by Chlorella vulgaris and Scenedesmus almeriensis in a multimetallic matrix,” *Ecotoxicol. Environ. Saf.*, vol. 182, no. January, p. 109393, 2019, doi: 10.1016/j.ecoenv.2019.109393.

[146] A. S. of A. Engineers, "Manure Production and Characteristics," 2003. [Online]. Available: http://www.agronext.iastate.edu/immag/pubs/manure-prod-char-d384-2.pdf.

[147] E. Posadas, M. del M. Morales, C. Gomez, F. G. Acién, and R. Muñoz, "Influence of pH and CO2 source on the performance of microalgae-based secondary domestic wastewater treatment in outdoors pilot raceways," *Chem. Eng. J.*, 2015, doi: 10.1016/j.cej.2014.12.059.

[148] I. S. Bădescu, D. Bulgariu, I. Ahmad, and L. Bulgariu, "Valorisation possibilities of exhausted biosorbents loaded with metal ions – A review," *J. Environ. Manage.*, 2018, doi: 10.1016/j.jenvman.2018.07.066.