



**Universidade de
Aveiro**

Ano 2020/2021

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**Otimização do processo de remoção de
mercúrio, cobalto e níquel em mistura pela
macroalga viva *Ulva lactuca***

**Process optimization of the removal of
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Doutora Eduarda Pereira, Professora Associada do Departamento de Química da Universidade de Aveiro e do Doutor Bruno Henriques, Investigador do Departamento de Química da Universidade de Aveiro.

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palavras-chave

Sorção, biomarcadores, elementos potencialmente tóxicos, água, metodologia de superfície de resposta, biotecnologia

resumo

Fatores como o crescimento populacional e económico, e alterações climáticas têm consequências e são uma ameaça aos sistemas aquáticos, resultando na contaminação da água, o que contribui para a sua escassez enquanto recurso. Este trabalho teve como objetivo estudar a capacidade da macroalga *Ulva lactuca* viva para funcionar como biossorvente na remoção de mercúrio, cobalto e níquel de água salina real. Um primeiro estudo de avaliação de toxicidade demonstrou que estes três elementos causam alterações bioquímicas nos mexilhões *Mytilus galloprovincialis*, sendo responsáveis pela ativação de defesas antioxidantes, redução da atividade metabólica e gasto de energias de reserva. Estes resultados reforçam a importância de desenvolver técnicas eficazes e sustentáveis para a remoção destes contaminantes de água. Nos ensaios realizados, a macroalga em estudo apresentou resultados promissores na remoção dos três elementos, alcançando no final da exposição (120 h) remoções na gama 65 - 98 % para o mercúrio, 83 – 97 % para o cobalto e 78 – 95 % para o níquel, sendo que estas gamas de percentagens de remoção incluem diferentes condições iniciais. Através da metodologia de superfície de resposta, combinada com desenho experimental de Box-Behnken, as condições de operação foram otimizadas em termos de massa inicial de macroalga, salinidade da água, e concentração inicial dos elementos. Em termos de tempo, foi concluído que, para as condições testadas, a extensão do processo das 48 para as 120 h não traz vantagens relevantes em termos de eficiência. No global, o trabalho dá um complemento importante à literatura a nível de contaminantes removidos, matrizes usadas e mistura estudada.

keywords

Sorption, biomarkers, potentially toxic elements, water, surface response methodology, biotechnology

abstract

Factors such as populational and economic growth, and climate changes have consequences and are threats to aquatic systems, leading to water contamination, that contributes to its scarce as a resource. This work aimed to study the capacity of the living macroalgae *Ulva lactuca* to act as a biosorbent in the removal of mercury, cobalt and nickel from real saline water. A first toxicity evaluation study showed that these three elements cause biochemical changes in mussel *Mytilus galloprovincialis*, being responsible for activating antioxidant defenses, reducing metabolic activity, and spending energy reserves. These results reinforce the importance of developing efficient and sustainable techniques to remove these contaminants from water. In the trials performed, the macroalgae under study showed promising results in the removal of the three elements, presenting at the end of exposure (120 h) removals in the range of 65 – 98 % for mercury, 83 – 97 % for cobalt and 78 – 95 % for nickel, being that these ranges of removal percentages include different initial conditions. Through the surface response methodology, combined with the experimental design of Box-behnken, the operational conditions were optimized in terms of initial macroalgae dosage, water salinity, and initial concentration of elements. In terms of time, it was concluded that, to the tested conditions, the extension of the process from 48 to 120 h does not present relevant advantages in terms of efficiency. Globally, this work provides an important complement to the literature in terms of removed contaminants, used matrices and mixture studied.

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1 INTRODUCTION

1.1 Water: importance and contaminants sources

Water covers 70 % of our planet and it is classified by United Nations as the core of sustainable development, being crucial for human survival, food and energy production, ecosystems and socio-economic issues of the countries. However, only 3 % of world's water can be used to drink, irrigate farm fields or bathe in. Of those 3 %, two thirds represents water in frozen glaciers or in unavailable sites for human use (Blanco et al., 2019; Tsani et al., 2020). Changes in different factors such as population or economic growth, beyond the climate changes, will lead to consequences on the future of water resources (Gude, 2017). Government leaders, also referred as decision-makers, face diverse challenges when it relates to water management. They usually try to reduce risks of water scarcity in a sustainable way, by applying methods of water reuse or using treated wastewater for diverse purposes. However, there are risks related to the quality of the reused water and it is peremptory to focus how to mitigate them (Müller et al., 2020). Water looks like a local issue instead of a global threat because wastewater treatment plants prevent the contamination of local rivers, treating industrial and municipal wastewater (Bey et al., 2021). However, we live in an interconnected world. Water is used to irrigate fields to grow crops and because of the globalization of the food market, a local contaminated river does not only affects local people, but it can also affect people from the other side of the world (Borsellino et al., 2020).

In nature there is a whole range of chemical elements that are crucial to life. The term trace elements refer to those that are present in concentrations lower (or much lower) than 100 mg/L and include cobalt (Co), nickel (Ni), lead (Pb), chromium (Cr), selenium (Se), magnesium (Mg), among others (Al-fartusie & Mohssan, 2017). Micronutrients are trace elements which have functions on organisms when in low concentrations (usually in $\mu\text{g}/\text{day}$) (Carr, 2020). Nevertheless, when their concentration exceeds the recommended one, they can become toxic (Helser & Cappuyns, 2021). Examples of those are copper (Cu), zinc (Zn), Se and iron (Fe) (Al-fartusie & Mohssan, 2017; Noor et al., 2020; Xiong et al., 2020). However, there are some elements like mercury (Hg), cadmium (Cd), Ni and Pb that are non-essential, show toxic effects at low concentrations and only have a harmful role in living organisms (Demir et al., 2020; Esposito et al., 2020).

The population growth has some consequences such as the expansion of urbanization, food production and industrial activities. All these lead to an exponential

increase of waste that, if inappropriately disposed, can cause damage to the environment. These wastes always have some potentially toxic elements in their constitution. One of the many characteristics of these constituents is the higher water solubility which, together with its discharge in large quantities, can cause water contamination (Ahamad et al., 2019; Francisco et al., 2019).

Throughout the entire food production process, there are many sources of environment contamination. Inorganic and organic fertilizers, pesticides, and inappropriate irrigation water (deeps wells, rivers, and irrigation channels (Picó et al., 2020; Souza et al., 2018)) are some sources of contamination on agricultural soils that can cause life-threatening cancers or other human diseases (Hou et al., 2020; Souza et al., 2018). Phosphate fertilizers have in their constitution elements like Cd, chromium (Cr), Ni, Pb, and Zn depending on their sources (Latifi & Jalali, 2018). The danger is not on occasional uses but on the constant use of those fertilizers over long period of time. Cadmium is one of the most biotoxic elements and the most representative pollutant in rice paddy soils, leading to concerns on Cd contaminated rice (Hou et al., 2020; Luo et al., 2020). Moving on to the industry, there are several well-identified sources of contamination such as mining and refining (Farjana et al., 2019). Extensive mining, the source of mineral that all countries find essential, is considered a threat by environment specialists because of the release of potentially toxic elements to aquatic ecosystems (Beck et al., 2020). The type of extraction will define the elements that are released. Metals like As, Cd, and Fe, for example, are from coal mines, while Hg was historically used in gold mining, although it is still used in some developing countries (Gyamfi et al., 2020). Smelting is a high temperature metal processing that emit metals in the form of particles and steam (Xing et al., 2019), which can lead to the formation of As, Cd, Pb (and others) aerosols when combined with the water in the atmosphere, that can be precipitated causing soil and/or water contamination (Souza et al., 2018). Dispersion of As because of geological phenomena is an example of a natural source, leading to high levels of As in water, soil, and air in both organic and inorganic form (Taheri et al., 2017). Because As is a Class I carcinogen, it requires attention (Bhutiani et al., 2016; Taheri et al., 2017). Volcanic eruptions (Elburg & Smet, 2020) and forest fires are also responsible for a large release of potentially toxic elements into the environment.

1.2 Priority List of Hazardous Substances

The Agency for Toxic Substances and Disease Registry (ATSDR) of the United States of America is responsible for the “Priority List of Hazardous Substances”. This list is revised and published every 2 years, and it prioritizes substances based on occurrence, toxicity, and human exposure to these substances at National Priorities List (NPL) sites. Of this list of 275 substances, there are 3 that will be emphasized below: Hg, Co and Ni (ATSDR, 2019).

Table 1 shows the maximum admissible concentration of Hg, Co, and Ni in effluents and drinking water according to the American and European legislations.

Table 1 - Maximum admissible concentration of Hg, Co and Ni in effluents and drinking water (American and European regulations) [$\mu\text{g/L}$] (adapted from (Chojnacka, 2009; Portuguese Decree-Law 236/98; Henriques, 2014)

Metal	Wastewater discharge limits		Drinking water standards	
	European	American	European	American
Hg	50	50	1	2
Co	1000	-	-	-
Ni	2000	5000	20	100

There are no wastewater discharge limits for Co in the American legislation, neither in drinking water for both the American and European legislations. The Co limit present in **Table 1** is the German requirement for discharges (Chojnacka, 2009). In general, the American legislation is less restrictive. Portuguese law follows the values presented in **Table 1** for Europe, except for Co that has no limit (Portuguese Decree-Law 236/98).

Mercury, Co and Ni are present in various products from a wide range of industries. This leads to many sources of possible contaminations by these elements, both individually and in mixture. The products used in our daily routine are domestic sources of these potentially toxic elements in urban wastewater. The pair Hg and Ni are found together in cosmetics and shampoos (Chen & Thyssen, 2018). The pair Co and Ni are crucial for national defense equipment, as well for new technology (Qiu et al., 2021). Lithium-ion batteries, used in new energy vehicles, contain Co and Ni in high quantities, which are increasingly an emerging danger to the environment with the growth of the battery industry (Qiu et al., 2021; Sun et al., 2020). Both Hg and Co are present in the

field of medicine (Atta et al., 2019; Biswas et al., 2018). The trio Hg, Co and, Ni can be found especially in paints and pigments (Vlachou-Mogire et al., 2020).

1.2.1 Uses and environmental concentrations for mercury

Mercury (Hg, CAS no. 7439-97-6) is ranked No. 3 in the ATSDR Priority List of Hazardous Substances (ATSDR, 2019). It is widespread in the environment in all spheres of the earth – atmosphere, hydrosphere, lithosphere, and biosphere (Knezović et al., 2016). Because of its high volatility, low solubility and low chemical activity, mercury can last 0.5 to 2 years on atmosphere and travel long distances (Li et al., 2020; Zhu et al., 2020). Here, Hg is present in particulate Hg and gaseous Hg. Particulate Hg species are those sorbed onto particles or combined with them, and include Hg(0), HgCl₂, HgBr₂, Hg(OH)₂, among others (some authors refers to these as HgP – particulate-bound mercury species) (Kwon et al., 2020; Li et al., 2020). These species are soluble in water, leading to water contamination, and can also suffer wet deposition on terrestrial ecosystems. Gaseous Hg mainly exists in the Hg(0) form, and it is referred to as reactive gaseous Hg (Li et al., 2020). Mercury in the environment is not biodegradable and can have natural and anthropogenic sources (Knezović et al., 2016; Pirrone et al., 2010).

Global mercury emissions come from both natural and anthropogenic sources. Natural sources are almost all related to the geochemical composition of sites, and it includes volcanic eruptions, soil erosion and release from the rocks (Knezović et al., 2016; Kwon et al., 2020; Li et al., 2020). The emission from volcanoes varies over time, depending on what phase they are (degassing or eruption). Pirrone et al. (2010) says that, on average, volcanoes and other geothermal activities, like calderas, are responsible for the release of about 90 tonnes/year of Hg to the atmosphere, accounting for 2 % of the natural sources. The source with the biggest contribution (52 %) are oceans, with a release of about 2682 tonnes/year to the atmosphere (Pirrone et al., 2010) that is largely due to the re-emission process of historically deposited Hg over sea surfaces. Another natural source of Hg emission to the environment, among an extensive list, is the bacterial decomposition of organometallic mercury compounds (Knezović et al., 2016). Overall, on an annual basis, natural sources were referred by Pirrone et al. (2010) as responsible for 5207 tonnes of Hg released to the global atmosphere. Anthropogenic sources of Hg are diverse and mainly related to industry. These sources are responsible for 2320 tonnes of Hg emitted annually (Pirrone et al., 2010). Fossil-fuel fired power plants (coal and oil), mining, industrial and municipals sewage treatments, ferrous and non-ferrous metals

manufacturing facilities, caustic soda and vinyl chloride production plants, ore processing facilities, incineration for different wastes, cement plants, chemical production facilities are some examples (Knezović et al., 2016; Kwon et al., 2020; Li et al., 2020; Reis et al., 2010; RoyChowdhury et al., 2018; Zhu et al., 2020). Coal burning is the major source when related to the industry use, as households' contribution is negligible. The cement industry is responsible for about 232 tonnes of Hg released and depends on the types of waste used (e.g., old tires and biomass); pig iron and steel production, with about 43 tonnes annually; and chlor-alkali plants are responsible for 163 tonnes of Hg released. Pirrone et al. (2010) further estimated a global Hg emission of nearly 7527 tonnes per year for both natural and anthropogenic sources.

Kim et al. (2016) published an extensive and detailed review about the route of Hg exposure to humans and its health impacts, the associated risk assessment and treatment. The potential of Hg to cause adverse effects on human health and the amounts of Hg released to the environment are the main reasons to the special attention given to this subject. To expose the problem of Hg water contamination, Kim et al. (2016) compiled concentrations of Hg in different water medium, like rainwater, drinking water or river water in different parts of the world, reinforcing that Hg is a world threat. Starting with China, concentrations of Hg of 0.05 to 0.06 µg/L were found in rainwater (Zhu et al., 2014). In México (Martínez-Trinidad et al., 2013), Hg dissolved in drinking water was measured and it reached concentrations from 0.01 to 0.17 µg/L, much lower than the US EPA maximum contaminant guidance levels for Hg in drinking water (2 µg/L) (Table 1). However, high levels of Hg were reported in sites surrounding abandoned cinnabar mines: 0.003 to 0.32 µg/L in Slovenia (Kocman et al., 2011), 0.02 to 0.36 µg/L in China (Qiu et al., 2006) and a big range of concentration of 0.01 to 20.3 µg/L in Spain (Nevado et al., 2003; Kim et al., 2016). Lillebø et al. (2011), studied the distribution of Hg in water from the Sado Estuary, Portugal. The median concentration of total dissolved Hg was 0.013 µg/L, with a maximum of 0.079 µg/L and a minimum of 0.004 µg/L (Lillebø et al., 2011).

1.2.2 Uses and environmental concentrations for cobalt

Cobalt (Co, CAS no. 7440-48-4) is ranked No. 52 in the ATSDR Priority List of Hazardous Substances (ATSDR, 2019). It is a ferromagnetic metal, used for magnetic alloys, hard metal and chemical industries, and it is also used for energy storage and electric mobility related to electric vehicles batteries, among others uses (Campbell, 2020;

John et al., 2020; Mohapatra et al., 2020; Monge & Gil-Alana, 2019). Cobalt is an essential element for diverse organisms including humans, in the form of vitamin B12. Cobalt and its compounds are widely distributed in nature and are part of numerous anthropogenic activities related with diverse industries (Leysens et al., 2017).

Unice et al. (2020) divided Co into 4 exposure categories: occupational, environmental, dietary, and medical. Cobalt has numerous industrial applications that leads to an occupational exposure. According to Leysens et al. (2017), almost 15 % of cobalt is used for hard metal production, and the Co uptake results are mainly from inhalation of dust, but it can also occur by dermal uptake. In the construction industry, exposure to this element can occur through skin contact with cement. Similar ways of exposure can also occur in the electronic-waste recycling industry, diamond industry and pigment production and paint industry (Leysens et al., 2017). The environmental exposure is related with the different Co compounds that are widely dispersed in nature. Usually, Co contamination occurs near heavily industrialized zones, being the incineration of combustible municipal solid waste a good example of a source. Mining activities are also largely responsible for the contamination of the surrounding aquatic environments. The Co release from electronic devices, the use of cosmetics and the disposal of Co containing jewellery are also know sources of this contaminant (Leysens et al., 2017; Monge & Gil-Alana, 2019). Dietary exposure is believed to be the main source of Co, as humans already consume Co in the vitamin B12 form (2.4 µg of this vitamin - recommended daily intake - contains 0.1 µg of Co) in addition to the unintentional dietary Co intake (Leysens et al., 2017). Cobalt is also present in medicine. In the 1950s and 1960s, Co preparations were used to treat anemia. Due to the side effects, it is not applied anymore. However, Co supplements are prescribed to treat hyperestrogenism and this element is also present on metal-on-metal hip prostheses (Leysens et al., 2017; Unice et al., 2020). Those are not only direct Co sources to humans, but also can be sources of Co contamination to the environment when the discharge of waste does not follow the law.

In 2019, mine production of Co reached 140 000 metric tons (Statista, 2020) and approximately 55 % of the world's Co is sourced from The Democratic Republic of Congo (Cobalt Institute, 2016).

The Cobalt Institute is a non-profit trade association composed of producers, users, recyclers and traders of cobalt that promote the sustainability of Co related processes, and

is responsible for an continuous and extensive research to characterise environmental exposure (Cobalt Institute, 2016). This Institute presents several studies, having identified the highest Co concentration in rainwater in highly industrial areas (1.7 µg/L) and found a wet and dry deposition level of 12 µg/m² in Massachusetts Bay (Golomb et al., 1997). Stubblefield et al. (2020) mentioned a big range of Co concentrations such as 0.002 – 64 µg/L and 0.025 – 2028 µg/L in surface waters from highly contaminated sites, all this in Canada. It also has values from the United States of America, where Co concentrations were measured from surface waters (< 0.1 to > 1000 µg/L), being the highest values from streams contaminated from mining. Some of these values are higher than the maximum permissible for wastewater discharge in Europe (**Table 1**).

1.2.3 Uses and environmental concentrations for nickel

Nickel (Ni, CAS no. 7440-02-0) is ranked No. 58 in the ATSDR Priority List of Hazardous Substances (ATSDR, 2019). It is a ferromagnetic metal resistant to oxidation and corrosion (Mai Oanh et al., 2020; Unutulmazsoy et al., 2017). Nickel is an essential trace metal for many living species. If Ni is taken up in quantities below the recommended, it can cause a deficiency. However, the uptake of this metal in big quantities can induce toxicity. The release of dangerous levels of Ni to the environment can be from both natural and anthropogenic sources (Michaeli et al., 2021).

Cempel and Nikel, (2006) summarized the sources of Ni in the environment, as well as some effects of the exposure to this metal and its compounds to some living organisms. Natural sources of Ni are like those observed for Hg, including erosion of rocks, volcanic emissions, and forest fires. Dust is formed after erosion of rocks, and wind spreads this dust into the environment, ending up in water or soil. Because of Ni and Ni compounds uses on industry, this element is released in many ways. Examples of anthropogenic sources are the production of stainless steel, nickel alloys with high resistance to corrosion and temperature, wastes of metallurgical, chemical and food processing industries, mining, and others (Abd Ali et al., 2020; Cempel & Nikel, 2006). Hussain et al. (2016) evaluated the Ni release from stainless steel and Ni titanium archwires in artificial saliva over three months. Although the quantity of Ni released in this case is not significant, the use of NiTi archwires should be limited in patients that are sensitive to Ni, as small amounts of Ni in saliva can cause reactions. So, a simple production of dental brackets can release amounts of Ni through wastes/effluents

disposal, direct oral exposure, and bad discharge of these equipment (Hussain et al., 2016).

There are many studies about Ni in the environment, dealing with the release of this element (Michaeli et al., 2021), and the amounts of it in ecosystems (Cempel & Nickel, 2006; Iyaka, 2011; Nickel Institute, 2021). The Nickel Institute is a global association that promotes and support the correct use of Ni in its applications and this organization also compiles studies and information about Ni, values for freshwater, marine water, soil, bioavailability in aquatic and terrestrial compartment and even some information about effects on living organisms such as birds and mammals (Nickel Institute, 2021). The total Ni emissions in Europe to the air, soil and water were estimated and compiled by European Union Environmental Risk Assessment of Nickel, (2015), showing that water is the most affected, with nearly 750 tons of Ni released per year. Waste management is the main source of water pollution with about 544 tons of Ni/year, followed by industry with 170 tons of Ni/year. Usually, drinking water contains Ni with a concentration below 10 µg/L. Cempel & Nickel (2016), referred that, in USA, 97 % of a large number of drinking water samples had a concentration of Ni less than 20 µg/L, with 80 % of these below 10 µg/L. However, in areas affected by industrial emissions or mining, values can be as higher as 75 or 200 µg/L. In rivers, Ni is presented in a form of a precipitated coating on particles or associated with organic matter. In South of Iran, values of river contamination ranged from 41.0 to 110.7 µg Ni/L (Diagomanolin et al., 2004).

1.3 Biomarkers as identifiers of contamination impacts

Contaminated aquatic ecosystems lead to a consequent exposure of aquatic organisms to PTEs. This exposure can cause toxicity or even death (Kolarova & Napiórkowski, 2021), depending on type and concentration of PTE, organism cellular resilience, water temperature, among other factors (Smirnova, 2015). Biomarkers and bioindicators have been used to study the impacts of water contamination in aquatic species like fish (Lee et al., 2019; Malhotra et al., 2020), mussels (Coppola et al., 2017; Freitas et al., 2020; Parisi et al., 2021), seagrass (Ferrat et al., 2002), oysters (Fitzgerald et al., 2020; Patra et al., 2021), algae (Almeida et al., 2017; Henriques et al., 2021) or cockles (Magalhães et al., 2018).

1.3.1 Impact of mercury exposure to aquatic organisms

The consequences of Hg exposure to different aquatic species have been studied. In fish species, Harayashiki et al. (2018) studied the effects of oral exposure to inorganic Hg in yellowfin bream (*Acanthopagrus australis*). The feeding behaviour became different after 4 days of exposure, but these differences became less significant over time, showing that fish can recover from the initial effects of Hg in the feeding behaviour. Vieira et al. (2018) studied the protein biomarkers of Hg exposure in jaraqui fish (*Semaprochilodus spp.*), measuring Hg concentrations in tissues of fish and protein spots, concluding that some proteins like ubiquitin-40S ribosomal protein S27a can be used as biomarkers for this PTE. In mussel species, Coppola et al. (2017) used the *Mytilus galloprovincialis* to study biochemical impacts of Hg exposure and it was concluded that Hg exposure can cause effects such as reduction of mussels' metabolic capacity and increase on energy reserves. Another specie of mussels, *Perna viridis* was exposed to Hg, studying the biochemical markers of oxidative stress (Verlecar et al., 2007). Results showed that both analysed tissues (gills and digestive glands) were active in generation of oxidative stress and antioxidant responses. Ferrat et al. (2002) evaluated some biochemical markers in the seagrass *Posidonia oceanica* after Hg exposure, showing that low Hg concentrations induce oxidative stress and that it leads to a quick response from the antioxidant defenses. Studies with aquatic species exposed to Hg have been done, but there is a lack of information about mixtures, that are closer to what happens in the environment.

1.3.2 Impact of cobalt exposure to aquatic organisms

Studies on aquatic species exposed to Co are less comparing with Hg in terms of number and variety of species. The consequences to Co exposure have been studied mainly in fish. Marr et al. (1998) studied the toxicity of Co in the rainbow trout (*Oncorhynchus mykiss*) and observed that Co concentrations higher or equal to 250 µg/L are lethal to the studied organism. Kubrak et al. (2011) used the goldfish *Carassius auratus* to study the oxidative stress in brain, liver and kidney induced by exposure to Co. Were measured the concentration of lipid peroxides, the content of carbonyl proteins, and SOD, Catalase, GR and G6PDH activity, showing that exposure to Co resulted in the increase of oxidative stress and the activation of defense systems in goldfish tissues. Sun et al. (2020) used the Japanese flounder fish (*Paralichthys olivaceous*) to study the toxicity of Ni and Co, giving focus on oxidative stress, histopathological changes and

gene expression. The lethal concentration for 50 % mortality (LC₅₀) in 3 and 8 cm fish exposed to Co for 96 h was found to be about 48 and 180 mg/L, respectively. The Co exposure caused oxidative stress, erythrocyte deformations, pathological changes, and differences in genes regulations. There are almost no studies about Co toxicity in bivalves, comparing to Hg ones, except on *Mytilus galloprovincialis*. However, these two studies only refers to lipid and sterol metabolism (Nechev et al., 2006) and metabolism on some polar and volatile compounds (Nechev et al., 2007), lacking information on many interesting biomarkers.

1.3.3 Impact of nickel exposure to aquatic organisms

Studies of Ni exposure to aquatic animals follow the same pattern as in Co exposure studies: most of them refers to fish species. Kubrak et al. (2012) observed that Ni exposure in goldfish *Carassius auratus L.* induces hyperglycemia and glycogenolysis, as well as causes oxidative stress in liver and white muscle. Sun et al. (2020) used the Japanese flounder fish (*Paralichthys olivaceous*) to study the toxicity of Ni and Co, giving focus on oxidative stress, histopathological changes and gene expression. The lethal concentration for 50 % mortality (LC₅₀) in 3 and 8 cm fish exposed to Ni for 96 h was found to be about 86 and 151 mg/L, respectively. As in the Co exposure, the Ni exposure caused oxidative stress, erythrocyte deformations, pathological changes, and differences in genes regulations. Palermo et al. (2015) studied the biochemical and genotoxic effects on fish *Prochilodus lineatus*. Samples of gills, liver, kidney, and muscle were analysed, and the results showed that kidney tissue was the one with higher Ni accumulation. Antioxidant defenses were affected, lipid peroxidation increased, and DNA was damaged. The study of Ni exposure on mussels was done by Dallas et al. (2013) and Millward et al. (2012), and was observed that Ni-induced genotoxicity may not be a consequence of oxidative DNA damage and that Ni caused impacts at cellular and genetic levels, respectively.

1.4 Contaminants removal from waters

The techniques used for decontamination of waters allow to remove potentially toxic elements from the contaminated system, lowering its concentration to legal levels or removing almost all of it from the solution. This way, potentially toxic elements are not available to living organisms. Carolin et al. (2017) reviewed the treatment

technologies used for decontamination of industrial effluents, and divided the technologies in seven groups: precipitation, ion exchange, coagulation/flocculation, flotation, membrane filtration, electrochemical treatment, and sorption. This is a division normally used by several authors (Carolin et al., 2017; Fu & Wang, 2011; Henriques, 2014).

In precipitation, chemicals are added to the water so they can react with the potentially toxic elements and form insoluble precipitates (Fu & Wang, 2011), or pH is changed to obtain the same result (Carolin et al., 2017). To remove the precipitates from water it is necessary to use additional methods such as filtration or sedimentation. There are several types of precipitation such as hydroxide precipitation, where is used an alkaline agent to raise the pH and trace metal ions are precipitated as hydroxide; sulphide precipitation, where the added sulphide precipitate metals as metal sulphides; carbonate precipitation, where it can be used a carbonate reagent to perform direct precipitation of metals, or by using carbon dioxide to convert hydroxides into carbonates (Matsui, 1996). Precipitation is a simple and low-cost method, however it requires a large amount of chemicals, producing sludge rich in metals. The regulation is strict and it is expensive to dispose these sludge (Carolin et al., 2017; Henriques, 2014).

Ion exchange is a separation process where ions to be removed are exchanged between the contaminated solution and an ion-exchange resin (Fu & Wang, 2011). After the process, the ion exchange resin usually can be easily recovered and then reused. The functional groups will define the type of ion exchange resin, that can be cationic, anion and chelating exchange resin. In industry, this removal process occurs in fixed bed or fluidized bed columns (Zewail & Yousef, 2015). Although this is a process characterized by its high removal efficiency and fast kinetics, it can only be called cost-effective if low-cost ion exchangers are used (Fu & Wang, 2011; Henriques, 2014).

Coagulation and flocculation occur by adding coagulants and flocculants to the solution, to allow a future separation process like sedimentation and filtration (Fu & Wang, 2011). In the beginning of the process the solution contains colloids – molecules/atoms with low density that do not settle. Coagulants are added to increase the particles density and the success of the treatment is dependent on the type of coagulant, pH, dosage, and other variables. Flocculants added next to coagulants, will lead to the agglomeration of this particles, forming larger particles and allowing to separate them more easily. The process needs constant mixing (Carolin et al., 2017). This is a treatment

technology used a lot in wastewater treatment plants, however it needs to be followed by other treatment processes and produce contaminated wastes (Henriques, 2014; Matsui, 1996).

Flotation is a solid-liquid separation technique using bubble attachment. Potentially toxic elements present in solution stick to the bubbles, and then go to the top of the surface and are removed (Carolin et al., 2017; Matsui, 1996). The three main categories are dissolved air flotation, characterized by the ascending of the complex bubbles/metals and consequent removal; ion flotation, using surfactants to join metals with wastewaters hydrophobic species and then remove them with air bubbles; precipitate flotation, that implies a first formation of precipitates and then an attachment of these to air bubbles (Fu & Wang, 2011). The formation of sludge inherent to this process is low and separation efficiency is high, however it is not only an expensive procedure, but also needs and highly cost maintenance (Carolin et al., 2017).

Membrane filtration are physical processes for separating metals from water by using semipermeable membranes. The separation occurs based on particle size, pH, concentration, or pressure applied. The most used in wastewater treatment are ultrafiltration, reverse osmosis, nanofiltration, and electrodialysis (Carolin et al., 2017; Fu & Wang, 2011). Ultrafiltration is used to perform the removal of dissolved and colloidal material from the water. Being the pore sizes of these membranes larger than the dissolved metal ions, surfactants are added so they can bind metal ions, form large structures and then allow the retention of these structures by the ultrafiltration membrane (Fu & Wang, 2011; Henriques, 2014). Reverse osmosis only allows water to pass through the semi-permeable membrane, rejecting the contaminants (Carolin et al., 2017; Fu & Wang, 2011). Nanofiltration is a process that, due to its characteristics, be an intermediate process between ultrafiltration and reverse osmosis. The separation mechanism of this process is size and charge exclusion (Fu & Wang, 2011). Electrodialysis is another membrane filtration process used in wastewater treatment. The membranes used in this process are charged and the ions pass across them using an electric field as driving force (Fu & Wang, 2011). When applied in industry, electrodialysis produces two streams in two different sites of the process, using alternatively anion and cation exchange membranes (Gurreri et al., 2020). These removal technologies offer high efficiency (almost total removal of metals, depending on the process and initial concentration) and easy operation, however, there are some drawbacks. The main problems of these

membrane filtration processes are: cost by energy consumption and membrane replacement, type of material used on membranes (dependent on the general process), membrane clogging and/or fouling, use of additional elements such as surfactants cause secondary contamination, requirements as low flow rate, among others (Carolin et al., 2017; Fu & Wang, 2011; Gurreri et al., 2020; Henriques, 2014).

Electrochemical methods implies the reduction of the metals to their zero oxidation state by using an electrochemical cell consisting of two electrodes, anode and cathode, and an external power source (Henriques, 2014). There are three well established types of electrochemical methods in the wastewater treatment industry: electrocoagulation, electroflotation, and electrodeposition (Fu & Wang, 2011), similar to those referred above, but with the addition of a magnetic field. Electrocoagulation has as main principle, the destabilization of suspended particles, coagulation together with the potentially toxic elements and subsequent removal as the coagulation method described above. However, the coagulants are generated by a reactor composed of two electrodes and a cathode with external energy being applied (Carolin et al., 2017). Electroflotation separates trace metals from water by bubbles attachment. These bubbles are generated from water electrolysis (Carolin et al., 2017; Fu & Wang, 2011). Electrodeposition occurs by applying electricity to the surface of one of the electrodes. Metal ions exchange to the oppositely charged electrode in the solution to be treated (Carolin et al., 2017). These described electrochemical methods are selective and versatile. The disadvantages are the large capital investment needed to start, the expensive electricity supply, needing of high maintenance, constant renovation of the electrode material, among others (Carolin et al., 2017; Fu & Wang, 2011; Henriques, 2014).

Sorption is the process where potentially toxic elements to be removed bind to the sorbent in solution (Matsui, 1996). This accumulation in the surface or interface of the sorbent occurs by chemical and physical factors, and it is different from absorption processes, where the substances to be removed (e.g., trace metals) are incorporated (Henriques, 2014). Activated carbon is a sorbent widely used in potentially toxic elements removal, but it is not the only one. Carbon nanotubes and biosorbents are also well reported in literature (Carolin et al., 2017; Fu & Wang, 2011). Activated carbon presents high surface area, with many pores on its constitution. Usually, this kind of sorbents comes from carbonaceous material, being coal the most used for activated carbon production (Carolin et al., 2017). Although the quick and effective removal, the costly

regeneration of the material after the sorptive capacity has been reached, and the increase of price of this material are two main drawbacks (Carolin et al., 2017; Fu & Wang, 2011; Henriques, 2014). Carbon nanotubes are made up of cylindrical graphite sheets folded into a tube-like structure and can be classified as single-walled or multi-walled carbon nanotubes. Potentially toxic elements ions interact with carbon nanotubes via electrostatic attraction, sorption-precipitation, ion exchange and chemical interaction (Carolin et al., 2017). This process has good efficiency, however, the presence of it in wastewater treatment plants can affect microorganisms metabolically and affect human beings through the food chain (Upadhyayula et al., 2009).

1.4.1 Biosorption and bioaccumulation as a water remediation technology

Considering the drawbacks for the conventional treatment methods as the high cost and high sludge produced, there is a need of finding alternatives. Biosorption is defined by the ability of some material of biological origin to bind substances (e.g., potentially toxic elements) to the surface of biosorbents. These biosorbents can also perform bioaccumulation, where trace metals enter the organism, if they are living organisms (Chojnacka, 2009). Biosorption and bioaccumulation processes have been studied to take advantage of the capacity of biomass to bind and concentrate substances as trace metals from water (Chojnacka, 2009; Henriques, 2014; Qin et al., 2020). The biomass used to perform metals removal are available in large quantities and have no economic value (Arora, 2019), what solves the high cost disadvantage of almost other used wastewater treatment technologies.

In the 80s, it was studied the capacity of biosorbents to remove Uranium and Thorium (Tsezos & Volesky, 1982; Tsezos & Volesky, 1981). The popularity of biosorption increased in the world of Science in the 90s. Volesky et al. (1993) and Holan et al. (1993), studied the removal of Cd from aqueous solutions. Holan et al. (1993) used biomass of marine algae, studying the impact of variables in the process, such as pH, drying temperatures, initial Cd concentration, and crosslinking of algae biomass with substances such as formaldehyde. The study included the comparison with commercial ion exchange resins existing at that time, and the biosorbents outperformed them. Volesky et al. (1993) tested non-living and resting cells of *Saccharomyces cerevisiae*. In 3 minutes, 73 % of the biosorption was already completed. Later, biosorption has been dubbed a possible solution to water contamination problem by toxic trace metal contamination (Vieira & Volesky, 2000). Over the last years, biosorbents have been the focus for many

researchers that tried live or dead biomass (Javanbakht et al., 2014), dry or wet (Pinto et al., 2020; Ramasamy et al., 2019), different dosages of biosorbents (Aly & Amasha, 2019; Fabre et al., 2020), with or without pre-treatment (Daneshvar et al., 2017), among others variables that could influence the process.

De Freitas et al. (2019) reported an overview of the research on sorption studies, regarding the removal of potentially toxic elements (Cd, Cr, Cu, Pb, Hg, Ni, and Zn). **Table 2** is adapted from De Freitas et al. (2019), selecting the five best (first five in descending order) and the two worst (the two last, where the penultimate represents the worst) sorbents for each contaminant, according to the respective q_{\max} , excluding any chemical modification. To better understand the table, it is necessary to consider that q_{\max} (mg/g) represents the maximum uptake values for each method, that is, the maximum quantity of contaminant removed (in mg) per each gram of sorbent. The M is the biomass dosage in g/L, and R the concentration range of each toxic metal studied, in mg/L. When there was no information about some of the topics, the table was filled with a NA. According to **Table 2**, the best sorbents were the bacteria and algal-based ones, except for Hg, where a fungal specie (*Saccharomyces cerevisiae*) performed better. However, this classification as “the best” and “the worst” considers only the values of q_{\max} . Depending on what problem to solve, and the contaminant concentration used, this classification can vary. For example, if the goal is to produce a non-expensive method, not only matters the q_{\max} , but also cost-related variables like the acquisition of the raw material (sorbent), the temperature used and additional pre-treatments. If the goal is to produce a green removal technology, it is wanted high contaminants removal using industry wastes (e.g., husks) or using high available sorbents in the environment (e.g., algae) that produce low contaminated sludge. Chemically modified sorbents are not shown in **Table 2** because this pre-treatment is an additional step that leads to bigger costs of the removal technology, and the goal was to have a general comparison of the sorbents near its raw state. However, the information presented by de Freitas et al. (2019), allows to discuss the effect on this modification. Some sorbents were only studied as chemically modified, therefore, even with good results, it does not allow a comparison with non-modified sorbents. On the other hand, some authors have studied both ways. For the Cd removal, *Ulva lactuca* sp. waste (after solvent extraction of oil) was used with chemical modification (alkaline treatment) (Bulgariu & Bulgariu, 2016) and without (Bulgariu & Bulgariu, 2012), and this modification increased q_{\max} from 33.7 to 41.9 mg/g. Chitosan

was used for Cr removal, and the natural form presented a lower q_{\max} than the crosslinked (chemically modified) (65.7 and 98.2 mg/g, respectively) (Baroni et al., 2008). For the Cu removal, there are three different studies with *Sargassum sp.*, by three different authors (Barquilha et al., 2017; Chen & Yang, 2005; Sheng et al., 2004). One of them used this sorbent after a chemical modification (0.2 % of formaldehyde), unlike the others. One of the non-modified *Sargassum sp.* presented higher q_{\max} (94.2 mg/g) (Barquilha et al., 2017) than the modified one (87.1 mg/g) (Chen & Yang, 2005). The other study with a non-modified *Sargassum sp.* presented the lowest q_{\max} (62.9 mg/g) (Sheng et al., 2004). Nevertheless, it should be noted that these studies were performed in different years, with different temperatures, dosages, range of metal concentration and by different persons, making the comparison unfeasible. In another study with Cu, a chemical modification (acidified) and a rise in temperature on dealginated residue increased q_{\max} from 50.9 (de Freitas et al., 2017) to 227.5 mg/g (de Freitas et al., 2018). For the Hg removal with chitosan there was an increase in q_{\max} from 25.3 to 75.5 mg/g with a chemical modification (glutaraldehyde) (Vieira & Beppu, 2006). *Sargassum sp.* was also used on Ni removal, by three different authors, with different conditions. The one with the chemical modification (0.2 % of formaldehyde) presented the best q_{\max} (71.6 mg/g) (Chen & Yang, 2005) than the others without (53.6 (Barquilha et al., 2017) and 35.8 mg/g (Sheng et al., 2004)).

Table 2- Examples of sorbents and respective experimental conditions found in the literature (adapted from de Freitas et al., 2019)

Toxic metal	Sorbent	Classification	pH / T(°C)	M (g/L) / R (mg/L)	q_{\max} (mg/g)
Cadmium	<i>Pseudomonas sp.</i>	Bacteria	7.0 / NA	2.0 / 10-1000	278
	<i>Staphylococcus xylosum</i>	Bacteria	6.0 / NA	2.0 / 10-1000	250
	<i>Fucus spiralis</i>	Algae	6.0 / NA	0.5 / 10-150	115
	<i>Sericin-alginate particles</i>	Industrial waste (cocoon of silkworm silk)	NA / 20	10.0 / 2-787	93.4

Table 2 – Continuation.

Toxic metal	Sorbent	Classification	pH / T(°C)	M (g/L) / R (mg/L)	qmax (mg/g)
Cadmium (cont.)	<i>Ascophyllum nodosum</i>	Algae	6.0 / NA	0.5 / 10-150	87.7
	<i>S. cerevisiae</i>	Fungi	7.2 / 25	2.13 / 0-27	6.71
	<i>Mucor rouxii</i>	Fungi	6.0 / NA	0.67 / 0-10	20.3
Chromium	<i>Sargassum muticum</i>	Algae	2.0 / NA	2.0 / 10-150	196
	<i>Staphylococcus xylosus</i>	Bacteria	1.0 / NA	8.0 / 5-1100	143
	<i>Pseudomonas sp.</i>	Bacteria	4.0 / NA	1.0 / 5-450	95
	<i>Chitosan natural</i>	Polysaccharide	6.0 / 25	6.0 / 250-1000	65.7
	<i>Polysiphonia lanosa</i>	Algae	2.0 / 21	2.0 / 4-250	45.8
	<i>Sericin- alginate particles</i>	Industrial waste (cocoon of silkworm silk)	3.5 / 40	10.0 / 1-442	5.3
	<i>Pistia stratiotes</i>	Aquatic herbs	4.0 / 25	2.0 / 1-208	16.5
Copper	<i>Extracellular polymeric substances</i>	Protein, polysaccharides, nucleic acid (Bacteria from activated sludge)	5.5 / 35	NA / 5-50	700
	<i>Ca-alginate</i>	Commercial polysaccharide	5.0 / 30	1.33 / 0-445	118
	<i>Pseudomonas putida</i>	Bacteria	4.5 / 30	1.0 / 5-286	96.9

Table 2 – Continuation.

Toxic metal	Sorbent	Classification	pH / T(°C)	M (g/L) / R (mg/L)	qmax (mg/g)
Copper (cont.)	<i>Sargassum sp.</i>	Algae	5.0 / 22	1.0 / 0-135	94.2
	<i>Padina sp.</i>	Algae	5.0 / 22	1.0 / 0-135	72.4
	<i>Calcined rice husk</i>	Food waste	4.0 / 25	10.0 / 0-445	3.6
	<i>Silk sericin-alginate blend</i>	Industrial waste (cocoon of silkworm silk)	4.5 / 25	10.0 / 0-65	5.1
Lead	<i>Extracellular polymeric substances</i>	Protein, polysaccharides, nucleic acid (Bacteria from activated sludge)	6.0 / 35	NA / 5-150	512
	<i>Waste Activated sludge</i>	Mainly Bacteria	6.0 / 65	0.04 / 0-20	363
	<i>Ulva sp.</i>	Algae	5.0 / 22	1.0 / 0-373	303
	<i>Padina sp.</i>	Algae	5.0 / 22	1.0 / 0-373	259
	<i>P. purpurogenum</i>	Fungi	5.0 / 20	2.0 / 10-750	253
	<i>Calcined rice husk</i>	Food waste	4.5 / 25	10.0 / 0-725	11.0
	<i>Pistia stratiotes</i>	Aquatic herbs	4.0 / 25	2.0 / 5-830	46.6
Mercury	<i>S. cerevisiae</i>	Fungi	5.0 / 25	1.0 / 10-200	270

Table 2- Continuation.

Toxic metal	Sorbent	Classification	pH / T(°C)	M (g/L) / R (mg/L)	qmax (mg/g)
Mercury (cont.)	<i>C. reinhardtii</i>	Algae	6.0 / 25	0.8 / 0-100	72.2
	<i>P. purpurogenum</i>	Fungi	5.0 / 20	2.0 / 10-750	70.4
	<i>Bacillus subtilis</i>	Bacteria	5.0 / 20	5.0 / 5-50	68.5
	<i>P. canescens</i>	Fruit	5.0 / 20	2.0 / 10-750	54.8
	<i>Bacillus sp.</i>	Bacteria	6.0 / 25	2.0 / 0-10	7.9
	<i>Chitosan natural</i>	Polysaccharide	6.0 / 25	12.0 / 37-375	25.3
Nickel	<i>Ca-alginate</i>	Commercial polysaccharide	5.0 / 30	1.33 / 0-411	71.7
	<i>Dealginated residue</i>	Algae	3.5 / 50	2.0 / 29-1057	61.1
	<i>Sargassum sp.</i>	Algae	5.0 / 30	1.33 / 0-411	53.6
	<i>Fucus spiralis</i>	Algae	6.0 / NA	0.5 / 10-150	50.0
	<i>S. cerevisiae</i>	Fungi	5.0 / 25	1.0 / 10-200	46.3
	<i>Activated sludge</i>	Mainly Bacteria	5.0 / 25	1.0 / 10-150	8.8
	<i>P. fluorescens</i>	Bacteria	5.0 / 27	1.0 / 0-100	12.4

Table 2 – Continuation.

Toxic metal	Sorbent	Classification	pH / T(°C)	M (g/L) / R (mg/L)	q _{max} (mg/g)
Zinc	<i>Extracellular polymeric substances</i>	Protein, polysaccharides, nucleic acid (Bacteria from activated sludge)	6.0 / 35	NA / 5-150	322
	<i>Waste activated sludge</i>	Mainly Bacteria	6.0 / 65	0.04 / 0-20	219
	<i>P. simplicissimum</i>	Fungi	5.0 / 28	1.0 / 0-300	65.6
	<i>Mucor rouxii</i>	Fungi	6.0 / NA	0.67 / 0-10	53.9
	<i>Fucus spiralis</i>	Algae	6.0 / NA	0.5 / 10-150	53.2
	<i>Silk sericin-alginate blend</i>	Industrial waste (cocoon of silkworm silk)	4.5 / 25	10.0 / 0-65	4.1
	<i>Cocoa pod husk</i>	Food waste	6.0 / 30	5.0 / 5-100	14.1

In general, chemically modified sorbents can improve the removal, leaving it up to the authors or the end users of the process (when applied) to decide whether this improvement is significant and necessary depending on the main goal.

Algae were also used with promising results and, when using macroalgae, the separation of the sorbents from solution after bioaccumulation seems to be a non-existing problem (Henriques et al., 2017). Henriques et al. (2015) used living marine macroalgae (*Ulva lactuca*, *Gracilaria gracilis* and *Fucus vesiculosus*) to remove Hg from saline waters, with three concentrations (10, 50 and 100 µg/L). The dosage of each algae per bottle was 1.6 g/L and the uptake of Hg was tested for 6 days, at 20 °C and initial pH of 7.9. At the end of exposure, removal values of about 89 to 99 % were reached, being *U. lactuca* the fastest and *F. vesiculosus* the slowest to remove Hg. The values of

bioaccumulation capacity were in the range of 20.8 – 208.5 µg/g. The same experiment was performed using dead biomass of *U. lactuca* as sorbent, and it was proved that living *U. lactuca* was better, reaching higher removals. This is justified by Henriques et al. (2015), with the accumulation process that only occurs in living organisms. Henriques et al. (2017) studied the bioaccumulation of Hg, Cd, and Pb by *F. vesiculosus* in mono-elemental conditions and mixtures. The experiment lasted 7 days, using a dosage of 1.7 g/L (fresh weight) of algae and concentrations of 10 – 200 µg/L for Cd, 50 – 1000 µg/L for Pb, and 10 - 100 µg/L for Hg, in the mono-elemental essays. Two mixtures were also studied: mixture 1 with 50 µg/L of each contaminant, and mixture 2 with 200 µg/L of Cd, 1000 µg/L of Pb and 50 µg/L of Hg. Removal percentages range was 62 – 67 % for Pb, and 93-97 % for Hg. In the Cd essays, initial concentrations affected the process and removal percentages range was 25 – 76 %. In mixture 1, removals were 94, 76 and 63 % for Hg, Pb and Cd, respectively, while for mixture 2, removal percentages were 97, 68 and 28 % for Hg, Pb and Cd, respectively. The bioremediation potential of living macroalgae was demonstrated (De Freitas et al., 2019; Henriques et al., 2015; Henriques et al., 2017) and the steps that have been taken concern the different types of contaminants that can be removed, and the optimization of the process. Following this logic, Henriques et al. (2019) studied the use of living macroalgae *U. lactuca* to perform the simultaneous removal of some trace elements, using three different algal doses (1.5, 3.0 and 6.0 g/L – fresh weight) and two different ionic strengths (salinity 15 and 35). The concentrations of each element were the maximum allowable concentration of those contaminants in effluent discharges. No significant differences were observed between the salinity 15 and 35. This fact was classified as promising by Henriques et al. (2019), because wastewaters are rich in salts and this can interfere with the efficiency of some of the convention treatment methods (Adeli et al., 2017). Removal percentage increased with the algae dose reaching water quality for human consumption for Hg. Quality for irrigation water was achieved for Cu, Pb and Ni (Henriques et al., 2019).

The vast majority of studies used distilled water as a matrix for the sorption assays (Ahmad et al., 2017; Annadurai et al., 2003; Dakiky et al., 2002; DeMessie et al., 2015; Lee & Yang, 1997; Senthilkumar et al., 2012; Singha & Das, 2011; Thirumavalavan et al., 2010).

The bioremediation, biosorption and bioaccumulation are areas that deserves more attention, optimization, and investment so it can be possible to apply all this knowledge

in practice in a near future. Biomass comprises a huge variety of materials that can be used as sorbents. The different characteristics between them allow different methods of removal to be created depending on the contaminants and their concentrations to be removed. The relative ease (compared to conventional methods) with which some types of sorbents are separated at the end of exposure, the low cost of obtaining these same materials, the smaller amount of contaminated waste at the end and the possibility of using industry waste promoting sustainability, make this an area that, sooner or later, will be applied in large scale in industry.

1.5 Design of experiments

In order to optimize processes, authors choose to study some factors that have influence on the process. Design of Experiments (DOE) allows to study different factors and has been used for efficient experiments and to analyse the given results (Anderson, 1997). One of the used methods is the Response Surface Method (RSM), a set of techniques that allows to establish a relation between independent factors and the respective responses (Fabre et al., 2019). The techniques used are mathematical and statistical, and a series of n experiments is performed, measuring the response for each one (Khuri & Mukhopadhyay, 2010).

Fabre et al. (2019) used the RSM while studying the removal of Hg using *Eucalyptus globulus* bark and the different factors that the RSM allowed to study were pH, salinity and biosorbent dosage. Ghani et al. (2017) studied the optimization of an adsorptive treatment of landfill using activated carbon from banana. The author used the RSM to study the activation temperature, activation time and impregnation ratio. These are two examples found that illustrate the use of RSM for reach optimal conditions.

2 OBJECTIVES

The main goal of this is to optimize the removal of mercury, cobalt, and nickel in mixture by the living macroalgae *Ulva lactuca*.

The specific objectives are:

- Evaluate the toxicity of Hg, Co and Ni in mussel *Mytilus galloprovincialis*;

- Use the macroalgae *Ulva lactuca* as biosorbent to remove mercury, cobalt, and nickel both in mono-elemental solutions and in mixture, using concentrations that mimic environmental and discharge conditions and real water matrices (e.g., seawater);

- Evaluate the influence of experimental parameters on sorption process and proceed to the optimization of the process.

3 MATERIALS AND METHODS

3.1 Cleaning procedure for laboratory material

Before the beginning of the experimental work, all the material used in the laboratory was properly cleaned to avoid unwanted contaminations. The first step was to rinse all the glassware material with tap water. Then, the same material was rinsed with distilled water and filled with HNO₃ (65 %, m/m) for 24 h. The next step was to submerge the material in HNO₃ (25 %, v/v) for 48 h. Finally, all the material was again rinsed with distilled water and dried at room temperature.

3.2 Chemical reagents

All the chemicals used in this study were of analytical grade and purchased from chemical suppliers. The certified standard solution of Mercury (1000 mg/L) was purchased from Merck Certipur®. The certified standard solution of Cobalt (1000 mg/L) was purchased from VWR Chemicals®. The certified standard solution of Nickel (10.000 ± 0.031 mg/L) was purchased from Inorganic Ventures™. All the working solutions were obtained by diluting the stock solution to the desired concentrations.

3.3 Evaluation of the toxicological effects of Hg, Co, and Ni in *Mytilus galloprovincialis*

3.3.1 Experimental conditions

To perform this experiment, mussel *Mytilus galloprovincialis* was chosen as biological indicator, because it is well known that this species responds with biochemical alterations when in contact to contaminants (Andrade et al., 2019; Coppola et al., 2018; Dallas et al., 2013; Freitas et al., 2020; Henriques et al., 2019; Pinto et al., 2019; Teixeira et al., 2017). Organisms were collected at the Ria the Aveiro lagoon (northwest Atlantic

coast of Portugal), in September 2020. This sampling site was studied by several authors, measuring the Hg (Coelho et al., 2005; Pereira et al., 2009; Rodrigues et al., 2010), Co and Ni (Geraldes et al., 2017; Rodrigues et al., 2010) concentrations in water (dissolved) and in sediments. In order to prevent differences in biological responses, mussels with



Figure 1- Cleaning mussels after sampling (left) and depuration/acclimation to the experimental conditions (right).

similar size (5.6 ± 0.9 cm length; 2.8 ± 0.7 cm width) were selected. After sampling, organisms were cleaned and placed in aquaria for 2 weeks for depuration and acclimation to the experimental conditions (**Figure 1**). The temperature chosen was 17 ± 1 °C and the salinity of artificial seawater was 30 ± 1 , resembling the sampling site. This artificial seawater was prepared with reverse osmosis water and Tropic Marin® SEA SALT (commercial salt). Mussels were fed every 2-3 days with Algamac protein plus (150,000 cells/animal/day) and the artificial seawater was often renewed during this period.

After the depuration/acclimation period, mussels were exposed for 28 days to the following 5 treatments (all at 17 ± 1 °C and salinity 30 ± 1): biological control (CTL, non-contaminated artificial seawater), 25 µg/L of Hg, 200 µg/L of Co, 200 µg/L of Ni and the mixture of these three PTEs (MIX, 25 µg/L of Hg, 200 µg/L of Co, 200 µg/L of Ni) (**Figure 2**).

These concentrations were chosen to mimic environmental conditions by diluting (2 times for Hg and 10 times for Ni and Co) the maximum admissible concentrations in effluents for each one of the elements (for Co it was used the same limit as Ni). It was also studied in mixture because the environment it is normally a multi-elementary

scenario. Each treatment had 3 replicates (3 L aquaria) and 6 mussels in it (6 organisms per aquarium, 18 per condition). A parallel study without mussels was conducted to evaluate the stability of the studied PTEs in water, and guarantee that during the 28 days the conditions were the pretended, discarding losses of PTEs concentrations due to external factors like aquarium walls, precipitation or others. For this, aquaria with 3 L of artificial seawater (17 ± 1 °C and salinity 30 ± 1) were contaminated with Hg, Co, Ni and MIX with the same concentrations as the experiment containing mussels. Two aquaria were used per treatment and aliquots of 5 mL were collected immediately after spiking, to assess the initial conditions, and before water renewal (1 time per week), to check if there was decrease in concentration.

During all the study, organisms were fed 3 times per week with Algamac protein plus (150,000 cells/animal/day). Artificial seawater was renewed weekly, and the experimental conditions re-established. Temperature and salinity were checked every day and aquaria were continuously aerated (**Figure 2**). Aliquots of 5 mL were collected in the contaminated conditions immediately after spiking (as in the parallel study) to assess the initial concentrations and check if it were the pretended concentrations – real vs nominal concentrations. For the Co and Ni analysis, aliquots were put in test tubes closed with parafilm, and stored at 4° C.



Figure 2 – Some close-ups from the conducted experiment.

For the Hg analysis, aliquots were put in Schott flasks, closed with the respectively screw cap and reinforced with parafilm. All samples were acidified using HNO₃ (Suprapur®, 65 % v/v) until pH ≤ 2. Then, this samples were immediately analysed or stored at -20 °C. Mortality was assessed during the 28 days experiment.

At the end of the 28 days experiment, organisms were frozen with liquid nitrogen and kept at -80 °C. Then, the whole soft tissue was homogenized manually using a mortar and a pestle under liquid nitrogen (**Figure 3**). Each individual organism was separated into aliquots with 0.5 g fresh weight (FW) for further biochemical study.



Figure 3 – Organisms being frozen with liquid nitrogen (left) and homogenization of the whole soft tissue (right).

3.3.2 Quantification of Hg, Co, and Ni in water

The quantification of Hg in water was made by cold vapour atomic fluorescence spectroscopy (CV-AFS), on a PSA 10.025 Millennium Merlin Hg analyser, using SnCl₂ (2 % m/v in HCl 10 % v/v) as reducing agent. Every day, standard solutions of Hg were prepared (0.0, 0.1, 0.2, 0.3 and 0.5 µg/L) to obtain a calibration curve ($r^2 > 0.995$). This calibration curve step was done three or four times a day. Blank measurements were performed (n = 20) to obtain the detection (0.007 µg/L) and quantification (0.02 µg/L) limits in this range (**Figure 4**).

The quantifications of Co and Ni in water were performed by inductively coupled plasma mass spectrometry (ICP-MS), on a Thermo ICP-MS XSeries equipped with a Burgener nebuliser at the *Laboratório Central de Análises* (LCA). The limits of quantification of the method were 0.1 µg/L and 2 µg/L for Co and Ni respectively (**Figure 5**).



Figure 4 - Cold vapour atomic fluorescence spectroscopy (CV-AFS) (top) and daily standard solutions (bottom).



Figure 5 – Inductively coupled plasma mass spectrometry (ICP-MS) – Thermo ICP-MS XSeries equipped with a Burgener nebuliser.

3.3.3 Biochemical parameters

To evaluate the biochemical responses in mussels after exposure to Hg, Co and Ni in the mono-elemental condition and mixture, 11 biochemical markers were studied. For each one was used 0.5 g FW (soft tissue) per mussel, studying 3 mussels per aquarium and 9 per treatment. The biochemical markers analysed can be divided in groups: energy reserves (glycogen content, GLY; total protein content, PROT), antioxidant defences (activities of superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx), biotransformation defences (glutathione S-transferases, GSTs; carboxylesterase, CbEs), cellular damage (lipid peroxidation levels, LPO; protein carbonyl levels, PC; reduced glutathione, GSH) and neurotoxicity (acetylcholinesterase, AChE). Each sample refers to each individual organism and was measured in duplicate. Three different extraction buffers were used: phosphate buffer (50 mmol/L potassium dihydrogen phosphate; 50 mmol/L potassium phosphate dibasic; 1 mmol/L ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (w/v) polyvinylpyrrolidone (PVP); 1 mmol/L dithiothreitol (DTT); pH 7.0) for GLY, PROT, SOD, CAT, GPx, GSTs,

CbEs, PC and AChE; trichloroacetic acid (20 % (w/v)) for LPO and KPE buffer (0.1 % Triton X-100 and 0.6 % sulfosalicylic acid in KPE (0.1 mol/L potassium phosphate buffer, 5 mmol/L EDTA, pH = 7.5)) for GSH. Every sample was sonicated for 15 s at 4 °C using a TissueLyser II (Qiagen) and then centrifuged for 20 min at 10,000 g. Supernatants were stored at – 80 °C when not immediately used. All biochemical markers were read in the same microplate reader (Biotek).

Metabolic capacity and energy reserves

For Gly quantification was used the sulphuric acid method, as described by DuBois et al. (1956). Was created a calibration curve with glucose standards (0 – 10 mg/mL) and the absorbance was measured at 492 nm after 30 min incubation time at room temperature. Results were expressed in mg per g FW.

The PROT content was determined based on the spectrophotometric Biuret method described by Robinson & Hogden (1940). Was created a calibration curve with bovine serum albumin (BSA) as standard (0 – 40 mg/mL) and the absorbance was measured at 540 nm. Results were expressed in mg per g FW.

Antioxidant defences

The SOD activity was determined using the method of Beauchamp & Fridovich (1971) after adaptations by Carregosa et al. (2014). Was created a standard curve with SOD standards (0.25 – 60 U/mL) and the absorbance was read at 560 nm after 20 min incubation time at room temperature. Results were expressed in U per g FW (U means one unit and corresponds to the quantity of the enzyme that catalyses the conversion of 1 µmol of substrate per min).

The CAT activity was determined by the Johansson & Borg (1988) method after adaptations performed by Carregosa et al. (2014). Was created a standard curve with formaldehyde standards (0 – 150 µmol/L) and the absorbance was read at 540 nm. Results were expressed in U per g of FW (U corresponds to the amount of enzyme that caused the formation of 1.0 nmol formaldehyde per min at 25 °C).

The GPx activity was determined following Paglia & Valentina (1967) methodology. The absorbance was read at 340 nm, every 10 s during 5 min. The enzymatic activity was determined using molar extinction coefficient (ϵ) = 6.22

(mmol/L)⁻¹ cm⁻¹, and the results were expressed in U per g FW (U corresponds to the amount of enzyme that caused the formation of 1.0 μmol NADPH oxidized per min).

Biotransformation defences

The GSTs activity was determined following Habig et al. (1974) methodology after modifications performed by Carregosa et al. (2014). The absorbance was read at 340 nm and the enzymatic activity was determined using $\epsilon = 9.6 \text{ (mmol/L)}^{-1} \text{ cm}^{-1}$. Results were expressed in U per g of FW (U corresponds to the amount of enzyme that catalysis the formation of 1 μmol of dinitrophenyl thioether per min).

The CbEs were measured following Hosokawa & Satoh (2001) methodology. Were used 2 different commercial substrates: ρ-nitrophenyl acetate (ρNPA) and ρ-nitrophenyl butyrate (ρNPB). The absorbance was read at 405 nm and was used $\epsilon = 18 \text{ (mmol/L)}^{-1} \text{ cm}^{-1}$ for the calculations. Results were expressed in nmol per min per g FW.

Cellular damage

The LPO was quantified based on the methodology described by Ohkawa et al. (1979). The absorbance was read at 535 nm and was used $\epsilon = 156 \text{ (mmol/L)}^{-1} \text{ cm}^{-1}$ for the calculations. Results were expressed in nmol of MDA formed per g of FW (MDA is the abbreviation of malondialdehyde, a by-product of lipid peroxidation).

The PC was quantified following the 2,4-dinitrophenylhydrazina (DNPH) alkaline method, described by Mesquita et al. (2014). The absorbance was read at 450 nm and was used $\epsilon = 23.308 \text{ (m,mol/L)}^{-1} \text{ cm}^{-1}$ for the calculations. Results were expressed in nmol per g of FW.

The GSH was quantified following the methodology described by Moron et al. (1979) after modifications performed by Carregosa et al. (2014). Was created a standard curve with glutathione standards (0 – 500 μmol/L) and the absorbance was read at 412 nm. Results were expressed as μmol per g of FW.

Neurotoxicity

The AChE activity was quantified following the Ellman et al. (1961) methodology after modifications performed by Mennillo et al. (2017). The absorbance was read at 412 nm for 5 min. The enzymatic activity was determined using $\epsilon = 13.6 \text{ (mmol/L)}^{-1} \text{ cm}^{-1}$. Results were expressed in nmol per min per g FW.

3.3.4 Statistical analyses

After all the calculations from the biochemical markers, results were submitted to statistical hypothesis testing. This was performed through permutational analysis of variance, using the software PERMANOVA + add-on in PRIMER v6 (Anderson et al., 2008). After the main test and when significant differences were observed, pairwise comparisons were performed among treatments. When $p < 0.05$, compared values were considered significantly different and identified in the figures with different lowercase letters.

3.4 Study of the removal of Hg, Co, and Ni with the macroalgae *Ulva lactuca*

3.4.1 Experimental conditions of the preliminary study

Marine macroalgae *Ulva lactuca* was collected in the Ria de Aveiro lagoon (northwest Atlantic coast of Portugal, 40°38'39''N, 8°44'43''W) (**Figure 6**). After sampling, macroalgae were plenty washed in the laboratory with fresh water to get rid of impurities, sand or even animal shells. Then, macroalgae were maintained in an aerated aquarium under natural light and at room temperature of 20 ± 2 °C. Seawater used for macroalgae maintenance and for the removal experiments was collected at Barra beach (Aveiro, Portugal) (**Figure 6**) (salinity 35), and filtered through 0.45 μm Millipore membrane.



Figure 6 – Macroalgae was collected at Ria de Aveiro lagoon (left) and seawater was collected at Barra beach (right).

Removal trials were carried out in glass Schott bottles of 0.5 L, covered up with parafilm with a few holes to allow only gas exchange. The mass of biosorbent (*U. lactuca*)

chosen to evaluate the removal of Hg, Co and Ni in the preliminary essay was 1.5 g FW per L. Macroalgae was weighed, and put in contact with the spiked solutions (**Figure 7**). It was exposed to 0.5 L of mono-elemental Hg (0.05 mg/L), Co (2 mg/L), Ni (2 mg/L) and mixtures 1 (Hg, 0.05 mg/L; Co, 2 mg/L; Ni, 2 mg/L) and 2 (Hg, 0.005 mg/L; Co, 0.2 mg/L; Ni, 0.2 mg/L). Concentrations of 0.05 mg/L for Hg and 2 mg/L for Ni, corresponds to the limits for these PTEs in wastewater discharges (Portuguese Decree-Law 236/98). There is no limit for Co in wastewater discharges (except in Germany), so it was used the same as in Ni because of the similarity between them in uses and concentrations in the environment described above in this work. The initial pH for all the solutions was within the range 7.8 – 8.0, and no pH adjustments were performed during the experiment. All the experiments were done in duplicate and a parallel experiment without macroalgae (controls) was performed to study the stability of each one of the elements in the mono-elemental conditions and mixtures, and to evaluate potential losses to external factors like precipitation or sorption to bottles walls. One blank solution (only with macroalgae and real seawater) was also studied to check the physiological condition of the macroalgae during all the process (**Figure 7**).



Figure 7 – Set up of the preliminary study of Hg, Co and Ni removal. On the top right corner it is represented the disks of macroalgae used in the essay.

This experiment lasted 72 h and had the same natural light and temperature as the aquariums where macroalgae were maintained. Aliquots of 5 mL were taken at 0, 1, 6, 24, 48 and 72 h for the experiments with macroalgae, and at 0, 24, 48 and 72h for the experiments without macroalgae (controls). For the Co and Ni analysis, aliquots were put

in polystyrene tubes closed with parafilm, and stored at 4° C. For the Hg analysis, aliquots were put in small Schott borosilicate glass flasks, closed with the respectively screw cap and reinforced with parafilm. All samples were acidified using HNO₃ (Suprapur®, 65 % m/m) until pH ≤ 2. Then, samples were immediately analysed or stored at -20 °C.

3.4.2 Optimization using the response surface methodology

Response Surface Methodology (RSM) is a tool for optimizing processes using proper methods of Design of Experiments (DoE). In this work, a Box–Behnken design (BBD) was used, which considers 3 factors in 3 levels (Fabre et al., 2019). RSM establishes a relation between different and independent factors and a final response, using linear and quadratic polynomial functions to describe the experimental results (Fabre et al., 2019). The analysis of variance (ANOVA) is one of the tools of RSM, giving p-values to each one of the factor. When the p-value is below 0.05, it is considered significant (Jokar et al., 2021). The goal of this experimental step is to have the maximum results with the minimum number of experiments possible, gaining time, decreasing costs (Witek-Krowiak et al., 2014), and making all the process more sustainable. The software used to analyse the data was *Design-experiment 13*.

To optimize the removal of Hg, Co, and Ni by the macroalgae *Ulva lactuca*, the 3 variables and 3 levels chosen were: sorbent (macroalgae) dosage (1.5, 3, 4.5 g/L), initial concentration of each element (Hg (5, 27.5, 50 µg/L), Co (200, 1100, 2000 µg/L), Ni (200, 1100, 2000 µg/L)) and salinity (15, 25, 35) (**Table 3**). The values of the extreme levels are equally distant from the mean (level 0) value. Then, a matrix following Box–Behnken design was created (**Table 4**). Finally, the response variable given by this methodology was the Hg, Co, and Ni removal (%) (results for each one of the element in mixture).

Table 3- Variables description and levels of work.

Variable	Level		
	-1	0	+1
Macroalgae dosage (g/L)	1.5	3	4.5
Initial concentration (µg/L)	Hg – 5	Hg – 27.5	Hg – 50
	Co – 200	Co – 1100	Co – 2000
	Ni - 200	Ni - 1100	Ni - 2000
Salinity	15	25	35

Table 4- Experimental condition of the Box-Behnken design.

Experiment	Macroalgae dosage (g/L)	Initial Concentration ($\mu\text{g/L}$)	Salinity
1	3	Hg - 5/ Co - 200 / Ni - 200	35
2	3	Hg - 27.5/ Co - 1100 / Ni - 1100	25
3	1.5	Hg - 27.5/ Co - 1100 / Ni - 1100	15
4	4.5	Hg - 50/ Co - 2000 / Ni - 2000	25
5	3	Hg - 5/ Co - 200 / Ni - 200	15
6	3	Hg - 27.5/ Co - 1100 / Ni - 1100	25
7	4.5	Hg - 27.5/ Co - 1100 / Ni - 1100	35
8	3	Hg - 27.5/ Co - 1100 / Ni - 1100	25
9	4.5	Hg - 5/ Co - 200 / Ni - 200	25
10	4.5	Hg - 27.5/ Co - 1100 / Ni - 1100	15
11	1.5	Hg - 27.5/ Co - 1100 / Ni - 1100	35
12	3	Hg - 50/ Co - 2000 / Ni - 2000	15
13	3	Hg - 50/ Co - 2000 / Ni - 2000	35
14	1.5	Hg - 5/ Co - 200 / Ni - 200	25
15	1.5	Hg - 50/ Co - 2000 / Ni - 2000	25

According to **Table 4**, there were 15 experiments to perform. Beside these, 4 parallel experiments were done: same conditions as the experiment 1, 5, 12 and 13 but without the macroalgae (control) to study the stability of the PTEs in solution. The stability of the macroalgae was also studied by putting the macroalgae in contact with non-contaminated water.

The optimization experiment lasted 120 h and had the same natural light and temperature as the aquariums where macroalgae were maintained. Aliquots of 5 mL were taken at 0, 24, 48, 72 h and 120 h for all the experiments. For the Co and Ni analysis, aliquots were put in test tubes closed with parafilm, and stored at 4° C. For the Hg analysis, aliquots were put in Schott flasks, closed with the respectively screw cap and reinforced with parafilm. All samples were acidified using HNO₃ (Suprapur®, 65 % v/v) until pH \leq 2. Then, samples were immediately analysed or stored at -20 °C.

3.4.3 Quantification of Hg, Co, and Ni in water

The quantifications of Co and Ni in water in this experiment was performed by inductively coupled plasma optical emission spectrometry (ICP-OES), at the *Laboratório Central de Análises* (LCA). The limits of quantification of the method were 5 µg/L and 10 µg/L, for Co and Ni respectively, with precision and accuracy.

The quantification of Hg in this experiment was performed as already described above.

3.5 Quality control

To guarantee that all the results meet the quality standards, control charts were elaborated with the values from the control experiments in all the essays. Using control charts, it is possible to observe the behaviour of the elements in study, that is, the variation of concentration along time. Warning and rejection lines help to understand what values meet the quality standards (**Figure 8**).

Values used in control charts represent a mean of 2 values ($n=2$) and were expressed in normalized concentration (C_t/C_0). What is expected is that the control values stay inside the warning line (10% deviation) or at least inside the rejection line (20 % deviation). When values are out the rejection line (losses in concentration when decreases or contamination when increases), they are not accepted and are discharged.

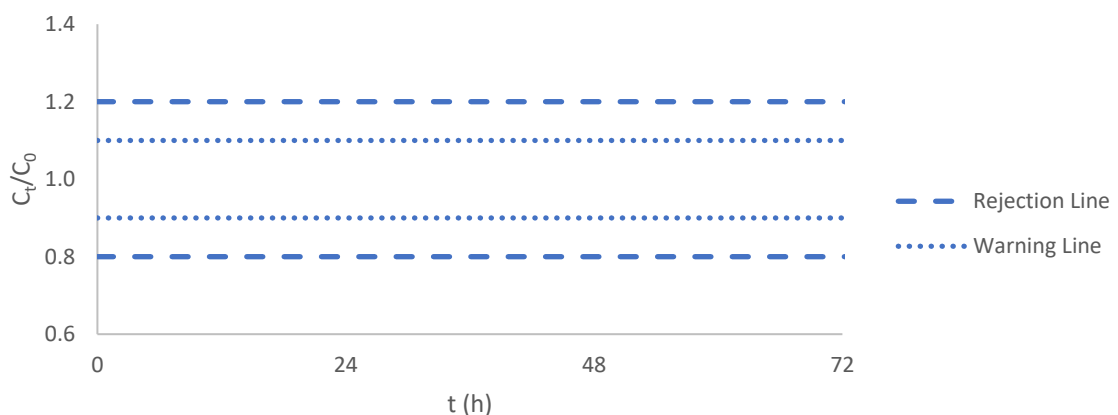


Figure 8 – Template of a control chart with the warning lines and the rejection lines.

4 RESULTS

The results obtained within this work will be presented, starting with the evaluation of the toxicological effects of Hg, Co and Ni in *Mytilus galloprovincialis*. Then, the results from a preliminary study on Hg, Co, and Ni removal by the macroalgae *U. lactuca* will follow and then the findings on the optimization of this removal process will be shown.

4.1 Toxicological effects of Hg, Co and Ni in *Mytilus galloprovincialis*

4.1.1 Mortality

During the 28 days of the experiment, none of the tested treatments induced mortality, achieving a 100 % survival rate.

4.1.2 Concentration of Hg, Co and Ni at the initial solutions

The nominal concentration for Hg was 25 µg/L and the real concentration was 25.6±5.1 µg/L. The nominal concentration for Co was 200 µg/L and the real concentration was 218±42 µg/L. The nominal concentration for Ni was 200 µg/L and the real concentration was 218±22 µg/L.

4.1.3 Biochemical markers

Metabolic capacity and energy reserves

For GLY content, no significant differences were observed among the CTL and mono-elementary conditions of Hg, Co and Ni. However, significantly lower GLY content was found in organisms exposed to MIX in comparison to all the others treatments, except mono-elementary Co exposed organisms (**Figure 9A**).

No significant differences were observed among all the treatments in terms of PROT content. However, slightly higher values were observed especially in mono-elementary Ni exposed organisms (**Figure 9B**).

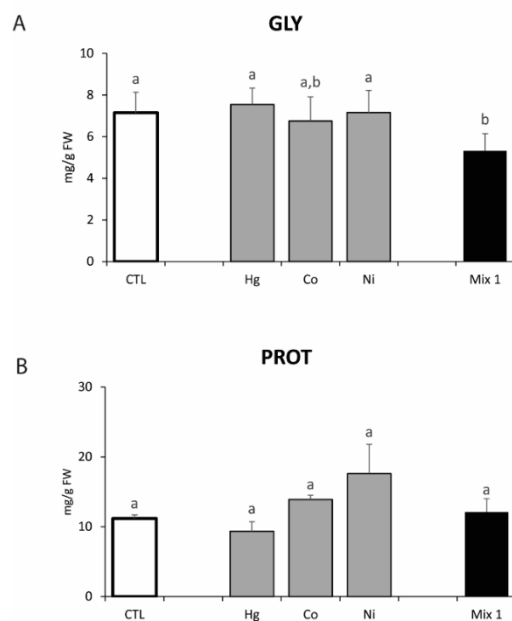


Figure 9 - A: Glycogen (GLY) content; B: Total protein (PROT) content, in *Mytillus galloprovincialis* exposed to different conditions at the end of the experiment. Results are mean + standard deviation. Different letters mean significantly differences between conditions.

Antioxidant defences

The activity of SOD increased in organisms exposed to Hg, Co and Ni, with significantly higher values in Hg and Co treatments in comparison to CTL, with no significant differences between Hg and Co treatments. No significant differences were observed between organisms under CTL and Mix treatments (**Figure 10A**).

The activity of CAT increased in Ni and Mix exposed organisms, but significantly higher values were only observed in mussels exposed to Mix compared to CTL, Hg and Co treatments. No differences were observed among CTL, Hg, Co and Ni conditions (**Figure 10B**).

The GPx activity was significantly higher in contaminated organisms compared to CTL ones. No significant differences were observed between all the contaminated organisms (**Figure 10C**).

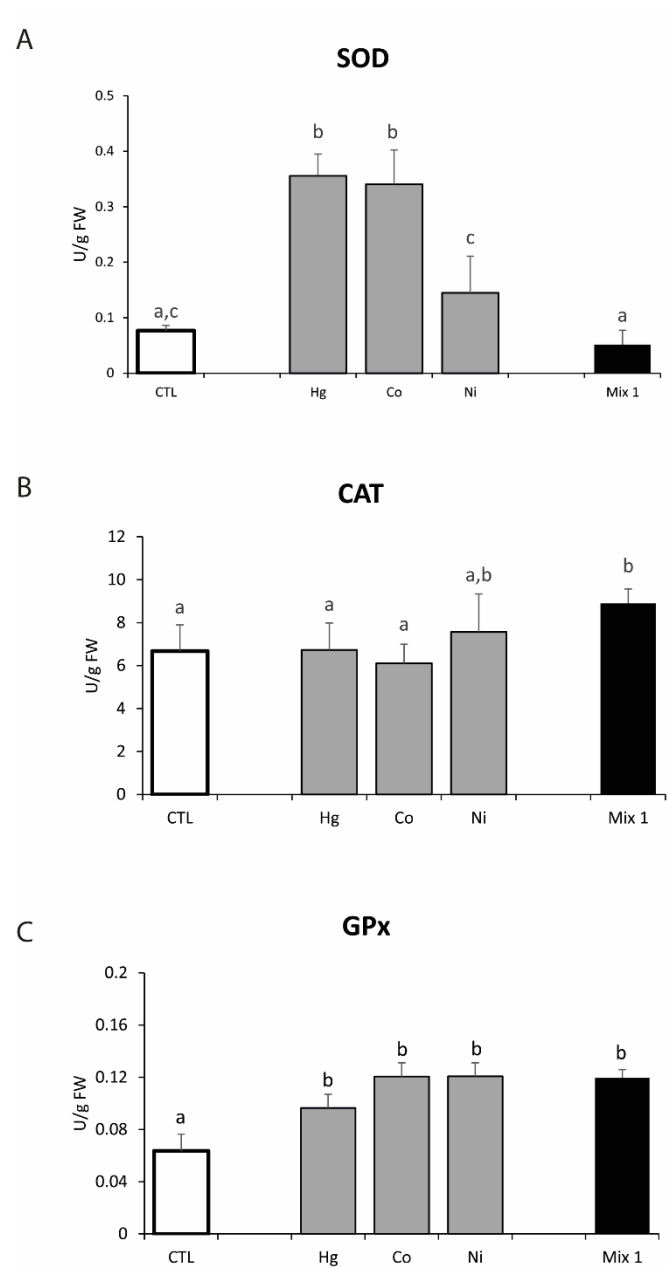


Figure 10. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione peroxidase (GPx) activity, in *Mytilus galloprovincialis* exposed to different conditions at the end of the experiment. Results are mean + standard deviation. Different letters mean significant differences between conditions.

Biotransformation defences

The activity of GSTs decreased significantly in Hg and Mix treatments in comparison to the remaining ones. No significant differences were found among CTL, Co and Ni treatments (**Figure 11A**).

The CbEs activities showed a similar pattern (**Figures 11B** and **11C**). In both cases, there was a significantly lower activity in organisms contaminated with Hg, Co or Ni in comparison to CTL ones. In CbEs_pNPA no significant differences were observed between organisms in CTL and organisms exposed to Mix, while in CbEs_pNPB there was a significant increase in organisms exposed to Mix in comparison to CTL (**Figure 11C**).

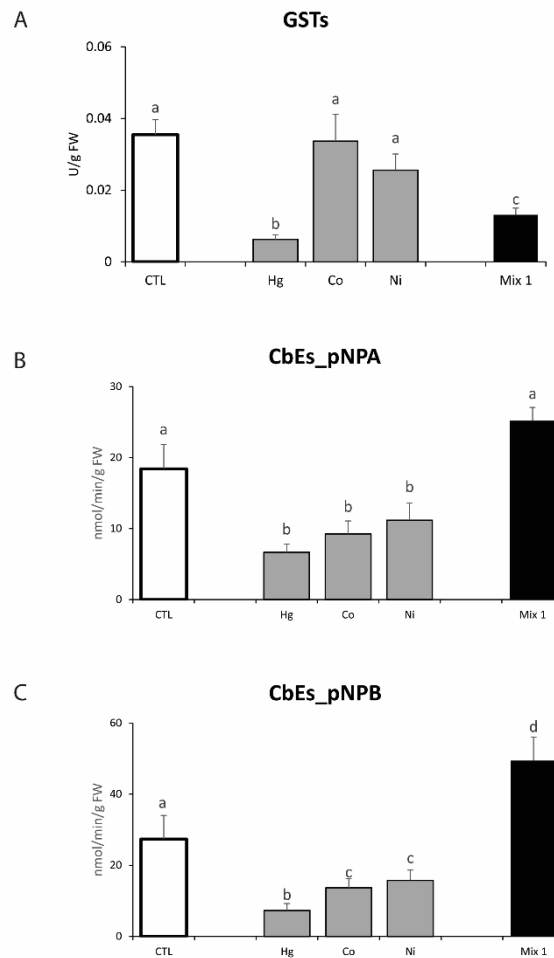


Figure 11. A: Glutathione S-transferases (GSTs) activity; B: Carboxylesterase activity measured using pNPA (CbEs_pNPA); C: Carboxylesterase activity measured using pNPB (CbEs_pNPB), in *Mytilus galloprovincialis* exposed to different conditions at the end of the experiment. Results are mean + standard deviation. Different letters mean significant differences between conditions.

Cellular damage

Levels of LPO were significantly lower in contaminated organisms, except in the ones contaminated with Hg that presented no significant differences compared to CTL. No significant differences were observed between Co and Ni exposed organisms. Levels

of LPO showed significant differences between Co and Mix conditions, with the lowest values in mussels exposed to the combined effect of the 3 elements. No significant differences were observed between Ni and Mix treatments (**Figure 12A**).

The PC levels were higher in Mix exposed organisms while the lowest values were observed in Ni exposed organisms, but differences to the CTL were only found to Mix organisms. No significant differences were found between CTL and mono-elementary treatments (**Figure 12B**).

In GSH levels no significant differences were observed among all the treatments (**Figure 12C**).

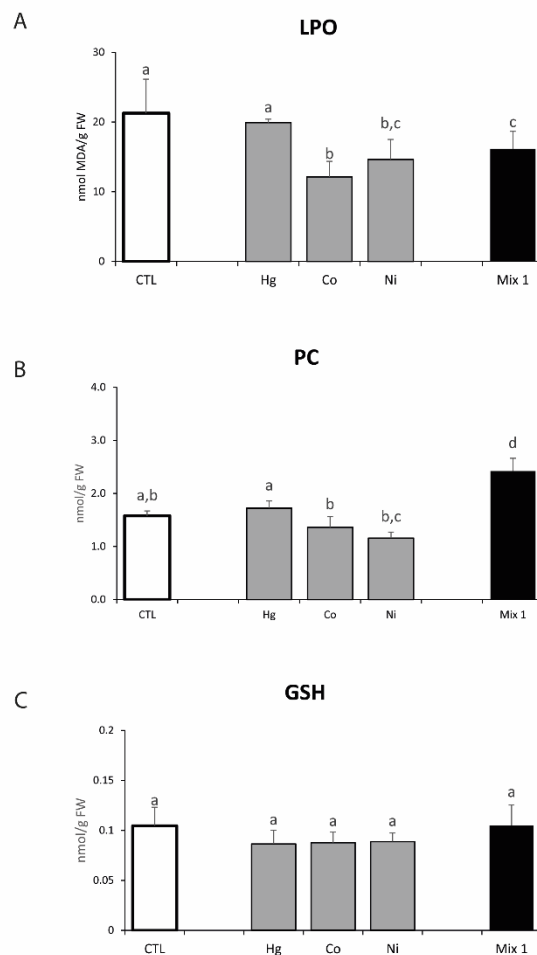


Figure 12. A: Lipid peroxidation (LPO) levels; B: Protein carbonylation (PC) levels; C: Reduced glutathione (GSH) content, in *Mytilus galloprovincialis* exposed to different conditions at the end of the experiment. Results are mean + standard deviation. Different letters mean significantly differences between conditions.

Neurotoxicity

The AChE activity decreased significantly in contaminated organisms. The lowest values were found in Hg and Co exposed organisms, with no significant differences between these treatments (**Figure 13**).

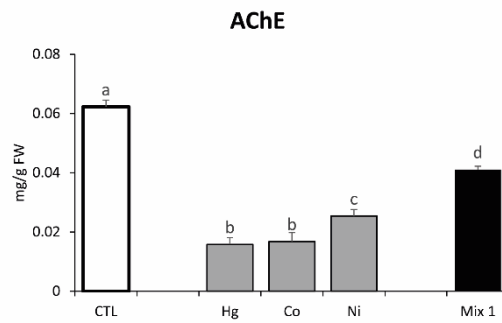


Figure 13. Acetylcholinesterase (AChE) activity, in Mytilus galloprovincialis exposed to different conditions at the end of the experiment. Results are mean + standard deviation. Different letters mean significantly differences between conditions.

4.2 Preliminary experiment on the removal of Hg, Co, and Ni with the macroalgae

4.2.1 Control charts

Figure 14 shows the control charts for Co (**A-C**), Ni (**D-F**) and Hg (**G-I**), where the normalized concentrations of the elements in solution over time, in the absence of macroalgae, are presented.

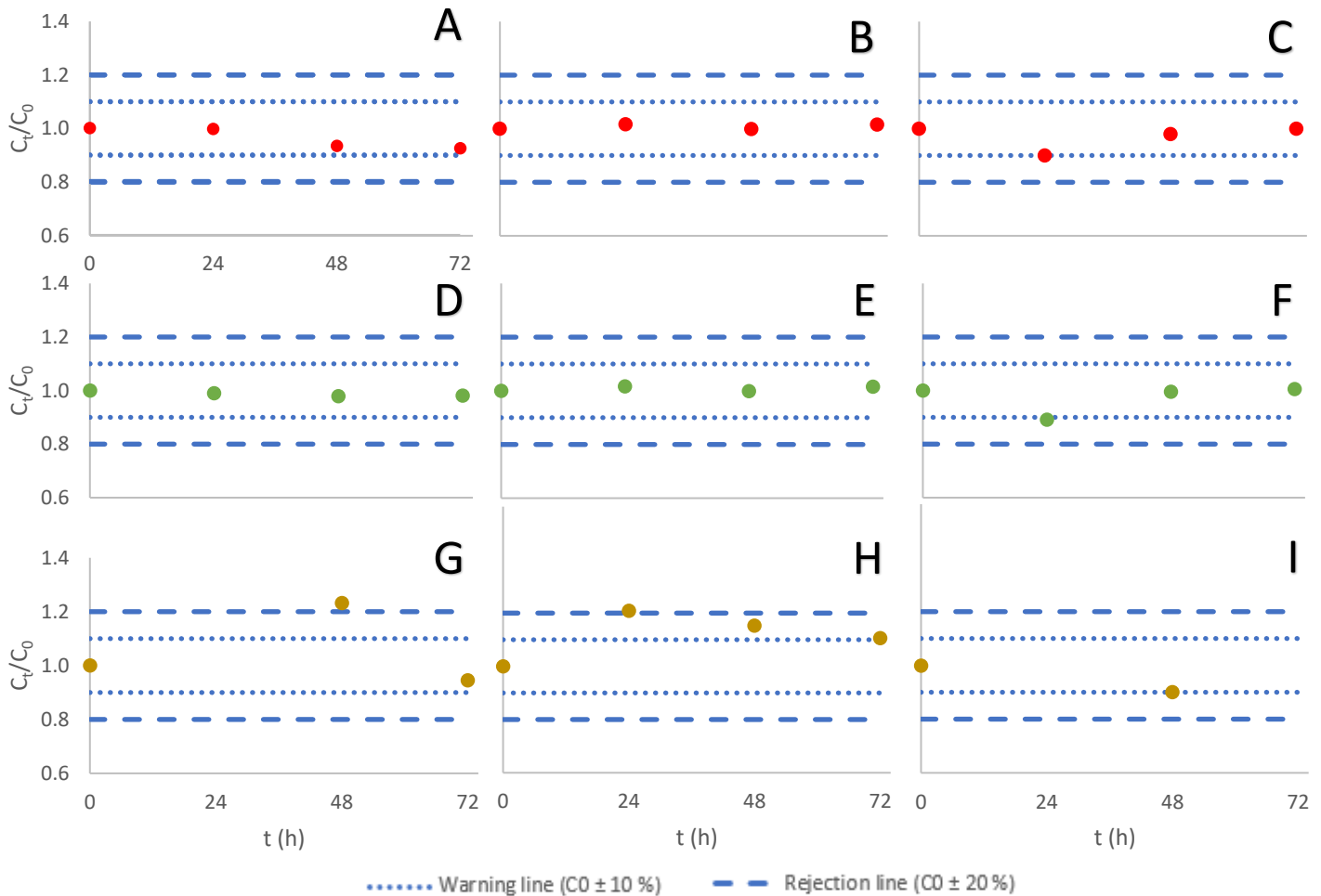


Figure 14 – Control charts for normalised concentrations for 0, 24, 48 and 72 h, for the follow conditions: A) Co in mono-elemental condition; B) Co in mixture with discharge conditions; C) Co in diluted mixture condition; D) Ni in the mono-elemental condition; E) Ni in mixture with discharge conditions; F) Ni in diluted mixture condition; G) Hg in mono-elemental condition; H) Hg in mixture with discharge conditions; I) Hg in diluted mixture condition. Co is in red, Ni is in green and Hg is in brown. Dash lines on $y = 0.9$ and $y = 1.1$ represent the warning line with an error of 10 %. Dash lines on $y = 0.8$ and $y = 1.2$ represent the rejection line with an error of 20 %.

All the values for Co and Ni are either inside the 10 % error line (warning) or inside the 20 % error line (rejection) (**Figure 14A-F**), pointing to the absence of relevant losses or contamination of the element due to external factors. The values of Hg in the mixture mimicking the discharge conditions (**Figure 14H**) are also within both lines, while in the mono-elemental condition (**Figure 14G**) and in the diluted mixture (**I**)

(Figure 14I) one and two points, respectively, are out of the rejection line (not represented). The overall stability of the elements in the controls allows us to assume that the potential decrease of the elements in solution, in the presence of algae, is due to their uptake by the organism.

4.2.2 Removal experiments

The results from the preliminary experiment on the removal of Hg, Co, and Ni by the macroalgae *U. lactuca* are presented in Figure 15, showing the normalized concentrations (C_t/C_0) for different times (0, 1, 6, 24, 48 and 72 h), in different conditions (mono-elemental, discharge value, and diluted mixture). In the mono-elemental condition, there are no results for the removal of Co after 1 and 6 h due to sample losses.

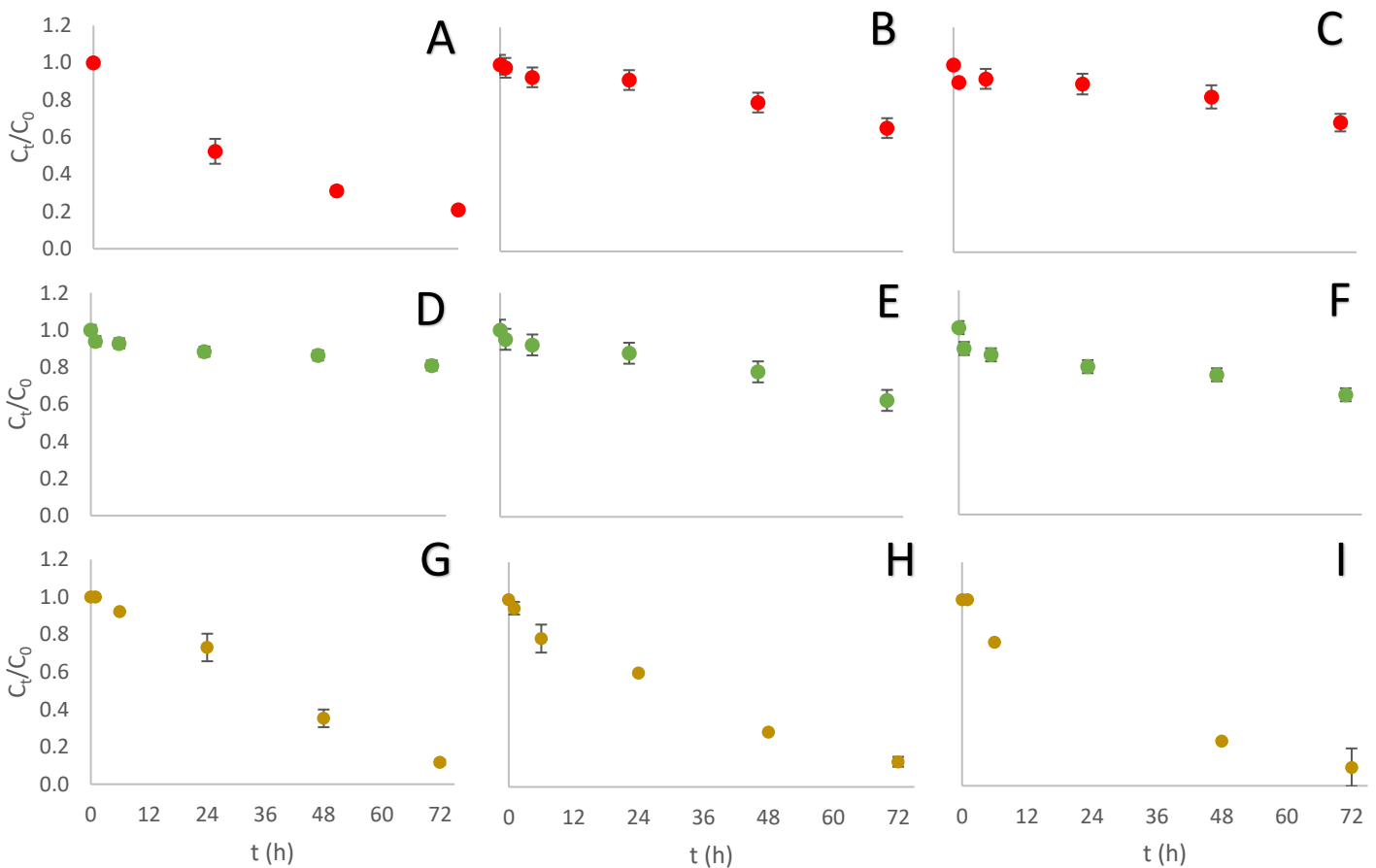


Figure 15 – Normalized concentration for 0, 1, 6, 24, 48 and 72 h, for the follow conditions: A) Co in the mono-elemental condition; B) Co in mixture with discharge conditions; C) Co in diluted mixture condition; D) Ni in the mono-elemental condition; E) Ni in mixture with discharge conditions; F) Ni in diluted mixture condition; G) Hg in the mono-elemental condition; H) Hg in mixture with discharge conditions; I) Hg in diluted mixture condition. Co is in red, Ni is in green and Hg is in brown.

It is possible to observe different removal patterns for the different conditions. After 72 h, *U. lactuca* removed Hg more efficiently than Co and Ni in all the 3 conditions. Removal of Hg led to C_t/C_0 values below 0.20 (removal percentages higher than 80 %) at the end of the experiment, being similar among the 3 conditions studied (**Figure 15G, H and I**). Decrease in C_t/C_0 for Co reached values between 0.66 and 0.69 (removal percentages between 31 and 34 %) at the end of the experiment, for the two mixtures (**Figure 15B and C**, respectively). However, in the mono-elemental condition, the removal of Co led to C_t/C_0 values near 0.20 (**Figure 15A**). Removal of Ni allowed to reach C_t/C_0 values between 0.62 and 0.81 (removal percentages between 19 and 38 %) at the end of the experiment. However, in the case of Ni, the removal was higher in the mixture conditions (**Figure 15E and F**) comparing to the mono-elemental condition that represented removal percentages lower than 20 % (**Figure 15D**).

4.3 Optimization of the removal of Hg, Co, and Ni with the macroalgae

4.3.1 Quality control

The control charts for the optimization experiments are presented in **Figure 16**. All the values are either inside the 10 % error line (warning) or inside the 20 % error line (rejection) (**Figure 16A-D**), evidencing lack of contamination or element loss (Co – red; Ni – green; Hg – orange).

To evaluate if the experimental design is correct it is necessary to assess the precision of the central point of BBD, that is, to assess the magnitude of the standard deviation associated with the mean of the three replicates, whose factor levels are fixed at the midpoint (experiments 2, 6 and 8, **Table 5**). In **Figure 17**, there are presented the removal patterns for these replicates (mean values of normalized concentration) for Hg (orange), Co (red) and Ni (green). It is possible to notice that the error bars are small (lower than 5%). This means that the 3 replicates in study are close enough to validate the experimental design followed. It is also possible to notice that the removal for all the 3 PTEs follows a similar pattern, being Ni (green) removed a little slower than the other 2 elements. The first 24 h are characterized by a fast removal of all the three elements in solution. Along time, the removal of elements becomes slower, reaching almost total removal for Hg, and removal of about 90 % for Co and Ni.

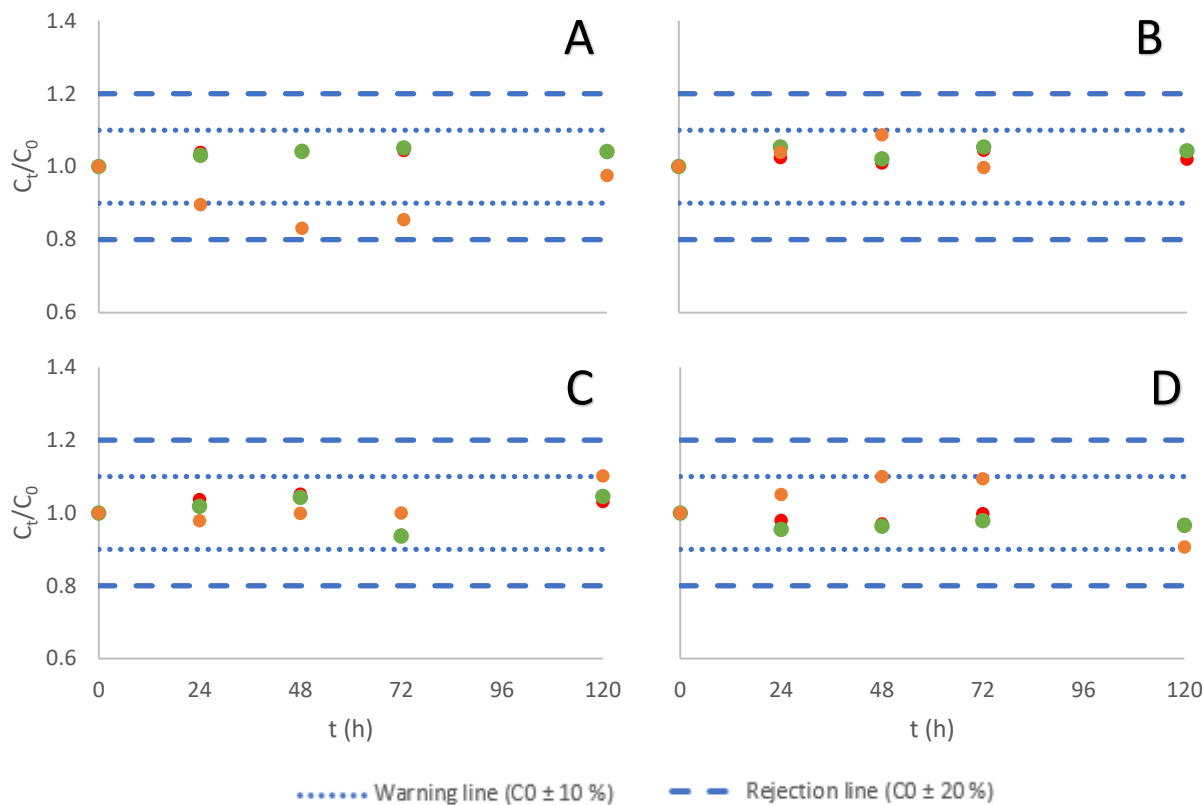


Figure 16 - Control charts for normalised concentrations for 0, 24, 48, 72 and 120 h, for the follow conditions: A) mixture (Hg – 5 $\mu\text{g/L}$; Co – 200 $\mu\text{g/L}$; Ni – 200 $\mu\text{g/L}$) and salinity 35; B) mixture (Hg – 5 $\mu\text{g/L}$; Co – 200 $\mu\text{g/L}$; Ni – 200 $\mu\text{g/L}$) and salinity 15; C) mixture (Hg – 50 $\mu\text{g/L}$; Co – 2000 $\mu\text{g/L}$; Ni – 2000 $\mu\text{g/L}$) and salinity 15; D) mixture (Hg – 50 $\mu\text{g/L}$; Co – 2000 $\mu\text{g/L}$; Ni – 2000 $\mu\text{g/L}$) and salinity 35; Co is in red, Ni is in green and Hg is in orange. Dash lines on $y = 0.9$ and $y = 1.1$ represent the warning line with an error of 10 %. Dash lines on $y = 0.8$ and $y = 1.2$ represent the rejection line with an error of 20 %.

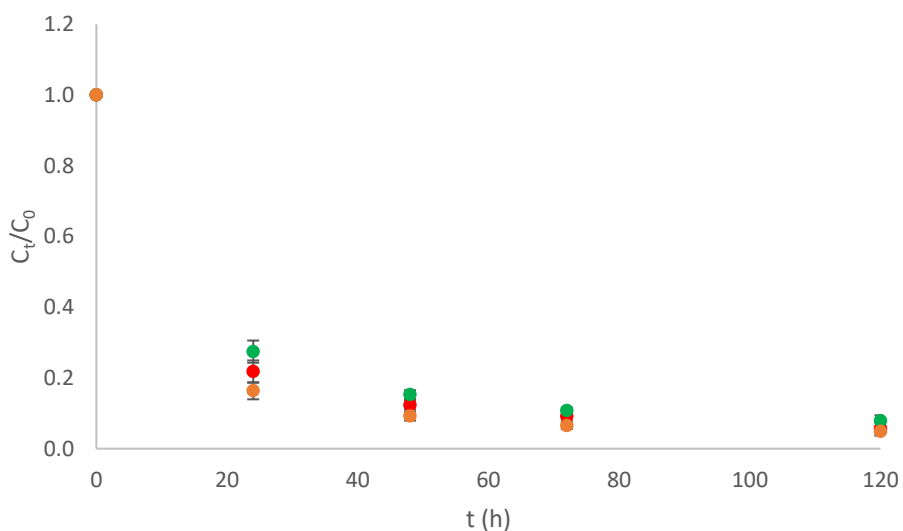


Figure 17 - Normalized concentration for 0, 24, 48 and 72 h, for each studied element at the central point of the experiments. Co is in red, Ni is in green and Hg is in brown.

4.3.2 3D removal response surfaces for Co, Ni and Hg

In order to obtain information on the optimal conditions that allow to achieve the highest Hg, Co, and Ni removal (%), the results the Box-Behnken design created with 15 experiment are represented in **Table 5**.

Table 5 – Removal (%) for the 15 experiments for Hg, Co and Ni.

Experiment	Removal (%) (Hg)				Removal (%) (Co)				Removal (%) (Ni)			
	24 h	48 h	72 h	120 h	24 h	48 h	72 h	120 h	24 h	48 h	72 h	120 h
1	75	82	85	95	83	89	91	91	75	83	85	84
2	82	92	94	97	80	89	92	96	74	86	90	93
3	77	90	91	94	62	83	88	93	59	82	89	93
4	86	92	96	97	83	92	95	97	79	90	93	95
5	66	88	76	89	72	84	86	87	57	76	81	79
6	86	91	94	94	80	88	91	95	74	85	90	93
7	89	93	93	97	82	89	91	93	76	86	89	91
8	83	90	92	94	75	86	89	92	69	83	88	90
9	73	79	86	93	78	85	86	86	70	80	82	79
10	83	89	93	94	77	88	91	93	69	84	88	89
11	59	85	88	89	50	74	80	84	46	70	80	85
12	73	92	94	95	65	89	94	96	63	87	93	94
13	87	93	95	97	78	88	93	96	77	88	93	94
14	54	64	59	65	47	75	86	89	37	53	73	80
15	56	78	88	89	41	71	86	94	40	69	87	95

It is possible to notice that the removal percentage was higher at the end of exposure (120 h). It is also possible to observe that in the first 24 h, the macroalgae removed the elements in some significant percentages, reaching values higher than 70 and 80 % for some cases. Other experiments showed removal percentages lower than 60 and 50 % at the first 24 h, but this removal increased to much higher values during all the process.

To evaluate which factors (macroalgae dosage, salinity, element concentration) influence the removal process and if the interaction between them has a significant role, on **Table 6** there are compiled the linear and quadratic influences, as well as the different ways of interaction on the removal process. The green values represent a positive impact, meaning that if the green factor increases, the response (removal percentage) will increase too. The red values represent a negative impact, meaning that if the red factor increases, the response (removal percentage) will decrease. The black values represent non-

significant factors (p -value > 0.05). To allow a better understanding, each factor is represented by a letter (A – Macroalgae dosage, B – Element concentration, C – Salinity), and an interaction between two factors is represented by the two letters that corresponds to each factor. The times chosen were 48 and 120 h, as explained below.

Table 6 – Factors with the correspondent p -values for times 48 and 120 h.

Model	Mercury		Cobalt		Nickel	
	48 h	120 h	48 h	120 h	48 h	120 h
A-Macroalgae dosage	0.0357	0.0413	0.0235	0.2376	0.0101	0.9145
B-Concentration	0.0176	0.0651	0.0016	0.0116	0.0485	0.0004
C-Salinity	0.6472	0.7764	0.4430	0.5535	0.9507	0.8245
AB	0.9742	0.1441	0.6727	0.3250	0.6013	0.7592
AC	0.3549	0.5229	0.1357	0.1405	0.2809	0.0904
BC	0.5072	0.7385	0.1284	0.5446	0.6209	0.2422
A ²	0.0406	0.1618	0.3746	0.1752	0.0507	0.2099
B ²	0.0339	0.2137	0.0150	0.6106	0.2207	0.0368
C ²	0.1001	0.3405	0.4435	0.3973	0.3457	0.5042

In **Table 6**, it is possible to notice that macroalgae dosage and element concentration influence the process in almost all the cases, being characterized by a positive influence. Salinity had no influence in all the experiments. Some factors lose their influence along time.

To create the 3D response surfaces, there were only considered the significant ($p < 0.05$) factors (green and red values). These factors are the ones presented in each equation of **Table 7**, where salinity was never a significant factor. The proximity between the coefficient of determination (R^2) and the adjusted value of the same coefficient (adjusted R^2), indicates the validity of the adjust, what happened in all the cases.

Table 7 – Equations with relevant parameter and with the respective coefficient of determination and the adjusted coefficient of determination for Co, Ni e Hg removal (%).

Equation in terms of actual factors	R ²	Adjusted R ²	Figure	PTE
Removal (%) = 53.14 + 18.74Macroalgae dosage – 2.42Macroalgae dosage ²	0.78	0.74	18 (A)	Co
Removal (%) = 87.35 + 0.004*Concentration	0.50	0.46	18 (B)	
Removal (%) = 31.32 + 25.51Macroalgae dosage + 0.0058Concentration – 3.35Macroalgae dosage ²	0.74	0.67	19 (A)	Ni
Removal (%) = 77.46 + 0.016Concentration – 3.93Concentration ²	0.85	0.83	19 (B)	
Removal (%) = 45.73 + 20.00Macroalgae dosage + 0.97Concentration – 2.86Macroalgae dosage ² – 0.013Concentration ²	0.77	0.68	20 (A)	Hg
Removal (%) = 81.05 + 3.63Macroalgae dosage	0.21	0.21	20 (B)	

Figure 18 shows the 3D response surfaces for Co removal obtained by plotting the variables macroalgae dosage and element concentration, keeping constant the value of salinity. Only the results for 48 h (**Figure 18A**) and 120 h (**Figure 18B**) were chosen to be shown here, because the removal observed at 48 h was already relevant, and 120 h corresponds to the end of the exposure.

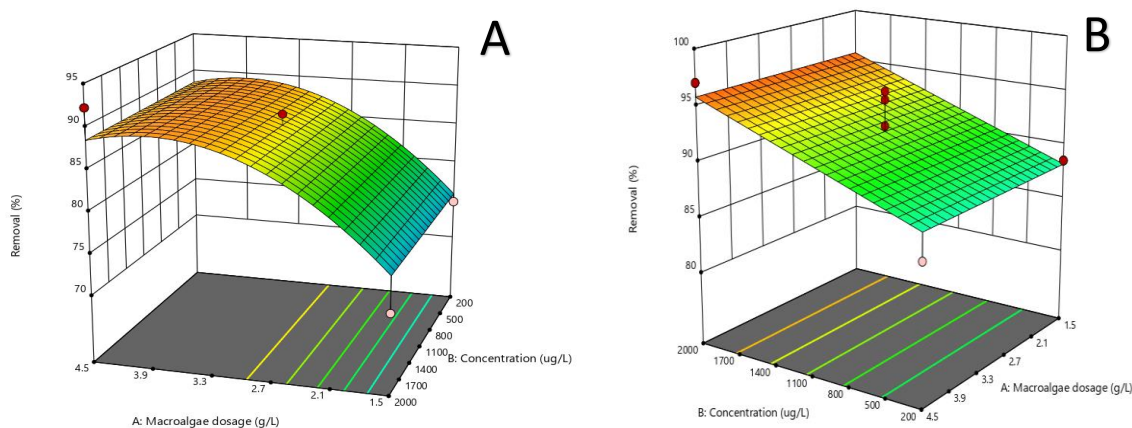


Figure 18 – 3D response surfaces for Co removal for 48 (A) and 120 h (B). For both times, it represents the interaction between macroalgae dosage (g/L) and element concentration (µg/L). Dots represent experimental values.

Salinity was the variable chosen to remain constant because the statistical analysis showed that this variable is no significant ($p > 0.05$) for the model that describes the removal. It can be observed that, for 48 h (**Figure 18A**), the increase in macroalgae dosage has a positive impact in the removal process. However, for this time, the concentration of Co has little or no impact in the removal process ($p > 0.05$). For the 120 h (**Figure 18B**), as the concentration of Co is increased, the removal percentage increases too. However, the macroalgae dosage is not significant at that time ($p > 0.05$). Using the RSM it was also possible to determine the values of the factors that conduct to the maximization of the removal percentage (optimization of the process) (**Table 8**).

Figure 19 shows the 3D response surfaces for the removal of Ni obtained by plotting the variables macroalgae dosage and concentration, while keeping constant the value of salinity. Only the results for 48 h (**Figure 19A**) and 120 h (**Figure 19B**) were chosen to be presented here, for the same reason as described above.

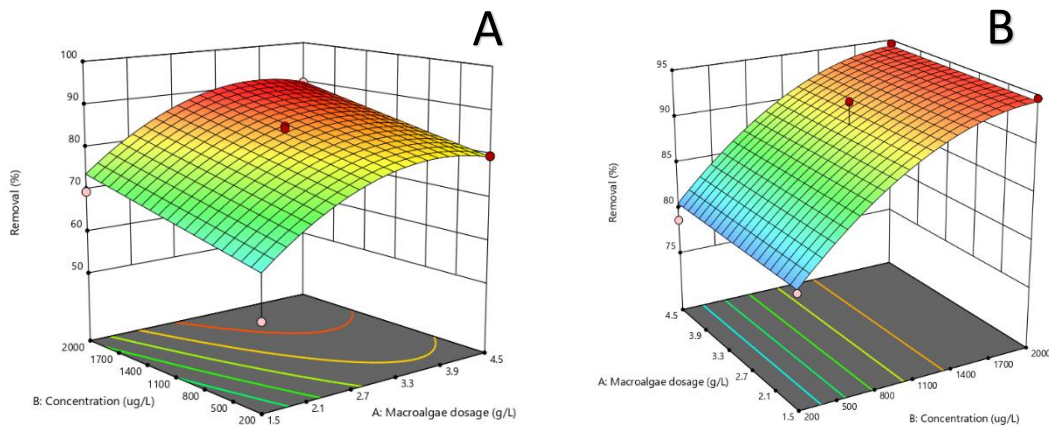


Figure 19 - 3D response surfaces for Ni removal for 48 (A) and 120 h (B). For both times, it represents the interaction between macroalgae dosage (g/L) and element concentration ($\mu\text{g/L}$). Dots represent experimental values.

Salinity remains constant because the statistical analysis showed again that it is not as significant as the other two variables. Like for Co, it can be observed that, for 48 h (**Figure 19A**), the increase in the macroalgae dosage has a positive impact in the removal process, reaching an optimal condition (at 3.8 g/L), after which the removal percentage decreases. For this time, the initial concentration of Ni has impact on the removal, showing that higher concentrations lead to higher removal percentages (between near 80 % for 200 $\mu\text{g/L}$ and near 90 % for 2000 $\mu\text{g/L}$). At 120 h (**Figure 19B**), the initial concentration of Ni is more impactful than at 48 h, evidencing a great removal

enhancement with the rise of Ni concentration in solution. However, at this time (end of the exposure), the macroalgae dosage is not significant ($p > 0.05$), as seen in the Co case.

Figure 20 displays the 3D response surfaces for the removal of Hg, obtained by plotting the variables macroalgae dosage and concentration, while the value of salinity, which was not considered significant ($p > 0.05$), is kept constant at its central point value (salinity 25). Like for Ni and Co, only the results for 48 h (**Figure 20A**) and 120 h (**Figure 20B**) were chosen to be presented here. It can be observed that, for the 48 h (**Figure 20A**), both the increase in the macroalgae dosage and the increase in the initial concentration of Hg have a positive impact in the removal process, reaching an optimal condition for 3.4 g/L and 32 $\mu\text{g/L}$, respectively. Increasing the values of these factors beyond the optimal leads to a reduction on Hg removal percentage. For the 120 h (**Figure 20B**), as the macroalgae dosage is increased, the removal percentage increases too. However, at that time the concentration of Hg is not significant ($p > 0.05$).

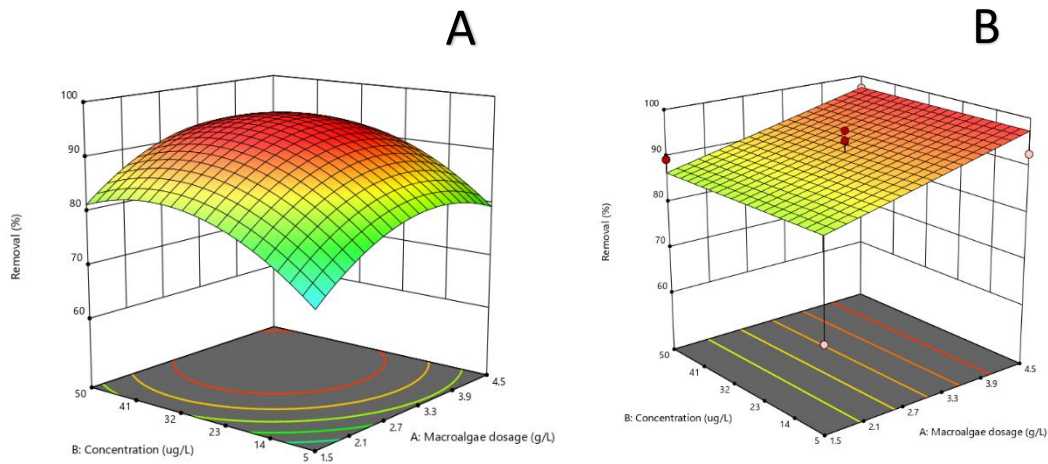


Figure 20 - 3D response surfaces for Hg removal for 48 (A) and 120 h (B). For both times, it represents the interaction between macroalgae dosage (g/L) and element concentration ($\mu\text{g/L}$). Dots represent experimental values.

The optimal conditions and the respectively maximum removal value for Hg, Co, and Ni at 48 and 120 h are compiled in **Table 8**.

Table 8 – Optimal conditions and maximum removal value for Hg, Co, and Ni at 48 and 120 h.

Time (h)	Variable	PTE		
		Hg	Co	Ni
48	Macroalgae dosage (g/L)	3.5	3.9	3.8
	Concentration ($\mu\text{g/L}$)	36.3	1100	2000
	Maximum removal value (%)	95	89	92
120	Macroalgae dosage (g/L)	4.5	3.0	3.0
	Concentration ($\mu\text{g/L}$)	27.5	2000	2000
	Maximum removal value (%)	97	96	94

In **Table 8** it is possible to notice that the maximum removal values increase from near 2 to near 7 %, when comparing 48 to 120 h to each one of the studied element.

5 DISCUSSION

5.1 Toxicological effects of Hg, Co and Ni in *Mytilus galloprovincialis*

During the experiment, all mussels survived. However, biochemical impacts in the organisms were observed and were related to the Hg, Co and Ni exposure. For the biomarkers related to the metabolic capacity and energy reserves of the *Mytilus galloprovincialis*, this study showed that organisms were capable to maintain the GLY content in the mono-elemental conditions. However, when exposed to the mixture of the 3 elements, GLY decreased comparing to control. These results indicate that the studied organisms tried to prevent the impacts, decreasing their metabolic activity and, thus, limiting the expenditure of GLY. This strategy is common when the stress conditions are not too high, with bivalves reducing their metabolism to reduce filtration rate and, therefore, accumulation of contaminants. Similar behaviour was reported by Leite et al. (2020) in *Mytilus galloprovincialis* exposure to rutile and anatase. This response worked in the mono-elemental condition. When elements were mixed, mussels had to use energy reserves to minimize the impacts, leading to a decrease of GLY content. These results indicate a higher stressful condition, with organisms increasing their metabolism to fight against stress (namely to activate enzymatic defences) with expenditure of energy

reserves. Similar results were reported by Cruz et al. (2016) when the clam *Ruditapes philippinarum* was exposed to caffeine: with the increase of concentration, bivalves were forced to increase their metabolism and their GLY content decreased. Nevertheless, in the present study, no differences were observed in the PROT content among treatments, indicating that either the mussels were not using proteins or were using but the production of them was not inhibited under these stressful conditions. Similar results were obtained by Freitas et al. (2020) while studying the impacts of salicylic acid (SA) in the same species under warming conditions: in organisms exposed to SA the strategy used was preserved the proteins, maintaining the PROT content.

The activity of antioxidant defenses does not followed a regular pattern. The activity of SOD increased in the mono-elemental conditions when compared to control, but was not significantly different when comparing control with mussels exposed to the mixture of elements. This means that when exposed to higher stress, organisms were not able to increase SOD. The activity of CAT was not significantly different when comparing control with the mono-elemental conditions, but increased in mussels exposed to the combination of all elements. The activity of GPx was significantly higher in all the contaminated scenarios comparing to the control. These results suggest that the antioxidant defenses (SOD, CAT, GPx) where activated, being SOD activated in the mono-elemental conditions, CAT in the mussels exposed to mixture, while GPx was increased in contaminated mussels regardless the element. This suggest that some antioxidant defenses can be inefficient or inhibited in some conditions, like SOD in mixture and CAT in the mono-elemental condition. As there are no studies concerning the toxicity resulting from exposure to Ni in *Mytilus galloprovincialis* and because the existing studies of toxicity of Co in these organism only gave information on lipid and sterol metabolism (Nechev et al., 2006) and metabolism on some polar and volatile compounds (Nechev et al., 2007), there is no direct comparison to do with the literature. Coppola et al. (2017a) studied the exposure of *Mytilus galloprovincialis* to Hg and found similar patterns as in this study. The activity of SOD was also significantly higher than control when exposed to a mono-elemental condition of Hg, and the activity of CAT was also no significantly different to control when exposed to a mono-elemental condition of Hg. Freitas et al. (2019) studied the exposure of *Mytilus galloprovincialis* to Pb with different temperatures and salinities and observed also a difference in enzymes activation as in the current study: at temperature 17 °C and salinity 25, with the exposure to Pb SOD

activity increased, no significant differences were observed in CAT activity and GPx activity decreased. Figueira et al. (2012) studied the exposure of *Ruditapes decussatus* and *Ruditapes philippinarum* to Cd. For *R. philippinarum*, with the increase in Cd concentration from 10 to 18 μM , the CAT activity increased and the SOD activity decreased, showing a similar pattern to the current study.

In terms of biotransformation defenses, the activity of GSTs decreased significantly in Hg and mixture treatments in comparison to the remaining ones. A decrease of GSTs was also observed by Coppola et al. (2018) when *Mytilus galloprovincialis* were exposed to Hg. A similar decrease was reported by Leite et al. (2020) when mussels were exposed to anatase. In this study, GSTs decreased and antioxidant defenses increased, following a similar pattern as in the present study where an increase of SOD and GPx occurred while GSTs decreased. No significant differences were found among CTL, Co and Ni conditions in terms of GSTs activity, probably because of the activation of antioxidant defenses like SOD and GPx in these treatments. The CbEs decrease is a consequence of the exposure to the mono-elemental conditions of Hg, Co and Ni and was also reported by Rivera et al. (2021) in a study regarding tropical freshwater invertebrates (*Macrobrachium jelskii*) exposed to aluminized solid rocket fuel combustion. De Lima et al. (2013) studied the exposure of zebrafish *Danio rerio* to metals (Pb, Cd, Cu and Fe) and also reported a decrease of CbEs in contaminated organisms.

In terms of cellular damage, levels of LPO decreased in almost all the treatments comparing to control, except in mussels exposed to Hg that showed no differences to the control. These findings indicate that no cellular damage was induced in the presence of Co, Hg, Ni or the combination of all elements. This response might indicate low stress levels caused by each element due to the low concentrations tested but also the efficiency of the defense mechanisms activated. Leite et al. (2020) studied the exposure of *M. galloprovincialis* to rutile (TiO_2 form) and recorded values of LPO near 20 nmol MDA/g FW (similar to the ones obtained in the present study) meaning that no LPO occurred. The PC levels were higher in mussels exposed to mixture and no significant differences were observed between mussels from control and mussels exposed to mono-elementary conditions. The increase of PC levels in *Mytilus galloprovincialis* was observed by Freitas et al. (2020) when these organisms were exposed to dysprosium. This result was justified by the capacity of dysprosium to induce the oxidation of proteins. Comparing this study with our findings, it is possible to conclude that the exposure to mixture induced the

oxidation of proteins of the organism, indicating higher stress levels that the exposure to each element independently. In GSH levels, no significant differences were observed among all the treatments, indicating that concentrations tested were not stressful enough to cause the loss of redox balance in mussels. Similarly, Monteiro et al. (2019) reported no changes in GSH levels in mussels *Mytilus galloprovincialis* exposed to a certain range of concentrations of titanium for 28 days, followed by 14 days in uncontaminated seawater. For the same days, only the most concentrated treatment led to a decrease of GSH levels. Nunes et al. (2017) studied the exposure of the clam *Ruditapes philippinarum* to paracetamol and reported no changes in GSH levels for some concentrations of paracetamol, having only a decrease in GSH levels for the highest concentration.

In terms of neurotoxicity, the activity of AChE decreased significantly in all the contaminated organisms, revealing the neurotoxic capacity of the elements tested. A similar response was observed by Chalkiadaki et al. (2014) when studying cadmium bioaccumulation in the marine bivalve species *M. galloprovincialis*, *Callista chione* and *Venus verrucosa*.

5.2 Preliminary experiment on removal of Hg, Co, and Ni with macroalgae *Ulva lactuca*

U. lactuca has shown its high capacity to remove elements like Hg, Co, and Ni, from mono-elemental and mixture conditions, in different ranges of concentration and salinity (real saline water). Although the removal of Hg has already been studied in the recent years, the information on the removal of Co and Ni is scarcer. As these two elements are used extensively in batteries, a sector that is currently booming (Sun et al., 2020), it is to be expected that the frequency and concentration of these elements in industrial effluents and aquatic ecosystems will increase. Beyond that, most studies focus only on mixtures, and do not involve real waters. So, the results reported in this study are new and came to fill gaps in the literature in this area.

To facilitate the comparison between elements, for the results obtained in the preliminary assay, the graphs from **Figure 15** were overlapped in **Figure 21**. The removal of Hg, Co and Ni achieved in each one of the conditions studied along time are presented (mono-elemental condition **(A)**, mixture with discharge conditions **(B)** and diluted mixture **(C)**). The error bars were omitted to facilitate the interpretation and clarity.

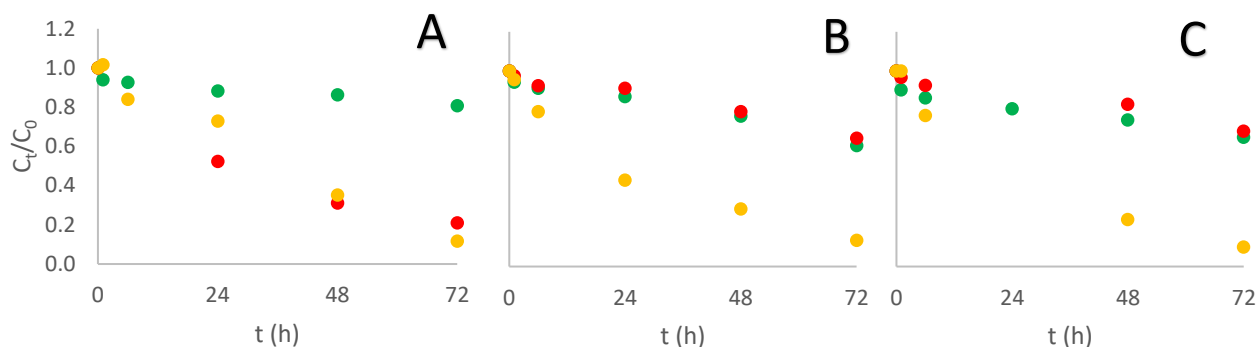


Figure 21 - Normalized concentration for 0, 1, 6, 24, 48 and 72 h for the follow conditions: A) Hg, Co and Ni in the mono-elemental condition; B) Hg, Co and Ni in mixture with discharge conditions; C) Hg, Co and Ni in diluted mixture. Co is in red, Ni is in green and Hg is in orange.

It is possible to notice that Hg is removed more easily than Co and Ni, except in the mono-elemental condition, where the removal of Co and Hg follow a similar pattern (**Figure 21**). In the mixture that mimics the discharge conditions, and in the diluted one, the removal of Co and Ni was lower than 40 %, suggesting that the macroalgae have higher affinity for Hg and/or the removal process is not optimized for those elements, comparing to the removal of Hg observed in the same conditions (higher than 80 %). The removal of Co was higher in the mono-elemental condition than in mixtures. One possible explanation for this behaviour is the competition for the binding sites of the macroalga *U. lactuca*. In mixtures, this competition increases with the increase of elements to be removed, like Hg and Ni. Vijayaraghavan et al., (2011) studied the interaction of lanthanides with *Turbinaria conoides* (brown marine alga), showing decreases in removal percentages from 65 to 76 %, when comparing mono-elemental systems to mixtures. Henriques et al. (2017) studied the removal of Hg, Cd e Pb by the macroalgae *U. lactuca* and observed that the removal of Cd and Pb decreased in the mixture condition comparing to the mono-elemental one. In the present study, the removal of Hg followed a similar pattern in all the conditions. Unlike Co, the removal of Ni was not negatively affected by the presence of potential competitors, like Hg and Co.

The analysis of the kinetic profiles (**Figure 21**), allow to conclude that the elements were not completely removed, and the surface of the macroalgae was not saturated, nor

was a steady state reached during the exposure period (a horizontal line in the profile is not evident). So, an increase in the exposure period would be favourable, as well as the rise on the amount of macroalgae used. The conclusions of this preliminary study were important to define the experimental design used to optimize the process, as the selection of the exposure time, and the ranges within factors.

5.3 Optimization of the removal of Hg, Co, and Ni with macroalgae *Ulva lactuca*

In order to optimize the conditions that allow to achieve the highest Hg, Co, and Ni removal, a Box-Behnken design (BBD) was created, consisting in 15 experiments (results in **Table 5**). Four parallel control experiments were also performed, which showed that the concentrations remained constant along time, discarding any losses or contamination of element due to external factors. Studies using RSM and BBD allow to study different variables while performing less experiments than the conventional way of performing individual studies, and it has been used by some authors (Fabre et al., 2019; Ghani et al., 2017).

All the experiments were characterized by a fast removal of the elements in the first 24 h of exposure, followed by a slowdown in the removal rate until 120 h. This could be explained with the binding sites present in the macroalgae. In the beginning of the process, these binding sites are available in large number, leading to a faster binding of the elements to the macroalgae. Similar removal patterns were observed in other studies. Henriques et al. (2015) observed this initial fast removal rate when studying three macroalgae for the removal of Hg. Henriques et al. (2019) reported a fast decline in concentration after 12 h, followed by a slowdown until the end of exposure, when using *U. lactuca* to remove several trace elements.

In almost all the experiments there was an increase in the removal percentage from 72 to 120 h, proving that the surface of the macroalgae was not saturated at 72 h. However, depending on the purpose of using this technology, it is wise to study if the increase in the removal efficiency pays off the two additional days of the process. Using the experiment 4 as an example, 96 % of Hg was removed after 72 h, leading to a residual concentration of Hg of 2.2 µg/L. If this treatment was done in an industrial effluent with the goal of reaching drinking water standards in America (2 µg/L for Hg) (hypothetical

case, disregarding variables such as the matrix), it would be necessary to extend the process to 120 h, where a final concentration of Hg in water of 1.4 $\mu\text{g/L}$ is reached.

The findings of the RSM analysis demonstrated that salinity does not have a key role in the removal of the contaminants, i.e. the macroalgae are able to achieve great removal rates at salinity 15, 25 or 35. Costa et al. (2020) studied the influence of salinity on the removal process of a diverse range of elements, concluding also that changes in salinity does not influence the process of Hg removal by *Ulva lactuca*, and it has little or non-influence in the Ni removal. Generally, salinity can influence biosorption by competing for the binding sites or forming complexes between salt and the elements in solution. However, this does not happen in the present study. One possible explanation can be the phosphate groups in *U. lactuca* that can have more affinity to the studied elements rather than chlorine ions.

The initial concentration of Hg, Co, or Ni, and macroalgae dosage influence all the removal process. For Co and Ni, the concentration has more influence at 120 h than at 48 h. For Hg, it is the opposite. In general, in the present study, when the concentration influences the removal process, the higher the concentration the higher the removal. However, in the case of Hg and for 48 h, the increase of concentration increases the removal until certain point (optimal condition). Passing that point, the removal decreases with the increase of element concentration. The macroalgae dosage played an important role in the first 48 h for most cases of the present study, not having great influence at 120 h, for Co and Ni. For Hg, the results highlight the influence of the macroalgae dosage during all the process. As for the initial concentration of the elements, in general, when the macroalgae dosage increases, the removal percentage increases too. However, also in the Hg case, there is an optimal condition that should not be changed. A possible explanation for the increase of removal when the macroalgae dosage increases can be due to the increase in binding sites in algae surface. However, at certain point, the number of free binding is very small being the limiting factor, a role assumed by the concentration gradient between the solution and the macroalgae. If the concentration of the element in solution is very low, the removal will not occur significantly.

Observing the optimal conditions and the respective maximum removal value (**Table 7**), it is possible to notice that the values at 48 h are high. When comparing to the ones at 120 h, the differences are in the range of 2-7 %. This increment in removal

percentage may not justify the extension of the process from 48 to 120 h, making the exposure process faster.

The optimization study presents promising results, showing that the macroalgae *U. lactuca* has the capacity to remove Hg, Co, and Ni from real matrices (collected seawater) in a big range of concentrations from the maximum permissible limits for discharge to ten times diluted concentrations, a closer value to values found in aquatic ecosystems. It also gave us information about the optimal conditions to maximize the removal of each one of the elements, allowing to save time and money in the laboratory.

5.4 Potential of this biotechnology: comparative analysis

5.4.1 Removal percentage and initial element concentration

In the last years there was an increase in studies related to biosorption/bioaccumulation of PTEs, highlighting its potential for water treatment, which was reinforced by the findings of this study.

For Hg, the removal percentages were between 54 and 89 % after the first 24 h, with a mean value of 75 %, considering all experiments. At 120 h, the removal percentages were between 65 and 97 % (mean value of 92 %). Rocha et al. (2014) used rice husk to remove Hg from water, achieving removal percentages of 56 and 65 % at 24 h. At 168 h, removal percentages were 81 and 93 %. In the present study, for the trials that included an initial concentration of 50 µg/L (4, 12, 13 and 15) *U. lactuca* achieved 75 % of removal (vs 56 % for rice husk) at 24 h, and 95 % (vs 81 % for rice husk) at 120 h (168 h for rice husk). Although the conditions of the two studies are not all equal, avoiding a direct comparison, it gives us an idea of the potential of the studied biotechnology.

Table 9 summarizes the removals of Hg, achieved by different biosorbents at 24 h and at the end of exposure, reported in other studies, as well as the type of biosorbent used and initial concentration of Hg. Related with the initial concentration of Hg used in the studies, it is possible to see a certain concern from the authors in studying environmental realistic concentrations, i.e., concentrations near to those that can be found in the environment or in industrial effluents. Being 50 µg/L the maximum admissible in effluents discharge for Hg (Portuguese Decree-Law 236/98), it is expected to find concentrations below that in the aquatic ecosystems due to natural dilution, so the values

of 5, 10 and 27.5 µg/L mimic real scenarios: in Spain it was found a range of concentration of Hg from 0.01 to 20.3 µg/L in water (Nevado et al., 2003). The Hg concentrations of 100 and 500 µg/L are a bit far from what is expected to find, but it is important to study the effect of the initial concentration on the removal performance of the proposed method.

Table 9 – Hg removal (%) from published studies with different sorbents vs the results in the present study.

Sorbent	Initial concentration of Hg (µg/L)	Removal at 24 h (%) (mean of all trials)	Removal at the end of exposure (%) (mean of all trials)	Reference
<i>Ulva lactuca</i>	5	67	85	Present study
	27.5	80	94	
	50	75	95	
Rice husk	50	56	81	(Rocha et al., 2014)
	500	65	93	
Banana peels	50	89	95	(Fabre et al., 2020)
<i>Ulva lactuca</i>	10	72	97	(Henriques et al., 2015)
	50	70	98	
	100	63	98	
<i>Gracilaria gracilis</i>	10	63	92	
	50	58	95	
	100	56	95	
<i>Fucus vesiculosus</i>	10	56	95	
	50	40	88	
	100	39	94	
<i>Ulva lactuca</i>	50	77	93	(Henriques, et al., 2019)

Observing the removal of Hg at 24 h (**Table 9**), it is possible to notice that *U. lactuca* can remove Hg faster than other types of macroalgae like *Gracilaria gracilis* and

Fucus vesiculosus, and faster than rice husk, being only slower than banana peels. However, the differences in removal observed in the beginning of the process are attenuated at the end.

It is important to reinforce that, as all the studies were not done using the same conditions (different sorbent dosages, different matrices, some are mono-elemental and others are mixtures) these comparisons are not as linear as it can look but are a great reference to evaluate our work and to think in future experiments.

For the removal of Co, there are less studies available in the literature. So, it was impossible to choose studies for comparison with conditions near the ones of the present study: initial concentrations are really higher, changes in pH, contact time between sorbent and solution of minutes or less hours than 24 h, carbonization of sorbents, among others different variables. The current study used concentrations to mimic real scenarios. The highest concentration were 2000 µg/L and once there are no Portuguese maximum admissible concentration for Co in effluents discharges, it was admitted the same value applied for Ni (Portuguese Decree-Law 236/98). The concentrations of 200 and 1100 µg/L are values that can represent concentrations found in aquatic ecosystems: there were reported concentrations in surface water from < 0.1 to > 1000 µg/L in the United States and concentrations from 0.025 to 2028 µg/L in Canada (Stubblefield et al., 2020). In the literature related to the removal of Co through bio-based sorbents, there is not a concern from the authors to mimic real scenarios as in this current study. Kumar et al. (2019) studied the removal of Co using three bio-based sorbents (native and carbonized form): groundnut seed cake powder, coconut cake powder and sesame seed cake powder. However, the initial concentrations of Co studied were in the range of 10000 to 90000 µg/L, 5 to 45 times higher than the maximum value admitted for Ni (same value used for Co in the present study), not being realist concentrations. Imessaoudene et al. (2020) studied the removal of Co using spent green tea leaves and an initial concentration of Co of 5000 µg/L, 2.5 times higher than the maximum value used in the present study. This concentration could be interesting to study the behaviour of the sorbent according to an increase of initial concentration if the author had also studied lower concentrations like 2000 µg/L and a more diluted one, which was not the case. Annadurai et al. (2003) studied the removal of Co (and other metals) using orange and banana peels. However, the initial concentrations used do not mimic real scenarios once again (5000 to 25000 µg/L). Krishnani et al. (2008) studied the removal of Co (and other metals) using rice husk but

proving again that there was no concern with realist concentrations of Co (50000 to 200000 µg/L, 25 to 100 times higher than the highest concentration of the present study). All cited studies were important to evaluate the use of bio-based sorbents to remove Co from water, but it would be interesting if the authors reproduced them with more realist concentrations.

For the removal of Ni, there are more studies than for Co, but the information in literature is also scarce. With the exception of the study of Henriques et al. (2019), all the studies using bio-based sorbents for the removal of Ni have the same problem pointed for the studies on Co removal: unrealistic concentrations. The highest initial concentration of Ni used in this study was 2000 µg/L, the maximum admissible in discharge of effluents (Portuguese Decree-Law 236/98). The concentrations of 200 and 1100 µg/L are values that can represent concentrations found in aquatic ecosystems: in South of Iran, values of river contamination ranged from 41.0 to 110.7 µg Ni/L (Diagomanolin et al., 2004), and in areas affected by industrial emissions or mining, values can be as higher as 75 or 200 µg/L (Cempel & Nikel, 2006). Annadurai et al. (2003) studied the removal of Ni (and other metals) using orange and banana peels. However, the initial concentrations used do not mimic real scenarios once again (5000 to 25000 µg/L). Krishnani et al. (2008) studied the removal of Ni (and other metals) using rice husk, with initial concentrations of Ni of 50000 to 200000 µg/L. Romera et al. (2007) studied the removal of Ni using six types of algae: *Codium vermilara*, *Spirogyra insignis*, *Asparagopsis armata*, *Chondrus crispus*, *Fucus spiralis* and *Ascophyllum nodosum*. The initial concentrations of Ni ranged from 10000 to 150000 µg/L, 5 to 75 times higher than the maximum permissible in effluent discharge. Henriques et al. (2019) studied the removal of Ni for an initial concentration of 2000 µg/L, the maximum permissible for effluents discharge. This was justified by the author as a way of mimic an industrial effluent (Henriques et al., 2019). In the cited study, after 24 h, 29 % of Ni was removed (mean of all trials) (vs 65 % in the present study for the same element concentration). The removal percentage of Ni at the end of the exposure was 43 % (mean of all trials) (vs 94 % in the present study for the same element concentration), with the highest removal value being 75 % (vs 95 % in the present study for the same element concentration). It is possible to conclude that the present study is promising in terms of Ni removal. It is important to know that both studies were done in mixtures, however the study of Henriques et al. (2019) had five additional contaminants in the mixture. Henriques et al. (2019) studied three dosages of macroalgae (1.5, 3 and 6

g/L) and the mean values of removal (%) decreased when lowering the dosage of macroalgae. The most important values for comparison in this case are the highest removal values at the end of both processes.

It is possible to conclude that the present study shows important results in terms of removal of Hg, Co and Ni and it can be important because it mimics real scenarios in terms of concentrations studied.

5.4.2 Innovative aspects of this study

Matrix used - The current study used real seawater as matrix with three different salinities: 15, 25 and 35. This is important because almost all the studies in literature used distilled water as matrix, as evident in the review of the state of art (introduction) done. The results of the present study showed that salinity has not a big influence on the process, being this biotechnology suitable for different salinity scenarios. Similar results about the influence of salinity was observed by Henriques et al. (2019) and Costa et al. (2020).

Removing elements from mixtures - many authors published about the removal of only one element from aqueous solution (Ali et al., 2020; Ahmad et al., 2017; Awual et al., 2019; Fabre et al., 2019, 2020; Huang et al., 2019; Imessaoudene et al., 2020; Lee & Yang, 1997; Niu et al., 1993; Zabihi et al., 2009). Others study the removal of different elements, although separately (mono-elemental conditions) (Annadurai et al., 2018; Sheng et al., 2004; Vázquez et al., 2009). However, in nature or in effluents, the common scenarios are mixtures. So, the current study focused on the simultaneously removal of Hg, Co, and Ni from a mixture, being closer to what happens in natural ecosystems or in effluents from industry. Recently, there has been an increase of concern in mimic real mixtures (Costa et al., 2020; Henriques et al., 2017; Henriques et al., 2019; Jacinto et al., 2018; Vijayaraghavan et al., 2011).

Separation of the biosorbents at the end of exposure - One of the goals of the current study was to create a biotechnology that allows an easy separation of the sorbent from the solution at the end of exposure. Using the macroalgae *U. lactuca*, and for the studied conditions, this goal was accomplished. The separation of the sorbent from the solution was done using a laboratory tong and in less than 20 seconds. Some bio-based sorbents present good results in terms of removal percentages, however the sorbent separation at the end of exposure can be hard, especially when the sorbents are used in the powder form or in small particles (Fabre et al., 2020; Kumar et al., 2019; Pehlivan et

al., 2009; Pehlivan & Altun, 2008; Rocha et al., 2014; Vázquez et al., 2009; Yao et al., 2010; Zabihi et al., 2009).

Sustainability - This is a sustainable biotechnology because of its goal - to remove contaminants from water - and the raw material and method behind, i.e., using a biosorbent available in large quantities in the environment (and that can also be cultivated for this purpose (Angelidaki et al., 2011; Nielsen et al., 2012)), and with low economical value. Besides that, it is a biotechnology that does not requires pre-treatment of the used sorbent as some techniques require, and all the process happens without any type of mechanical stirring or heating, wasting no energy and becoming a cheap technology.

6 CONCLUSIONS

A literature review evidenced that methods for removing PTEs from water based on biosorption have the potential to replace the conventional methods, after the necessary optimization. Different types of sorbents have been tested in different conditions, but there is still a lot to evolve. In order to respond to the challenges of the area, new essays were proposed, being the Response Surface Method important for this task, reducing the number of the experiments and allowing to study different variables.

As the current legislation in Portugal for the discharge of effluents containing Hg, Co, and Ni is relatively old (1998), or does not even indicate permitted values (Co), and the trend in recent years is to increasingly restrict the discharge of these PTEs, it was considered interesting to assess the toxicity of these elements at concentrations lower than legal criteria. Overall, the present study demonstrated that the exposure of the mussel *Mytilus galloprovincialis* to mono-elemental conditions of Hg (25 µg/L), Co (200 µg/L), Ni (200 µg/L) and to mixture (Hg – 25 µg/L; Co – 200 µg/L; Ni – 200 µg/L) induced limited alterations on mussel's biochemical performance, namely in terms of energy reserves, cellular damage, and redox balance, although the antioxidant defences were activated. Greater alterations were observed when all elements were tested together, with expenditure of GLY content, damage of proteins and higher CAT and GPx activities. Furthermore, a clear neurotoxic effect was observed, with lower AChE activity in contaminated mussels. These results show that the PTEs in study may influence the physiological performance of mussels, reducing their growth and reproduction capacity, being necessary to remove them from water in a sustainable way.

After optimization of the process, macroalgae *U. lactuca* showed great results in removing Hg, Co, and Ni, demonstrating once again the potential to be part of a biotechnology for the bioremediation of waters. The capacity to remove in different ranges of salinity, macroalgae dosage and initial concentration of the contaminant, proves its versatility for being applied in different scenarios. Some of the differentiating factors of this work are the study in real matrices (rare in literature), the study and comparison of mono-elemental condition vs mixtures, and the study of this process optimization. The optimal conditions were found for each one of the PTEs for 48 and 120 h, and it was concluded that an extension to 120 h may not be needed, as removal percentages of 95 % (Hg), 89 % (Co) and 92 % (Ni) can be achieved in just 48 h.

As future work, it will be interesting to quantify the concentrations of Hg, Co, and Ni in mussels and macroalgae tissues, as well as to try different types of biosorbents, following the same methodology, and making a direct comparison at the end. Another interesting study could be the recovery of these elements from the sorbent to be used again by industry, promoting a circular economy.

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