Universidade de Aveiro Departamento de Química
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**BRUNO MANUEL
GALINHO HENRIQUES**

Metais em plantas e algas: acumulação e potencial aplicação em processos de remediação

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica da Doutora Maria Eduarda da Cunha Pereira, Professora Associada do Departamento de Química da Universidade de Aveiro e do Doutor Miguel Ângelo do Carmo Pardal, Professor Associado com agregação do Departamento de Ciências da Vida da Universidade de Coimbra

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palavras-chave

Metais, plantas, macroalgas, bioacumulação, biossorção, remediação

resumo

O presente trabalho reporta o estudo da bioacumulação de elementos potencialmente tóxicos (cádmio, chumbo e mercúrio) por macroalgas marinhas *(Ulva lactuca*, *Fucus vesiculosus* e *Gracilaria gracilis*), abundantes nas costas e sistemas estuarinos a nível mundial. Estes organismos mostraram ser capazes de resistir a contaminação multi-metálica moderada (concentrações ambientalmente relevantes), incorporando uma elevada quantidade de metal nos seus tecidos. As elevadas percentagens de remoção alcançadas, em especial para o mercúrio (99%), demonstram a potencialidade destas algas como base de uma nova biotecnologia de tratamento de águas salinas contaminadas com metais (mais eficiente, de menor custo e mais amiga do ambiente que os métodos convencionais). A *U. lactuca* foi considerada a mais promissora pelo melhor desempenho apresentado. A comparação entre os processos de bioacumulação e biossorção sugeriu que em alguns casos a utilização do organismo vivo terá vantagens sobre a aplicação da sua biomassa, pela maior simplicidade de aplicação do processo global, e pelas menores concentrações residuais de metal alcançadas na solução (em especial para o Cd).

A transferência e acumulação de Hg por plantas terrestes (*Lolium perenne* e *Brassica Juncea*) em campos agrícolas perto de uma zona industrial contaminada foi também estudada. Apesar dos baixos fatores de bioacumulação encontrados (<1), registaram-se elevados conteúdos de Hg nas plantas (até 84 mg Kg^{-1} nas raízes e 6.9 mg Kg^{-1} na parte aérea, peso seco). Estimativas da ingestão diária por animais de pasto (vacas e ovelhas) apontaram para o potencial risco para a saúde humana derivada do consumo da sua carne.

Os resultados evidenciaram o importante papel que as plantas e as algas estudadas poderão ter na proteção, avaliação de risco e remediação de sistemas ambientais contaminados com metais.

keywords

Metals, plants, macroalgae, bioaccumulation, biosorption, remediation

abstract

The present work reports the study of the bioaccumulation of potentially toxic elements (cadmium, lead and mercury) by marine macroalgae (*Ulva lactuca*, *Fucus vesiculosus* and *Gracilaria gracilis*), abundant in the coast and estuarine systems worldwide. These organisms proved to be capable of withstanding moderate multi-metallic contamination (environmentally relevant concentrations), incorporating high amounts of metal in their tissues. The high removal percentages achieved, in particular for mercury (99%), demonstrate the potential of these algae as a basis for a new biotechnological treatment of saline waters contaminated with metals (more efficient, cost-effective and environmentally friendly than conventional methods). *U. lactuca* was considered the most promising due to the better performance presented. The comparison between the bioaccumulation and biosorption processes suggested that in some cases the use of the living organism will have advantages over the application of biomass, due to the simplicity of the overall process, and the lower residual concentration of metal achieved in the solution (especially for Cd).

The transfer and accumulation of Hg by terrestrial plants (*Brassica juncea* and *Lolium perenne*) in agricultural fields near a contaminated industrial area was also studied. Despite the low bioaccumulation factors found (<1), there were high Hg content in plants (up to 84 mg kg^{-1} in roots and up 6.9 mg kg^{-1} in shoots, dry weight). Daily intake estimates for grazing animals (cows and sheep) pointed to the potential risk to human health derived from consumption of their meat.

The results highlighted the important role that plants and algae may have in protection, risk assessment and remediation of environmental systems contaminated with metals.

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Chapter 1

Introduction

1 Introduction

1.1 Ecosystems: importance and pressures

Healthy and naturally functioning ecosystems are the backbone of all life on the planet, offering fundamental resources and services such as food, water, clean air, shelter, climate regulation, raw materials and energy to all forms of life (Eftec 2005, Harley et al. 2006). The Man's ability to gather those resources and process them in different ways allowed the human population to thrive and flourish like no other in our planet. However the rapid demographic growth and economic development, observed since the beginning of the last century, led to an enormous demand for food, water, timber, metal, fuel and other raw materials, resulting in dramatic pressures on global ecosystems.

The “Millenium Ecosystem Assessment’s Report” (2005) states that natural ecosystems have changed more rapidly and extensively in the former 50 years than in any period of human history. Stress and effects of anthropogenic origin include: overexploitation of natural resources; deforestation and habitat destruction; expansion of areas reserved for agriculture and livestock; release of a major greenhouse gas (CO_2), and its consequent contribution to global warming; and decrease of availability of fresh water by discharges of agricultural, domestic and industrial effluents. Available data shows that cropland areas increased by 466% over the last 300 years (Goldewijk and Ramankutty 2004) and nowadays approximately 25% of Earth’s terrestrial surface is already being used for food production (Millennium Ecosystem Assessment 2005). Estimates for the available amount of water per person (a key resource for human health) indicate a decrease from 16 800 m^3 in 1950 to 6800 m^3 in 2000, and currently one third of the world's population is under moderate-to-high water scarcity stress (Millennium Ecosystem Assessment 2005). It should be notice that of all the earth’s water, 97% is salt water, 2% is frozen and only 1% is fresh water available for humans to drink (Meindersma et al. 2006). It is also known that the distribution of fresh water on Earth is not uniform, there exist regions with large rivers and lakes but also areas with extensive desert, where little oasis turn into true paradises. These scenarios can become even worse if we look at projections which reveal that during the next 5 decades the demand for food will increase by 70-80% and for water 30-85% (Millennium Ecosystem Assessment 2005).

The growing and continuous stress on ecosystems can lead to the complete rupture of their structure and consequently to the degradation of their support and regulation services. Biodiversity, food production, climate regulation and water quality regulation will be inevitably affected, yielding major adverse impacts on human health.

In order to avoid ecosystem disruption is imperative to prevent and reduce the environmental damage caused by unsustainable anthropogenic actions (e.g. avoiding the use of potentially toxic elements, or reducing their levels in industrial discharges). Moreover, the identification, risk assessment, and mitigation of injured ecosystems (e.g contaminated soils) is crucial for animal and human well-being.

1.2 Contamination of ecosystems by metals: issues and challenges

Nature offers a whole range of chemical elements that make life possible on Earth as we know it, mostly through the soil overlying the Earth's outer layer (Kabata-Pendias 2007). Some of them are designated as trace elements (TE's) because of their occurrence at concentrations lower than 100 ppm (Hooda 2010). Actually, many of these elements are present in much lower concentrations than this (Sparks 2003). TE’s have also been termed “toxic metals”, “trace metals” or “heavy metals”, although none of the terms is entirely satisfactory from a chemical viewpoint. In fact, most of them are metals, for example Cd, Cr, Co, Cu, Pb, Hg, Ni and Zn, but metalloids like As and B (Sparks 2003), non-metals (for example Se) and halogen (for example I and F) are also included (Hooda 2010). The term “heavy metal” commonly used to describe metals that are toxic has never been defined by any authority e.g., IUPAC (Duffus 2002). In fact, there are numerous definitions of “heavy metals” classifying them according to certain properties, including density (specific gravity), atomic weight and number, and no relationship can be found between them and the toxicity attributed to “heavy metals” (Chojnacka 2009a). Regarding the term "toxic metals", it is not always clear whether a given element should be considered as toxic (Chojnacka 2009a).

There are elements essential to life, known as micronutrients, which are needed, in low quantities, for the proper functioning of an organism (Table 1.1). They contribute for protein structure stabilization, facilitate electron transfer reactions (Ash and Stone 2003), or like Cu, Zn and Fe are constituents of catalytic sites of several enzymes (Torres et al. 2008). However, when the concentration of these elements becomes higher than required they can become toxic. In some cases the difference between essential and harmful concentration is very narrow. Another group of elements plays only harmful role in living organisms, such as carcinogenicity and negative effects on kidneys, nervous system, endocrine system, reproduction, and respiratory system (Hodgson 2004). Mercury, Cd and Pb are examples of toxic elements that have no known metabolic or beneficial function on organisms, cause no problem if missing from a diet and thus are considered non-essential (Table 1.1) (Siegel 2002). This so-called “toxic-trio” may displace or substitute essential elements on enzymes, interfering with their proper functioning (Torres et al. 2008). Furthermore, they may trigger oxidative stress, i.e., lead to enhanced generation of reactive oxygen species, which can overwhelm cell’s antioxidant defenses, causing damage on subcellular organelles (Ali et al. 2013). They can enter in the human beings via food chain and often exceed the toxic levels before they produce visible effects (Srivastava and Goyal 2010).

Metals are among the most common environmental contaminants along with dyes, phenols, insecticides, pesticides and a wide spectrum of aromatics (Dadhaniya, Patel et al. 2007). However, when compared with the majority of contaminants, metals stand out since they are not biodegradable. They persist in the environment indefinitely, circulating within or between ecosystems, being subject to phenomena of bioconcentration in organisms and biomagnification along food chain (Ernst et al. 2008, He and Chen 2014). In fact, living organisms incorporate in their tissues, in a gradual manner over time, amounts of metals greater than those of the surrounding medium (Goel 2006). This problem unleashes along the trophic levels (Selin 2009) and, consequently, the man is one of the most affected living beings since it occupies the top of the food chain (Chojnacka 2010).

Table 1.1: Essential elements for optimal functioning of biological processes and organs in humans and non-essential elements (Siegel 2002).

Essential metal/metalloid micronutrients (a few mg* or µg per day):**	Co**, Cr**, Cu*, Fe*, Mn*, Mo**, Ni**, Se**,V*, Zn*
Non-essential elements	Be, Cd, Hg, Pb, Sb, Ti
Macronutrients (~100 mg or more per day):	Ca, Cl, Mg, P, K, Na, S
Other essential micronutrients	F, I, Si

The health hazards of metals in food chains, even at low concentrations, have been recognized by the World Health Organization (WHO), by the Environmental Protection Agency of the United States (Srivastava and Goyal 2010), as well as by a large number of governmental institutions worldwide. The Agency for Toxic Substances and Disease Registry of the United States of America (ATSDR 2011) periodically revises and publishes the “Priority List of Hazardous Substances”, taking into account their toxicity, quantity released and the ease of exposition. In this list, the “toxic-trio” is highlighted: lead is ranked as 2nd, mercury 3rd and cadmium 7th (arsenic is the “king of poisons”). A brief description of major sources, applications and health adverse effects of these three metals will be made below.

1.2.1 Cd, Pb and Hg: properties, applications and harmful effects on human health

1.2.1.1 Cadmium

Cadmium (Cd, CAS No. 7440-43-9) is a soft and malleable bluish white metal (Herber 2008). It was discovered by Friedrich Strohmeyer at 1817, as an impurity in zinc carbonate. In fact, its name came from the Latin word "*cadmia*" meaning "calamine" (zinc carbonate, $ZnCO_3$) (Winter 2012). Some of the physical and chemical properties of Cd are referred in Table 1.2.

Although scarce, this element occurs naturally in the earth's crust, in its elemental form and in solid state. As mineral, Cd may be combined with other elements, such as sulphur (greenockite, CdS) or oxygen (monteponite, CdO). Other simple compounds formed during weathering processes are $Cd(OH)_2$, $CdCl_2$ and CdF_2 (Kabata-Pendias 2007, Herber 2008). In soils, Cd may form several types of complex ions and organic chelates, existing mostly (99%) associated with soil colloids (Kabata-Pendias 2007).

In the aquatic systems, Cd exists as the hydrated ion or as ionic complexes with other inorganic or organic substances (ATSDR 2012). For pH < 7, the hydrated ion Cd^{2+} is the dominant species in solution, while for higher pH values, Cd^{2+} coexists with hydroxides, which will gain growing expression. Soluble forms of cadmium migrate in water whereas insoluble forms will deposit and absorb to sediments (Kabata-Pendias 2007). The mean concentration of Cd in unpolluted natural fresh water is approximately 1 $\mu g\ L^{-1}$ while for seawater it is between 0.04-0.30 $\mu g\ L^{-1}$ (Reimann and Caritat 1998). In 2000, the main applications of Cd at global level included batteries (12 390 tons, 70%), pigments (2301 tons, 13%), coatings (1416 tons, 8%), stabilizers (1239 tons, 7%) and alloys and others (354 tons, 2%) (Økland et al. 2005, Kabata-Pendias 2007). It also plays a critical role in several cutting-edge technologies such as solar cells, due to its remarkable characteristics as great resistance to corrosion, and excellent electrical conductivity (Srivastava and Goyal 2010).

Table 1.2: Main properties of cadmium (adapted from Winter (2012)).

Symbol	Cd
Melting point	321ºC
Boiling point	767ºC
Atomic number	48
Molar mass	112.41 g mol^{-1}
Density	8.65 g cm^{3}
Atomic radius (calculated)	1.61 Å
Covalent radius	1.44 Å
Electronegativity	1.69

Cadmium is obtained mainly as a by-product of mining of other metals like zinc, lead and copper but it is also recovered from recycled materials such as Zn-Cd batteries (ATSDR 2012). The general trend in the overall consumption of cadmium in the last two decades has been the increase in their use in the production of batteries and a decrease in other applications.

Breathing high levels of cadmium can severely damage the lungs. Eating food or drinking water with very high levels severely irritates the stomach, leading to vomiting and diarrhea. Long-term exposure to lower levels of cadmium leads to a buildup of cadmium in the kidneys and possible kidney disease. Other long-term effect is the weakening of bones (ATSDR 2012). Cadmium and cadmium compounds are also listed as probable Human carcinogens (Otero et al. 2009).

1.2.1.2 Lead

Lead (Pb, CAS no. 7439-92-1) is a naturally occurring metal, with color bluish gray, ductile and soft, whose levels in different ecosystems result mainly from various anthropogenic activities. It is one of the oldest metals known to man and its name probably came from the Celtic word “*luaide*” meaning reddish (because of the colour of lead tetroxide), whereas its symbol Pb is originated from the Latin word "*plumbum*" (Winter 2012). Table 1.3 shows some of the physical and chemical properties of this metal.

Lead is rarely found in its elemental form, occurring mainly as Pb^{2+} and being a constituent of various minerals (Srivastava and Goyal 2010). Galena (lead sulfide, PbS; 87% Pb) is the most common lead ore, followed by anglesite (lead sulfate, $PbSO_4$; 68% Pb), and cerussite (lead carbonate, $PbCO_3$; 77.5% Pb) (Gerhardsson 2008). According to Kabata-Pendias (2007), the overall mean value of Pb for different unpolluted soils is 25 mg kg^{-1}. In soil solution the Pb concentrations are relatively low, varying from <1 to 60 µg L^{-1}, where it occurs as cationic species: Pb^{2+}, $PbCl^+$, $PbOH^+$, and anionic species: $PbCl_3^-$, $Pb(CO_3)_2^{2-}$.

In aqueous medium, lead may exist in multiple forms, depend especially on the pH and dissolved salt contents of the water. Its main forms in seawaters are $PbCO_3$ and $PbCl_2$ while in surface and ground waters its species are: Pb^{2+}, $PbOH^+$, $PbHCO_3^+$, and $PbSO_4$ (Kabata-Pendias 2007). In most of the aquatic environments (generally neutral or alkaline) it has low mobility and eventually ends to precipitate on the bottom sediments. The median concentration of Pb in worldwide ocean waters has been calculated as 0.03 µg L^{-1} whereas in river waters contents vary highly from 0.007 to 3.8 µg L^{-1} (Kabata-Pendias 2007).

Table 1.3: Main properties of lead (adapted from Winter (2012)).

Symbol	Pb
Melting point	327.5ºC
Boiling point	1750ºC
Atomic number	82
Molar mass	207.2 g mol^{-1}
Density	11.34 g cm^3
Atomic radius (calculated)	1.54 Å
Covalent radius	1.46 Å
Electronegativity	1.87

Usually, Pb is obtained from galena deposits where it is extracted together with zinc, silver and (most abundantly) copper (Srivastava and Goyal 2010). In 2001, about 325 000 tonnes were mined in Europe, 150 000 tonnes in Africa, 1100 000 tonnes in America, 725 000 tonnes in Asia, and 715 000 tonnes in Oceania (Gerhardsson 2008).

Lead has many different uses including production of batteries, ammunition, cables, fishing lures, devices to shield X-rays and as petrol additives (Økland et al. 2005). It is also used in paints and ceramic products, caulking, and pipe solder although these applications have been dramatically reduced in recent years, due to health concerns. Due to the gradual and increasing use of unleaded gasoline since the 1980s, exposure to lead has decreased dramatically (Gerhardsson 2008).

Lead can affect almost every organ and system in human body, however, his main target is the nervous system. It can also cause weakness in fingers, wrists or ankles and cause small increases in blood pressure. High-level exposure in men can damage the organs responsible for sperm production (ATSDR 2007). Lead accumulates in mammals and aquatic organisms and some lead compounds are considered possible carcinogens (Økland et al. 2005).

1.2.1.3 Mercury

Mercury (Hg, CAS no. 7439-97-6) is a high volatile element, bright silvery, and the only metal that is liquid at room temperature (Table 1.4). There are reports about mercury in Roman literature (Drasch et al. 2008), but its poisonous properties were known long before, perhaps since 2000 BC where it was used by Chinese alchemists (Kabata-Pendias 2007). Its name honors the Mercury planet and its symbol "Hg" came from the Latin word "*hydrargyrum*" meaning "liquid silver" (Winter 2012).

Table 1.4: Main properties of mercury (adapted from Winter (2012)).

Symbol	Hg
Melting point	-38.83ºC
Boiling point	356.73ºC
Atomic number	80
Molar mass	200.59 g mol^{-1}
Density	14.190 g cm^{3}
Atomic radius (calculated)	1.71 Å
Covalent radius	1.32 Å
Electronegativity	2.00

Several forms of mercury occur naturally in the environment – metallic mercury (also known as elemental mercury), inorganic mercury (mercurous and mercuric salts) and organometallic mercury (e.g. CH_3Hg^+) - and can be found in three oxidation states (0, +1, +2). Its distribution is the result of natural processes and human activity. Volcanic eruptions, erosion and volatilization from the surface of the oceans are the main natural sources of this element.

Mercury can gets in soil through natural, or anthropogenic deposition of ash (e.g. coal burning, municipal incineration) and precipitation. Other important sources are the use of pesticides, sewage sludge and disposal of industrial and domestic waste. Some argue that today, due to widespread pollution is not possible to speak in "natural levels" of Hg in soils, however it can be considered that these rarely exceed 1 mg kg^{-1} (Kabata-Pendias 2007). In the soil-solution, cationic species (Hg_2^{2+}, $HgCl^+$, and $HgCH_3^-$) coexists, but some anions ($HgCl_3^-$ and HgS_2^{2-}) may also be found.

In the aquatic system, mercury contents vary significantly depending on type and location of water, but they are generally low. In open sea the Hg levels are in the range of 0.5-3.0 ng L^{-1}, whereas coastal and bay waters contain from 2 to 65 ng Hg L^{-1} (Kabata-Pendias 2007). In surface fresh waters the concentration of Hg is well below 20 ng L^{-1} (Drasch et al. 2008). Mercury may reach and enter the water system in several ways: atmospheric deposition (wet and dry), transport water from runoff, or leaching of top layer of soils to groundwater. In the aquatic environment, Hg exists in several forms, Hg^0, Hg^{2+}, complexes of Hg^{2+} with different organic and inorganic ligands, and organometallic Hg forms, mostly methylmercury and dimethylmercury (Drasch et al. 2008); and it is associated with suspended particulate matters, organic matters and dissolved organic carbon (DOC) (Kabata-Pendias 2007). In oceanic waters, mercury exists mainly as chloro-complexes, $HgCl_4^{-2}$and $HgCl_3^-$, and thus Hg is bound to humic substances in less extent than occurs in freshwater environments. Changes in chemical, physical, biological and hydrological conditions can cause changes in its oxidation state and chemical forms, as for example, the reduction of Hg^{2+} to Hg^0 and, most important, the in-situ bacterial conversion of inorganic mercury species to the much more toxic methylmercury.

Most of the virgin mercury is produced by the mining of its most representative mineral, Cinnabar (HgS). The EU was traditionally a major exporter of mercury, generating around 3000 tonnes per year (about 25% of the total global supply of Hg) (European Commission 2013). The old Hg mine in Almadén, Spain was the largest Hg producer in the world, but meanwhile it ceased functions. Due to its toxic properties and strict regulations, globally the use of mercury has decreased (from 680 tonnes in 1999 to 320 tonnes in 2007 in EU) (Kabata-Pendias 2007, European Commission 2013). According to the Portuguese

Environmental Agency (APA 2012), in 2010 the total amount of mercury emitted in Portugal from anthropogenic sources was 2 tons (no available data regarding natural emissions).

Nowadays, mercury is still used in a variety of applications: fluorescent lamps, batteries, measuring and control equipment, electric conductors, metal recovering processes, dental fillings, bactericides, fungicides, insecticides, pharmaceuticals (merbromin and thiomersal) and also in paints (Økland et al. 2005). Some industrial plants in the chlor-alkali sector, which produces chlorine and caustic soda were also known to use large amounts of Hg (about 160-190 tonnes in EU, 2007), but this use is being phased out.

Exposure to Hg in humans occurs primarily via food, with fish being a major source of methyl mercury (Sakamoto et al. 2001), which is the most toxic form of it (Challa et al. 2008). In humans, concentrations > 10 µg g^{-1} of Hg in the liver or > 6 µg g^{-1} of Hg in the brain can cause death whereas > 20 µg dL^{-1} of Hg in the blood can cause chromosome damage. A body burden of 55 mg Hg for a 51 kg person can cause the onset of loss of motor control (ataxia) (Seiler et al. 1988). Other effects of exposure to mercury in humans, namely on brain functioning are irritability, shyness, tremors, changes in vision or hearing and memory problems (ATSDR 1999). Mutagenic and teratogenic effects of Hg are recognized, although data on the mechanisms of action are scarce and controversial in the available literature (Cardoso et al. 2013).

Due to production-oriented policies, the mobilization and release of these metals into the environment by anthropogenic action increased significantly. As result, levels and bioavailability of these toxic elements in soils and waters grew considerably, which poses a risk to the environmental and human health. Within this thematic, some issues and challenges, that have attracted the scientific community attention in the last years, will be presented below.

1.2.2 The Ecosystem soil

Soil, being a fundamental part of the terrestrial ecosystem, is the main source of metals for plants (micronutrients and contaminants). As all trace elements, metals in soils may have various origins: (i) lithogenic – inherited from the lithosphere (parent material), (ii) pedogenic – from lithogenic sources but forms changed due to soil-forming processes, and

(iii) anthropogenic – elements deposited onto and/ or into soils as results of human's activities.

It has been assumed that the behavior of metals in soils and in consequence their availability to organisms and plants (or bioavailability) differ as to their origin (Kabata-Pendias 2007). Metals originated from Earth's formation, exist occluded within the mineral or strongly bounded to it, in the solid phase of the soil, and their bioavailability is very slight. By contrary, metals that came from anthropogenic sources are moderately and easily available to plants or to other organisms. This is due to the fact that the metals originated from human activities, generally exist in the solid phase weakly bound, for example adsorbed to soil organic matter or to the surface of soils minerals, being potentially exchangeable.

Inputs via natural sources (e.g. weathering of minerals, erosion and volcanic activity) constitute a significant burden of metals in the soil, nevertheless the contribution from anthropogenic sources in polluted soils for many of them can be several times that from natural source. Studies of metals distribution in some Portuguese ecosystems showed that soils near industrial and mining sites contain much greater levels of these elements compared with their local background concentrations (Da Silva et al. 2005, Reis et al. 2009). Soils with high levels of metals pose a long-term risk of increased plant uptake and leaching (Xue et al. 2003, Álvarez-Ayuso et al. 2008, Zhao et al. 2009).

The food chain is considered a major pathway to transfer many metals from soil to humans, although there are several possible routes to transfer metals including drinking water, air/ dust inhalation or absorption through skin.

In the last three decades there has been a real "explosion" of research data and various studies on the occurrence and behavior of almost all trace elements have been published (Kabata-Pendias 2007). This has provided a better understanding on the concentrations at which plants and organisms are protected from "excessive accumulation" of potentially toxic elements. These "safe" concentrations of PTE's are the basis of current regulations (for example EU sludge Directive limits 86/278/EEC), for their inputs to soils, as for example through the application of sewage sludge in soils in Europe, North American and other areas. However, the current regulatory framework must be in continuous improvement to better protect the wider environment, which is only possible through continued scientific study. For example, in the UK recent field experiments indicated that the current soil total Cd limit of 3 mg kg^{-1} (maximum possible concentrations (MPC) in Sludge Directive) may not be adequately protective against producing wheat grain with concentrations of Cd above the EU grain Cd MPC of 0.235 mg kg^{-1}, unless the pH is maintained above 6.8 (Chaudri et al. 2007).

Recent studies also showed that in order to assess the environmental risk of metals to ecosystems and human health, the evaluation of total contaminant concentrations may not be sufficient (Rodrigues et al. 2010b). For example, in the 1950s, a major incidence of severe cadmium toxicity, causing the itae-itae disease, occurred in Toyama, Japan (Nogawa 1981, Nogawa and Kido 1993). The total Cd contents of Toyama rice fields was very low (5 mg kg^{-1}) when compared with those of Shipham in the UK or Stolberg in Germany (50–150 mg kg^{-1}). However, contrary to Toyama, few or no effects of Cd were observed in the latter two cases (Inskip et al. 1982, Ewers et al. 1985, Alloway et al. 1988). The difference in soil chemistry was a main factor responsible for this paradoxical outcome. The low soil pH at Toyama favored Cd mobilization and uptake by rice, whereas neutral pH of the Shipham and Stolberg soils restricted Cd uptake by vegetables.

1.2.2.1 Pools of metals in soil

Soil is a dynamic system that consists of heterogeneous mixtures of different organic and organic-mineral substances, clay minerals, oxides and hydroxides of Fe, Mn, and Al, and other solid components as well as a variety of soluble substances (Kabata-Pendias 2007). The binding mechanisms for metals and forms of their occurrence in the different soil compartments (Figure 1.1) are manifold, and are determined by soil physical, chemical and biological processes (Hooda 2010), which can vary between nanoseconds and centuries or more (Kabata-Pendias 2007).

The speciation and transfer of metals between the soil compartments should be considered as the main process controlling their behavior and bioavailability. According to the direct chemical availability, a partitioning approach well-accepted by the scientific community considers 3 fractions of metals in soils: available, reactive and inert fractions (Rodrigues et al. 2010a). It should be emphasized that other conceptual fractions can be defined, for example according to the metal speciation, e.g. inorganic and organometallic fractions.

In upland soils of England and Wales, the maximum percentages in soil solution in an individual sample were 4% for Cu, 19% for Zn, 9% for Cd, 0.7% for Pb (Tipping et al. 2003). Yet this tiny soluble fraction might have important environmental significance, because it represents the direct available pool of metals in soils for groundwater, plants, and other living organisms (Saxe et al. 2001, Lofts et al. 2004, Hough et al. 2005, Peijnenburg et al. 2007, Koopmans et al. 2008).

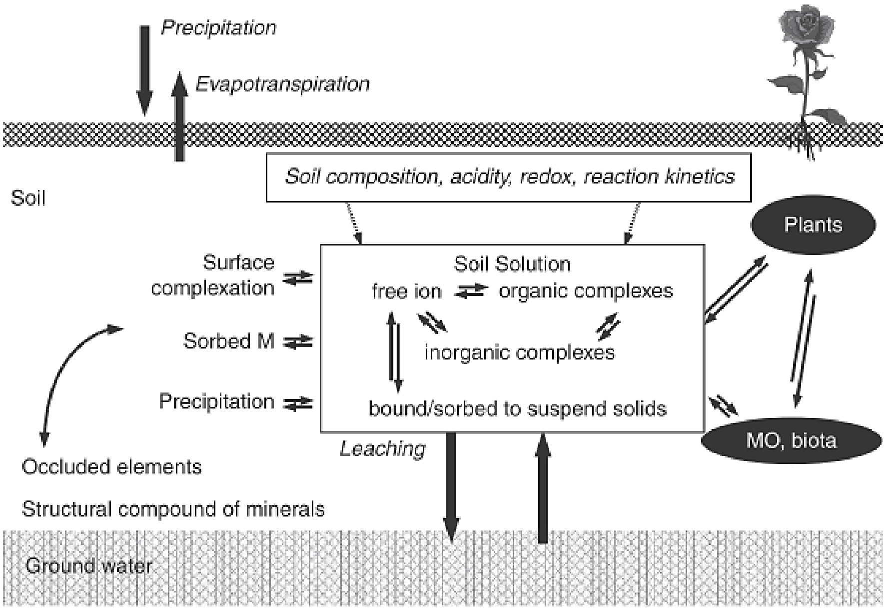

Figure 1.1: Schematic representation of various pools of metals in the soil system. The soil solution is the central gateway through which various forms of metals interact with the soil solid phase and soil biological activity (Hooda 2010).

When in the soil solid part, metals can be superficially adsorbed or complexed with soil components such as clay minerals, hydrated oxides of iron and manganese, or organic matter, being in equilibrium with the soil solution phase. This fraction is commonly referred to as the reactive pool (Rodrigues et al. 2010a, Tack 2010). This fraction of metals is in equilibrium with the directly available pools, which can be leached from soils by transport through the soil solution, be detrimental to soil (micro)organisms, and be incorporated into trophic chains (Sastre et al. 2007).

When occluded within soil solid matrix, strongly bound within the crystalline matrix of the solid phase, metals become unavailable for transport or plant uptake, at least in the short term. The release from this pool usually occurs only within a large time frame, particularly in aerobic soils since it generally depends on slow weathering processes. This fraction is commonly referred to as the inert pool of the soil (Rodrigues et al. 2010a, Tack 2010).

1.2.2.2 Evaluation of metals pools in soil

The determination of various fractions of these elements are broadly used to link their soils contents with potential bioavailability/uptake by plants, and to predict their mobility and potential transport to groundwater (Kabata-Pendias 2007). Several methodologies to assess and quantify the conceptual fractions in soils have been developed and tested, particularly during the last decade.

Most of the analytical techniques to assess total (or pseudo-total) metal contents in soils are dissolution based, i.e., decomposition techniques that involve the use of strong acids and an external heat source to decompose the sample matrix. Nondestructive techniques such as particle-induced X-ray emission and X-ray fluorescence spectrometry have also been used for metal analysis in soils (Du Laing 2010).

Soil chemical extractions can also be used to determine reactive pools of metals although at present only operationally defined protocols exist (Römkens et al. 2004, Rodrigues et al. 2010a). Single extraction procedures using strong complexing agents (chelating agents such as EDTA - 0.05/0.1 M) and diluted solutions of strong acids (such as HCl – 0.1 M and HNO_3 – 0.43 M) have been applied to assess metal reactive pools (Tipping et al. 2003, Römkens et al. 2004, Peijnenburg et al. 2007, Romkens et al. 2009) with comparable results

The use of artificial soil solutions, and weak extractants such as $NaNO_3$, NH_4NO_3, $Ca(NO_3)_2$ or $CaCl_2$ have been commonly used to estimate the available pool of Cd, Cu, Ni, Pb and Zn in soils (Houba et al. 2000, Degryse et al. 2007, Meers et al. 2007, Romkens et al. 2009). The use of 0.01 M $CaCl_2$ as an extraction reagent was proposed by Novozamsk et al. (1993) to estimate the bioavailability of metals and nutrients in air dried soil samples, being considered at present the extractant that gives better indication of the bioavailable pools of cationic metals such Cd and Zn (Du Laing 2010).

1.2.2.3 Metals behavior modelling

The use of single extractions is important to ascertain chemically distinguishable pools of metals within the soil matrix and to assess their availability. However to perform a more accurate assessment of the current and future risk levels it is necessary to develop tools that are able to explain the relationship between the soil and the soil solution (and the role of soil properties). Tools to predict available pool of metal contaminants, to estimate for example leaching losses, uptake by crops or exposure to soil micro-organisms are therefore necessary to evaluate if soils can be used safely for example, for food production.

During the last three decades numerous models (both empirical and mechanistic) have thus been proposed and developed (Sauvé et al. 2000, Tipping et al. 2003, Romkens et al. 2009), revealing the role of soil properties such as clay, organic matter, pH, dissolved organic carbon and ionic strength in controlling solid solution partition of metals (Yin et al. 2002, Gustafsson et al. 2003, Rieuwerts et al. 2006). For example, certain studies showed that an increase in both clay and organic matter, increases the capacity of the soil to retain metals (McBride 1994). Similarly, with an increase in pH, metal retention for cationic metals like Cd, Zn, Cu and Pb also increases (Selim and Sparks 2001).

Although there are studies focused on the assessment of the concentrations of metals in plants, and derivation of soil to plant transfer functions (Al-Masri et al. 2008, Vandenhove et al. 2009), little information is available about the contaminant mercury. The transfer and accumulation of organo-metallic mercury (OrgHg) in terrestrial ecosystems is poorly documented, and information about the exposure of grazing animals to OrgHg from soils and feed as well as on risks of exposure to animal and humans is also very scarce.

1.2.2.4 Metals in Portuguese soils

In Portugal, there is no global and comprehensive legislation on soil protection (DGADR 2009). The Decree-Law 276/2009 on the agricultural use of sewage sludge, proves to be an exception, although insufficient because there is no specific legislation governing the assessment and/or remediation of contaminated soils (Rodrigues et al. 2009b). Actually, only some of the EU Member States, have developed and implemented gradually over the past 20-30 years, a policy of soil protection, namely England, Holland and Germany (Rodrigues et al. 2009a). However, a scenario change is expected in the medium term, with the possible approval of the Framework Directive for Soil Protection, since to date the soil is the only environmental component that is not protected at Community level by specific legislation (DGADR 2009). Also, information on soil contamination in Portugal, soil risk assessment and animal/human exposure is generally scarce. Past soil studies include mostly soil fertility and agricultural productivity investigations (Abreu et al. 1993, Fernandes et al. 2000). In the last decade, research on soil contamination at mining areas and (eco) toxicity of soils gained particular attention (da Silva et al. 2004, Loureiro et al. 2005, Pereira et al. 2006). More recently, a study on the background concentrations of metals and other PTE's in the entire country provided relevant information that can be used as baseline data for future studies (Inácio et al. 2008). A few studies have also been undertaken in Portugal on the effectiveness of phytoremediation technologies of historical contamination at mining sites (Alvarenga et al. 2004, Díez Lázaro et al. 2006). A more limited number of studies have focused on diffuse and line sources of contamination such as the impacts of urban development on soil systems (Rodrigues et al. 2006). An important research on metallic contaminant levels in various areas of Portugal was recently executed, following an approach which to date have been applied only in temperate regions (Rodrigues et al. 2010a, Rodrigues et al. 2010b). Through the application of well-accepted soil extraction procedures and empirical Freundlich-type models in combination with mechanistically based models, the authors were able to predict the "reactive" and the "available" content of some metals and other PTEs successfully.

1.2.3 The Ecossytem Water

All life on the Earth emerged from water and may disappear by the water itself, because it is undeniably the most valuable and indispensable natural resource existing on our planet. Maintaining water quality is critical to secure all forms of life and crucial for health and for a good environmental management. Thus, water is certainly the most studied environmental compartment, and part of the research seeks to understand the distribution of chemical elements and their forms, in different types of water, and its uptake into biota (Raspor 2008).

In aquatic systems, Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-} and HCO_3^- are the major ions, and their concentration is usually much higher in sea than in river waters. These ions may also occur as different species adsorbed by inorganic and organic colloidal particles (Kabata-Pendias 2007). Metals and the so-called secondary elements, such as C, N, P, S, and Si are also present in all water systems.

As for soils, human pollution plays a significant role in increasing the concentration of metals in the different water resources (Torres et al. 2008). Metallurgical industry, paints and tanneries, mineral extraction and fertilizers generate high amounts of metal-rich effluents whose final destination is usually aquatic ecosystems (Afkhami et al. 2010, Fu and Wang 2011, He and Chen 2014). This phenomenon is particularly significant at estuaries and coastal areas, since they are the main disposal sites for anthropogenic contaminants (Torres et al. 2008).

In the aquatic environment, metals tend to be associated with the particles in suspension due to its high affinity for particulate matter (Kim et al. 2004). In most cases, they are rapidly absorbed either by clay or organic compounds, and deposited in estuarine and marine sediments (Ramalhosa 2001), or they are caught by plankton and aquatic plants (Kabata-Pendias 2007). Thus, in most situations, metal concentrations in bottom sediments and/or in plankton constitute good indicators of water contamination. The speciation of metals in sediments is generally very similar to that in soil.

Although immobilized in sediments, even for years, the metals remain a hazard since, at any time, they may be released to the water column (Kabata-Pendias 2007), and thus become available to aquatic organisms and transport (Kim et al. 2004, Cardoso et al. 2008). This release may occur due to sediment resuspension, or result from redox changes or microbial activity (Kabata-Pendias 2004). Burrowing organisms as well as organisms feeding on benthic invertebrates also contribute to transport metals from sediments to the water column. Another important factor to take into account are nuisances induced by extreme weather events.

Several biotic and abiotic parameters (salinity, temperature, light, pH, Eh, and ligand concentration) influence metal species distribution in aquatic systems, and their uptake and accumulation by organisms (Raspor 2008). For example, inorganic mercury may be methylated, by anaerobic organisms in aquatic systems to methylmercury, which is more easily assimilated and bioaccumulated by the aquatic invertebrates than the inorganic form (Lawson and Mason 1998).

Nowadays, environmental policies related to water quality are starting to reflect the character of non-renewable resource, whose availability may be compromised by human activity. In order to protect the environment and the humans from the adverse effects of metal contaminants, a large number of governmental institutions worldwide have set maximum limits for their levels in the aquatic systems as well for effluent discharges (He and Chen 2014). It should be noticed that environmental law has become more stringent every day (Yetilmezsoy et al. 2009).

1.2.3.1 Legislation regulating water quality

In 2001 (roughly one year after the approval of the EU Water Framework Directive - 2000/60/EC), the European Union classified Hg, Cd and Pb as "priority hazardous substances" in the field of water policy (Decision No 2455/2001/EC), and therefore, their discharges should be ceased or eliminated progressively within 20 years. The final Directive on Priority Substances, released in 2008 (Directive 2008/105/EC), excluded lead and its compounds of the list, which however remain in the List II of substances whose discharge into the aquatic environment are subject to prior authorization and emission values set (Directive 2006/11/EC). List II refers to substances, which despite having a detrimental effect on the aquatic environment, this effect may be limited to a certain area, and be dependent on the characteristics and location of the water in which these substances are released. Recently, the new Directive 2013/39/EU brought some amendments to Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. New substances have been added to the list of priority hazardous substances (e.g. dicofol, perfluorooctane sulfonic acid), which should now be reviewed at least every six years. There were no changes related to the classification of Hg, Cd and Pb. Table 1.5 shows the levels of some PTEs in raw effluents and their maximum admissible concentration in effluents and drinking water (American and European regulations) (Chojnacka 2009a). It is possible to see that in general the European law is more demanding.

At the national legislation level, in the field of water resources and water quality, two of the most important decrees in Portugal are: the Decree-Law 236/98, establishing standards, criteria and quality goals, in order to protect the aquatic environment and to improve the quality of water in terms of its main uses; and the Decree-Law 306/2007, regulating the quality of water used for human consumption. More recently, Decree-Law

103/2010, transposes Directive 2008/105/EC and partly, Commission Directive 2009/90/EC, establishing environmental quality standards (EQS's) in the field of water policy.

Table 1.5: Levels of some PTEs in raw effluents, maximum admissible concentration in effluents and drinking water (American and European regulations) [µg L^{-1}] (adapted from (Chojnacka 2009a).

PTE	Domestic wastewater	Wastewater discharge limits		Drinking water standards	
		European	**American**	**European**	**American**
As	1-5	100	2000	10	10
Cd	1-4	not allowed[1]	100	5	5
Cu	30-100	500	5000	2000	1300
Hg	1-3	not allowed[1]	5	1	2
Ni	10-40	Variable [2]	5000	20	100
Pb	25-80	Variable [2]	2000	10	15
Se	-	1000	-	10	50
Zn	80-300	2000	5000	-	-

1 - priority hazardous substance whose discharge must be stopped; 2 – priority substance in the field of water policy, whose discharge is subject to prior authorization and emission values set (Directive 2013/39/EU).

Table 1.6 summarizes the wastewater discharge limits for Cd, Hg and Pb, as well as the EQS's for surface waters and limit-values for these metals in water for human consumption, in Portugal. Following the trend, also in Portugal the legislation has become increasingly restrictive. This is notorious for the maximum allowable concentrations for surface waters, which about 4 years ago were 1, 10 e 50 µg L^{-1} for Hg, Cd and Pb, respectively.

The control of metal discharges and the removal of metals from aqueous solutions have become a challenge for the twenty-first century (Srivastava and Goyal 2010). A quick search on the academic citation indexing service "*Web of Knowledge*", using the key words "*metal removal*" and "*water*" revealed that the number of studies performed regarding this thematic more than triplicated in the last decade (from approximately 600 citations in 2003 to approximately 2000 citations in 2013).

Table 1.6: Limit-values for the concentration of Pb, Hg and Cd in wastewater discharges, EQS's in surface waters and limit-values in water for human consumption according to Decrees-law 236/98, 306/2007 and 103/2010.

	Wastewater discharges	**Fresh surface waters**		**Other surface waters**		**Human consumption**	
	ELV (µg L^{-1})	AA-EQS (µg L^{-1})	MAC-EQS (µg L^{-1})	AA-EQS (µg L^{-1})	MAC-EQS (µg L^{-1})	MRV (µg L^{-1})	MPV (µg L^{-1})
Cd and its compounds	200	≤ 0.08 C1 0.08 C2 0.09 C3 0.15 C4 0.25 C5	≤ 0.45 C1 0.45 C2 0.6 C3 0.9 C4 1.5 C5	0.2	≤ 0.45 C1 0.45 C2 0.6 C3 0.9 C4 1.5 C5	1	5
Hg and its compounds	50	0.05	0.07	0.05	0.07	0.5	1
Pb and its compounds	1000	7.2	--	7.2	--	--	10

ELV – emission limit-value; EQS - environmental quality standard; AA – annual average; MAC – maximum allowable concentration; MRV – maximum recommended value; MPV – maximum permissible value; C1, C2, C3, C4 and C5 are classes of water hardness according to Directive 2008/105/EC.

Currently there are several processes that can be applied to the removal and recovery of metals from aquatic systems, namely ion exchange (Korngold et al. 2001), membrane filtration (reverse osmosis, ultrafiltration) (Landaburu-Aguirre et al. 2009), electrochemical techniques (Chen 2004), precipitation (Matlock et al. 2002), coagulation and flocculation (El Samrani et al. 2008), and adsorption using activated carbon (Yanagisawa et al. 2010). An overview on these techniques will be presented in the next section. Some aspects common to the studies reported in the literature will be also presented.

1.3 Metal removal from waters: techniques used so far

Since it is impossible to degrade the metals, the only way forward will be their safe disposal, i.e. their removal from the contaminated system, their concentration and immobilization in a solid state, not available to living organisms and biological cycles. The commonly used processes for the treatment of waters contaminated with metals are based on this principle.

1.3.1 Precipitation

Chemical precipitation is commonly employed as a pre-treatment for other treatment technologies of raw wastewaters. By changing the pH of the medium or adding other chemical species to the solution, metals precipitate typically as hydroxides, sulfides, or carbonates (Tchobanoglous et al. 2003). The forming insoluble precipitates are separated from the water by sedimentation or filtration. In the precipitation process, apart from the addition of chemical precipitants, coagulants and flocculants are also used to increase particle size through aggregation (Dabrowski et al. 2004). This process is relatively simple and by far the most widely used process in industry (Fu and Wang 2011). A major disadvantage of this method is the requirement of large doses of alkaline materials to increase and maintain pH values typically from 4.0 to above 6.5 for optimal metal removal (Matlock et al. 2002). Other disadvantages of this technique are the production of a large amount of sludge rich in metals which are highly regulated, and have costly disposal requirements (Farooq et al. 2010), and the low efficiency when the target contaminant is present in low concentrations.

1.3.2 Coagulation and flocculation

Just as chemical precipitation, coagulation and flocculation followed by sedimentation or filtration can be used for the removal of most of the metals. Through the addition of coagulants, such as aluminium, ferrous sulfate and ferric chloride, the forces that keep the metals in solution are neutralized leading to their aggregation/coagulation. Flocculation uses polymers to form bridges between the flocs and bind the particles into large agglomerates, which are then removed or separated by filtration or floatation (Fu and Wang 2011). Coagulation-flocculation must be followed by other treatment techniques since generally they cannot treat the metal wastewater completely. Furthermore these methods produce concentrated and toxic wastes, which lead to disposal problems (Farooq et al. 2010).

1.3.3 Electrochemical techniques

Electrochemical methods involve the use of an electrochemical cell, which allows the reduction of the metals to their zero oxidation state. Thus metals are recovered in their elemental state and with a high degree of purity. The cell consists of two electrodes, positive (anode) and negative (cathode) which are kept at a given potential through an external power source. Metallic ions in solution are attracted to the electrode due to electrostatic forces between opposite charges. Electrochemical technologies have the great advantage of being selective, however they involve a relatively large capital investment and the expensive electricity supply (Fu and Wang 2011). Maybe for that they have not been widely applied. In addition they require a neutral medium which implies pre-treatment (Farooq et al. 2010).

1.3.4 Membrane filtration

Membrane filtration technologies are no more than physical procedures for separating particles by means of semipermeable membranes. Depending on the size of the particles/contaminants which are to be removed, it may be applied: microfiltration, ultrafiltration, nanofiltration and reverse osmosis. These techniques promise high efficiency and easy operation (Fu and Wang 2011).

Ultrafiltration (UF) techniques use low transmembrane pressures to achieve metal removal and are based on the addition of surfactants, such as sodium dodecyl sulfate (SDS) to wastewater. Surfactant molecules will aggregate into micelles that can bind metal ions to form large metal-surfactant structures, which will be retained by the UF membrane. The efficiency of this process is dependent of the characteristics and concentrations of the metals and surfactants, solution pH, ionic strength, and parameters related to membrane operation. The main disadvantages of UF are the required low flow rate and high costs (Farooq et al. 2010).

Since the development of the first practical cellulose acetate membranes in the early 60's and the subsequent development of thin-film, composite membranes, the uses of reverse osmosis (RO) have expanded to a wide variety of wastewater treatment applications (Williams 2003). Reverse osmosis (Figure 1.2) is a pressure-driven process whereby a semi-permeable membrane rejects dissolved constituents present in the feed water. This rejection is due to size exclusion, charge exclusion and physical–chemical interactions between solute, solvent and membrane (Malaeb and Ayoub 2011).

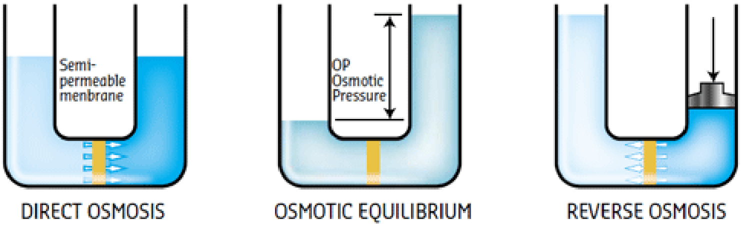

Figure 1.2: Reverse osmosis operating principle.

Reverse osmosis has become an increasingly popular treatment technology, being responsible for more than 20% of the world's desalination capacity (Fu and Wang 2011). A major concern for reverse osmosis systems remains to be the cost dictated by both energy consumption and membrane replacement costs (Malaeb and Ayoub 2011).

1.3.5 Adsorption using activated carbon

Adsorption is a process in which a particular component is accumulated on the surface or interface between two phases as a result of chemical and physical factors. It should be distinguished from absorption, where the component is "incorporated" in another phase. The overall process, which includes adsorption, absorption and ion exchange is referred as sorption. It should be noted that the term adsorption is often found in the literature on studies where a given material is used to promote the removal of metals, but where the mechanism is not the subject of study. In such cases the term sorption would be more suitable.

Activated carbon (AC) is an adsorbent widely used in the removal of metal contaminants. Its extensive surface area, result of large volume of micro and mesopores, enables a quick and effective removal (Fu and Wang 2011). The major problem with the activated carbon based treatment process is the regeneration of the material after its adsorptive capacity has been reached, which makes this process costly (Farooq et al. 2010). In addition, the acquisition cost AC is becoming increasingly high.

1.3.6 Ion-exchange

Ion exchange is a process of purification where ions to be removed are exchanged between the contaminated solution, and a solid polymeric or mineral "ion exchanger" (Figure 1.3). The most wide-spread use of this technology is in domestic water softening, where sodium ions from a cationic-exchange resin replace the calcium and magnesium ions in the treated water thus reducing the hardness. However, ion exchange has been also used in wastewater applications for the removal of metals, nitrogen and total dissolved solids, due to their many advantages, such as high treatment capacity, high removal efficiency and fast kinetics (Tchobanoglous et al. 2003, Fu and Wang 2011). A vast variety of ion exchangers have been used for metal and other PTEs removal, including natural or synthetically produced polymers, clays, zeolites, several types of biomaterials and more recently nanomaterials (Gonzalez et al. 2010). Ion-exchange is considered to be cost-effective if only low-cost ion exchangers such as natural zeolites or other materials that are abundantly available in nature are used (Entezari and Tahmasbi 2009). Ion exchangers can be either cation exchangers or anion exchangers. There are also amphoteric exchangers which are able to exchange both cations and anions simultaneously. However, better results are obtained in mixed beds that contain a mixture of anion and cation exchange resins or passing the treated solution through several different ion exchange materials (Srivastava and Goyal 2010).

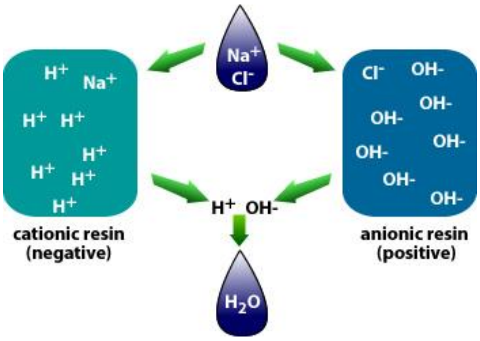

Figure 1.3: Ion Exchange process

Table 1.7 summarizes the main advantages and disadvantages associated with each of the treatment methods described. Although some methodologies provide good removal rates, high selectivity and possibility of regeneration/reuse of the sorbent material, all of them present several negative aspects. Formation of toxic by-products, high generation of sludge which require a proper disposal, and high initial and running costs are some of the weaknesses that jeopardize the viability of existing methods (Brinza et al. 2009, Liu et al. 2009).

Table 1.7: Summary of some methods to remove metals from contaminated waters and their main advantages and disadvantages (adapted from Faroq et al. 2010).

Process	Advantages	Disadvantages
Precipitation	• Simple • Inexpensive • Most of metals can be removed	• Large amounts of sludge produced • Disposal problems
Coagulation and flocculation	• Sludge settling • Dewatering	• High cost • Incomplete removal • Large consumption of chemicals • Further toxic wastes and disposal problems
Electrochemical techniques	• Metal selective • No consumption of chemicals • Pure metals can be achieved	• High capital cost • High running cost • Initial solution pH
Membrane filtration	• Less solid waste produced • Less chemical consumption • High efficiency (>95% for single metal)	• High initial and running cost • Low flow rates • Removal negatively affected by the presence of other ions
Adsorption using activated carbon	• Most of metals can be removed • High efficiency (>99%)	• Cost of activated carbon • No regeneration
Ion exchange	• Metal selective • High regeneration of materials	• High cost • Less number of metal ions removed

Thus, it becomes crucial to develop alternative techniques, taking into account not only its effectiveness, but also the materials employed, the simplicity and the associated cost (Farooq et al. 2010). To achieve this goal, the scientific community has mainly followed two different approaches: the development and application of new synthetic materials, such as nanomaterials, highly efficient and selective (Savage and Diallo 2005, Theron et al. 2008, Shan et al. 2009, Bao et al. 2013); the development of biotechnologies, which are based on processes of biosorption and bioaccumulation to achieve metal removal (Arief et al. 2008, Chojnacka 2010, Flouty and Estephane 2012, Bulgariu and Bulgariu 2014), and which are more environmentally friendly, less expensive, without neglecting efficacy.

Although promising, the application of nanotechnology for remediation of contaminated ecosystems still raises many questions related to the possible toxicity of these materials, both for humans and for ecosystems. It is therefore indispensable a thorough research about the direct and indirect effects that engineered nanoparticles may have on living organisms (Sanchez et al. 2011).

1.4 Biosorption and bioaccumulation as remediation technologies

Considering the very low values required by law for the concentration of metals in water, and the need for alternatives to conventional treatment methods, processes such as biosorption and bioaccumulation have been intensively studied (Romera et al. 2007, Mata et al. 2009, Farooq et al. 2010). These processes occur freely and continuously in the environment (Chojnacka 2010), but under controlled and optimized conditions of operation, they can be the basis of biotechnologies for remediation of contaminated systems. Biotechnologies making use of biological material from plants, bacteria, fungi, yeast or algaeto promote the removal and recovery of metal contaminants.

Biosorption is a physical-chemical process involving the separation of a contaminant in liquid phase by using a solid phase consisting of inactive or non-living biomass (Farooq et al. 2010). It is a process very similar to conventional adsorption and/or ion exchange, being the biological nature of the sorbent the main distinguishing feature (Cho and Kim 2003, Diniz and Volesky 2005, Chojnacka 2009a). Nevertheless unlike mono functional ion exchange resins, biosorbents feature a range of functional groups (carboxyl groups, sulfates, phosphates, etc.) in their cell wall, with high capacity to attract metal ions present in the fluid, which provides them a remarkable removal efficiency (Flouty and Estephane 2012).

Biosorption is relatively quick, metabolically passive, and reversible process (Chojnacka 2010). Thus, contrary to the adsorption by activated carbon, most of the biosorbents may be regenerated (Flouty and Estephane 2012).

The first research on biomass-metal interactions dates back to 1960 (Chojnacka 2010). However, only in the 90s biosorption assumed greater importance in science, largely due to the contribution given by prof. Bohumil Volesky of McGill University in Canada (Volesky 2007). He provided much of the theoretical basis of the process and made the first attempts for its marketing (Volesky 2001, Volesky and Naja 2007), showing that biomass was capable of concentrate metal ca. 1000 fold (Chojnacka 2010).

Since then, a significant number of studies on biosorption of metals from aqueous solutions have been conducted worldwide (Arief et al. 2008). Efforts have been made to the use of different types of biological materials, widely available and inexpensive, and to the optimization of biosorption parameters, in order to achieve maximum efficiency of removal (Kar and Misra 2004). Some examples of the most studied biomasses include anaerobically digested sludge (Tokcaer and Yetis 2006), biomass of moss and lichens (Tuzen and Sari 2009, Tuzen et al. 2009), crab shells and eggs (Park et al. 2007, Rae et al. 2009, Vijayaraghavan et al. 2011), and dead biomass of algae (Romera et al. 2007, Liu et al. 2009, Huang et al. 2010). Agricultural waste (Rocha et al. 2013, Jaishankar et al. 2014) and industry by-products (Akar et al. 2009, Lopes et al. 2014) have also been used.

Non-living biomass may be applied directly, or after pre-treatment (Klimmek et al. 2001, O'Connell et al. 2008), which in most cases comprises, besides drying and grinding, acid (Choi and Yun 2004, Niu et al. 2007) or alkaline (Bulgariu and Bulgariu 2014) treatment (activation).

Trends in current research on biosorption also include the study of the removal mechanism, i.e. identification of main processes that occur in the surfaces of the biosorbents and characterization of existing functional groups (Fourest E. and Volesky B. 1996, Davis et al. 2003, Volesky 2007, Sud et al. 2008). To this end, the combination of techniques such as FTIR, SEM-EDX, TEM as well as classical methods such as titrations has proven very useful (Arief et al. 2008). Currently it is thought that there are different processes which contribute to biosorption mechanism (Figure 1.4): adsorption on the surface and pores, ion-exchange, surface precipitation, complexation and chelation, and entrapment in capillaries and spaces of polysaccharide network (Ahluwalia and Goyal 2007, Farooq et al. 2010). Nevertheless, in most cases adsorption and ion exchange are identified as the dominant processes.

The mechanism by which metals bind to the biomass is closely related to the solution composition and structure of the cell wall of biomass (Demirbas 2008). The most common functional groups are: hydroxyl (-OH), carboxyl (R-COOH), sulfonate ($R\text{-}SO_3^-$), thiol (R-SH), amines ($R\text{-}NH_2$), amides ($R\text{-}CONH_2$) and phosphonates (PO_4^{3-}) (Volesky 2007, Wang and Chen 2009).

Process configurations are also studied extensively, including batch and column studies (Volesky 2007, Apiratikul and Pavasant 2008), as well as carrying out the process in membrane bioreactors (Beolchini et al. 2006).

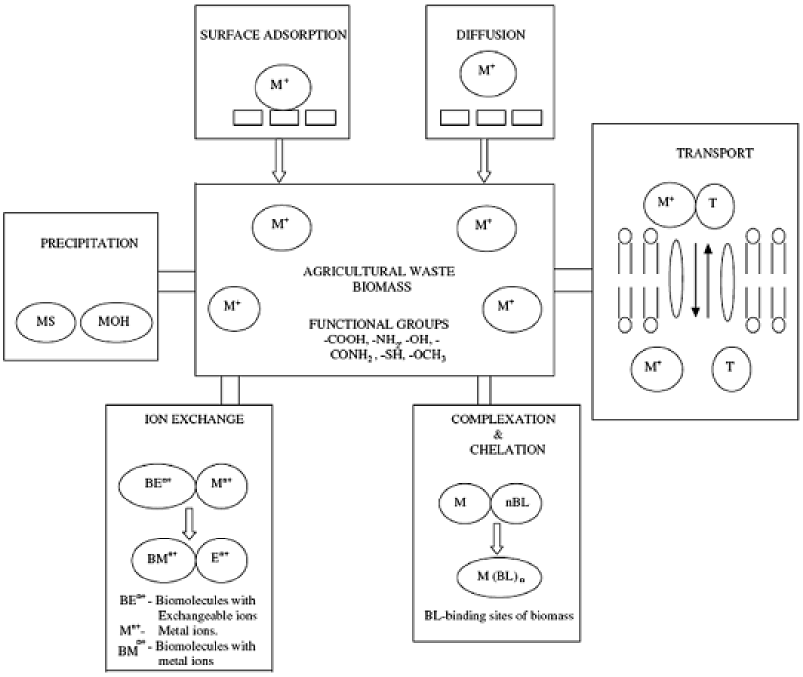

Figure 1.4: Mechanism of biosorption (Sud et al. 2008, Farooq et al. 2010).

Research on biosorption has focused primarily on the removal of PTEs commonly used in industry, such as Cu, Zn, Cr, Pb, Cd, Ni and in less extent Hg, Al, Co, As and Th (Figure 1.5) (Chojnacka 2009a). Despite being part of the "toxic-trio", Hg is not as documented as Pb and Cd, probably due to the difficulties inherent to the study of this metal, such as its laboratory handling, analytical determination and complexity of solution chemistry (Volesky 2007).

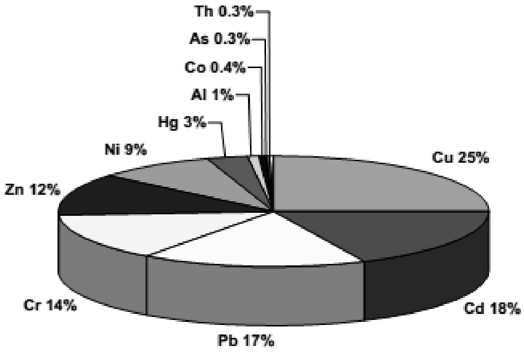

Figure 1.5: PTEs more common in biosorption studies (Chojnacka 2009a).

All scientific development has culminated in a series of review papers on biosorption, condensing all available information on the vast array of biological materials studied (Ho et al. 2000, Babel and Kurniawan 2003, Davis et al. 2003, Arief et al. 2008, Sud et al. 2008, Wang and Chen 2009, Farooq et al. 2010, He and Chen 2014). However, the evaluation of the performance of metal removal for a given biosorbent over other sorbents is not an easy task, since experimental conditions, such as pH, temperature and sorbate/sorbent ratio are very different among studies, and thus results are not entirely comparable.

Overall, biosorption promises to fulfil the main requirements for a successfull biotechnology, which are efficiency, competitiveness and economic viability. Some authors argue that the application of biosorption processes can reduce capital costs by 20%, operation costs by 36% and total treatment costs by 28%, when compared with suitable ion exchange systems (Volesky 2001, Zouboulis et al. 2004).

Biosorption is already commercialized and accepted by the EPA as biotechnology (EPA/540/S5-90/005), however only a few biosorbents are available on the market such as AlgaSORB® and AMT-BIO-CLAIM® (Chojnacka 2010). AlgaSORB® consists of powdered *Spirogyra* alga, which is immobilized on solid column of silica gel (Singh and Prasad 2000), while AMT-BIO-CLAIM® is based on bacteria of the genus Bacillus treated with caustic soda.

Bioaccumulation process uses living biomass. Apart from binding to the surface, contaminants are also accumulated inside the cells through the organism metabolic cycle (intracellular accumulation) – Figure 1.6. In a first phase (I), which is fast and reversible, contaminants bind to the cell surface, due to various passive metabolic processes similar to those occurring in biosorption. For that reason this step is commonly referred to as biosorption, or passive accumulation. Then transport of contaminants into the cell occurs (II) primarily through active transport systems, which require energy consumption (metabolically active process) (Kadukova and Vircikova 2005, Torres et al. 2008, Chojnacka 2009a).

A major advantage of bioaccumulation process, in addition to the greater number of binding sites provided (surface and inner), is the use of growing cells (cell multiplication – III), which leads to a multiplication of those binding sites (Malik 2004). Thus, by using bioaccumulation, lower residual concentrations in treated aqueous solution will be potentially achieved (Chojnacka 2007).

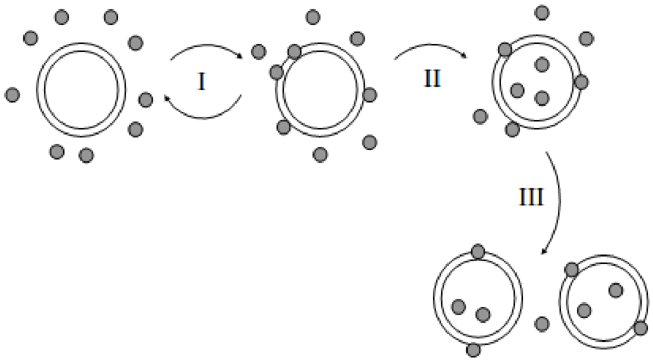

Figure 1.6: Stages on the bioaccumulation of metals (adapted from Chojnacka (2009a)).

Table 1.8 presents a comparison between biosorption and bioaccumulation processes (Chojnacka 2010). One of the main disadvantages of using living organisms, in relation to biosorption, is the need for a nutrient source, in order to maintain the stability and constant growing of the biomass. However, this limitation may be overcomed by using photosynthetic organisms such as algae and other aquatic plants – phytoremediation (Kara 2004, Prasanna et al. 2008), whose nutritional requirements are minor and require a source of inorganic carbon as carbon dioxide (Chojnacka 2010). Thus, companies who adopt this biotechnology will concomitantly decrease the levels of contaminants in their effluents (metals and/or compounds of phosphorus and nitrogen, which are responsible for eutrophication) and reduce CO_2 footprint (as well as avoid carbon taxes if applicable).

When considering the operational aspects, bioremoval by living organisms is simpler since biomass is placed directly in contact with the contaminated fluid on containers or constructed wetlands (Babatunde et al. 2008, Marchand et al. 2010, Guittonny-Philippe et al. 2014). There is no need for complex processes of stirring, and separation such as filtration, which are indispensable for biosorption, since in that case biomass is usually applied in powder form (in order to increase its contact surface) (Aksu and Dönmez 2000). In special cases, if the removal mechanism consists only of the uptake and accumulation, in contrast to the transformation of a contaminant, living organisms may be harvested and removed after remediation for disposal, or possible recovery of contaminants (Susarla et al. 2002, Sooksawat et al. 2013). The use of live cultures also reduces the number of additional unit processes, related to the preparation of biomass for this kind of applications, including harvesting, drying, processing and activation of the material (Aksu and Dönmez 2005).

Table 1.8: Comparison between biosorption and bioaccumulation processes (adapted from Chojnacka (2010))

Biosorption	Bioaccumulation
Metabolically passive process	Metabolically active process
Sorbent dead biomass	Sorbent living biomass
Metals are bound with cellular surface	Metals are bound with cellular surface and interior
Main process: Adsorption/ion exchange	Main process: Absorption
Reversible process	Partially reversible process
Nutrients are not required	Nutrients are required
Single-stage process	Double-stage process
Quicker than bioaccumulation	Slower than biosorption
Not controlled by metabolism	Controlled by metabolism
No danger of toxic effect to biomass	Danger of toxic effects caused by contaminants to biomass
No cellular growth	Cellular growth occurs
Intermediate equilibrium concentration of metal ions	Very low equilibrium concentration of metal ions

External factors governing bioaccumulation performance are basically the same which influence the cultivation of organisms: composition of growth medium, pH, temperature and the presence of other contaminants. These factors may inhibit the growth of cells and also bioaccumulation itself (Chojnacka 2010). In fact, a severe limitation of this process is the range of concentrations where it can be used. Theoretically it is impossible to treat solutions with high load of contaminants since it will lead to biomass decay and consequent death. However, if the method is intended to be used as the final polishing step (where most existing methods fail or become prohibitively expensive), the concentration of contaminants in solution would be of a magnitude of few mg L^{-1} (Chojnacka 2010).

Organisms likely to be used in bioremediation processes should have some resistance to considerable levels of contaminants, not having protection mechanisms against excessive accumulation within the cells (Deng and Wilson 2001, Kocberber and Donmez 2007). Some living organisms, whose capacity for bioaccumulation of metals has been studied, are yeasts, fungi, bacteria and microalgae (Kadukova and Vircikova 2005, Dwivedi et al. 2006, Chojnacka 2007, Farooq et al. 2010, Flouty and Estephane 2012).

Within bioremediation, phytoremediation is also an emerging "green" technology, where terrestrial or aquatic plants are used to remove, transform or store toxic chemicals which are found in soils, sediments, groundwater, surface water and even the air (Raskin et al. 1997, Susarla et al. 2002, Fereshteh et al. 2007, Gomes and Asaeda 2013, Krems et al. 2013, Liu et al. 2013, Tak et al. 2013).

For the reasons mentioned above, bioremoval and bioremediation, based on the processes of biosorption and bioaccumulation, have major advantages over traditional methods of treatment. Besides the low cost of operation, since biological wastes or living biomass, which are widely available are used, minimization of the volume and toxicity of produced sludge, as well as high efficiency in detoxification of complex and diluted effluents make this biotechnologies very promising.

1.5 Common aspects on the studies of metal removal

There are some common aspects to most of the studies reported in the literature concerning the removal of metals: researchers seek to understand the experimental factors which control and affect the removal, in order to optimize the process. Furthermore, they attempt to translate the results into mathematical functions that ensure rapid characterization of the kinetics and extent of the process. Basically, the ultimate goal is to be able to predict the course of the process at given conditions in order to design a complete technique and to make a practical use of it. Some factors affecting sorption processes such as sorbent nature, initial concentration of metal in solution, pH, presence of foreign ions, and temperature (Kadirvelu and Goel 2005), are now enumerated with findings of some available research studies.

1.5.1 Influence of experimental parameters

1.5.1.1 Effect of pH

Through the literature review it is possible to observe that in most cases metal recovery by different types of sorbents is strongly affected by pH. The extent of that influence is determined by the type of sorbent and metal.

In 2002, Manju and co-authors found that the percentage removal of metal ions by polyacrylamide-grafted iron(III) oxide (PGHyFeO–COOH) increased with the increase in pH up to a certain value and then decreased with further increase in pH. The authors reported that the maximum removal of Pb was observed at an optimum pH of 6.0 (95.7%), whereas, the maximum removal of Hg and Cd was observed at pH 5.0, 84.9% and 81.0%, respectively. Below and above this pH, a decreasing trend in removal was observed. The possible sites on

PGHyFeO–COOH for specific adsorption in acidic pH includes H^+ ions in –COOH functional groups (Manju et al. 2002). Hence, the effect of pH on metal ions can be explained as due to the exchange behaviour of H^+ ions from peripheral –COOH groups. At very low pH, the competition between M^{2+} (M = Pb, Hg, Cd) and the higher concentration of H^+ ions for sorption sites is in favour of H^+, and as a result, less removal efficiency was observed at low pH; with increase of the pH (up to optimum pH), the enhancement of sorption is apparently due to the hydrolysis of the exchanging cations, since the hydroxyl-metal complex, that is $M(OH)^+$ is sorbed in preference to the uncomplexed cations (Manju et al. 2002).

In 2010 Huang et al. found that biosorption of Cd from aqueous solution onto *Hydrilla verticillata*, a perennial submerged macrophyte, was highly dependent on hydrogen ion concentration of solution. Maximum sorption efficiency (96%) was obtained at pH 5–7, while at lower pHs, there was clear competition between Cd and protons for biomass sorption sites, decreasing sorption efficiency. At pH values higher than 7, cadmium hydroxide precipitation was observed (Huang et al. 2010). Tuzen and Sari (2009) reported that biosorption efficiency of Hg ions onto moss Drepanocladus revolvens increased from 70 to 91.5% as pH increased from 2 to 5. The maximum biosorption was found to be 96% at pH 5.5 (Tuzen and Sari 2009).

1.5.1.2 Effect of temperature

Depending on the structure and/or surface functional groups of a particular sorbent, temperature could have more or less impact on the sorption capacity, since temperature change alters the adsorption equilibrium in a specific way determined by the endothermic or exothermic nature of the process (Arief et al. 2008). Thermodynamic parameters like Gibbs free energy (ΔG^0), enthalpy (ΔH^0) and entropy (ΔS^0) provide valuable information about the sorption process and allow addressing the possibility and feasibility of a certain reaction.

In 2005, Tüzün et al. found that the biosorption capacity of the microalgae *Chlamydomonas reinhardtii* for Hg, Cd and Pb did not significantly change for different temperatures. These results indicate that the biosorption of Hg, Cd and Pb on the algal biomass appears to be temperature independent over the temperature range tested by the authors (5 to 35ºC) (Tuzun et al. 2005).

Later, Ghodbane and Hamdaoui (2008) observed that the biosorption of Hg onto eucalyptus bark increased with the increase in temperature, indicating the endothermic nature of the process. The enhancement in the sorption capacity was explained by the authors due to the interaction between sorbate ions and sorbent, creation of some new sorption sites or the increased rate of intraparticle diffusion of Hg ions into the pores of the sorbent at higher temperatures (Ghodbane and Hamdaoui 2008). By contrary, Huang et al. (2010) observed a slow decrease in the sorption of Cd with the rise in temperature, and suggested that was due to the increasing tendency to desorb from the interface to the solution. This result also indicated that the sorption process of Cd onto *Hydrilla verticillata* was exothermic.

1.5.1.3 Effect of initial metal concentration

The initial metal concentration in solution is another important parameter of removal studies, mainly due to factors such as availability of specific surface functional groups and the ability of surface groups to bind metals, especially at high concentrations (Arief et al. 2008).

The effect of initial concentration on uptake percentage of Cd by methly thiourea and phenyl thiourea supporting resins was studied by Sharaf et al. (2007). The uptake increases as the initial concentration increases until reach a plateau, after which, the concentration no longer affects the uptake capacity (Sharaf et al. 2007). The load capacity of the resins was 3.2 and 3.0 mmol g^{-1} for the methly thiourea resin and phenyl thiourea resin, respectively.

The biosorption capacity of the microalgae *Chlamydomonas reinhardtii* biomass also increased first with increasing initial metal concentration and reached a saturation value, which were 200 mg L^{-1} for Hg and Cd and 300 mg L^{-1} for Pb (Tuzun et al. 2005). In the biosorption of Hg onto eucalyptus bark, Ghodbane and Hamdaoui (2008) noticed that an increase in initial metal concentration leads to an increase in the sorption capacity of eucalyptus bark, consequence of the increase in the driving force.

1.5.1.4 Effect of coexisting-ions

Since the industrial effluents, far from being single-component, are complex solutions (containing several PTEs simultaneously) and the removal of a given metal may be influenced by the presence of other metals (Srivastava and Goyal 2010), several authors include this factor on their removal studies.

The competition among Pb, Hg and Cd for the sorption sites of PGHyFeO–COOH was evaluated by Manju et al. (2002). The values of sorption reported by the authors were: Pb=14. 6 mg g^{-1}, Hg=9.76 mg g^{-1} and Cd=8.31 mg g^{-1}, who concluded that for ions of the same valence, sorbent prefers the metal with higher atomic number (Manju et al. 2002). In 2005, Lv, Hor et al. studied the competitive sorption characteristics of binary and ternary mixtures of metal ions Pb, Cu and Cd on microporous titanosilicates ETS-10. The authors observed that ETS-10 displays a high selectivity towards one metal in a two-component or a three-component system with affinity order of Pb>Cd>Cu. The authors suggest that such behaviours are determined by the hydrated ionic radii and the hydration energy of the metals species (Lv et al. 2005). In the same year, Payne and Abdel-Fattah (Payne and Abdel-Fattah 2005) investigated the competition between Pb ions and cations such as potassium, and verified that ionic competition reduced Pb removal by natural zeolites chabazite and clinoptilolite. More recently, Zhang et al. (2010) studied the effects of Cl^{-}, SO_4^{2-}, and PO_4^{3-} on Hg sorption onto PANI/HA nanocomposite. The authors observed that neither the presence of SO_4^{2-} nor the PO_4^{3-} has any significant influence on the removal of Hg but the effect of Cl^{-} is notable (Figure 1.7). Possibly Cl^{-} competed favourably with OH^{-} for Hg to form Hg–Cl complexes and these complexes have less affinity to the PANI/HA, causing a substantially decreased on Hg removal (Zhang et al. 2010).

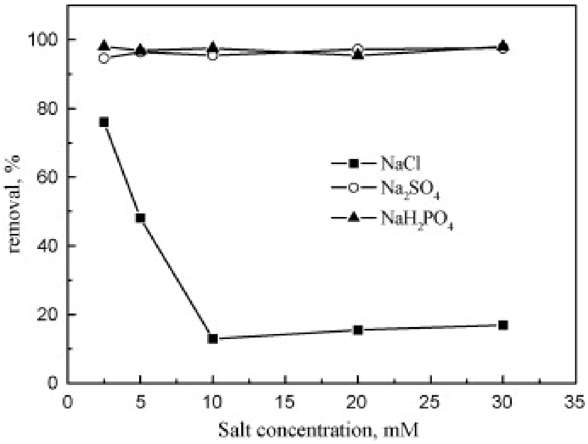

Figure 1.7: Effect of coexisted ions on mercury removal by PANI/HA. Hg initial concentration = 50 mg L^{-1}; ionic strength = 0.2 M $NaNO_3$; pH 5.0 buffered by 2 mM NaAc/HAc solution; PANI/HA = 0.5 g L^{-1} (data from (Zhang et al. 2010))

1.5.1.5 Regeneration of materials

The regeneration of the sorbent, although not always studied, is an important factor for keeping the process cost down, and to open the possibility of recovering valuable metals extracted from liquid phase.

In 2000, Denizli et al. reported that the regeneration of the dithiocarbamate-incorporated microspheres loaded with Hg ions is feasible by the use of strong acids. Desorption ratio was very high (up to 99%) when using a desorption medium containing 1M HNO_3. The authors reported that chelate forming interactions between incorporated dithiocarbamate groups and Hg ions are weaker with decreasing pH. The sorption capacity of the recycled dithiocarbamate-incorporated microspheres can still be maintained at 98% level at the fifth cycle (Denizli et al. 2000).

Using a solution of 0.2M NaCl, Manju et al. (2002) obtained good desorption efficiency of various metals from PGHyFeO–COOH: 98.3% Pb, 93.1% Hg and 90.1% Cd. Authors reported that Na^+ ions are easily displaced by metal ions bonded to sorbent during adsorption experiments indicating an ion-exchange process. The small fraction of sorbed metals not recoverable by regeneration presumably represents the metal which is bound through strong interaction and, as a result, the sorption capacity is reduced in subsequent cycles (Manju et al. 2002).

In 2006 Lodeiro et al. studied eleven types of eluting solutions at different concentrations to find the most appropriate eluting agent for Cd loaded biomass. The authors reported high efficiency of chloride and nitrate calcium salts as desorbing agents, for the release of the Cd previously bound to protonated *Sargassum muticum* alga, causing no structural damage in the biomass or even reinforcing it. HNO_3 and HCl were found to have similar desorption capacity, but were more aggressive for the stability of the algae; nevertheless, acid desorption contributed to sweep soluble biomass material, which could block a fixed-bed column employed in metal sorption–regeneration cycles (Lodeiro et al. 2006).

1.5.2 Mathematical modelling and interpretation of results

Kinetics and equilibrium are two important aspects to consider when it comes to evaluating a particular removal process. A diverse set of mathematical models is usually applied to the results by the researchers in order to describe the rate and extent of sorption processes. A brief presentation of the most common models found in the literature will be made bellow.

1.5.2.1 Kinetic modelling

Analysis of the uptake rate of a particular contaminant by a sorbent is a fundamental step for assessing the applicability of that removal process in contamination control. Furthermore, it is essential for the optimization of operating conditions in full-scale, since sorption kinetics determines the residence time required for completion of the removal process (Ho et al. 2000). The kinetic models can be categorized as reaction models, which assume the sorption process as a whole, or diffusion models that presuppose different stages in the process where mass transfer phenomena occur (Ho et al. 2000, Qiu et al. 2009, Malash and El-Khaiary 2010) (Table 1.9).

Lagergren's first-order equation and Ho's second-order expression (Wang and Chen 2009) are the most widely used among kinetic reaction models, which are based on the capacity of the sorbent. In addition to these two, the Elovich model is also very popular in describing chemisorption kinetics (Ho and McKay 2004).

Table 1.9: Sorption kinetic models.

Kinetic model	Equation	Notes
Pseudo-first-order (Lagergren)	$q_t = q_e(1-e^{-k_1 t})$	Adsorption capacity
Pseudo-second-order (Ho)	$q_t = \frac{q_e^{2} k_2 t}{1+q_e k_2 t}$	Adsorption capacity
Elovich	$q_t = \frac{1}{\beta}\ln\ (1+\alpha\beta t)$	Chemisorption
Weber-Morris	$q_t = k_{id} t^{1/2}$	Intraparticle diffusion
Boyd	$F = 1-\frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp(-n^2 Bt); F = \frac{q_t}{q_e}$	Film diffusion

Note: q_t and q_e are the amount of solute sorbed per gram of sorbent at time t and at equilibrium, respectively; k_1 and k_2 are rate constants; α is the initial sorption rate and β the Elovich desorption constant; k_i is the intraparticle-diffusion parameter; F is the fractional attainment of equilibrium, at different times, t, and Bt is a function of F.

Boyd’s film-diffusion (Boyd et al. 1947) and Webber’s pore-diffusion (Weber and Morris 1963), two widely known diffusion-based models, are often used in order to analyse the sorption mechanism and the rate-controlling step involved in the sorption process (film or external diffusion, pore diffusion, surface diffusion, sorption on the pore surface, or by combination of more than one step (Rocha et al. 2013)).

1.5.2.2 Equilibrium models

The sorption isotherm gives information about the sorbate´s distribution between the liquid phase (solution) and the solid phase (sorbent) when the sorption process reaches an equilibrium state, for a fixed temperature and pH. Its accurate mathematical description could provide some insight into the sorption mechanism and the surface properties (Ho et al. 2002). However, the determination of the isotherms is not quite feasible when using living organisms, since the equilibrium is not truly achieved, given the continued growth of the organism.

As for the kinetics, there are several mathematical models which can be used to describe the equilibrium sorption, usually classified into empirical equations or into mechanistic models (Wang and Chen 2009).The mechanistic models are based on the mechanism of metal sorption, which are able not only to represent but also to explain and predict the experimental behaviour. However, empirical models such as the two-parameters Freundlich (Freundlich 1906) and Langmuir (Langmuir 1918) sorption models, and the three-parameters Sips or Langmuir-Freundlich isotherm model (Sips 1948), among others (Dubinin–Radushkevich, Redlich-Petersen), are those that recur over the available literature (Table 1.10).

Table 1.10: Isotherm models.

Isotherm	**Equation***	**Notes**
Langmuir	$q_e = \frac{q_m b_L C_e}{1 + b_L C_e}$	Assumes a monolayer sorption, i.e. a fixed number of energetically equivalent sites are available on the sorbent surface and once a sorbate molecule occupies a site no further adsorption takes place; Forces of interaction between adsorbed molecules are negligible (Cho et al. 2010)
Freundlich	$q_e = K_F C_e^{1/n}$	Comprises heterogeneous adsorptive energies on the adsorbent surface and therefore may be applied to describe equilibrium of nonideal sorption on heterogeneous surfaces as well as multilayer sorption (Ho et al. 2002)
Sips	$q_e = \frac{q_m (b_S C_e)^{1/n}}{1 + (b_S C_e)^{1/n}}$	Results from the combination of the Langmuir and the Freundlich isotherms; is capable of modelling both homogeneous and heterogeneous binding surfaces; At low sorbate concentrations Sips equation reduces to a Freundlich isotherm, while at high sorbate concentrations it predicts a monolayer adsorption capacity characteristic of the Langmuir isotherm (Ho et al. 2002)

*q_m is the maximum sorption capacity and b_L is the Langmuir constant related to the free energy of adsorption; K_F is a constant related to the adsorption capacity of the sorbent and n is the adsorption intensity or the heterogeneity of the sorbent.; b_S is the Sips constant related to the energy of adsorption

1.5.2.3 Data analysis in sorption studies: Common errors

Although nowadays an appreciable amount of mathematical models be available to researchers describe their experimental results, and thus study the kinetics, equilibrium and sorption mechanism, not always its application is well performed.

Recently, El-Khaiary and Malash (2011) highlighted some common errors in sorption data analysis, which are often found in the literature, e.g., misuse of linearization, misapplication of models for a given situation, or abuse of R^2 for model comparison. Authors point out that these errors render the results "not reliable", and argue that their abundance and proliferation in literature has granted them some credibility erroneously, leading researchers to accept them, inadvertently, as scientifically correct practices.

Misuse of linearization

Linearization is a procedure that aims to simplify the resolution of the mathematics, having emerging in an era where computers were not available. However, this procedure is still very popular today, being possible to find countless articles in the literature where the linearization of sorption models (kinetic and equilibrium) is performed, or even revision papers, where the linearization of several kinetic models is presented and explained (Qiu et al. 2009). Nevertheless, the linearization of an inherently nonlinear equation by use of various transformations must be careful, since it may bring problems, such as the destruction of assumed distributional properties, and the introduction of bias which often are not detected by the subsequent statistical analysis of results (Crini et al. 2008, El-Khaiary and Malash 2011). As an example, Table 1.11 shows four linear forms of the original equation of Ho's pseudo-second-order kinetic model (Eq. (4)), as well as the effects of such transformations, according to El-Khaiary and Malash (2011). Moreover, to demonstrate the effect of linearizing Ho's equation on the accuracy of parameter estimates, the authors made use of published experimental data of Kononova et al. (2007), and estimated kinetic parameters q_e and k_2 by means of linear and nonlinear least squares regressions. Then the obtained parameters were used to plot Eq. (4) - Figure 1.8.

Table 1.11: Linearized forms of the pseudo-second-order kinetic model (El-Khaiary and Malash 2011).

Type	Linearized form	Plot	Effects of linearization
Lin 1	$\frac{t}{q}=\frac{1}{k_2q_e^2}+\frac{1}{q_e}t$	$\frac{t}{q}\ vs.\ t$	Reversal of relative weights of data points because *1/q* in the dependent variable *t* in both dependent and independent variables, leading to spurious correlation
Lin 2	$\frac{t}{q}=\frac{1}{q_e}+\left(\frac{1}{k_2q_e^2}\right)\frac{1}{t}$	$\frac{1}{q}\ vs.\ \frac{1}{t}$	Reversal of relative weights of data points because of *1/q* in dependent variable. Independent variable is *1/t*, leading to distortion of error distribution.
Lin 3	$q=q_e-\left(\frac{1}{k_2q_e}\right)\frac{q}{t}$	$q\ vs.\ \frac{q}{t}$	*q* in both dependent and independent variables, leading to spurious correlation. The presence of *q* in the independent variable (*q/t*) introduces experimental error, violating a basic assumption in the method of least squares. *1/t* in independent variable, leading to distortion of error distribution
Lin 4	$\frac{q}{t}=k_2q_e^2-k_2q_eq$	$\frac{q}{t}\ vs.\ q$	*q* in both dependent and independent variables, leading to spurious correlation. The presence of *q* in the independent variable introduces experimental error, violating a basic assumption in the method of least squares

It is clear that, despite the common origin, linearized forms of Ho's equation led to very different estimates of the parameters (and consequently, to different curve fittings). Furthermore, it is obvious that nonlinear regression produced the best fit. Thus, linearization of data should be avoided in sorption studies, especially given the diversity of software that is available these days, which can be very useful in modelling the results.

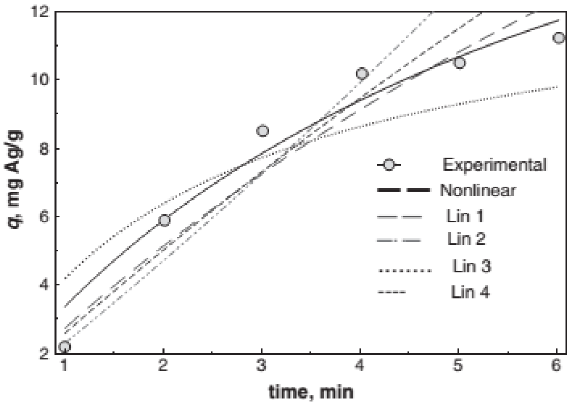

Figure 1.8: Comparison of the pseudo-second-order parameters estimated by linear and nonlinear regressions for the adsorption of silver thiocyanate complexes on anion exchanger AV-17-10P (El-Khaiary and Malash 2011).

Abuse of R^2 for model comparison

Another common practice in research is the use of coefficient of determination, R^2, to assess and compare the goodness of fit of several models to experimental data. R^2 is generated in the regression output of virtually all statistical software

Nevertheless, there are some problems associated with the use of this parameter, as the possibility of its value be artificially high when a model has a small degree of freedom for error (El-Khaiary and Malash 2011). Thus, evaluation of the performance of a particular model should not rely solely on this parameter. Conventional statistical tests should also be used, in order to test the significance of estimated regression parameters. The addition of the standard deviation of residuals ($S_{y.x}$) and/or the relative error (Er) between experimental and predicted values, leads to improvements in assessing the goodness of fit. The mathematical equations that translate R^2, $S_{y.x}$ and Er are displayed ahead, in the chapters 3, 4 and 5.

Another important issue is its frequent use, erroneously, to compare models that have different degrees of freedom. For comparing models with different degrees of freedom, El-Khaiary and Malash (2011) suggest the use of Akaike's Information Criterion (AIC), which is based on information theory and maximum likelihood theory, and as such, it determines which model is more likely to be correct and quantifies how much more likely

1.6 Algae: potential application in bioremoval and bioremediation

Algae are cosmopolitan organisms, marking their presence in every medium that provides them enough light and humidity, temporarily or permanently. Thus, it is possible to find algae in fresh or salt water, in damp soil or even on snow (Vidotti 2004).

The classification of algae takes into account several characteristics, including nature of the chlorophyll(s), cell wall chemistry and flagellation. Table 1.12 summarizes algal divisions belonging to Plantae kingdom as well as their most important characteristics (Davis et al. 2003).

Table 1.12: Algal divisions and significant characteristics (Davis et al. 2003)

Division	Common name	Pigments	Storage product	Cell wall	Flagella
Chlorophyta	Green algae	Chlorophyll *a*, *b*; α-, β- and γ-carotenes and several xanthophylls	Starch (amylose and amylopectin) (oil in some)	Cellulose in many (β-1,4-glucopyroside), hydroxyproline glucosides; xylans and mannans; or wall absent; calcified in some	Present
Rhodophyta	Red algae	Chlorophyll *a* (*d* in some Florideophyceae); R- and C-phycocyanin, allophycocyanin; R- and B-phycoerythrin. *α*- and *β* carotene and several xanthophylls	Floridean starch (amylopectin-like)	Cellulose, xylans, several sulfated polysaccharides (galactans) calcification in some; alginate in corallinaceae	Absent
Phaeophyta	Brown algae	Chlorophyll *a*, *c*; β-carotene and fucoxanthin and several other xanthophylls	Laminaran (β-1,3-glucopyranoside, predominantly); mannitol	Cellulose, alginic acid, and sulphated mucopolysaccharides (fucoidan)	Present

The presence of chlorophyll a is a common characteristic to the three types of algae. Additionally, in all cases, cellulose is present as the most common fibrillar material in the skeleton of cell walls. However other components are part of the fibrillar skeleton and of the amorphous embedding matrix, which together constitute the typical cell wall of an algae. Components, such as xylan in the red algae, xylans and mannans in the green algae, and alginic acid or alginate, with a smaller amount of sulfate polysaccharide (fucoidan) in the brown algae are of major importance for the performance and mechanism of the biosorption process (Davis et al. 2003, Romera et al. 2007). Besides cell wall chemistry, also its presence or absence greatly influences the sorption process. For example, Cryptophyta, which does not have a cell wall, does not perform very well as metal sorbents (Davis et al. 2003).

Due to the complexity of marine environmental conditions, algae evolved becoming an abundant and rich source of biologically active substances (Kumar et al. 2013). Perhaps for this reason, historically they always had some economic importance, being long used for different purposes. For example, carrageenan, a hydrocolloid extracted from red seaweeds, is used for centuries as a thickener and stabilizer in various food products (Van de Velde 2002). The use of seaweed as fertilizer, especially in agricultural fields near the sea (which is the case of Aveiro with its "moliço" - seagrass, and "sargaço" - algae of the genus *Sargassum*) comes from the fourteenth century, and its commercial harvest was firstly regulated in 1309 by the Portuguese king D. Dinis (Pereira 2007). Agar, another hydrocolloid extracted from seaweed, has wide application in studies of microbiology and in the separation of macromolecules by electrophoresis, chromatography, and DNA sequencing (Pereira 2007).

In the last years, new applications in several areas have been studied tirelessly for this natural resource, which is available in virtually unlimited quantities in the seas and oceans. In the energy sector, algae seem to create a strong enthusiasm in the pursuit of "greener" energy sources (Kovacevic and Wesseler 2010). Since they are able to replicate their biomass several times daily, and to produce at least 15 times more oil per hectare compared to competing food crops (Antunes and Silva 2010), microalgae have a considerable potential for the production of biofuels, in particular biodiesel. More recently the use of macroalgae have earned its space in this field (Allen et al. 2014).

An increasing number of scientific studies conducted in recent years has shown that algae have high ability to bind metals and other trace elements (Romera et al. 2006), due to presence of polysaccharides, proteins and lipids on the cell wall surface, which is rich in functional groups (Romera et al. 2007). This ability to bind microelements, and accumulate them internally may be useful for the production of new food products - "biofortified food" (Chojnacka 2010). In fact, through processes of biosorption and/or bioaccumulation, biomass of algae can be enriched with essential microelements (Chojnacka 2009b), such as iron, iodine and zinc, whose deficiency in human and animal diet is of particular concern nowadays (so-called hidden hunger) (Kennedy et al. 2003). This "biofortified" biomass can be consumed directly or be used as feed additive in livestock nutrition, avoiding the use of traditional mineral diet supplements (for animals and human) and mineral fertilizers (for plants) (Michalak et al. 2011).

Another potential application of algae, which has recently been evaluated is its use as "chemical reagents", in processes of pre-concentration on analytical chemistry (Romero-Gonzalez et al. 2000, Vidotti 2004). As example, algae *Spirulina major* was successfully applied to the separation and pre-concentration of Se(IV) and Se(VI) from water and river sediment by Shunxin et al. (1999). After pre-concentration, determination was performed on alga biomass by graphite furnace atomic absorption spectrometry.

The ability of the algae to remove elements from diluted solutions and to concentrate them in their tissues has also been tested for the recovery of precious metals like gold, silver, platinum and palladium (Das 2010).

Despite the potential applications presented previously, is in the environmental domain that the study of metal ion-alga interactions has gained more interest recently. Biosorption and bioaccumulation of metals by algae may be efficiently used in the assessment of environmental pollution (as biomonitors) (Das 2010) as well as in pollution prevention (wastewater treatment) (Gavrilescu 2004) and remediation of contaminated environments (phytoremediation) (Ali et al. 2013).

Several studies conducted in recent years have shown that algae possess the ability to attract and bind metals and dyes from aqueous solutions (Romera et al. 2007, Wang and Chen 2009, Jayakumar et al. 2014). However, scientific articles involving macroalgae are in less number when compared with other types of biomass (mainly bacteria and fungi) (Romera et al. 2006) or even with publications concerning microalgae. Studies carried out in multi-metal systems, which more closely resemble the real aquatic systems are even rarer. Virtually non-existent are studies about the potential use of living macroalgae on bioremediation of aquatic ecosystems and effluents rich in metals (Costa et al. 2011). In fact, despite phytoremediation has recently become an object of intense public debate and scientific interest, and also an issue for some review articles (Lasat 2002, Gerhardt et al. 2009), little information can be found in the literature about the potential use of algae on phytoremediation. This is somewhat surprising given that several field studies reported high levels of contaminants in the algal biomass obtained from contaminated sites (Giusti 2001, Coelho et al. 2005, Sekabira et al. 2011). A small exception have been the studies that put under test their bioaccumulation capabilities on bioremediation of wastewaters from aquaculture, by removing nutrients (nitrates and phosphates) (da Costa 2006, Nielsen et al. 2012).

The use of living estuarine and marine macroalgae for the remediation of saline systems contaminated with metals, can bring a particular advantage over conventional methods of ion exchange and even biosorption. It has already been reported loss of performance in metal removal when a method is applied to a salty water (due to the effect of the presence of other ions, such as Na^+ or Cl^-) (Payne and Abdel-Fattah 2005). Estuarine macroalgae, being perfectly adapted to saline environment and to wide variations in salinity possess osmoregulation mechanisms, and specific mechanisms that allow them to capture and concentrate essential and non-essential metals under such conditions (Turner et al. 2008).

The accumulation of metals by living algae depends on the type of algae and metabolic activity, of the speciation and concentration of the metals, on the exposure time, and on other environmental factors, such as salinity, pH and temperature (Kamala-Kannan et al. 2008). Understanding these parameters is essential for the optimization of biosorption and bioaccumulation processes, and consequently, to develop a new biotechnology remediation, more advantageous than existing methods.

1.7 General aims and thesis structure

During the last century, environmental protection policies were ignored in favour of industrialization and economic interests, resulting in severe contamination of ecosystems.

In order to protect ecosystems and human well-being against the hazardous effects of metal contaminants, it is imperative to identify and assess the risks associated with contaminated sites, as well as to develop strategies for their mitigation. Moreover, unsustainable anthropogenic actions should cease. The use of those potentially toxic elements should be avoided, and/or their concentrations in industrial discharges should be reduced to levels as close as possible to zero, by using more efficient and cost-effective treatment technologies. In this context, information about the potential transfer and consequent accumulation of metals by primary producers (such as terrestrial plants and algae) in contaminated environments, is extremely valuable. This knowledge can be applied not only for the development of better tools for the assessment of risks to trophic chains, but also for the development of remediation biotechnologies, more green and effective than conventional ones.

Thus, the work developed under the scope of this PhD project aimed the study and description of the accumulation of metals, namely Cd, Pb and Hg, by primary producers with special focus to marine macroalgae. More attention was also given to Hg, since this metal is not as documented as the other two, probably due to the difficulties inherent to the study of this metal. Moreover, the major concern associated with Hg, is related to its methylated forms (organo-metallic Hg), which are more toxic and less studied in terrestrial systems.

The results obtained may be of outmost importance for environmental contamination biomonitoring, and particularly for bioremediation of contaminated saline waters.

This thesis was structured by chapters, corresponding to manuscripts published or in process of submission to international scientific journals. At the end, brief final considerations will be made. Below are presented the abstracts of each chapter, including objectives and findings.

Chapter 2:

Although the transfer of organo-metallic mercury (OrgHg) in aquatic food webs has long been studied, it has only been recently recognized that there is also accumulation in terrestrial systems. There is still however little information about the exposure of grazing animals to OrgHg from soils and feed as well as on risks of exposure to animal and humans. In this study we collected 78 soil samples and 40 plant samples (*Lolium perenne* and *Brassica Juncea*) from agricultural fields near a contaminated industrial area and evaluated the soil-to-plant transfer of Hg as well as subsequent trophic transfer. Inorganic Hg (IHg) concentrations ranged from 0.080 to 210 mg kg^{-1} d.w. in soils, from 0.010 to 84 mg kg^{-1} d.w. in roots and from 0.020 to 6.9 mg kg^{-1} d.w. in shoots. OrgHg concentrations in soils varied between 0.20 and 130 µg kg^{-1} d.w. representing on average 0.13% of the total Hg (THg). In root and shoot samples OrgHg comprised on average 0.58% (roots) and 0.66 % (shoots) of THg. Average bioaccumulation factors (BAFs) for OrgHg in relation to soil concentrations were 3.3 (for roots) and 1.5 (for shoots).

The daily intake (DI) of THg in 33 sampling sites exceeded the acceptable daily intake (ADI) of THg of both cows (ADI= 1.4 mg d-1) and sheep (ADI= 0.28 mg d-1), in view of food safety associated with THg in animal kidneys. Estimated DI of OrgHg for grazing animals were up to 220 µg d-1 (for cows) and up to 33 µg d-1 (for sheep).

This study suggested that solely monitoring the levels of THg in soils and feed may not allow to adequately taking into account accumulation of OrgHg in feed crops and properly address risks associated with OrgHg exposure for animals and humans. Hence, the inclusion of limits for OrgHg in feed quality and food safety legislation is advised.

Chapter 3:

In this chapter, the bioaccumulation capabilities of three different macroalgae species (green, red and brown), very common on temperate coasts and estuaries, for the removal of Hg from salt water, using environmental realistic concentrations was assessed and explored. Levels of Hg accumulated by all seaweeds varied between 20.8-22.7, 93.5-103.2 and 202.3-208.5 µg g^{-1}, for initial concentrations of Hg in solution of 10, 50 and 100 µg L^{-1}, respectively, representing bioconcentration factors of c.a. 2000. A comparative evaluation of bioaccumulation (live biomass) and biosorption (dead biomass) processes was conducted for *U. lactuca*, which had displayed the best performance in accumulating Hg. By using only c.a. 500 mg L^{-1} of dead macroalga (d.w) it was possible to obtain removal efficiencies at equilibrium (achieved after 24 to 72h of contact time) between 73 and 91%, for all concentrations studied. The removal of Hg by living seaweed, although slower, was more promising, since for an equal dose ($m_{macroalgae}/V_{solution}$) all Hg levels were reduced by about 99%. Thus, through bioaccumulation, European legal criteria for drinking water quality (1 µg Hg L-1) was fulfilled. Pseudo-second-order and Elovich models described quite well experimental data, assuming a biosorption essentially of chemical nature instead of physical, assumption which was corroborated by FTIR spectra analysis. Determination of total mercury content in algal biomass over time, allowed us to confirm and to follow the incorporation of this metal by the organism. Organo-metallic Hg concentrations in *U. lactuca* at the end of bioaccumulation trials were very low, corresponding to very small conversion rates of inorganic Hg (0.02-0.05%) which suggests that methylation is a slow process.

Overall, results intended to be a contribution for the development of an efficient and cost-effective remediation biotechnology, based on the use of widely available live macroalgae, for saline waters contaminated with Hg.

Chapter 4:

A worldwide available, green marine macroalga, with fast growth was applied for the removal of Cd, Pb and Hg from contaminated saline waters. Metal bioaccumulation by living *Ulva lactuca* was studied during 6 days, under different contamination scenarios, mimicking those found in real world. In single-contaminant systems, with concentrations ranging from 10-100 µg L^{-1} for Hg, 10-200 µg L^{-1} for Cd, and 50-1000 µg L^{-1} for Pb, biomasses of macroalgae around 500 mg L^{-1} (d.w.) were able to remove, in most cases 93 to 99% of metal, allowing to achieve water quality criteria regarding both surface and drinking waters. Under multi-contaminant systems, comprising simultaneously the three metals, macroalgae still performed well, with Hg removal (c.a. 99%) not being significantly affected by the presence of Cd and Pb, even when those metals were in higher concentrations. At the same time, removal efficiencies for Cd and Pb varied between 57-96%, and 34-97%, respectively, revealing a gradient of affinity of *U. lactuca* toward metals: Hg>Cd>Pb. Chemical quantification of metal content in macroalgae, after bioaccumulation assays demonstrated that all Cd and Hg removed from solution was really bound in macroalgae tissues, while only half of Pb showed to be sorbed on the biomass. Overall, *U. lactuca* accumulated up to 209 µg g^{-1} of Hg, up to 347 µg g^{-1} of Cd and up to 1641 µg g^{-1} of Pb, which correspond to bioconcentration factors ranging from 500 to 2200, in a dose-dependent accumulation. Pseudo-first order, pseudo-second order and Elovich models showed a good performance in describing the kinetics of bioaccumulation, in the whole period of time. No mortality was observed during the bioaccumulation assays. In the range of experimental conditions used, *U. lactuca* relative growth rate was not significantly affected by the presence of metals, which is in accordance to its known tolerance to contaminants.

Results may be an important contribution for developing a macroalgae-based biotechnology, applied for contaminated saline water remediation, more “green” and cost-effective than conventional treatment methods.

Chapter 5:

This work aimed the assessment of the bioaccumulation of metal contaminants in salt water and its effect on the growth rate by *Fucus vesiculosus,* species very common on temperate coasts and estuaries. Results obtained in a 7-day study of exposure to metals of, using environmental realistic conditions, evidenced its high potential for the removal of Pb, Hg and Cd from contaminated saline waters. For monometallic solutions, by using only ca 450 mg/L^{-1} (dry weight) of the living organism, it was possible to reduce the initial concentrations of Pb in 65%, 95% of the Hg levels, and between 25 and 76% the concentrations of Cd. The bioremoval carried out by the macroalgae was quicker for Hg, and led to residual concentrations of this metal in solution below or very close to the maximum permissible value (VMA) for water of human consumption. Overall, bioconcentration factors ranged from 600 to 2300. The removal efficiency was proven in multi-contaminant systems, and the performance of the process was not affected by the presence of the three metals simultaneously. Elovich kinetic model described very well the bioaccumulation of Pb and Cd over time, while pseudo-second-order model adjusted better to experimental data regarding Hg. *F. vesiculosus* showed different affinity toward studied metals, following the sequence order: Hg>Pb>Cd. Analysis of metal content after bioaccumulation, proved that al metal removed from solution was bound to the biomass, and depuration experiments, conducted immediately after bioaccumulation assays reveled no significant loss of metal back to solution along time. Exposure to metal contaminants only adversely affected the organism’s growth for the highest concentrations of Cd and Pb.

Overall, results intended to be a contribution for the development of a remediation biotechnology for confined saline waters contaminated with metals, more efficient and with lower costs than the traditional treatment methods.

Chapter 6:

The biosorption capability of two different marine macroalgae (green *Ulva lactuca* and brown *Fucus vesiculosus*) was evaluated in the removal of toxic metals (Hg, Cd and Pb) from saline waters, under different contamination scenarios.

Results showed that, independently of the contamination level, both green and brown macroalgae have a remarkable capacity to biosorb Hg and Pb.

In single-contaminant systems, by using c.a. 500 mg of non-pre-treated algae biomass (size <200 µm) per litter, it was possible to achieve removal efficiencies between 96 and 99% for Hg, and up to 86% for Pb. Despite the higher removal of Hg, equilibrium was always reached more quickly for Pb (after 24-72h of contact time for Hg and after 8h of contact time for Pb). Under multi-contaminant systems, comprising simultaneously the three metals, both macroalgae still performed well, although Pb removal by *U. lactuca* was more inhibited than that achieved by *F. vesiculosus*.

Under the experimental conditions used, neither the green nor the brown macroalgae were effective to remove Cd from saltwater (maximum removal of 20%). Moreover, both algae showed similar equilibrium selectivity toward the target metals: Hg>Pb>>Cd. Pseudo-first order, pseudo-second order and Elovich models have been used successfully to describe the kinetics of Hg and Pb biosorption, in the whole period of time, and for the full range of concentrations tested.

Overall, results demonstrated that *F. vesiculosus* and *U. lactuca* may be part of an efficient and cost-effective technology for removing metals from contaminated salt waters. However, the brown algae has greater potential, since always presented higher initial sorption rates, higher removal percentages and higher amounts of metals sorbed than the green one.

This thesis led to the following scientific publications:

- **Risks associated with the transfer of toxic organo-metallic mercury from soils into the terrestrial feed chain**.
  Henriques B., Rodrigues S.M., Coelho C., Cruz N., Duarte A.C., Römkens P.F.A.M., Pereira E. Environment International. (2013) 59, 0, 408-417.
  DOI: 10.1016/j.envint.2013.07.006

- **Bioaccumulation and biosorption of Hg by living marine macroalgae: prospecting for a new remediation biotechnology applied to saline waters**.
  Bruno Henriques, Luciana S. Rocha, Cláudia B. Lopes, Paula Figueira, A. C. Duarte, M.A. Pardal, E. Pereira.
  Submitted to Bioresource Technology

- **Developing a macroalgae-based biotechnology for water remediation: simultaneous removal of Cd, Pb and Hg by living *Ulva lactuca***
  Bruno Henriques, Luciana S. Rocha, Cláudia B. Lopes, Paula Figueira, A. C. Duarte, M.A. Pardal, E. Pereira
  To be Submitted to Water Research

- **Bioaccumulation of Hg, Cd and Pb by *Fucus vesiculosus* in seawater: study on single and multi-metal systems**
  Bruno Henriques, Cláudia B. Lopes, Paula Figueira, A. C. Duarte, M.A. Pardal, E. Pereira
  To be Submitted to Marine Pollution Bulletin.

- **Comparative study on metal biosorption by two macroalgae in saline waters: mono and multi-metal systems**
  Bruno Henriques, Cláudia B. Lopes, Paula Figueira, A. C. Duarte, M.A. Pardal, E. Pereira
  Submitted to Journal of Hazardous Materials

1.8 References

Abreu, J. P. D. M. E., I. Flores, F. M. G. Abreu and M. V. Madeira (1993). "Nitrogen uptake in relation to water availability in wheat." Plant and Soil **154**(1): 89-96.

Afkhami, A., M. Saber-Tehrani and H. Bagheri (2010). "Simultaneous removal of heavy-metal ions in wastewater samples using nano-alumina modified with 2,4-dinitrophenylhydrazine." Journal of Hazardous Materials **181**(1-3): 836-844.

Ahluwalia, S. S. and D. Goyal (2007). "Microbial and plant derived biomass for removal of heavy metals from wastewater." Bioresource Technology **98**(12): 2243-2257.

Akar, T., I. Tosun, Z. Kaynak, E. Ozkara, O. Yeni, E. N. Sahin and S. T. Akar (2009). "An attractive agro-industrial by-product in environmental cleanup: dye biosorption potential of untreated olive pomace." J Hazard Mater **166**(2-3): 1217-1225.

Aksu, Z. and G. Dönmez (2000). "The use of molasses in copper(II) containing wastewaters: effects on growth and copper(II) bioaccumulation properties of Kluyveromyces marxianus." Process Biochemistry **36**(5): 451-458.

Aksu, Z. and G. Dönmez (2005). "Combined effects of molasses sucrose and reactive dye on the growth and dye bioaccumulation properties of Candida tropicalis." Process Biochemistry **40**(7): 2443-2454.

Al-Masri, M. S., B. Al-Akel, A. Nashawani, Y. Amin, K. H. Khalifa and F. Al-Ain (2008). "Transfer of 40K, 238U, 210Pb, and 210Po from soil to plant in various locations in south of Syria." Journal of Environmental Radioactivity **99**(2): 322-331.

Ali, H., E. Khan and M. A. Sajad (2013). "Phytoremediation of heavy metals-Concepts and applications." Chemosphere **91**(7): 869-881.

Allen, E., D. M. Wall, C. Herrmann and J. D. Murphy (2014). "Investigation of the optimal percentage of green seaweed that may be co-digested with dairy slurry to produce gaseous biofuel." Bioresource Technology **170**(0): 436-444.

Alloway, B. J., I. Thornton, G. A. Smart, J. C. Sherlock and M. J. Quinn (1988). "The Shipham report. An investigation into cadmium contamination and its implications for human health. Metal availability." Sci Total Environ **75**(1): 41-69.

Alvarenga, P. M., M. F. Araújo and J. A. L. Silva (2004). "Elemental Uptake and Root-Leaves Transfer in Cistus Ladanifer L. Growing in a Contaminated Pyrite Mining Area (Aljustrel-Portugal)." Water, Air, and Soil Pollution **152**(1-4): 81-96.

Álvarez-Ayuso, E., A. García-Sánchez, X. Querol and A. Moyano (2008). "Trace element mobility in soils seven years after the Aznalcóllar mine spill." Chemosphere **73**(8): 1240-1246.

Antunes, R. and I. C. Silva (2010). Utilização de algas para a produção de biocombustíveis C. d. C. E. Renováveis, Instituto Nacional da Propriedada Industrial.

APA. (2012). "PORTUGUESE INFORMATIVE INVENTORY REPORT 1990 – 2010." Retrieved 09/07/12, from http://www.apambiente.pt/_zdata/DPAAC/INERPA/IIR_20120515.pdf.

Apiratikul, R. and P. Pavasant (2008). "Batch and column studies of biosorption of heavy metals by Caulerpa lentillifera." Bioresource Technology **99**(8): 2766-2777.

Arief, V. O., K. Trilestari, J. Sunarso and S. Ismadjl (2008). "Recent progress on biosorption of heavy metals from liquids using low cost biosorbents: characterization, biosorption parameters and mechanism studies." Clean **36**(12): 937-962.

Ash, C. and R. Stone (2003). "A Question of Dose." Science **300**(5621): 925.

ATSDR. (1999). "ToxFAQsTM for Mercury." Retrieved Junho de 2014, from http://www.atsdr.cdc.gov/tfacts46.pdf.

ATSDR. (2007). "Public Health Statement, Lead." Retrieved Junho de 2014, from http://www.atsdr.cdc.gov/ToxProfiles/tp13-c1-b.pdf

ATSDR. (2012). "Public Health Statement, Cadmium." Retrieved Junho 2014, from http://www.atsdr.cdc.gov/ToxProfiles/tp5-c1-b.pdf.

Babatunde, A. O., Y. Q. Zhao, M. O'Neill and B. O'Sullivan (2008). "Constructed wetlands for environmental pollution control: A review of developments, research and practice in Ireland." Environment International **34**(1): 116-126.

Babel, S. and T. A. Kurniawan (2003). "Low-cost adsorbents for heavy metals uptake from contaminated water: a review." Journal of Hazardous Materials **97**(1-3): 219-243.

Bao, J., Y. Fu and Z. Bao (2013). "Thiol-functionalized magnetite/graphene oxide hybrid as a reusable adsorbent for Hg2+ removal." Nanoscale Research Letters **8**(1): 486.

Beolchini, F., F. Pagnanelli, L. Toro and F. Veglio (2006). "Ionic strength effect on copper biosorption by Sphaerotilus natans: equilibrium study and dynamic modelling in membrane reactor." Water Res **40**(1): 144-152.

Boyd, G. E., J. Schubert and A. W. Adamson (1947). "The Exchange Adsorption of Ions from Aqueous Solutions by Organic Zeolites .1. Ion-Exchange Equilibria." Journal of the American Chemical Society **69**(11): 2818-2829.

Brinza, L., C. A. Nygård, M. J. Dring, M. Gavrilescu and L. G. Benning (2009). "Cadmium tolerance and adsorption by the marine brown alga Fucus vesiculosus from the Irish Sea and the Bothnian Sea." Bioresource Technology **100**(5): 1727-1733.

Bulgariu, L. and D. Bulgariu (2014). "Enhancing Biosorption Characteristics of Marine Green Algae (Ulva lactuca) for Heavy Metals Removal by Alkaline Treatment." Bioprocessing & Biotechniques **4**(1).

Cardoso, P. G., A. I. Lillebo, C. B. Lopes, E. Pereira, A. C. Duarte and M. A. Pardal (2008). "Influence of bioturbation by Hediste diversicolor on mercury fluxes from estuarine sediments: A mesocosms laboratory experiment." Marine Pollution Bulletin **56**(2): 325-334.

Cardoso, P. G., S. C. Marques, M. D'Ambrosio, E. Pereira, A. C. Duarte, U. M. Azeiteiro and M. Â. Pardal (2013). "Changes in zooplankton communities along a mercury contamination gradient in a coastal lagoon (Ria de Aveiro, Portugal)." Marine Pollution Bulletin **76**(1–2): 170-177.

Challa, Y. R., L. R. d. Astudillo, A. Ramirez, A. Escalona and G. Martínez (2008). "Distribution of Total and Organic Mercury in Superficial Soils in the Upper Manzanares River Watershed, Sucre State, Venezuela." Air, Soil and Water Research **1**(ASWR-1-de-Astudillo-et-al).

Chaudri, A., S. McGrath, P. Gibbs, B. Chambers, C. Carlton-Smith, A. Godley, J. Bacon, C. Campbell and M. Aitken (2007). "Cadmium availability to wheat grain in soils treated with sewage sludge or metal salts." Chemosphere **66**(8): 1415-1423.

Chen, G. (2004). "Electrochemical technologies in wastewater treatment." Separation and Purification Technology **38**(1): 11-41.

Cho, D. H. and E. Y. Kim (2003). "Characterization of Pb2+ biosorption from aqueous solution by Rhodotorula glutinis." Bioprocess and Biosystems Engineering **25**(5): 271-277.

Choi, S. B. and Y. S. Yun (2004). "Lead biosorption by waste biomass of Corynebacterium glutamicum generated from lysine fermentation process." Biotechnology Letters **26**(4): 331-336.

Chojnacka, K. (2007). "Bioaccumulation of Cr(III) ions by Blue-Green alga *Spirulina sp.* Part I. A Comparison with Biosorption." American Journal of Agricultural and Biological Sciences **2**(4): 218-223.

Chojnacka, K. (2009a). Biosorption and bioaccumulation in practice. New York, Nova Science Publishers, Inc.

Chojnacka, K. (2009b). "Technological use of algae in the food and chemical industries." Przemysl Chemiczny **88**(5): 414-418.

Chojnacka, K. (2010). "Biosorption and bioaccumulation - the prospects for practical applications." Environment International **36**(3): 299-307.

Coelho, J. P., M. E. Pereira, A. Duarte and M. A. Pardal (2005). "Macroalgae response to a mercury contamination gradient in a temperate coastal lagoon (Ria de Aveiro, Portugal)." Estuarine, Coastal and Shelf Science **65**(3): 492-500.

Commission Directive 2009/90/EC (2009). Commission Directive 2009/90/EC of 31 July 2009 laying down, pursuant to Directive 2000/60/EC of the European Parliament and of the Council, technical specifications for chemical analysis and monitoring of water status., Official Journal of the European Union. **201:** 36-38.

Costa, S., D. Crespo, B. M. G. Henriques, E. Pereira, A. C. Duarte and M. A. Pardal (2011). "Kinetics of Mercury Accumulation and Its Effects on Ulva lactuca Growth Rate at Two Salinities and Exposure Conditions." Water Air and Soil Pollution **217**(1-4): 689-699.

Crini, G., F. Gimbert, C. Robert, B. Martel, O. Adam, N. Morin-Crini, F. De Giorgi and P.-M. Badot (2008). "The removal of Basic Blue 3 from aqueous solutions by chitosan-based adsorbent: Batch studies." Journal of Hazardous Materials **153**(1–2): 96-106.

da Costa, V. M. F. (2006). Utilização da macroalga *Ulva lactuca Linnaeus* a redução de nutrientes (NH_4^+, NO_3^- e PO_4^{-2}) provenientes da carcinicultura. PÓS-GRADUAÇÃO EM BIOECOLOGIA AQUÁTICA, Universidade Federal do Rio Grande do Norte.

Da Silva, E. F., E. C. Fonseca, J. X. Matos, C. Patinha, P. Reis and J. M. Santos Oliveira (2005). "The effect of unconfined mine tailings on the geochemistry of soils, sediments and surface waters of the lousal area (Iberian Pyrite Belt, Southern Portugal)." Land Degradation & Development **16**(2): 213-228.

da Silva, J. M., J. M. C. N. Soares and D. L. Karlen (2004). "Implement and soil condition effects on tillage-induced erosion." Soil & Tillage Research **78**(2): 207-216.

Dabrowski, A., Z. Hubicki, P. Podkoscielny and E. Robens (2004). "Selective removal of the heavy metal ions from waters and industrial wastewaters by ion-exchange method." Chemosphere **56**(2): 91-106.

Das, N. (2010). "Recovery of precious metals through biosorption — A review." Hydrometallurgy **103**(1–4): 180-189.

Davis, T. A., B. Volesky and A. Mucci (2003). "A review of the biochemistry of heavy metal biosorption by brown algae." Water Research **37**(18): 4311-4330.

Decision No 2455/2001/EC (2001). Decision No 2455/2001/EC of the European Parliament and of the Council of 20 November 2001 establishing the list of priority substances in the field of water policy and amending Directive 2000/60/EC Official Journal of the European Union. **311:** 1-5.

Decree-Law No. 103/2010 (2010). Decree-Law No. 103/2010 of the Portuguese Ministry of Environment and Spatial Planning of 24 September on pollution reduction, Diário da República : I Série. **187:** 4289-4298.

Decree-Law No. 236/98 (1998). Decree-Law No. 236/98 of the Portuguese Ministry of the Environment of 1 August establishing water quality standards, Diário da República : I Série. **176:** 3676-3722.

Decree-Law No. 276/2009 (2009). Decree-Law No. 276/2009 of the Portuguese Ministry of Environment, Spatial Planning and Regional Development of October 2 on the agricultural use of sewage sludge, Diário da República : I Série. **192:** 7154 -7165.

Decree-Law No. 306/2007 (2007). Decree-Law No. 306/2007 of the Portuguese Ministry of Environment, Spatial Planning and Regional Development of 27 August regulating quality of water used for human consumption., Diário da República : I Série. **167:** 5747 – 5765.

Degryse, F., V. Vlassak, E. Smolders and R. Merckx (2007). "Mobilization of Cd upon acidification of agricultural soils: column study and field modelling." European Journal of Soil Science **58**(1): 152-165.

Demirbas, A. (2008). "Heavy metal adsorption onto agro-based waste materials: A review." Journal of Hazardous Materials **157**(2–3): 220-229.

Deng, X. and D. B. Wilson (2001). "Bioaccumulation of mercury from wastewater by genetically engineered Escherichia coli." Applied Microbiology and Biotechnology **56**(1-2): 276-279.

Denizli, A., K. Kesenci, Y. Arica and E. Piskin (2000). "Dithiocarbamate-incorporated monosize polystyrene microspheres for selective removal of mercury ions." Reactive and Functional Polymers **44**(3): 235-243.

DGADR. (2009). "JORNADA SOBRE PROTECÇÃO DO SOLO - Resumo das conclusões." Retrieved Junho 2011, from http://www.spcs.pt/Jornada_Protec_Solo.pdf.

Díez Lázaro, J., P. S. Kidd and C. Monterroso Martínez (2006). "A phytogeochemical study of the Trás-os-Montes region (NE Portugal): Possible species for plant-based soil remediation technologies." Science of The Total Environment **354**(2-3): 265-277.

Diniz, V. and B. Volesky (2005). "Biosorption of La, Eu and Yb using Sargassum biomass." Water Research **39**(1): 239-247.

Directive 2000/60/EC (2000). Directive 2000/60/EC of the European Parl

iament and of the Council establishing a framework for the Community action in the field of water policy, Official Journal of the European Communities. **327**.

Directive 2008/105/EC (2008). Directive 2008/105/EC of the European Parliament and the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council, Official Journal of the European Union. **348:** 84-97.

Directive 2013/39/EU (2013). Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy, Official Journal of the European Union. **226:** 1-17.

Drasch, G., M. Horvat and M. Stoeppler (2008). Mercury. Elements and Their Compounds in the Environment, Wiley-VCH Verlag GmbH**:** 931-1005.

Du Laing, G. (2010). Analysis and Fractionation of Trace Elements in Soils, John Wiley & Sons, Ltd.

Duffus, J. H. (2002). ""Heavy metals" - A meaningless term? (IUPAC technical report)." Pure and Applied Chemistry **74**(5): 793-807.

Dwivedi, S., R. D. Tripathi, U. N. Rai, S. Srivastava, S. Mishra, M. K. Shukla, A. K. Gupta, S. Sinha, V. S. Baghel and D. K. Gupta (2006). "Dominance of Algae in Ganga Water Polluted Through Fly-Ash Leaching: Metal Bioaccumulation Potential of Selected Algal Species." Bulletin of Environmental Contamination and Toxicology **77**(3): 427-436.

Eftec (2005). The Economic, Social and Ecological Value of Ecosystem Services: A Literature Review London

El-Khaiary, M. I. and G. F. Malash (2011). "Common data analysis errors in batch adsorption studies." Hydrometallurgy **105**(3-4): 314-320.

El Samrani, A. G., B. S. Lartiges and F. Villiéras (2008). "Chemical coagulation of combined sewer overflow: Heavy metal removal and treatment optimization." Water Research **42**(4–5): 951-960.

Entezari, M. H. and M. Tahmasbi (2009). "Water softening by combination of ultrasound and ion exchange." Ultrasonics Sonochemistry **16**(3): 356-360.

Ernst, G., S. Zimmermann, P. Christie and B. Frey (2008). "Mercury, cadmium and lead concentrations in different ecophysiological groups of earthworms in forest soils." Environmental Pollution **156**(3): 1304-1313.

European Commission. (2013). "MEMO/13/871 " Retrieved September 2014, from http://europa.eu/rapid/press-release_MEMO-13-871_en.htm.

Ewers, U., A. Brockhaus, R. Dolgner, I. Freier, E. Jermann, A. Bernard, R. Stiller-Winkler, R. Hahn and N. Manojlovic (1985). "Environmental exposure to cadmium and renal function of elderly women living in cadmium-polluted areas of the Federal Republic of Germany." International Archives of Occupational and Environmental Health **55**(3): 217-239.

Farooq, U., J. A. Kozinski, M. A. Khan and M. Athar (2010). "Biosorption of heavy metal ions using wheat based biosorbents - A review of the recent literature." Bioresource Technology **101**(14): 5043-5053.

Fereshteh, G., B. Yassaman, A. Moghaddam, M. Reza, Zavar, and M. A. & Hossein (2007). "Phytoremediation of Arsenic by Macroalga: Implication in Natural Contaminated Water, Northeast Iran." Journal of Applied Sciences **7**(2): 1614-1619.

Fernandes, M. L., Calouro, F., Indiati, R., Barros and A. M. (2000). Evaluation of soil test methods for estimation of available phosphorus in some portuguese soils : A greenhouse study. Philadelphia, PA, ETATS-UNIS, Taylor & Francis.

Flouty, R. and G. Estephane (2012). "Bioaccumulation and biosorption of copper and lead by a unicellular algae Chlamydomonas reinhardtii in single and binary metal systems: A comparative study." Journal of Environmental Management **111**: 106-114.

Fourest E. and Volesky B. (1996). "Contribution of sulphonate groups and alginate to heavy metal biosorption by the dry biomass of *Sargassum fluitans*." Environmental Science Technology **30**: 277-282.

Freundlich, H. (1906). "Over the adsorption in solution." J Phys Chem **57**: 385–470.

Fu, F. and Q. Wang (2011). "Removal of heavy metal ions from wastewaters: A review." Journal of Environmental Management **92**(3): 407-418.

Gavrilescu, M. (2004). "Removal of heavy metals from the environment by biosorption." Engineering in Life Sciences **4**(3): 219-232.

Gerhardsson, L. (2008). Lead. Elements and Their Compounds in the Environment, Wiley-VCH Verlag GmbH**:** 879-900.

Gerhardt, K. E., X. D. Huang, B. R. Glick and B. M. Greenberg (2009). "Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges." Plant Science **176**(1): 20-30.

Ghodbane, I. and O. Hamdaoui (2008). "Removal of mercury(II) from aqueous media using eucalyptus bark: Kinetic and equilibrium studies." Journal of Hazardous Materials **160**(2-3): 301-309.

Giusti, L. (2001). "Heavy metal contamination of brown seaweed and sediments from the UK coastline between the Wear river and the Tees river." Environment International **26**(4): 275-286.

Goel, P. K. (2006). Water pollution : causes, effects and control. New Delhi, New Age International.

Goldewijk K. K. and Ramankutty N. (2004). Land use changes during the past 300 years. Encyclopedia of Life Support Systems (EOLSS). W. Verheye. Oxford, UK, Eolss Publishers.

Gomes, P. I. A. and T. Asaeda (2013). "Phytoremediation of heavy metals by calcifying macro-algae (Nitella pseudollabellata): Implications of redox insensitive end products." Chemosphere **92**(10): 1328-1334.

Gonzalez, C. M., J. Hernandez, J. G. Parsons and J. L. Gardea-Torresdey (2010). "A study of the removal of selenite and selenate from aqueous solutions using a magnetic iron/manganese oxide nanomaterial and ICP-MS." Microchemical Journal **96**(2): 324-329.

Guittonny-Philippe, A., V. Masotti, P. Höhener, J.-L. Boudenne, J. Viglione and I. Laffont-Schwob (2014). "Constructed wetlands to reduce metal pollution from industrial catchments in aquatic Mediterranean ecosystems: A review to overcome obstacles and suggest potential solutions." Environment International **64**(0): 1-16.

Gustafsson, J. P., P. Pechová and D. Berggren (2003). "Modeling Metal Binding to Soils: The Role of Natural Organic Matter." Environmental Science & Technology **37**(12): 2767-2774.

Harley, C. D. G., A. R. Hughes, K. M. Hultgren, B. G. Miner, C. J. B. Sorte, C. S. Thornber, L. F. Rodriguez, L. Tomanek and S. L. Williams (2006). "The impacts of climate change in coastal marine systems (vol 9, pg 228, 2006)." Ecology Letters **9**(4): 500-500.

He, J. and J. P. Chen (2014). "A comprehensive review on biosorption of heavy metals by algal biomass: Materials, performances, chemistry, and modeling simulation tools." Bioresource Technology **160**(0): 67-78.

Herber, R. F. M. (2008). Cadmium. Elements and Their Compounds in the Environment, Wiley-VCH Verlag GmbH**:** 689-708.

Ho, Y. S. and G. McKay (2004). "Sorption of copper(II) from aqueous solution by peat." Water Air and Soil Pollution **158**(1): 77-97.

Ho, Y. S., J. C. Y. Ng and G. Mckay (2000). "Kinetics of pollutant sorption by biosorbents: Review." Separation and Purification Methods **29**(2): 189-232.

Ho, Y. S., J. F. Porter and G. Mckay (2002). "Equilibrium isotherm studies for the sorption of divalent metal ions onto peat: Copper, nickel and lead single component systems." Water Air and Soil Pollution **141**(1-4): 1-33.

Hodgson, E. (2004). A textbook of modern toxicology. New York, John Wiley and Sons.

Hooda, P. S. (2010). Assessing Bioavailability of Soil Trace Elements, John Wiley & Sons, Ltd.

Houba, V. J. G., E. J. M. Temminghoff, G. A. Gaikhorst and W. van Vark (2000). "Soil analysis procedures using 0.01 <i>M</i> calcium chloride as extraction reagent." Communications in Soil Science and Plant Analysis **31**(9): 1299 - 1396.

Hough, R. L., A. M. Tye, N. M. J. Crout, S. P. McGrath, H. Zhang and S. D. Young (2005). "Evaluating a ‘Free Ion Activity Model’ applied to metal uptake by <i>Lolium perenne</i> L. grown in contaminated soils." Plant and Soil **270**(1): 1-12.

Huang, L.-z., G.-m. Zeng, D.-l. Huang, L.-f. Li, C.-y. Du and L. Zhang (2010). "Biosorption of cadmium(II) from aqueous solution onto *Hydrilla verticillata*." Environmental Earth Sciences **60**(8): 1683-1691.

Inácio, M., V. Pereira and M. Pinto (2008). "The Soil Geochemical Atlas of Portugal: Overview and applications." Journal of Geochemical Exploration **98**(1-2): 22-33.

Inskip, H., V. Beral and M. Mcdowall (1982). "Mortality of Shipham Residents - 40-Year Follow-Up." Lancet **1**(8277): 896-899.

Jaishankar, M., B. B. Mathew, M. S. Shah, K. M. T.P. and S. G. K.R. (2014). "Biosorption of Few Heavy Metal Ions Using Agricultural Wastes." Journal of Environment Pollution and Human Health **2**(1): 1-6.

Jayakumar, R., M. Rajasimman and C. Karthikeyan (2014). "Sorption of hexavalent chromium from aqueous solution using marine green algae Halimeda gracilis: Optimization, equilibrium, kinetic, thermodynamic and desorption studies." Journal of Environmental Chemical Engineering **2**(3): 1261-1274.

Kabata-Pendias, A. (2004). "Soil-plant transfer of trace elements - an environmental issue." Geoderma **122**(2-4): 143-149.

Kabata-Pendias, A., Mukherjee, Arun B. (2007). Trace Elements from Soil to Human, Springer.

Kadirvelu, K. and J. Goel (2005). Ion Exchange and Inorganic Adsorption. Water Encyclopedia, John Wiley & Sons, Inc.

Kadukova, J. and E. Vircikova (2005). "Comparison of differences between copper bioaccumulation and biosorption." Environment International **31**(2): 227-232.

Kamala-Kannan, S., B. Prabhu Dass Batvari, K. J. Lee, N. Kannan, R. Krishnamoorthy, K. Shanthi and M. Jayaprakash (2008). "Assessment of heavy metals (Cd, Cr and Pb) in water, sediment and seaweed (Ulva lactuca) in the Pulicat Lake, South East India." Chemosphere **71**(7): 1233-1240.

Kar, P. and M. Misra (2004). "Use of keratin fiber for separation of heavy metals from water." Journal of Chemical Technology & Biotechnology **79**(11): 1313-1319.

Kara, Y. (2004). "Bioaccumulation of copper from contaminated wastewater by using Lemna minor." Bulletin of Environmental Contamination and Toxicology **72**(3): 467-471.

Kennedy, G., G. Nantel and P. Shetty (2003). "The scourge of “hidden hunger”: global dimensions of micronutrient defieciencies." Food, Nutrition and Agriculture **32**: 8-16.

Kim, E. H., R. P. Mason, E. T. Porter and H. L. Soulen (2004). "The effect of resuspension on the fate of total mercury and methyl mercury in a shallow estuarine ecosystem: a mesocosm study." Marine Chemistry **86**(3-4): 121-137.

Klimmek, S., H. J. Stan, A. Wilke, G. Bunke and R. Buchholz (2001). "Comparative analysis of the biosorption of cadmium, lead, nickel, and zinc by algae." Environmental Science & Technology **35**(21): 4283-4288.

Kocberber, N. and G. Donmez (2007). "Chromium(VI) bioaccumulation capacities of adapted mixed cultures isolated from industrial saline wastewaters." Bioresource Technology **98**(11): 2178-2183.

Kononova, O. N., A. G. Kholmogorov, N. V. Danilenko, N. G. Goryaeva, K. A. Shatnykh and S. V. Kachin (2007). "Recovery of silver from thiosulfate and thiocyanate leach solutions by adsorption on anion exchange resins and activated carbon." Hydrometallurgy **88**(1–4): 189-195.

Koopmans, G. F., P. F. A. M. Römkens, M. J. Fokkema, J. Song, Y. M. Luo, J. Japenga and F. J. Zhao (2008). "Feasibility of phytoextraction to remediate cadmium and zinc contaminated soils." Environmental Pollution **156**(3): 905-914.

Korngold, E., N. Belayev and L. Aronov (2001). "Removal of arsenic from drinking water by anion exchangers." Desalination **141**(1): 81-84.

Kovacevic, V. and J. Wesseler (2010). "Cost-effectiveness analysis of algae energy production in the EU." Energy Policy **38**(10): 5749-5757.

Krems, P., M. Rajfur, M. Waclawek and A. Klos (2013). "The Use of Water Plants in Biomonitoring and Phytoremediation of Waters Polluted with Heavy Metals." Ecological Chemistry and Engineering S-Chemia I Inzynieria Ekologiczna S **20**(2): 353-370.

Kumar, P., M. Govindaraju, S. Senthamilselvi and K. Premkumar (2013). "Photocatalytic degradation of methyl orange dye using silver (Ag) nanoparticles synthesized from Ulva lactuca." Colloids and Surfaces B: Biointerfaces **103**(0): 658-661.

Landaburu-Aguirre, J., V. García, E. Pongrácz and R. L. Keiski (2009). "The removal of zinc from synthetic wastewaters by micellar-enhanced ultrafiltration: statistical design of experiments." Desalination **240**(1–3): 262-269.

Langmuir, I. (1918). "THE ADSORPTION OF GASES ON PLANE SURFACES OF GLASS, MICA AND PLATINUM." Journal of the American Chemical Society **40**(9): 1361-1403.

Lasat, M. M. (2002). "Phytoextraction Of Toxic Metals." J. Environ. Qual. **31**(1): 109-120.

Lawson, N. M. and R. P. Mason (1998). "Accumulation of mercury in estuarine food chains." Biogeochemistry **40**(2-3): 235-247.

Liu, W. T., J. C. Ni and Q. X. Zhou (2013). "Uptake of Heavy Metals by Trees: Prospects for Phytoremediation." Energy and Environment Materials **743-744**: 768-781.

Liu, Y., Q. Cao, F. Luo and J. Chen (2009). "Biosorption of Cd2+, Cu2+, Ni2+ and Zn2+ ions from aqueous solutions by pretreated biomass of brown algae." Journal of Hazardous Materials **163**(2–3): 931-938.

Lodeiro, P., R. Herrero and M. E. Sastre de Vicente (2006). "Batch desorption studies and multiple sorption-regeneration cycles in a fixed-bed column for Cd(II) elimination by protonated Sargassum muticum." Journal of Hazardous Materials **137**(3): 1649-1655.

Lofts, S., D. J. Spurgeon, C. Svendsen and E. Tipping (2004). "Deriving Soil Critical Limits for Cu, Zn, Cd, and Pb: A Method Based on Free Ion Concentrations." Environmental Science & Technology **38**(13): 3623-3631.

Lopes, C. B., J. R. Oliveira, L. S. Rocha, D. S. Tavares, C. M. Silva, S. P. Silva, N. Hartog, A. C. Duarte and E. Pereira (2014). "Cork stoppers as an effective sorbent for water treatment: the removal of mercury at environmentally relevant concentrations and conditions." Environ Sci Pollut Res Int **21**(3): 2108-2121.

Loureiro, S., A. L. G. Ferreira, A. M. M. Soares and A. J. A. Nogueira (2005). "Evaluation of the toxicity of two soils from Jales Mine (Portugal) using aquatic bioassays." Chemosphere **61**(2): 168-177.

Lv, L., M. P. Hor, F. Su and X. S. Zhao (2005). "Competitive adsorption of Pb2+, Cu2+, and Cd2+ ions on microporous titanosilicate ETS-10." Journal of Colloid and Interface Science **287**(1): 178-184.

Malaeb, L. and G. M. Ayoub (2011). "Reverse osmosis technology for water treatment: State of the art review." Desalination **267**(1): 1-8.

Malash, G. F. and M. I. El-Khaiary (2010). "Piecewise linear regression: A statistical method for the analysis of experimental adsorption data by the intraparticle-diffusion models." Chemical Engineering Journal **163**(3): 256-263.

Malik, A. (2004). "Metal bioremediation through growing cells." Environment International **30**(2): 261-278.

Manju, G. N., K. Anoop Krishnan, V. P. Vinod and T. S. Anirudhan (2002). "An investigation into the sorption of heavy metals from wastewaters by polyacrylamide-grafted iron(III) oxide." Journal of Hazardous Materials **91**(1-3): 221-238.

Marchand, L., M. Mench, D. L. Jacob and M. L. Otte (2010). "Metal and metalloid removal in constructed wetlands, with emphasis on the importance of plants and standardized measurements: A review." Environmental Pollution **158**(12): 3447-3461.

Mata, Y. N., M. L. Blázquez, A. Ballester, F. González and J. A. Muñoz (2009). "Biosorption of cadmium, lead and copper with calcium alginate xerogels and immobilized Fucus vesiculosus." Journal of Hazardous Materials **163**(2-3): 555-562.

Matlock, M. M., B. S. Howerton and D. A. Atwood (2002). "Chemical precipitation of heavy metals from acid mine drainage." Water Research **36**(19): 4757-4764.

McBride, M. B. (1994). Environmental chemistry of soils. New York, USA, Oxford University Press.

Meers, E., G. Du Laing, V. Unamuno, A. Ruttens, J. Vangronsveld, F. M. G. Tack and M. G. Verloo (2007). "Comparison of cadmium extractability from soils by commonly used single extraction protocols." Geoderma **141**(3-4): 247-259.

Meindersma, G. W., C. M. Guijt and A. B. de Haan (2006). "Desalination and water recycling by air gap membrane distillation." Desalination **187**(1-3): 291-301.

Michalak, I., K. Chojnacka, Z. Dobrzanski, H. Gorecki, A. Zielinska, M. Korczynski and S. Opalinski (2011). "Effect of macroalgae enriched with microelements on egg quality parameters and mineral content of eggs, eggshell, blood, feathers and droppings." J Anim Physiol Anim Nutr (Berl) **95**(3): 374-387.

Millennium Ecosystem Assessment (2005). Ecosystems and human well-being : synthesis. Washington, DC.

Nielsen, M. M., A. Bruhn, M. B. Rasmussen, B. Olesen, M. M. Larsen and H. B. Moller (2012). "Cultivation of Ulva lactuca with manure for simultaneous bioremediation and biomass production." Journal of Applied Phycology **24**(3): 449-458.

Niu, C. H., B. Volesky and D. Cleiman (2007). "Biosorption of arsenic (V) with acid-washed crab shells." Water Research **41**(11): 2473-2478.

Nogawa, K. (1981). Itai-Itai disease and follow-up studies. Cadmium in the environment. N. JO. New York-Chichester-Brisbane-Toronto-Singapore, John Wiley & Sons. **II Health effects:** 1-37.

Nogawa, K. and T. Kido (1993). "Biological monitoring of cadmium exposure in itai-itai disease epidemiology." International Archives of Occupational and Environmental Health **65**(0): S43-S46.

Novozamsky, I., T. M. Lexmond and V. J. G. Houba (1993). "A Single Extraction Procedure of Soil for Evaluation of Uptake of Some Heavy Metals by Plants." International Journal of Environmental Analytical Chemistry **51**(1): 47 - 58.

O'Connell, D. W., C. Birkinshaw and T. F. O'Dwyer (2008). "Heavy metal adsorbents prepared from the modification of cellulose: A review." Bioresource Technology **99**(15): 6709-6724.

Økland, T. E., E. Wilhelmsen and Ø. Solevåg (2005). A study of the priority substances of the Water Framework Directive, Monitoring and need for screening. Norway, Bergfald & Co.

Otero, M., C. B. Lopes, J. Coimbra, T. R. Ferreira, C. M. Silva, Z. Lin, J. Rocha, E. Pereira and A. C. Duarte (2009). "Priority pollutants (Hg2+ and Cd2+) removal from water by ETS-4 titanosilicate." Desalination **249**(2): 742-747.

Park, H. J., S. W. Jeong, J. K. Yang, B. G. Kim and S. M. Lee (2007). "Removal of heavy metals using waste eggshell." Journal of Environmental Sciences **19**(12): 1436-1441.

Payne, K. B. and T. M. Abdel-Fattah (2005). "Adsorption of Divalent Lead Ions by Zeolites and Activated Carbon: Effects of pH, Temperature, and Ionic Strength." Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering **39**(9): 2275 - 2291.

Peijnenburg, W. J. G. M., M. Zablotskaja and M. G. Vijver (2007). "Monitoring metals in terrestrial environments within a bioavailability framework and a focus on soil extraction." Ecotoxicology and Environmental Safety **67**(2): 163-179.

Pereira, L. (2007). "As Algas Marinhas e Respectivas Utilidades." Retrieved 10-07-2012, from http://br.monografias.com/trabalhos913/algas-marinhas-utilidades/algas-marinhas-utilidades.pdf.

Pereira, R., J. P. Sousa, R. Ribeiro and F. Goncalves (2006). "Microbial indicators in mine soils (S. Domingos Mine, Portugal)." Soil & Sediment Contamination **15**(2): 147-167.

Prasanna, R., P. Jaiswal and B. D. Kaushik (2008). "Cyanobacteria as potential options for environmental sustainability - promises and challenges." Indian Journal of Microbiology **48**(1): 89-94.

Qiu, H., L. Lv, B. C. Pan, Q. J. Zhang, W. M. Zhang and Q. X. Zhang (2009). "Critical review in adsorption kinetic models." Journal of Zhejiang University-Science A **10**(5): 716-724.

Rae, I. B., S. W. Gibb and S. Lu (2009). "Biosorption of Hg from aqueous solutions by crab carapace." Journal of Hazardous Materials **164**(2–3): 1601-1604.

Ramalhosa, E., Monterroso, P., Abreu, S., Pereira, E., Vale, C. & Duarte, A. (2001). "Storage and export of mercury from a contaminated bay (Ria de Aveiro, Portugal)." Wetlands Ecology and Management **9**: 311–316.

Raskin, I., R. D. Smith and D. E. Salt (1997). "Phytoremediation of metals: using plants to remove pollutants from the environment." Current Opinion in Biotechnology **8**(2): 221-226.

Raspor, B. (2008). Elements and Elemental Compounds in Waters and the Aquatic Food Chain. Elements and Their Compounds in the Environment, Wiley-VCH Verlag GmbH**:** 127-147.

Reimann and Caritat (1998). Chemical elements in the environment. Berlin Heidelberg, Springer-Verlag.

Reis, A. T., S. M. Rodrigues, C. Araújo, J. P. Coelho, E. Pereira and A. C. Duarte (2009). "Mercury contamination in the vicinity of a chlor-alkali plant and potential risks to local population." Science of The Total Environment **407**(8): 2689-2700.

Rieuwerts, J. S., M. R. Ashmore, M. E. Farago and I. Thornton (2006). "The influence of soil characteristics on the extractability of Cd, Pb and Zn in upland and moorland soils." Science of The Total Environment **366**(2-3): 864-875.

Rocha, L., C. Lopes, J. A. Borges, A. C. Duarte and E. Pereira (2013). "Valuation of Unmodified Rice Husk Waste as an Eco-Friendly Sorbent to Remove Mercury: a Study Using Environmental Realistic Concentrations." Water, Air, & Soil Pollution **224**(7): 1-18.

Rodrigues, S., M. E. Pereira, L. Sarabando, L. Lopes, A. Cachada and A. Duarte (2006). "Spatial distribution of total Hg in urban soils from an Atlantic coastal city (Aveiro, Portugal)." Science of The Total Environment **368**(1): 40-46.

Rodrigues, S. M., B. Henriques, E. F. da Silva, M. E. Pereira, A. C. Duarte, J. E. Groenenberg and P. F. A. M. Romkens (2010a). "Evaluation of an approach for the characterization of reactive and available pools of 20 potentially toxic elements in soils: Part II - Solid-solution partition relationships and ion activity in soil solutions." Chemosphere **81**(11): 1560-1570.

Rodrigues, S. M., B. Henriques, E. F. da Silva, M. E. Pereira, A. C. Duarte and P. F. A. M. Romkens (2010b). "Evaluation of an approach for the characterization of reactive and available pools of twenty potentially toxic elements in soils: Part I - The role of key soil properties in the variation of contaminants' reactivity." Chemosphere **81**(11): 1549-1559.

Rodrigues, S. M., M. E. Pereira, E. F. da Silva, A. S. Hursthouse and A. C. Duarte (2009a). "A review of regulatory decisions for environmental protection: Part I -- Challenges in the implementation of national soil policies." Environment International **35**(1): 202-213.

Rodrigues, S. M., M. E. Pereira, E. F. da Silva, A. S. Hursthouse and A. C. Duarte (2009b). "A review of regulatory decisions for environmental protection: Part II--The case-study of contaminated land management in Portugal." Environment International **35**(1): 214-225.

Romera, E., F. Gonzalez, A. Ballester, M. L. Blazquez and J. A. Munoz (2006). "Biosorption with algae: A statistical review." Critical Reviews in Biotechnology **26**(4): 223-235.

Romera, E., F. Gonzalez, A. Ballester, M. L. Blazquez and J. A. Munoz (2007). "Comparative study of biosorption of heavy metals using different types of algae." Bioresource Technology **98**(17): 3344-3353.

Romero-Gonzalez, M. E., C. J. Williams and P. H. E. Gardiner (2000). "The application of dealginated seaweed as a cation exchanger foron-line preconcentration and chemical speciation of trace metals." Journal of Analytical Atomic Spectrometry **15**(8): 1009-1013.

Romkens, P. F., H. Y. Guo, C. L. Chu, T. S. Liu, C. F. Chiang and G. F. Koopmans (2009). "Characterization of soil heavy metal pools in paddy fields in Taiwan: chemical extraction and solid-solution partitioning." Journal of Soils and Sediments **9**(3): 216-228.

Römkens, P. F. A. M., J. E. Groenenberg, L. T. C. Bonten and W. D. Vries and J. Bril (2004). Derivation of partition relationship to calculate Cd, Cu, Ni, Pb, and Zn solubility and activity in soil solutions. Report no. 305. Wageningen.

Sakamoto, M., A. Nakano and H. Akagi (2001). "Declining Minamata male birth ratio associated with increased male fetal death due to heavy methylmercury pollution." Environmental Research **87**(2): 92-98.

Sanchez, A., S. Recillas, X. Font, E. Casals, E. Gonzalez and V. Puntes (2011). "Ecotoxicity of, and remediation with, engineered inorganic nanoparticles in the environment." Trac-Trends in Analytical Chemistry **30**(3): 507-516.

Sastre, J., G. Rauret and M. Vidal (2007). "Sorption-desorption tests to assess the risk derived from metal contamination in mineral and organic soils." Environment International **33**(2): 246-256.

Sauvé, S., W. Hendershot and H. E. Allen (2000). "Solid-Solution Partitioning of Metals in Contaminated Soils: Dependence on pH, Total Metal Burden, and Organic Matter." Environmental Science & Technology **34**(7): 1125-1131.

Savage, N. and M. S. Diallo (2005). "Nanomaterials and water purification: Opportunities and challenges." Journal of Nanoparticle Research **7**(4-5): 331-342.

Saxe, J. K., C. A. Impellitteri, W. J. G. M. Peijnenburg and H. E. Allen (2001). "Novel Model Describing Trace Metal Concentrations in the Earthworm, Eisenia andrei." Environmental Science & Technology **35**(22): 4522-4529.

Seiler, H. G., H. Sigel and A. Sigel (1988). Handbook on Toxicity of Inorganic Compounds, Taylor & Francis.

Sekabira, K., H. O. Origa, T. A. Basamba, G. Mutumba and E. Kakudidi (2011). "Application of algae in biomonitoring and phytoextraction of heavy metals contamination in urban stream water." International Journal of Environmental Science and Technology **8**(1): 115-128.

Selim, H. M. and D. L. Sparks (2001). Heavy metals release in soils. Boca Raton, USA, Lewis Publishers.

Selin, N. E. (2009). "Global Biogeochemical Cycling of Mercury: A Review." Annual Review of Environment and Resources **34**: 43-63.

Shan, G. B., R. Y. Surampalli, R. D. Tyagi and T. C. Zhang (2009). "Nanomaterials for environmental burden reduction, waste treatment, and nonpoint source pollution control: a review." Frontiers of Environmental Science & Engineering in China **3**(3): 249-264.

Sharaf, M. A., S. A. Sayed, A. A. Younis, A. B. Farag and H. A. Arida (2007). "Removal of Trace Contaminants from Water Using New Chelating Resins." Analytical Letters **40**(18): 3443 - 3456.

Shunxin, L., Q. Shahua, H. Ganquan and H. Fei (1999). "Separation and preconcentration of Se(IV)/Se(VI) speciation on algae and determination by graphite furnace atomic absorption spectrometry in sediment and water." Fresenius' Journal of Analytical Chemistry **365**(5): 469-471.

Siegel, F. R. (2002). Environmental Geochemistry of Potentially Toxic Metals, Springer.

Singh, R. and B. B. Prasad (2000). "Trace metal analysis: selective sample (copper II) enrichment on an AlgaSORB column." Process Biochemistry **35**(9): 897-905.

Sips, R. (1948). "Combined form of Langmuir and Freundlich equations." Journal of Chemical Physics **16**(490-495).

Sooksawat, N., M. Meetam, M. Kruatrachue, P. Pokethitiyook and K. Nathalang (2013). "Phytoremediation potential of charophytes: Bioaccumulation and toxicity studies of cadmium, lead and zinc." Journal of Environmental Sciences **25**(3): 596-604.

Sparks, D. L. (2003). Environmental Soil Chemistry. California, Elsevier Science

Srivastava, S. and P. Goyal (2010). Novel Biomaterials: Decontamination of Toxic Metals from Wastewater, Springer

Sud, D., G. Mahajan and M. P. Kaur (2008). "Agricultural waste material as potential adsorbent for sequestering heavy metal ions from aqueous solutions - A review." Bioresource Technology **99**(14): 6017-6027.

Susarla, S., V. F. Medina and S. C. McCutcheon (2002). "Phytoremediation: An ecological solution to organic chemical contamination." Ecological Engineering **18**(5): 647-658.

Tack, F. M. G. (2010). Trace Elements: General Soil Chemistry, Principles and Processes, John Wiley & Sons, Ltd.

Tak, H. I., F. Ahmad and O. O. Babalola (2013). "Advances in the Application of Plant Growth-Promoting Rhizobacteria in Phytoremediation of Heavy Metals." Reviews of Environmental Contamination and Toxicology, Vol 223 **223**: 33-52.

Tchobanoglous, G., F. L. Burton and H. D. Stensel (2003). Wastewater Engineering: Treatment and Reuse, Inc. McGraw-Hill.

Theron, J., J. A. Walker and T. E. Cloete (2008). "Nanotechnology and water treatment: Applications and emerging opportunities." Critical Reviews in Microbiology **34**(1): 43-69.

Tipping, E., J. Rieuwerts, G. Pan, M. R. Ashmore, S. Lofts, M. T. R. Hill, M. E. Farago and I. Thornton (2003). "The solid-solution partitioning of heavy metals (Cu, Zn, Cd, Pb) in upland soils of England and Wales." Environmental Pollution **125**(2): 213-225.

Tokcaer, E. and U. Yetis (2006). "Pb(II) biosorption using anaerobically digested sludge." Journal of Hazardous Materials **137**(3): 1674-1680.

Torres, M. A., M. P. Barros, S. C. G. Campos, E. Pinto, S. Rajamani, R. T. Sayre and P. Colepicolo (2008). "Biochemical biomarkers in algae and marine pollution: A review." Ecotoxicology and Environmental Safety **71**(1): 1-15.

Turner, A., S. S. Pedroso and M. T. Brown (2008). "Influence of salinity and humic substances on the uptake of trace metals by the marine macroalga, Ulva lactuca: Experimental observations and modelling using WHAM." Marine Chemistry **110**(3-4): 176-184.

Tuzen, M. and A. Sari (2009). "Removal of mercury(II) from aqueous solution using moss (Drepanocladus revolvens) biomass: Equilibrium, thermodynamic and kinetic studies." Journal of Hazardous Materials **171**(1-3): 500-507.

Tuzen, M., A. Sari, D. Mendil and M. Soylak (2009). "Biosorptive removal of mercury(II) from aqueous solution using lichen (Xanthoparmelia conspersa) biomass: Kinetic and equilibrium studies." Journal of Hazardous Materials **169**(1-3): 263-270.

Tuzun, I., G. Bayramoglu, E. YalçIn, G. Basaran, G. Çelik and M. Y. ArIca (2005). "Equilibrium and kinetic studies on biosorption of Hg(II), Cd(II) and Pb(II) ions onto microalgae Chlamydomonas reinhardtii." Journal of Environmental Management **77**(2): 85-92.

Van de Velde, F. e. D. R., G.A. (2002). Carrageenan. Biopolymers v. 6. Polysaccharides II, polysaccharides from eukaryotes. S. D. B. e. A. S. E.J. Vandamme. Weinheim; Chichester, Wiley-VCH**:** 245-274.

Vandenhove, H., G. Olyslaegers, N. Sanzharova, O. Shubina, E. Reed, Z. Shang and H. Velasco (2009). "Proposal for new best estimates of the soil-to-plant transfer factor of U, Th, Ra, Pb and Po." Journal of Environmental Radioactivity **100**(9): 721-732.

Vidotti, E. C., & Rollemberg, M. C. (2004). "Algas: Da economia nos ambientes aquáticos à bioremediação e à química analítica." Quimica Nova **27**: 139–145.

Vijayaraghavan, K., H. Y. N. Winnie and R. Balasubramanian (2011). "Biosorption characteristics of crab shell particles for the removal of manganese(II) and zinc(II) from aqueous solutions." Desalination **266**(1–3): 195-200.

Volesky, B. (2001). "Detoxification of metal-bearing effluents: biosorption for the next century." Hydrometallurgy **59**(2-3): 203-216.

Volesky, B. (2007). "Biosorption and me." Water Research **41**(18): 4017-4029.

Volesky, B. and G. Naja (2007). "Biosorption technology: starting up an enterprise." International Journal of Technology Transfer and Commercialisation **6**(2): 196-211.

Wang, J. L. and C. Chen (2009). "Biosorbents for heavy metals removal and their future." Biotechnology Advances **27**(2): 195-226.

Weber, W. J. and J. C. Morris (1963). "Kinetics of adsorption on carbon from solution." Kinetics of Adsorption on Carbon from Solution.

Williams, M. E. (2003). "A Review of Wastewater Treatment by Reverse Osmosis " Retrieved May 2011, from www.eetcorp.com/heepm/RO_AppsE.pdf.

Winter, M. J. (2012). "WebElements: the periodic table on the web." Retrieved Junho de 2014, from http://www.webelements.com.

Xue, H., P. H. Nhat, R. Gächter and P. S. Hooda (2003). "The transport of Cu and Zn from agricultural soils to surface water in a small catchment." Advances in Environmental Research **8**(1): 69-76.

Yanagisawa, H., Y. Matsumoto and M. Machida (2010). "Adsorption of Zn(II) and Cd(II) ions onto magnesium and activated carbon composite in aqueous solution." Applied Surface Science **256**(6): 1619-1623.

Yetilmezsoy, K., F. Ilhan, Z. Sapci-Zengin, S. Sakar and M. T. Gonullu (2009). "Decolorization and COD reduction of UASB pretreated poultry manure wastewater by electrocoagulation process: A post-treatment study." Journal of Hazardous Materials **162**(1): 120-132.

Yin, Y., C. A. Impellitteri, S.-J. You and H. E. Allen (2002). "The importance of organic matter distribution and extract soil:solution ratio on the desorption of heavy metals from soils." The Science of The Total Environment **287**(1-2): 107-119.

Zhang, Y., Q. Li, L. Sun, R. Tang and J. P. Zhai (2010). "High efficient removal of mercury from aqueous solution by polyaniline/humic acid nanocomposite." Journal of Hazardous Materials **175**(1-3): 404-409.

Zhao, L. Y. L., R. Schulin and B. Nowack (2009). "Cu and Zn mobilization in soil columns percolated by different irrigation solutions." Environmental Pollution **157**(3): 823-833.

Zouboulis, A. I., M. X. Loukidou and K. A. Matis (2004). "Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal-polluted soils." Process Biochemistry **39**(8): 909-916.

Chapter 2

Risks associated with the transfer of toxic organo-metallic mercury from soils into the terrestrial feed chain

2 Risks associated with the transfer of toxic organo-metallic mercury from soils into the terrestrial feed chain

2.1 Introduction

Mercury (Hg) is an extremely toxic pollutant that poses global environmental and human health risks (Selin 2009). During the last two centuries anthropogenic emissions of Hg, e.g. from chlor-alkali plants (Biester et al. 2002) and coal power stations (Kabata-Pendias 2001) significantly exceeded the emission/inputs of natural sources (Peralta-Videa et al. 2009) resulting is severe soil contamination (Selin 2009). Despite such contamination many of these sites are still being used for agriculture and pasture without a proper evaluation of Hg distribution and speciation in soils and without an assessment of risks to animals and humans. To assess such risks, it is imperative to consider the speciation of Hg, both in soils and crops.

In the terrestrial and aquatic environment Hg occurs in both organic and inorganic forms (Challa et al. 2008), including elemental mercury (Hg^0), mercurous (Hg^+) and mercuric (Hg^{2+}) salts (Kabata-Pendias 2001), cinnabar (HgS) and organo-metallic compounds, such as methylmercury(II) ($MeHg^+$) and ethylmercury(II) ($EtHg^+$) (Fernández-Martínez et al. 2005). The most common Hg mineral is HgS which is quite insoluble rendering this species rather unavailable for transport or plant uptake, at least in the short term (Rodrigues et al. 2010). Mercury in soil usually is strongly adsorbed onto organic matter and/or metal oxides which generally results in a large pool of mercury being relatively immobile (Kabata-Pendias 2001). Inorganic mercury (IHg) species such as mercuric chloride ($HgCl_2$) are only weakly sorbed to soil components (Han et al. 2003) and can also be subject to mercury methylation processes resulting in highly mobile organic mercury species including methylmercury (MeHg) one of the most toxic organo-metallic forms of Hg (Han et al. 2003; Selin 2009). Organo-metallic mercury (OrgHg) compounds are more toxic than IHg salts due to their ability to form water-soluble complexes with cysteine in body tissues (Clarkson and Magos 2006) which increases the potential for uptake and accumulation by organisms (Fernández-Martínez et al. 2005).

Exposure to OrgHg mercury is of great concern due to its potential impact to the central nervous system that affects human and wildlife development and health (Mailman and Bodaly 2005; Meng et al. 2011). For grazing animals, environmental exposure can also result in accumulation of OrgHg in kidneys of cows and sheep as well as in the transfer of OrgHg into animals' milk (Olkowski 2009). Hence, along with animal health effects important aspects of OrgHg in livestock relate with potential risk of toxicity associated with the consumption of animal products by the human population.

This is relevant for terrestrial systems since recent studies showed that soil Hg can easily enter the food chain (Qiu et al. 2008; Meng et al. 2010; Rimmer et al. 2010; Shao et al. 2012). Risks for animals and humans particularly arise through dietary intake of organic forms of Hg present in edible plants (Gardea-Torresdey et al. 2005; Wang et al. 2011). Estimates indicate that in general, about 1 to 3% of total Hg (THg) in surface soils is in the organo-metallic form (Kabata-Pendias 2007) while in upland plants up to 2.5% of the THg can be in the organo-metallic form (Mailman and Bodaly 2005). Recent studies have shown that rice grain is an intensive bioaccumulator of MeHg (up to 10-100 times greater than other crop plants (Qui et al. 2008)) due to cultivation under flooded conditions where microbial Hg(II) methylation occurs (Rothenberg et al. 2011a) and that rice consumption is a significant pathway for human exposure to organic forms of Hg (Qiu et al. 2008; Rothenberg et al. 2011b; Zhang et al. 2010a,b). However, despite the recent documentation of uptake of OrgHg from contaminated soils for rice and other edible crops including white cabbage, spinach, radish and lettuce and maize (Qiu et al. 2008; Shao et al. 2012) as well as evidence of Hg bioaccumulation and trophic transfer to wildlife in forest ecosystems (Gnamus et al. 2000; Rimmer et al. 2010) relatively little is know about OrgHg uptake by feed crops and potential transfer and bioaccumulation for grazing animals or about OrgHg thresholds for animals' dietary intake to ensure food safety. To fill this gap studies on the exposure of grazing animals to organic forms of Hg in soils at polluted sites are necessary.

Hence, the scientific objectives of this study were: (1) to evaluate speciation of Hg in soils from an historically contaminated industrial site in Portugal and to assess factors affecting the geographical variability of OrgHg in soils (2) to evaluate soil-to-feed transfer of IHg and OrgHg; (3) to estimate the animal exposure to IHg and OrgHg from contaminated soils and feed; and (4) to assess potential risks for animal health and food safety.

2.2 Materials and methods

2.2.1 Study area

The Estarreja Chemical Complex (ECC) is located 1 km north from the village of Estarreja (North-West Portugal). According to the Köppen classification (Peel et al. 2007) the region has a warm-summer mediterranean climate (Cbs). The annual average temperature is 14.6 ºC and the average precipitation per year is 914 mm (Rodrigues et al. 2006). The fields surrounding the complex are mostly used for agricultural and livestock purposes. The predominant vegetation includes ryegrass and mustard greens. The ECC dates back to the 1950s and among other industrial units it includes a chlor-alkali plant with production of chlorine and sodium hydroxide from rock salt and which used electrolytic cells with mercury cathodes for several decades (Costa and Jesus-Rydin 2001). Since the 1950s and until the late 1970s, liquid effluents from the Chemical Complex were discharged into the “Esteiro de Estarreja”, which is a river branch of a nearby lagoon (“Ria de Aveiro”), through open streams that crossed the surrounding agricultural fields, such as “Vala de S. Filipe” (Figure 2.1). In 1986 the plant consumed 28 ton/year of Hg as a raw material and it was estimated that around 12% of annual consumption of Hg was released to the atmosphere (Rodrigues et al. 2006).

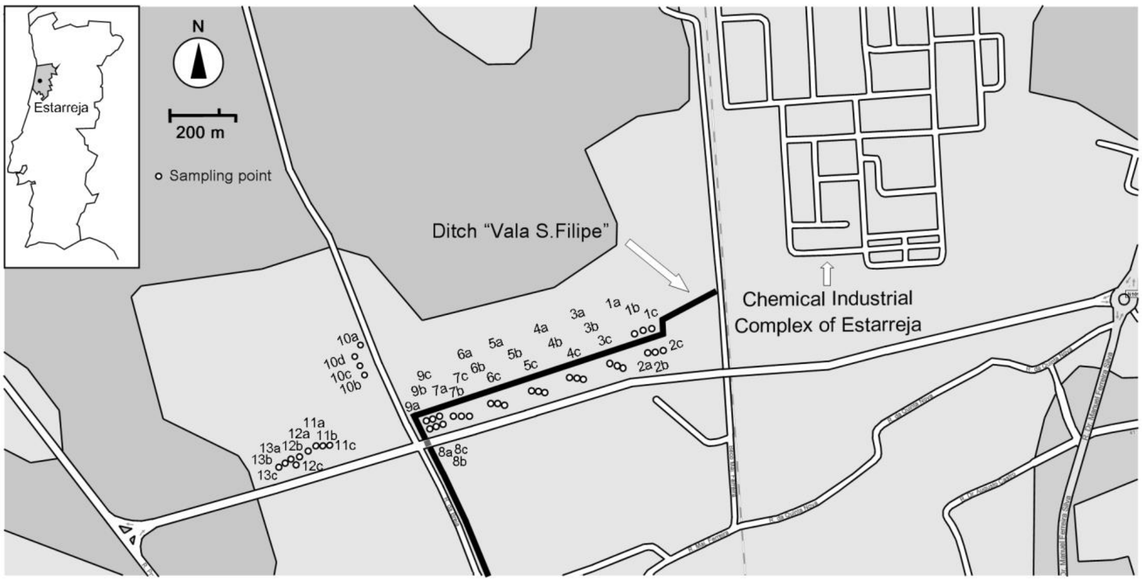

Figure 2.1: Map with the area of the study and the location of the sampling points.

Although the chlor-alkali plant completely ceased the use of mercury cathodes in 2002, severe contamination by this pollutant still remains in the surrounding environment (Reis et al. 2009; Rodrigues et al. 2012a).

2.2.2 Sampling and sample preparation

Sampling was carried out at 39 sites along agricultural/pasture fields located in the vicinity of the chlor-alkali plant, as showed in Figure 2.1. Samples were collected:

- along the former effluent stream (“Vala de S. Filipe”) – from site 1 to site 8,
- at the former effluent stream – site 9;
- away from the former effluent stream – from site 10 to site 13.

At each site, soil samples were taken at two depths (0-10 cm and 10-20 cm) using a plastic spade. Afterwards, soils were air dried at room temperature until constant weight and sieved to < 2 mm using a nylon sieve. Visible stones and biological debris were removed during the drying stage. The following soil properties were measured: pH (according to ISO 10390:1994 procedure), soil organic carbon content (Org C) (Elemental Analysis, LECO CNH-2000, according to ISO 10694:1995) and particle size distribution (using a Coulter LS230 laser diffraction particle size analyser).

A total of 40 plant samples, including ryegrass (*Lolium perenne,* n=35) and mustard greens (*Brassica Juncea,* n=5) were collected. All samples were placed in plastic bags during transport to the laboratory. At the laboratory, plant samples were washed with tap water and thoroughly rinsed with distilled water using a brush to remove all traces of dust and soil from the roots and shoots. Both root and shoot samples were dried at 40 ºC until constant weight, as several authors agree that this temperature can be applied to avoid loss of mercury due to volatilization (Loredo et al. 2003; Kamal et al. 2004), and milled.

2.2.3 Total mercury concentration

Total mercury (THg) concentrations in soil, roots and shoots were measured by thermal decomposition atomic absorption spectrometry with gold amalgamation (LECO model AMA-254), as described by Costley et al. (2000). The analysis is performed directly on the sample (sample mass varied in the range 5-150 mg d.w.), without digestion or specific pre-treatment, avoiding mercury losses or contamination as well as matrix interferences. At least three replicate measurements were carried out for each sample (acceptable relative standard deviation among replicates: <10%). Several blanks (i.e. an empty sample nickel boat) were run before and between sample analyses to ensure that mercury was not being carried over between samples. Detection and quantification limits obtained through blank measurements (n=20) were 0.01 ng Hg (defined as the mean value plus 3.3 standard deviation) and 0.03 ng Hg (defined as the mean value plus 10 standard deviation), respectively.

Analytical quality control was performed by using Certified Reference Materials (CRMs):

- BCR 060 - Aquatic plant (*Lagarosiphon major*); 0.34±0.04 mg kg^{-1} (total Hg)
- CRM 021 – USEPA Certified soil reference; 4.7±0.4 mg kg^{-1} (total Hg)
- TORT 2 - *Lobster Hepatopancreas*; 0.27±0.06 mg kg^{-1} (total Hg)

The CRMs were analysed every day prior to the beginning of the analysis and repeated at the end of the day. All percentages of recovery for THg were within the range of 87–113% (n=19).

2.2.4 Organic and inorganic mercury concentration

Organo-metallic mercury (OrgHg) concentrations in soil, roots and shoots were assessed through a method described by Válega et al. (2006). Extraction of OrgHg mercury compounds from the sample matrix was achieved through digestion of 100-200 mg d.w of each sample with a mixture of 18% KBr in 5% H_2SO_4 with $CuSO_4$ (1 mol L^{-1}), followed by extraction of OrgHg by toluene. The extractions were always performed in triplicate. The OrgHg mercury compounds retained in the toluene fraction were back extracted into an aqueous solution of thiosulphate. Mercury in liquid aliquots of the extracts (250-1000 µL) was quantified by thermal decomposition atomic absorption spectrometry with gold amalgamation (LECO model AMA-254). It was not possible to quantify OrgHg in 20 soil, 7 root and 13 shoot samples since their contents were below the limit of quantification (0.03

ng Hg). To ensure the quality of results procedural blanks (i.e. procedure with the reagents only) and reference material TORT-2 (*Lobster Hepatopancreas*; 0.152±0.013 mg kg^{-1} of methylmercury) were always carried out in parallel with the samples and analysed. The procedural blanks were always below the detection limit of the equipment (0.01 ng Hg). The recovery for OrgHg mercury (assuming that the dominant form in this fraction is methylmercury (Ullrich et al. 2007; Nam and Basu 2011) was in the range 80-86% (n=11). Inorganic mercury (IHg) was obtained subtracting OrgHg from THg.

2.2.5 Statistical analysis

Descriptive calculations and statistical analysis of data were performed using IBM SPSS Statistics 19 for Windows, whereas the graphics were plotted using OriginPro 8.5 for Windows.

Daily intake (DI) of THg and OrgHg for cows and sheep was estimated according to de Vries et al. (2007):

$$DI = [Hg]_{feed} \times I_{feed} + [Hg]_{soil} \times I_{soil} \qquad (1)$$

Where: DI is given in mg d^{-1}; $[Hg]_{feed}$ and $[Hg]_{soil}$ is the concentration of Hg (THg or OrgHg) in feed and soil, in mg kg^{-1} dry weight (d.w.); I_{feed} and I_{soil} are the daily ingestion rate of feed and soil by grazing animals in kg d^{-1} d.w.

Here, the following ingestion rates for soil and feed (dry weight) were used: 0.41 kg d^{-1} of soil and 16.9 kg d^{-1} of grass (for cows); 0.10 kg d^{-1} of soil and 2.5 kg d^{-1} of grass (for sheep) (de Vries et al. 2007). Furthermore it was assumed that animals remained in the field during the whole growing period and always graze at the same site.

We estimated the concentration of THg in animal (cow and sheep) organs (kidney, liver and muscle) according to the approach described by de Vries et al. (2007), as follows:

$$[THg]_{animal\_organ} = \left([THg]_{feed}\, x \frac{I_{feed}}{I_{feed} + I_{soil}} + [THg]_{soil}\, x \frac{I_{soil}}{I_{feed} + I_{soil}} \right) xBAF_{feed-animal} \qquad (2)$$

Where: $[THg]_{animal\_organ}$ = concentration of THg in the animal organ (kidney, liver or muscle), in mg kg^{-1} fresh weight (f.w.) (organ concentrations are calculated in f.w. to allow comparison with limit values from legislation)

$BAF_{feed-anima\ l}$= transfer coefficient or bioaccumulation factor from feed to animal organ for THg expressed as mg Hg kg^{-1} animal organ (f.w.)/ mg Hg kg^{-1} feed (d.w.). For THg the $BAF_{feed-animal}$ for cows is 0.638, 0.158 and 0.00092 for kidney, liver and muscle, respectively (de Vries et al. 2007). For sheep $BAF_{feed-animal}$ equals 0.468, 0.0572 and 0.00094 for kidney, liver and muscle, respectively (de Vries et al. 2007).

It was not possible to estimate levels of OrgHg in animal organs since no $BAF_{feed-animal}$ values were found in literature for these forms of the metal.

2.3 Results

2.3.1 Physical-chemical properties of soil

Table 2.1 summarizes physical-chemical properties of the studied soils. The pH of all soil samples was similar among depths, ranging between 4.5 and 5.8 for 0-10 cm layer and between 4.4 and 5.7 for 10-20 cm layer. These values are consistent with previous studies that evidenced the slightly acidic character of most of the Portuguese soil (Inácio et al. 2008). Furthermore, the pH range is comparable to that reported by Reis et al. (2009) for agricultural soils of the same region.

The soil organic carbon content ranged from 1.1% to 6.4% for the 0-10 cm depth although only four samples showed values above 3%. For the 10-20 cm depth, organic carbon values were relatively low (from 0.060% to 2.6%) with a median value of 1.6%. No significant ($p<0.05$) differences were found for organic carbon levels between depths 0-10 cm and 10-20 cm, with the exception of samples 9a, 9b and 9c that showed significantly higher values at 0-10 cm. Soil organic carbon values showed a tendency to increase with distance from the Chemical Industrial Complex (from site 1 to site 9), with maximum values of 4.5-6.4% at samples 9a, 9b and 9c (in the 0-10 cm layer). This can be associated with the pattern of sedimentation of organic matter along the stream.

Table 2.1: Physical-chemical properties of soils collected at two depths.

Soil property	Depth (cm)	nº of samples	Minimum	Maximum	Mean ± S.D.	Median
pH	0-10	39	4.5	5.8	5.0 ± 0.4	4.9
	10-20	39	4.4	5.7	4.9 ± 0.4	4.8
Org C (%)	0-10	39	1.1	6.4	2.1 ± 1.1	1.8
	10-20	39	0.06	2.6	1.3 ± 0.5	1.6
Sand (%)	0-10	39	21	87	68 ± 17	72
	10-20	39	23	94	70 ± 14	74
Silt (%)	0-10	39	11	69	28 ± 14	24
	10-20	39	4	66	25 ± 12	23
Clay (%)	0-10	39	2	11	4 ± 2	4
	10-20	39	1	11	4± 2	4

The clay content of these soils was generally low (median of 4.0% for both depths). With the exception of samples 9a, 9b and 9c soil texture did not vary considerably between depths and included sand, loamy sand, sandy loam, loam and silt loam soils. For samples 9a, 9b and 9c, sand percentages were relatively higher at the 10-20 cm layer (77-94 %) when compared to the 0-10 cm depth (53-69 %).

2.3.2 Inorganic and organic mercury in soils

Inorganic Hg concentrations in soils for the two depths are presented in Figure 2.2A. The IHg levels were highly variable, ranging from 0.080 to 210 mg kg^{-1} d.w. (with a mean of 27 mg kg^{-1} and a median of 5.0 mg kg^{-1}) in the 39 samples of 0-10 cm depth and from 0.080 to 83 mg kg^{-1} d.w. (with a mean of 17 mg kg^{-1} and a median of 5.0 mg kg^{-1}) in the 39 samples of 10-20 cm depth. The IHg concentration in soils varied between 97 and 100 % of their respective THg concentration.

The IHg levels were higher than those observed by Biester et al. (2002) at three different sites contaminated by emissions from chlor-alkali plants (up to 3 mg kg^{-1}). In 97% of the samples the IHg concentrations were above THg levels in non-contaminated “natural” Portuguese soils (THg 0.08 mg kg^{-1} d.w.) as reported by Inácio et al. (2008). No specific legislation regulating the assessment of soil contamination exists in Portugal, yet according to the Portuguese Decree-Law nº276/2009, which concerns sewage sludge application in agricultural soils, the limit for THg concentration in soils at pH<5.5 is 1 mg kg^{-1} d.w. This limit was exceeded in 65% of soil samples.

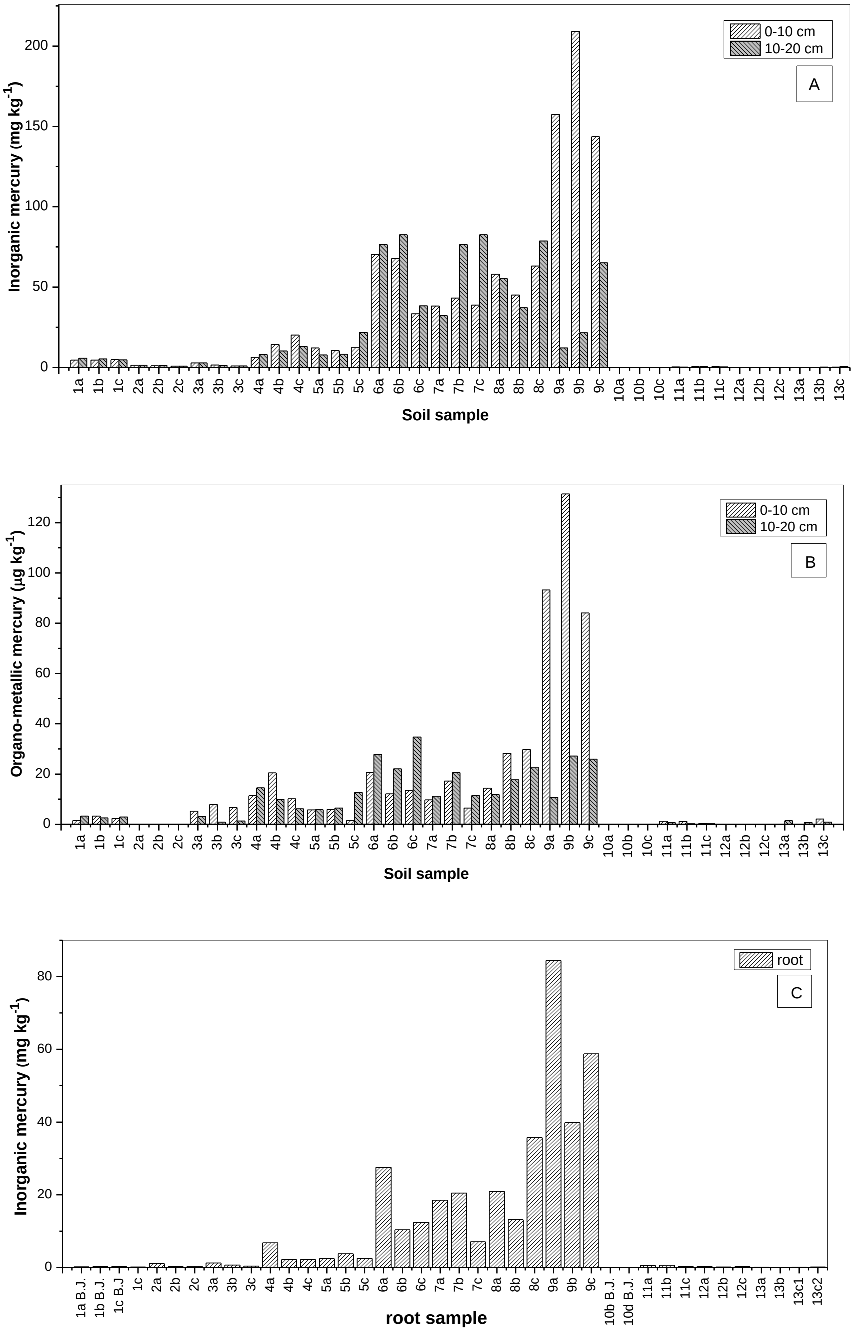

Figure 2.2: Inorganic and organo-metallic mercury concentrations in soils (A and B) for the two depths and in plants (C, D and E) for roots and shoots. Inorganic and organo-metallic mercury concentrations are expressed in mg kg^{-1} and µg kg^{-1} of dry weight, respectively. All samples in C, D and E are from *Lolium perenne* with the exception of 5 plants indicated by "B.J." (*Brassica Juncea*).

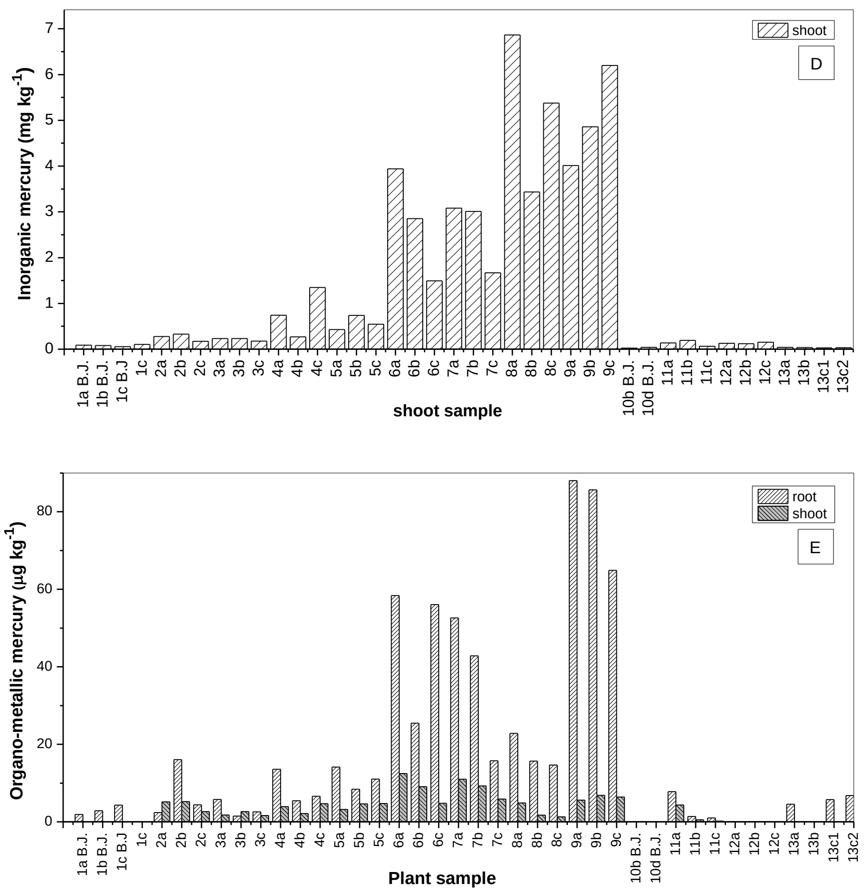

Figure 2.2 (cont.)

Organo-metallic mercury concentrations in soil are presented in Figure 2.2B. The concentration of OrgHg ranged between 0.20 and 130 µg kg^{-1} d.w. (with a mean of 14 and a median of 5.2 µg kg^{-1} d.w.), representing on average 0.13% of THg concentration in soil. The relatively low percentages of OrgHg in soils in relation to the respective THg are in line with those observed by Fernández-Martínez et al. (2005) near an abandoned mercury mining area in Spain (< 1%) as well as in contaminated and non-contaminated sites in Slovenia (0.003-0.17% OrgHg in relation to THg) (Gnamus et al. 2000).

As observed in Figure 2.2A there is a clear pattern of increase of IHg levels from site 1 to site 9. Such pattern shows a tendency to increasing IHg levels along the stream and with distance from the Chemical Complex. This is similar to what was observed for soil organic carbon. In fact there is a significant positive correlation between IHg and soil organic carbon ($r=0.85$, $p<0.01$). The main factor contributing to the distribution of IHg in soils is therefore not the distance to the primary Hg source but the pattern of distribution of soil organic carbon along the former effluent stream. For soils located at 300-500 m away from “Vala de S. Filipe” (sites 10 to 13), IHg levels were considerably lower and below 0.70 mg kg^{-1} d.w. indicating IHg levels decrease rapidly with distance from the stream.

With the exception of site 9, there was no significant difference in soil IHg concentrations between the two depths ($\rho<0.05$). These soils are regularly plowed by the local farmers contributing to this homogeneous distribution of the soil mercury pool within the surface soil layers. For samples 9a, 9b and 9c collected from inside “Vala de S. Filipe” relatively higher IHg levels were found at the 0-10 cm layer (140-210 mg kg^{-1} d.w.), compared to the 10-20 cm depth (12-65 mg kg^{-1} d.w.) that is related with the significantly higher organic carbon contents of the 0-10 cm layer as well as with the sandy nature of the 10-20 cm layer (> 90% sand). Such soil characteristics contribute to a higher retention of Hg in the 0-10 cm layer when compared with the 10-20 cm.

Similarly to IHg concentrations, there was an increase in OrgHg from site 1 to site 9, with samples 9a, 9b and 9c (0-10 cm) showing the highest levels of the dataset (84-130 µg kg^{-1} d.w.). The OrgHg concentration in soils was significantly positively correlated with the respective IHg concentration ($r=0.96$, $p<0.01$). A positive significant correlation was also observed between the organic carbon content of soils and the concentration of OrgHg ($r=0.91$, $p<0.01$). This is in line with previous studies reporting that OrgHg in soils have a great affinity for organic matter that contributes for the retention of the metal in the soil solid matrix (Jen-How, 2005).

Similarly to IHg, there were no statistically significant differences between OrgHg concentrations in both depths ($p>0.05$), with the exception of site 9 that showed significantly higher levels in the 0-10 cm depth (84-130 µg kg^{-1} d.w.), compared to the 10-20 cm layer (11-27 µg kg^{-1} d.w.).

2.3.3 Inorganic and organic mercury in plants

As shown in Figure 2.2C and 2.2D, IHg concentration in roots ranged from 0.010 to 84 mg kg^{-1} d.w. (with a mean of 9.4 mg kg^{-1} and a median of 0.85 mg kg^{-1}; n=40) whereas in shoots, IHg concentrations varied between 0.020 to 6.9 mg kg^{-1} d.w. (with a mean of 1.3 mg kg^{-1} and a median of 0.25 mg kg^{-1}; n=40). The percentage of IHg in relation to THg was from 85 to 100 % (in roots) and from 92 to 100 % (in shoots). Relatively higher IHg concentrations were obtained in roots when compared with shoots (Figure 2.2C and 2.2D). The IHg in plants (both roots and shoot) increased from site 1 to site 9, showing that high concentrations of IHg in plants were generally associated with high levels of IHg in soils. Almost 73 % of all shoot samples exceeded the current quality criteria for THg in feedstuff as proposed by the European Commission (2002) and which is 0.11 mg Hg kg^{-1} d.w. (for feed with relative moisture content of 12%). Such contaminated plant samples were collected either at the "Vala de S. Filipe" or at its immediate vicinity. These plants are commonly used as feed for local cattle.

Figure 2.2E presents the distribution of OrgHg concentrations in plant samples. The concentration of OrgHg mercury varied from 1.0 to 88 µg kg^{-1} d.w. in roots (with a mean of 18 and a median of 6.7 µg kg^{-1} d.w.) and from 0.20 to 13 µg kg^{-1} d.w. in shoots (with a mean of 4.1 and a median of 3.9 µg kg^{-1} d.w.), representing on average 0.58 % and 0.66 % of THg in the respective plant parts. The percentages of OrgHg in relation to THg obtained were comparable to the percentage of MeHg reported in vegetation from Canada (Mailman and Bodaly 2005) and Slovenia (Gnamus et al. 2000). It should be noticed that the OrgHg concentration in root samples (median of 6.7 µg kg^{-1} d.w.) was generally higher than the respective OrgHg concentration in soils (median of 5.2 µg kg^{-1} d.w.) (Figure 2.2B and 2.2E).

2.4 Discussion

2.4.1 Relationships between inorganic/organic mercury in soil, inorganic/organic mercury in plants and soil-plant bioaccumulation factors

As shown in Figure 2.3A, a positive significant relationship between IHg in roots of *Lolium perenne* and IHg in soil was observed (r^2=0.89; p<0.01, log-transformed) indicating that soil is the source of root IHg in this feed crop. The fact that there is an even stronger relationship between IHg in shoots of *Lolium perenne* and IHg in the respective roots (Figure 2.3D, r^2=0.92; p<0.01, log-transformed) suggests that the source of IHg in these plants is not only the sorption of IHg from small soil particles adhering to root/shoot plant surfaces but also the absorption of the metal into root cells and subsequent translocation to aerial parts. The much lower concentrations of IHg in ryegrass shoots compared to those in roots also suggest that roots act as a barrier and reduce translocation of IHg to the aboveground plant, as was recently observed for rice (Qiu et al. 2008; Meng et al. 2011; Meng et al. 2012). Although the process of IHg retention in plant roots is still not fully understood, Válega and co-authors (2009) concluded that for *Halimione portulacoides (L.) Aellen* in salt marshes, root cell wall immobilization of Hg is a major mechanism of metal resistance. Krupp et al. (2009) reported that the formation of metal–phytochelatin complexes under environmental exposure traps Hg(II) in plant roots and reduces its translocation into shoots.

For OrgHg, a significant positive relationship between levels in roots of *Lolium perenne* and corresponding concentrations in soils (r^2=0.48: p<0.01, log-transformed) was observed suggesting that soil can be a source of OrgHg to roots (Rothenberg et al. 2011a; Zhang et al. 2010b) (Figure 2.3D). However, the r^2 value of the soil-root relationship for OrgHg was relatively lower than that obtained for IHg indicating that OrgHg and IHg can have different sorption mechanisms. Differences in uptake and accumulation mechanisms for IHg and OrgHg were also suggested by Zhang et al. (2010b) and they reflect enhanced uptake of OrgHg from the soil to plants as a consequence of the different biochemical characteristics of OrgHg compared to IHg (Gnamus et al. 2000). Dissimilarities in the root uptake for OrgHg and IHg can also derive from variation in the availability of OrgHg and IHg from soils resulting from the interaction between Hg and soil constituents and which are affected by the chemical form of the metal (Gnamus et al. 2000). Previous studies showed that the availability of IHg in soils for root uptake is generally lower than that of OrgHg (Bernaus et al. 2005; Boszke et al. 2008). Furthermore, there is a strong and significant relationship between concentration of OrgHg and the respective IHg in roots (r^2=0.71; p<0.01 log-transformed) which suggests that methylation of IHg in the roots can also be a source of OrgHg. This is corroborated by the fact that root concentrations of OrgHg in our study were higher than the respective OrgHg levels in soils, contrary to what happened with IHg.

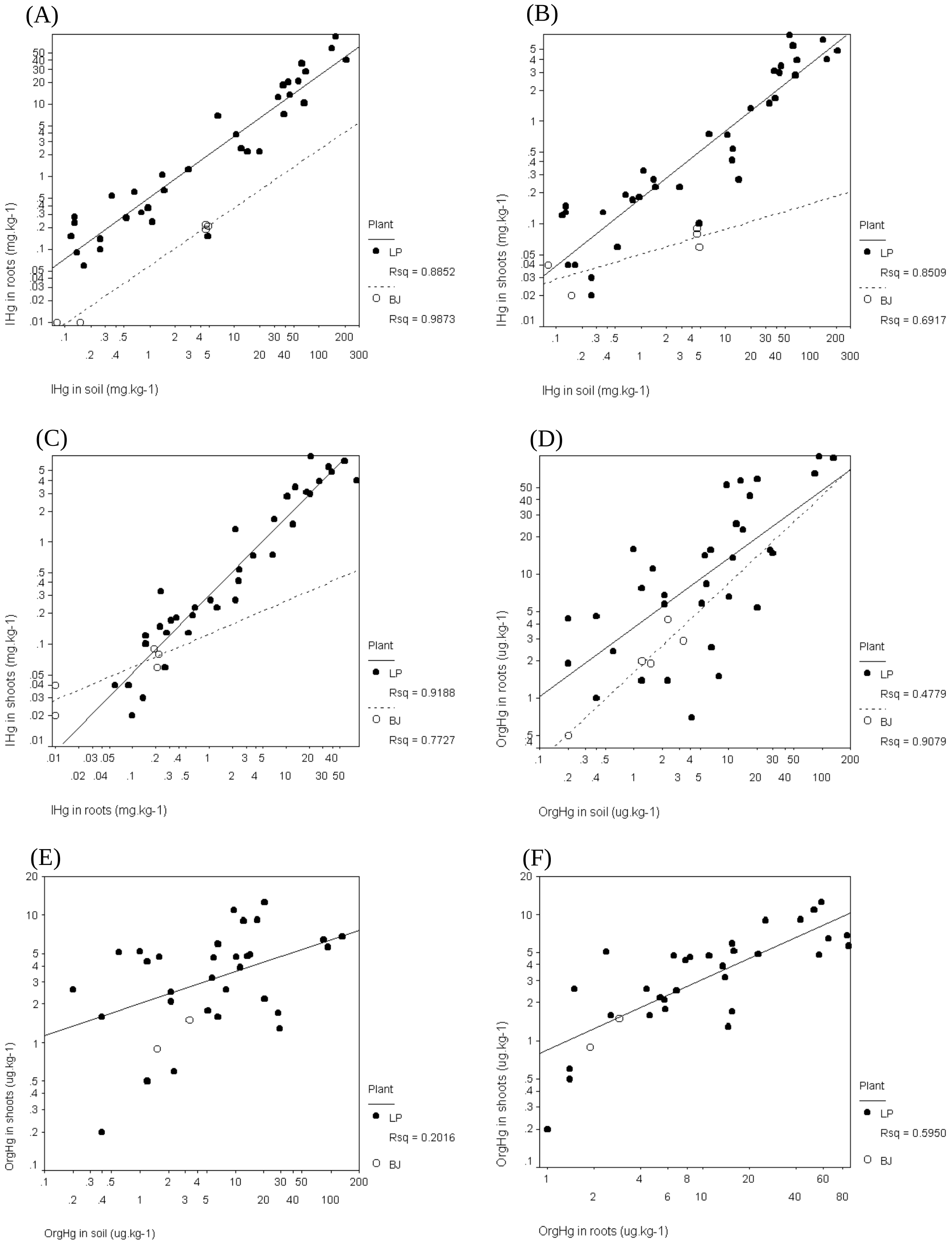

Figure 2.3: Scatterplots of relationships between IHg/ OrgHg in plants and IHg/OrgHg in soil (A-F). All values of concentrations were log-transformed.

For OrgHg in shoots of *Lolium perenne,* a significant and strong positive relationship was obtained with the respective root concentrations (r^2=0.60; p<0.01, log-transformed) indicating that the OrgHg levels observed in the aboveground plant parts derive from the translocation of OrgHg from the roots. This was also observed in studies with rice (Meng et al. 2011). In fact, Qiu et al. (2008) reported that OrgHg is more easily translocated to aboveground plant parts than IHg. According to Krupp et al. (2009) the easier translocation of OrgHg compared to IHg occurs because phytochelatins can sequester Hg(II) but not MeHg and therefore there is no retention of the organic forms of the metal in the roots as occurs for inorganic ones.

Since only five samples of *Brassica Juncea* were analysed it was not possible to infer on possible patterns of uptake and/or translocation of IHg or OrgHg for this plant. From Figure 2.3 these seem to be relatively lower than for *Lolium perenne*, but additional data would be necessary to draw conclusions on this.

To infer on the possible accumulation of Hg in plant tissues, bioaccumulation factors (BAFs, i.e. the root/soil and shoot/soil concentration ratios for IHg and OrgHg) were calculated (Figure 2.4). The BAFs for IHg varied between 0.030 and 2.2 with mean of 0.49 and median of 0.39 (for roots) and between 0.010 and 1.1 with mean of 0.19 and median of 0.080 (for shoots). With the exception of 3 samples (BAFs: 1.2-2.2), the BAFs for IHg were always lower than 1 suggesting that there is no accumulation of IHg in roots nor in shoots, relative to soil concentrations.

From Figure 2.4 is clear the BAFs for IHg in roots and shoots are not constant. It was possible to establish a clear and significant relationship between the variation of BAFs of IHg in shoots and the variation of IHg concentrations in both soils and roots, by linear regression analysis (Rodrigues et al. 2012b) as described by the following regression model:

$$LogBAF_{IHg\_shoots} = -0.55 - 0.91 \times Log[IHg_{Soil}] + 0.63 \times Log[IHg_{Roots}] \qquad (r^2=0.80,\ p<0.01)$$

This regression model suggests that BAFs for IHg in shoots decrease with increasing levels of soil IHg contamination and increase proportionally with concentration of IHg in roots.

The mean, median (and range) of BAFs for OrgHg were 3.3 and 1.8 (0.18-22) for roots and 1.5 and 0.46 (0.040-13) for shoots (Figure 2.4). Accumulation of OrgHg was observed at 26 sites in roots and at 8 sites in shoots where the BAF was higher than 1. These BAF values for OrgHg in shoots are comparable to those obtained in vegetation samples from Slovenia (mean BAF values for vegetation from different areas varied between 0.18 and 2.7) (Gnamus et al. 2000), and slightly lower than those reported by Zhang et al. (2010b) for rice (mean values: 4.4-6.9). The BAF values for OrgHg were on average 13 times higher than the respective values for IHg. Higher accumulation of OrgHg when compared with IHg was also previously observed in vegetation and rice samples (Gnamus et al. 2000; Zhang et al. 2010b).

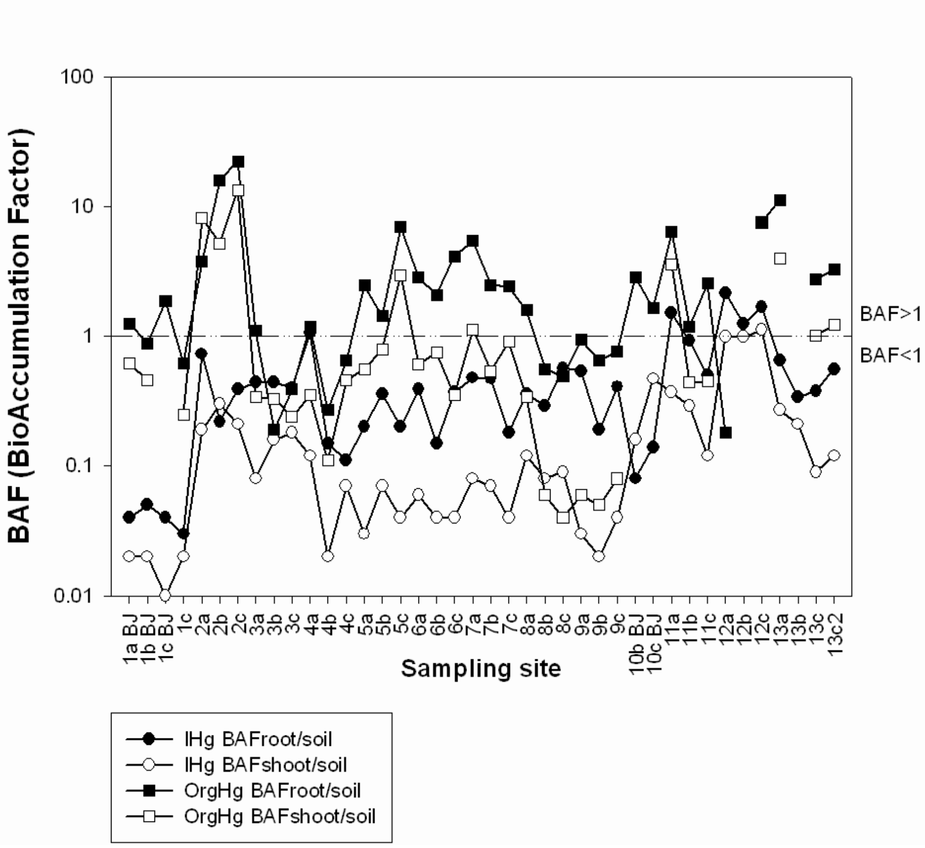

Figure 2.4: Bioaccumulation factors (root/ soil and shoot/soil concentration ratios for IHg and OrgHg) vs. sampling points. All samples are from Lolium perenne with the exception of 5 plants indicated by "B.J." (Brassica Juncea).

There was no significant relationship ($p>0.05$) between the BAF of OrgHg in roots and the respective BAF for IHg, which confirms that OrgHg has different accumulation mechanisms when compared with IHg. Furthermore, the BAF for OrgHg in roots is not constant. Hence, to assess which variables are responsible for the variability of BAFs we tested the relationship between the BAF for OrgHg in roots (log-transformed values) and the concentration of IHg in soils; the concentration of OrgHg in soils; soil properties (including pH, OrgC and clay); and the concentration of IHg in roots, by linear regression analysis. Results showed that the levels of IHg in soils and soil properties do not significantly affect the accumulation of OrgHg in roots ($p>0.05$) suggesting that the variation in the availability of OrgHg in soil in association with soil properties is not the main factor controlling its accumulation in roots. The linear regression model (Rodrigues et al. 2012b) that best describes the variability of BAF for OrgHg in roots is the following:

$$LogBAF_{OrgHg\_roots} = -0.68 - 0.39 \times Log[OrgHg_{Soil}] + 0.44 \times Log[IHg_{Roots}]$$

(r^2=0.58, p<0.01)

These results show that the accumulation of OrgHg in roots decreases with increasing levels of soil OrgHg indicating that these plants have an ability to reduce accumulation of OrgHg at the most polluted sites. By contrary, higher levels of IHg in roots lead to higher accumulation of OrgHg corroborating the hypothesis that both accumulation from soils and plant induced methylation are responsible for accumulation of OrgHg in roots.

Finally, the BAFs for OrgHg in shoots were significantly positively correlated with the corresponding BAFs in roots as described by the following linear regression model:

$$LogBAF_{OrgHg\_shoots} = -0.52 + 1.0 \times Log\lfloor BAF_{OrgHg\_roots} \rfloor$$ (r^2=0.68, p<0.01).

Furthermore, the BAFs for OrgHg in roots were negatively correlated with concentrations of OrgHg in soils (r^2=0.67, p<0.01, log-transformed values) which suggests that accumulation of OrgHg in ryegrass shoots increases proportionally to accumulation in roots and that it decreases with increasing levels of OrgHg in soil, as observed for roots.

2.4.2 Exposure of grazing animals to inorganic and organic mercury in soils and feed

The estimated DI of THg as calculated by eq.1 (Figure 2.5A) varied widely between 0.45 and 170 mg d^{-1} (median: 5.2 mg d^{-1}) for cows and between 0.073 and 33 mg d^{-1} (median: 0.85 mg d^{-1}) for sheep. This wide range reflects the large range in soil and feed levels of THg observed in this study. The DI in 33 sampling sites exceeded the acceptable daily intake (ADI) of THg of both cows (ADI= 1.4 mg d^{-1}) and sheep (ADI= 0.28 mg d-1) in view of food safety associated with THg in animal kidneys (de Vries et al. 2007).

The ingestion of contaminated feed had a greater contribution than soils to most of the estimated values of THg daily intake for both grazing animals. For cows the relative contribution of feed varied between 33 and 98% (median: 77%) whereas soil contributed in 2.0 to 67% (median: 23%). For sheep the relative contribution of soil for estimated THg daily intakes was slightly larger (from 3.0 to 77%, with a median value of 33%) yet the preponderance of feed contribution remained (from 23 to 97%, with a median value of 67%).

For OrgHg the estimated values of DI (Figure 2.5B) for cows were between 1.0 and 220 µg d^{-1} (median: 71 µg d^{-1}). For sheep the DI of OrgHg varied between 0.10 and 33 µg d^{-1} with a median value of 11 µg d^{-1}. The DI of OrgHg was between 0.020 and 3.0% of the DI of THg for both cows and sheep. No values for ADI of OrgHg were found in literature or legislation for grazing animals (cows and sheep) but given the high toxicity of these Hg compounds such ADI values should be established urgently. For OrgHg, soil contributed with 7.0% and 10% (median values) and feed with 94% and 91% (median values) for cows and sheep, respectively, suggesting that feed is the primary source of OrgHg to grazing animals.

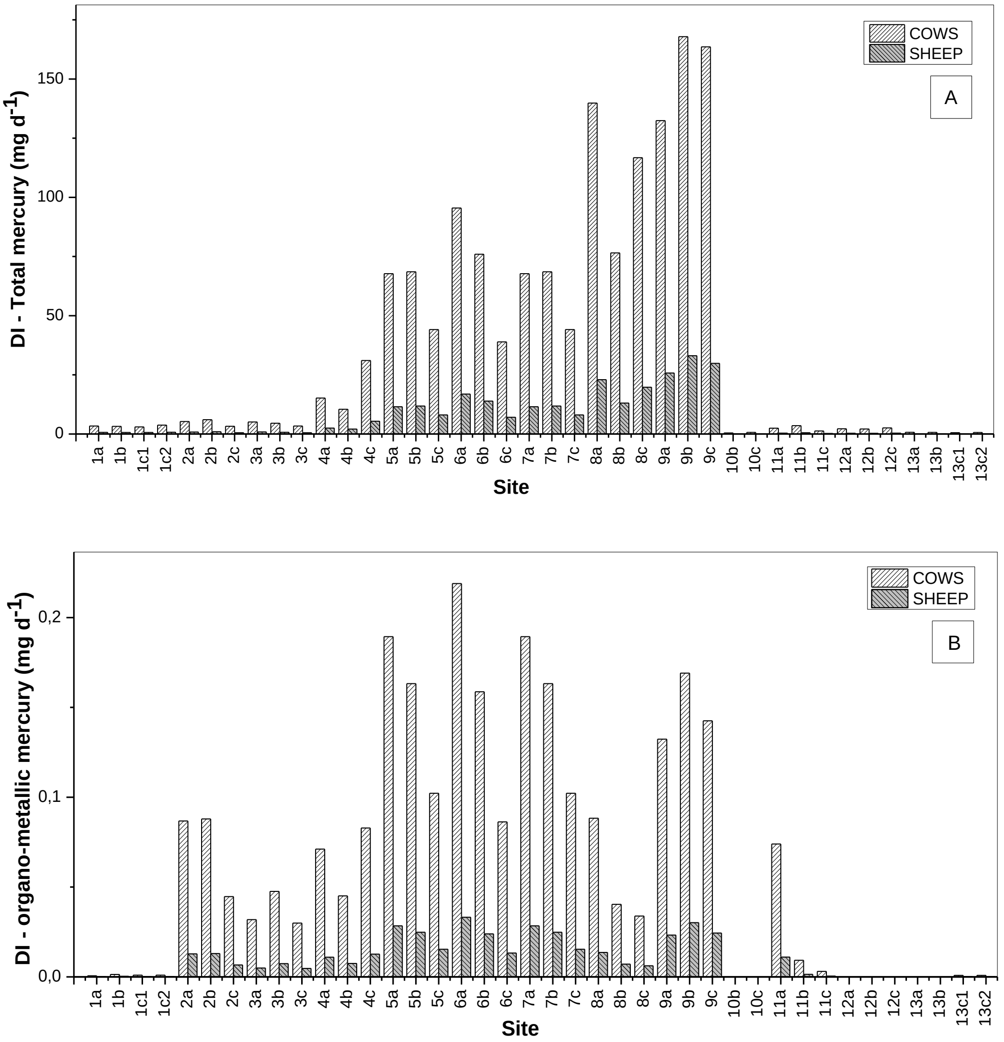

Figure 2.5: Daily intake of total mercury (5A) and organo-metallic mercury (5B) for cows and sheep at the various sampling sites (expressed in mg d^{-1}).

2.4.3 Estimated levels of total mercury in animal organs

The estimated concentration of THg in cow kidney calculated by eq.2 ranged widely from 0.017 to 6.2 mg kg^{-1} fresh weight (f.w.) (median: 0.19 mg kg^{-1} f.w.) whereas in sheep kidney levels of THg varied between 0.0080 and 2.9 mg kg^{-1} f.w., with a median of 0.090 mg kg^{-1}. The estimated concentrations of THg in liver were between 0.0040 and 1.5 mg kg^{-1} f.w. (median: 0.050 mg kg^{-1}) and between 0.0020 and 0.70 mg kg^{-1} f.w. (median: 0.020 mg kg^{-1}) for cow and sheep, respectively. Muscle was the organ with the lower values of estimated THg concentration for both animal species and the only organ for which all estimated levels were below critical limit of 0.05 mg kg^{-1} f.w., as defined in food quality criteria (de Vries et al. 2007). The estimated concentrations of THg in muscle were between 0.000020 and 0.0090 mg kg^{-1} f.w. in cow (median of 0.00030 mg kg^{-1}) and between 0.000030 and 0.010mg kg^{-1} f.w. in sheep, with a median of 0.00030 mg kg^{-1}. Almost 85% of all estimated levels of THg in cow kidney and 53% of all estimated concentrations in cow liver exceeded the current food quality criteria (0.05 mg kg^{-1} f.w.). For sheep almost 73% of all estimated levels in kidney and 45% of all estimated levels in liver were above critical limits. These results give indication of the potential risk to human health derived from the consumption of offal from animals that graze in this area.

Since $BAF_{feed\text{-}animal}$ values from literature are only available for THg it is not possible to estimate the relative percentage of IHg and OrgHg in animal organs.

2.5 Implications for risk assessment

Results from this environmental study indicate that there is uptake of IHg from soils by roots of *Lolium perenne* and subsequent translocation of IHg to above ground plant parts, although plant roots can to a certain extent retain IHg and reduce its translocation. No accumulation of IHg in shoots or roots in relation to soil IHg concentrations was observed (BAFs<1). On average, 99% of THg in shoots is in the form of IHg. Thereby, and since significant relationships between BAFs for IHg in edible plant parts and the corresponding concentration of IHg in soils were established from experimental data, it is possible to derive site-specific thresholds for IHg in soils from available quality criteria for feed crops (THg), as described by Rodrigues et al. 2012b. However, since IHg and OrgHg have different uptake and accumulation mechanisms in *Lolium perenne* (as also previously resported for other plants by Zhang et al. 2010b) and since OrgHg is known to be much more toxic than IHg, the establishment of soil thresholds for IHg (or THg) may not fully address the actual health risks for animals (and subsequently for humans).

This study indicated that despite the relatively low percentage of OrgHg in plants, this pool must not be overlooked since it represents a relatively high concentration in edible plant parts (up to 13 µg kg^{-1} d.w.) and includes the most toxic and mobile forms of Hg, primarily MeHg (Fernández-Martínez et al. 2005). Traditionally, feed control quality only included measurement of THg and disregarded organo-metallic forms of the metal despite their high toxicity (Hedegaard and Sloth 2011). However, results suggest that there is accumulation of OrgHg in roots (BAFs>1) which results from both root uptake and plant enhanced methylation of IHg in surrounding rhizosphere (Sun et al. 2011). It has also been suggested that there is no barrier to the translocation of OrgHg from roots to shoots as in the case of IHg. These processes result in accumulation of OrgHg also in shoots, relatively to soil concentrations (BAFs>1). Such BAFs for OrgHg in shoots increase with increasing BAFs for roots. But since no limits for OrgHg concentrations in animal feed were found in Portuguese or European legislation (European Commission 2002) it is not possible to effectively evaluate potential risks of exposure for livestock associated with dietary intake of feed at these OrgHg levels. Also, without such limits it is not possible to back-calculate threshold concentrations for OrgHg in soils, using soil-to-plant transfer relationships for OrgHg which could be derived from experimental data as described by Rodrigues et al. (2012a,b). Such site-specific thresholds for OrgHg in soils could then be used as a most robust way to identify those fields which in fact should not be used for raising cattle or for the production of food and fodder products.

Finally, since the toxicokinetics of Hg in animals depends on the chemical form of the metal, in order to properly ensure food safety it is necessary to further understand transfer and potential bioaccumulation of OrgHg in animal organs since currently no $BAF_{feed\text{-}animal}$ are available from literature.

2.6 Conclusions

The DI of THg in 33 sampling sites analysed in this study exceeded the acceptable daily intake (ADI) of THg of both cows (ADI= 1.4 mg d^{-1}) and sheep (ADI= 0.28 mg d^{-1}), in view of food safety associated with THg in animal kidneys. Furthermore, estimated daily intake of OrgHg for grazing animals was up to 220 μg d^{-1} (for cows) and up to 33 μg d^{-1} (for sheep). These results suggest that feed crops are an effective way of entry of OrgHg into the terrestrial feed and food chain which may represent a threat to animal and human health.

At the moment, feed quality control measures (particularly European Directive 2002/32/EC, EC 2002) only include the measurement of THg concentrations. This study suggested that solely monitoring the levels of THg in soils and feed may not allow to adequately taking into account accumulation of OrgHg in feed crops and properly address risks associated with the high toxicity of OrgHg required to obtain an accurate assessment of risks posed by metal mobilization from soils into the foodchain to animal (and human) health. In the future, ADIs for OrgHg for livestock should be set. Also, limits for OrgHg in feed and animal products for human consumption should be included in legislation for feed quality and food safety.

2.7 References

Bernaus, A.; Gaona, X.; Ivask, A.; Kahru, A.; Valiente, M. Analysis of sorption and bioavailability of different species of mercury on model soil components using XAS techniques and sensor bacteria. Anal Bioanal Chem 382:1541-1548; 2005

Biester, H.; Müller, G.; Schöler, H.F. Binding and mobility of mercury in soils contaminated by emissions from chlor-alkali plants. Sci Total Environ 284:191-203; 2002

Boszke, L.; Kowalski, A.; Astel, A.; Barański, A.; Gworek, B.; Siepak, J. Mercury mobility and bioavailability in soil from contaminated area. Environ Geol 55:1075-1087; 2008

Challa, Y.R.; Astudillo, L.R.d.; Ramirez, A.; Escalona, A.; Martínez, G. Distribution of Total and Organic Mercury in Superficial Soils in the Upper Manzanares River Watershed, Sucre State, Venezuela. Air Soil Water Res 1:21-29; 2008

Clarkson, T.W., Magos, L. The toxicology of mercury and its chemical compounds. Crit. Rev. Toxicol. 36:609-662; 2006

Costa, C.; Jesus-Rydin, C. Site investigation on heavy metals contaminated ground in Estarreja — Portugal. Eng Geol 60:39-47; 2001

Costley, C.T.; Mossop, K.F.; Dean, J.R.; Garden, L.M.; Marshall, J.; Carroll, J. Determination of mercury in environmental and biological samples using pyrolysis atomic absorption spectrometry with gold amalgamation. Anal Chim Acta 405:179-183; 2000

de Vries, W.; Römkens, P.F.A.M.; Schütze, G. Critical Soil Concentrations of Cadmium, Lead, and Mercury in View of Health Effects on Humans and Animals. Rev Environ Contam T Springer New York; 2007

European Commission. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. 2002

Fernández-Martínez, R.; Loredo, J.; Ordóñez, A.; Rucandio, M.I. Distribution and mobility of mercury in soils from an old mining area in Mieres, Asturias (Spain). Sci Total Environ 346:200-212; 2005

Gardea-Torresdey, J.L.; Peralta-Videa, J.R.; de la Rosa, G.; Parsons, J.G. Phytoremediation of heavy metals and study of the metal coordination by X-ray absorption spectroscopy. Coordin Chem Rev 249:1797-1810; 2005

Gnamus, A.; Byrne, A.R.; Horvat, M. Mercury in the soil-plant-deer-predator food chain of a temperate forest in Slovenia. Environ Sci Technol 34:3337-3345; 2000

Han, Y.; Kingston, H.M.; Boylan, H.M.; Rahman, G.M.M.; Shah, S.; Richter, R.C., et al. Speciation of mercury in soil and sediment by selective solvent and acid extraction. Anal Bioanal Chem 375:428-436; 2003

Hedegaard, R.V.; Sloth, J.J. Speciation of arsenic and mercury in feed: why and how? Biotechnol Agron Soc 15:45-51; 2011

Inácio, M.; Pereira, V.; Pinto, M. The Soil Geochemical Atlas of Portugal: Overview and applications. J Geochem Explor 98:22-33; 2008

Jen-How, H. Artifact formation of methyl- and ethyl-mercury compounds from inorganic mercury during derivatization using sodium tetra(n-propyl)borate. Anal Chim Acta 532:113-120; 2005

Kabata-Pendias, A. Trace Elements in Soils and Plants. Boca Raton: CRC Press LLC; 2001

Kabata-Pendias, A., Mukherjee, Arun B. Trace Elements from Soil to Human: Springer; 2007

Kamal, M.; Ghaly, A.E.; Mahmoud, N.; Côté, R. Phytoaccumulation of heavy metals by aquatic plants. Environ Int 29:1029-1039; 2004

Krupp, E.M.; Mestrot, A.; Wielgus, J.; Meharg, A.A.; Feldmann, J. The molecular form of mercury in biota: identification of novel mercury peptide complexes in plants. Chem Commun:4257-4259; 2009

Loredo, J.; Pereira, A.; Ordóñez, A. Untreated abandoned mercury mining works in a scenic area of Asturias (Spain). Environ Int 29:481-491; 2003

Mailman, M.; Bodaly, R.A. Total mercury, methyl mercury, and carbon in fresh and burned plants and soil in Northwestern Ontario. Environ Pollut 138:161-166; 2005

Meng, B.; Feng, X.; Qiu, G.; Wang, D.; Liang, P.; Li, P., et al. Inorganic mercury accumulation in rice (Oryza sativa L.). Environ Toxicol Chem 31:2093-2098; 2012

Meng, B.; Feng, X.B.; Qiu, G.L.; Cai, Y.; Wang, D.Y.; Li, P., et al. Distribution Patterns of Inorganic Mercury and Methylmercury in Tissues of Rice (Oryza sativa L.) Plants and Possible Bioaccumulation Pathways. J Agr Food Chem 58:4951-4958; 2010

Meng, B.; Feng, X.B.; Qiu, G.L.; Liang, P.; Li, P.; Chen, C.X., et al. The Process of Methylmercury Accumulation in Rice (Oryza sativa L.). Environ Sci Technol 45:2711-2717; 2011

Nam, D.H.; Basu, N. Rapid methods to detect organic mercury and total selenium in biological samples. Chem Cent J. 5; 2011

Olkowski, A.A. Livestock Water Quality, A Field Guide for Cattle, Horses, Poultry, and Swine: University of Saskatchewan/Minister of Agriculture and Agri-Food Canada; 2009

Peel, M.C.; Finlayson, B.L.; McMahon, T.A. Updated world map of the Koppen-Geiger climate classification. Hydrol Earth Syst Sc. 11:1633-1644; 2007Peralta-Videa, J.R.; Lopez, M.L.; Narayan, M.; Saupe, G.; Gardea-Torresdey, J. The biochemistry of environmental heavy metal uptake by plants: Implications for the food chain. Int J Biochem Cell B 41:1665-1677; 2009

Qiu, G.; Feng, X.; Li, P.; Wang, S.; Li, G.; Shang, L., et al. Methylmercury Accumulation in Rice (Oryza sativa L.) Grown at Abandoned Mercury Mines in Guizhou, China. J Agr Food Chem 56:2465-2468; 2008

Reis, A.T.; Rodrigues, S.M.; Araújo, C.; Coelho, J.P.; Pereira, E.; Duarte, A.C. Mercury contamination in the vicinity of a chlor-alkali plant and potential risks to local population. Sci Total Environ 407:2689-2700; 2009

Rimmer, C.C.; Miller, E.K.; McFarland, K.P.; Taylor, R.J.; Faccio, S.D. Mercury bioaccumulation and trophic transfer in the terrestrial food web of a montane forest. Ecotoxicology 19:697-709; 2010

Rothenberg, S.E.; Feng, X.B.; Dong, B.; Shang, L.H.; Yin, R.S.; Yuan, X.B. Characterization of mercury species in brown and white rice (Oryza sativa L.) grown in water-saving paddies. Environmental Pollution. 159:1283-1289; 2011a

Rothenberg, S.E.; Feng, X.B.; Li, P. Low-level maternal methylmercury exposure through rice ingestion and potential implications for offspring health. Environ Pollut 159:1017-1022; 2011b

Rodrigues, S.; Henriques, B.; Reis, A.; Duarte, A.; Pereira, E.; Römkens, P. Hg transfer from contaminated soils to plants and animals. Environ Chem Lett 10:61-67; 2012a

Rodrigues, S.; Pereira, M.E.; Sarabando, L.; Lopes, L.; Cachada, A.; Duarte, A. Spatial distribution of total Hg in urban soils from an Atlantic coastal city (Aveiro, Portugal). Sci Total Environ 368:40-46; 2006

Rodrigues, S.M.; Henriques, B.; da Silva, E.F.; Pereira, M.E.; Duarte, A.C.; Romkens, P.F.A.M. Evaluation of an approach for the characterization of reactive and available pools of twenty potentially toxic elements in soils: Part I - The role of key soil properties in the variation of contaminants' reactivity. Chemosphere 81:1549-1559; 2010

Rodrigues, S.M.; Pereira, M.E.; Duarte, A.C.; Römkens, P.F.A.M. Soil–plant–animal transfer models to improve soil protection guidelines: A case study from Portugal. Environ Int 39:27-37; 2012b

Selin, N.E. Global Biogeochemical Cycling of Mercury: A Review. Ann Rev Environ Res 34:43-63; 2009

Shao, D.D.; Wu, S.C.; Liang, P.; Kang, Y.; Fu, W.J.; Zhao, K.L., et al. A human health risk assessment of mercury species in soil and food around compact fluorescent lamp factories in Zhejiang Province, PR China. J Hazard Mater 221–222:28-34; 2012

Sun, X.J.; Wang, Q.C.; Ma, H.Y.; Wang, Z.C.; Yang, S.M.; Zhao, C.W.; Xu, L. Effects of plant rhizosphere on mercury methylation in sediments. J Soil Sediment. 11:1062-1069; 2011

Ullrich, S.M.; Ilyushchenko, M.A.; Kamberov, I.M.; Tanton, T.W. Mercury contamination in the vicinity of a derelict chlor-alkali plant. Part I: Sediment and water contamination of Lake Balkyldak and the River Irtysh. Sci Total Environ. 381:1-16; 2007

Válega, M.; Abreu, S.; Pato, P.; Rocha, L.; Gomes, A.; Pereira, M., et al. Determination of Organic Mercury in Biota, Plants and Contaminated Sediments Using a Thermal Atomic Absorption Spectrometry Technique. Water Air Soil Poll 174:223-234; 2006

Válega, M.; Lima, A.I.G.; Figueira, E.M.A.P.; Pereira, E.; Pardal, M.A.; Duarte, A.C. Mercury intracellular partitioning and chelation in a salt marsh plant, Halimione portulacoides (L.) Aellen: Strategies underlying tolerance in environmental exposure. Chemosphere 74:530-536; 2009

Wang, J.; Feng, X.; Anderson, C.W.N.; Qiu, G.; Ping, L.; Bao, Z. Ammonium thiosulphate enhanced phytoextraction from mercury contaminated soil - Results from a greenhouse study. J Hazard Mater 186:119-127; 2011

Zhang, H.; Feng, X.B.; Larssen, T.; Qiu, G.L.; Vogt, R.D. In Inland China, Rice, Rather than Fish, Is the Major Pathway for Methylmercury Exposure. Environ Health Persp 118:1183-1188; 2010a

Zhang, H.; Feng, X.B.; Larssen, T.; Shang, L.; Li, P. Bioaccumulation of Methylmercury versus Inorganic Mercury in Rice (Oryza sativa L.) Grain. Environ Sci Technol 44:4499-4504; 2010b.

Chapter 3

Bioaccumulation and biosorption of Hg by living marine macroalgae: prospecting for a new remediation biotechnology applied to saline waters

3 Bioaccumulation and biosorption of Hg by living marine macroalgae: prospecting for a new remediation biotechnology applied to saline waters.

3.1 Introduction

Increasing industrialization, which allowed Man to thrive and achieve high living standards, has resulted in the rise of the contamination levels in the environment at large-scale. Due to its high toxicity, temporal persistence (Chojnacka and Mikulewicz 2014), bioaccumulation and bioamplification along the trophic chain (Coelho et al. 2005), mercury (Hg) has aroused a great concern worldwide (Bulgariu and Bulgariu 2014).

In the aquatic environment, Hg is usually found as Hg^{2+} or as complexes of Hg^{2+} with different organic and inorganic ligands (Kabata-Pendias 2007). However, reduction of Hg^{2+} to elemental form Hg^0 may occurs, as well as methylation of inorganic Hg, resulting in highly mobile organo-metallic species, such as methylmercury, which is the most toxic form of Hg (Clarkson and Magos 2006). Organo-metallic species are more readily assimilated by organisms than inorganic forms (Henriques et al. 2013), which enhances the risks associated with the entrance of Hg into the food chain – the main route of human exposure to this contaminant (mainly through the consumption of fish).

Alerted to the toxic effects of Hg, governmental institutions around the world have imposed increasingly tight limits for its concentration in industrial effluents. Recently European Union (EU) even brought a paradigm shift, ranking Hg as "priority hazardous substance" in the field of water policy, whose discharges should be ceased or eliminated progressively until 2021 (Decision No 2455/2001/EC). So, instead of limits for wastewater discharges, legislation now considers environmental quality standards (EQS) for this metal in surface waters (Directive 2013/39/EU 2013).

Physicochemical processes, conventionally used for metal removal, often have high operating costs, generate large amounts of sludge which require a proper disposal, or are ineffective when aim is to achieve very low residual levels (Velasquez and Dussan 2009, Farooq et al. 2010). The development of alternative treatment technologies, is therefore essential. In this sense, methods based on the use of biomasses or organic wastes – biosorption (Farooq et al. 2010), or even of live organisms – bioaccumulation (Chojnacka

2010), such as plants or macroalgae, largely available, may be the solution for reducing levels of Hg to acceptable limits, in a cost-effective and environmentally friendly way.

In the literature there is a considerable number of studies reporting the successful application of several biomasses in metal removal (Cheung et al. 2000, Ho and McKay 2004, Chojnacka 2007, Farooq et al. 2010), although less have been devoted to Hg (Rocha et al. 2013, Lopes et al. 2014). Furthermore, oddly the majority of works deals with synthetic water or freshwater, although most industrial effluents ends into estuaries or coastal areas (Torres et al. 2008), which are complex dynamic systems rich in salts. The focus on the removal of a particular contaminant in unrealistic and overly high concentration may also be pointed as a failure of most research works (Rocha et al. 2013).

In the last years, marine macroalgae have gained special attention in the field of water remediation (Romera et al. 2007, Freitas et al. 2008, He and Chen 2014), due to their remarkably ability to bind metals, which is attributed to the structure and chemical composition of their cell walls, offering different metal-binding sites (Hamdy 2000, Romera et al. 2007). However, almost all of those studies concern the use of dead biomass, neglecting the potential advantages of bioaccumulation, such as metal intracellular binding, which may lead to lower residual concentrations (Chojnacka 2010).

The study of metal accumulation by living organisms has been limited to microalgae (Flouty and Estephane 2012) and other microorganisms (Chojnacka 2007), or relates with field studies (Varma et al. 2011, El-Said 2012). Comparison between bioaccumulation and biosorption of metals by macroalgae is also virtually non-existent in the literature (Flouty and Estephane 2012).

There is a need for laboratory studies, performed under controlled conditions, discussing metal bioaccumulation kinetics (Chojnacka 2007, Costa et al. 2011), since most works only reports initial and final concentrations of metal (Velasquez and Dussan 2009) or present data as a function of time, discarding the mathematical modelling of the results, which relate to short exposure periods (Wang and Dei 1999, Singh et al. 2000).

Marine macroalgae may be divided into brown algae (Phaeophyta), red algae (Rhodophyta) and green algae (Chlorophyta), with differences on the cell wall, which may dictate the binding to a particular metal. Carboxyl and sulphate are the predominant functional groups in brown algae cell walls, which are mainly composed by cellulose, alginic acid and sulphated polysaccharides (Romera et al. 2007). Besides cellulose, red and green algae contain, respectively, agar and carragenates, rich in sulphated polysaccharides, and glycoproteins, which comprise amino, carboxyl, sulphate and hydroxyl groups (Romera et al. 2007). In addition, intrinsic growth rate and potential production of exudates (Coelho et al. 2005, Torres et al. 2008) may also influence Hg removal by each macroalgae specie. A better understanding of the mechanisms and kinetics of Hg accumulation by macroalgae is essential to develop a full-scale biotechnology, to be applied to water remediation.

Thus, the main objective of this study was to evaluate and compare the uptake and accumulation of Hg by three different living macroalgae, *Ulva lactuca* (green macroalgae), *Gracilaria gracilis* (red macroalgae) and *Fucus vesiculosus* (brown macroalgae) from Hg spiked saline waters, using environmental realistic concentrations. In addition, for the macroalgae with best performance, the study and comparison of biosorption and bioaccumulation processes (in terms of removal efficiencies and kinetics), as well as the assessment of potential Hg methylation during removal were also addressed.

3.2 Materials and methods

3.2.1 Material and chemicals

All chemical reagents used in this work were of analytical reagent grade, obtained from chemical commercial suppliers and were used without further purification. The certified mercury standard stock solution, containing 1.001±2 mg L^{-1} of Hg(II) in nitric acid 0.5 mol L^{-1}, was purchased from Merck. All working solutions, including standards for the calibration curves, were obtained by diluting the stock solution. All material used in the experiments was previously washed in Derquim 5% rinsed in Milli-Q water (18 MΩ cm^{-1}), soaked in 25% HNO_3 for at least 24h and subsequently rinsed with Milli-Q water. All glass vessels used in the experiments as reaction vessels or in the storage of the water samples were additionally soaked in concentrated HNO_3 (65%) for at least 24h, before reuse.

3.2.2 Macroalgae collection and maintenance

The green alga *Ulva lactuca* (Chlorophyta), the red alga *Gracilaria gracilis* (Rhodophyta) and brown alga *Fucus vesiculosus* (Phaeophyta) were collected in the Mondego estuary (Figueira da Foz, Portugal, 40°08′N, 8°50′W) and transported to the laboratory in isothermal plastic bags containing some local water. After rinse with seawater to remove debris and epibionts a small part of the algae was immediately freeze dried for subsequent quantification of the natural (baseline) concentrations of total and organometallic Hg. The remaining part was transferred to 30 L clear glass tanks equipped with air pump and filled with filtered seawater enriched with Provasoli stock solution (Costa et al. 2011). Until the beginning of the experiments the algae were maintained in the aquariums (water was changed weekly) under natural light (approximately 12L:12D), at room temperature of 20±2°C. Seawater needed for algae maintenance and for the sorption experiments was collected at Vagueira beach (18 km southwest of Aveiro, Portugal), filtered through 0.45 µm pore size filters and stored in the dark at 4°C until further use.

A brief characterization of seawater at 22ºC, including pH, salinity, conductivity and multi-elemental analysis was carried out. The pH (8.0), salinity (35 g L^{-1}) and conductivity (54.5 mS cm^{-1}) were recorded on a WTW meter. Concentrations of major (Ca, K, Mg, Na and Si) and minor elements (Al, As, B, Ba, Be, Cd, Co, Cr, Cu, Fe, Li, Mn, Ni, P, Pb, Sr, Vand Zn), obtained by inductively coupled plasma spectroscopy, using a Jobin – Yvon JY70 Plus Spectrometer (data not show), were in line with those reported by Lopes et al. (2014), which correspond to the natural levels in seawater at Portuguese coast. The concentration of Hg in the natural seawater was also determined using the methodology described below on 2.5.1 and the value found (3.1 ng L^{-1}) is typical of a non-polluted water.

3.2.3 Bioaccumulation experiments

Bioaccumulation tests were conducted in Schott Duran® glass bottles of 1 L, where each macroalgae species (alive) was placed individually in contact with natural seawater spiked with Hg. Three initial concentrations of Hg in seawater were tested, namely 10, 50 and 100 µg L^{-1}. These concentrations were considered environmentally realistic, intending to mimic real cases: 50 µg L^{-1} represent the "old" maximum permissible value for effluent discharges from industrial sectors (Directive 84/156/EEC 1984); 100 µg L^{-1} may represent a situation of an accidental discharge of untreated effluent and 10 µg L^{-1} may be the concentration in some point of the receiving aquatic ecosystem after the dilution of the discharged effluent into the water body. The spiked solutions were obtained by adding the desired volume of the Hg standard solution to filtered seawater. All the solutions were left to pre-equilibrate during 24h before the beginning of the experiments. An aliquot of each solution was collected immediately before adding the seaweeds to confirm the real concentration of Hg. Only a portion of algae was used in the experiments (Wang and Dei 1999). Small pieces with uniform size were cut from the healthy thallus and added to the spiked seawater, in a total of approximately 1.6 g L^{-1}, fresh weight (small discs with diameter (Ø) of 18 and 56 mm were used in the case of *U. lactuca*). This algae biomass does not jeopardize the normal macroalgae growth during experiments, as studies on *U. lactuca* biomass production (which has the highest growth rate of the three) reported maximum biomass yield for a stocking density 10 times higher (4 kg FW m^{-2} or 16 g FW L^{-1}) (Nikolaisen 2011). On the other hand the chosen amount took into account the minimization of the final residue, reducing the issues associated with its safe disposal. All algal pieces were acclimatized in clean seawater for several hours prior to mercury exposure. The uptake of Hg by the living macroalgae was followed during 6 days by determining the concentration of this metal in solution samples (5-10 mL) collected at defined time intervals. All the samples were acidified to pH ≤ 2 using Suprapur HNO_3 and stored at 4ºC until analysis. In the tests involving *U. lactuca* an algal disc (Ø=18mm) was also removed from the solution, at each sampling time, washed in clean seawater and freeze dried for later mercury quantification. The combined mass of all discs removed along the experiment represent less than 10% of the initial total biomass. All bioaccumulation trials were performed in triplicate, under natural light at room temperature of 20±2°C. Blanks (macroalgae in clean seawater) and controls (Hg spiked seawater in the absence of macroalgae) were always running in parallel with the experiments. No pH buffer was added to the system and no pH adjustments were made, since this could interfere with the Hg uptake and cellular metabolism of the organisms (Vasconcelos and Leal 2001, Velasquez and Dussan 2009). The initial pH of the solutions was 7.9 and slightly variations, although not significant, were observed in the course of the experiments (8.1±0.3). At the end of the experiments, macroalgae were harvested, rinsed and freeze dried for later quantification of total and organometallic Hg.

3.2.4 Biosorption experiments

The biosorption of Hg by *U. lactuca* was evaluated by placing its dead biomass (≈500 mg L^{-1}, dry weight) in contact with seawater spiked with Hg, in the same range of concentrations used for bioaccumulation. At defined time intervals, aliquots (5-10 mL) were collected from the system and filtered through acid-washed 0.45-µm Millipore membranes. Filtrates were immediately adjusted to pH<2 with Suprapur nitric acid, and stored at 4ºC until Hg quantification. The experiments were held under the same laboratory conditions mentioned above, although solutions were maintained under constant stirring. Blanks and controls, as described above, were always running in parallel with the experiments. The *Ulva* biomass was used as biosorbent without any specific pre-treatment. Macroalgae were simply washed with distilled water, air-dried during several days, dried at 40ºC for 24h and milled using a domestic coffee grinder.

3.2.5 Mercury quantification

3.2.5.1 Mercury concentration in solution

Mercury quantification in all water samples was performed by cold vapour atomic fluorescence spectroscopy (CV-AFS), using a PSA 10.025 Millennium Merlin Hg analyser and $SnCl_2$ (2% m/v in HCl 10% v/v) as a reducing agent. This system provides low detection limits and enables determinations in the range of ng L^{-1} to µg L^{-1}. The concentration of Hg in the collected samples was determined through a calibration curve ($r^2 \geq 0.999$), plotted and checked daily, using five standard solutions (0.0 to 0.5 µg L^{-1}). At least three replicate measurements were carried out for each sample (relative standard deviation among replicates: <5%). In this range, detection and quantification limits obtained through blank measurements (n = 20) were 0.007 µg L^{-1} (defined as the mean value plus 2.86 standard deviation) and 0.021 µg L^{-1} (defined as the mean value plus 10 standard deviation), respectively. The natural baseline value for total mercury in seawater was quantified after addition of 500 µL of a saturated solution of potassium persulfate to 50 mL of filtered seawater and irradiation by a UV lamp (1000 W) for 30 min. Following irradiation, the excess of oxidant was reduced with 37.5 µL of hydroxylamine solution 12% (w/v) (Pato et al. 2010) . The measurement was then performed by CV-AFS, using a calibration curve from 0 and 60 ng L^{-1}. In this range, the detection and quantification limits, as defined above, were 1.6 and 3.0 ng L^{-1}, respectively (n=15, 99.5% confidence level).

3.2.5.2 Total mercury concentration in algal biomass

Total mercury concentrations in macroalgae biomass were measured by thermal decomposition atomic absorption spectrometry with gold amalgamation using a LECO© AMA-254, as described by Costley et al. (2000). The analysis is performed directly in the sample (1 to 20 mg, dry weight) without digestion or pre-specific treatment, avoiding mercury losses or contamination as well as matrix interferences. At least three replicate measurements were carried out for each sample with an acceptable relative standard deviation among replicates: <10%. Detection and quantification limits, as described previously, were 0.01 ng Hg and 0.03 ng Hg, respectively (n=20, 99.5% confidence level). Several blanks (i.e. an empty sample nickel boat) were run before and between sample analyses to ensure that mercury was not being carried over between samples. The quality of the results was assessed by using the Certified Reference Material (CRM) BCR60 (*Lagarosiphon major*; 0.34 ± 0.04 mg kg^{-1} of total Hg). The CRM was analyzed every day prior to the beginning of the analysis and repeated at the end of the day. All percentages of recovery for total Hg were within the range of 85–107% (n = 17).

3.2.5.3 Organometallic mercury concentration in algal biomass

The fraction of organometallic mercury (OrgHg) in living *U. lactuca* before and after Hg exposure was assessed through a method described by Válega et al. (2006). Extraction of OrgHg mercury compounds from the sample matrix was achieved through digestion of 100–200 mg d.w. of each sample with a mixture of 18% KBr in 5% H_2SO_4 with $CuSO_4$ (1 mol L^{-1}), followed by extraction of OrgHg by toluene. The extractions were always performed in triplicate. The OrgHg mercury compounds retained in the toluene fraction were back extracted into an aqueous solution of thiosulphate. Mercury in liquid aliquots of the extracts (250–1000 μL) was then quantified as a whole by thermal decomposition atomic absorption spectrometry with gold amalgamation (LECO model AMA-254). To ensure the quality of results procedural blanks (i.e. procedure with the reagents only) and reference material TORT-2 (*Lobster Hepatopancreas*; 0.152 ± 0.013 mg kg^{-1} of methylmercury) were always carried out in parallel with the samples and analysed. The procedural blanks were always below the detection limit of the equipment (0.01 ng Hg). The recovery for OrgHg mercury (assuming that the dominant form in this fraction is methylmercury (Ullrich et al. 2007, Nam and Basu 2011)) was in the range 87–90% (n = 10).

3.2.6 FTIR spectra measurements

FTIR spectra of *U. lactuca* before and after exposure to Hg were recorded by using a Bruker optics tensor 27 spectrometer coupled to a horizontal attenuated total reflectance (ATR) cell using 256 scans at a resolution of 4 cm^{-1}.The samples were examined directly and data were obtained as transmittance. The analysis of FTIR spectra was carried out by examining the spectral bands that are modified (Bulgariu and Bulgariu 2014).

3.2.7 Analysis of experimental data

When macroalgae are exposed to seawater contaminated with Hg, uptake and accumulation of this metal by macroalgae is expected to occur, leading to a decrease of its concentration in solution over time (and to simultaneous increase of Hg content in the biomass). Uptake will persist until all the Hg be removed from the solution or until an equilibrium state be attained, assuming that necessary time for this has elapsed. Thus mercury uptake/accumulation by macroalgae defined as the amount of Hg bound by unit of mass at a given time t (q_t, μg g^{-1}) was deduced from the mass balance between the initial Hg concentration in the solution (C_0, μg L^{-1}) and the concentration after a particular period of contact time t (C_t, μg L^{-1}):

$$q_t = \frac{(C_0 - C_t)V}{m} \qquad (1)$$

where V (L) is the volume of the solution and m (g) is the macroalga biomass, in dry weight. When equilibrium was attained, $t=t_e$, $q_t=q_e$ and $C_t=C_e$ (residual Hg concentration in solution). The performance of the removal process was also evaluated and compared using the Hg removal percentage (R, %), which at time t is defined by:

$$R_t\ (\%) = \frac{(C_0 - C_t)}{C_0} \times 100 \quad (2)$$

3.2.7.1 Biosorption and bioaccumulation kinetic models

Kinetics of Hg removal process using non-living biomass (biosorption) and using live biomass (bioaccumulation) were described by applying three of the most common kinetic reaction models, in their non-linear forms (El-Khaiary and Malash 2011), namely, Lagergren pseudo-first-order model (Lagergren 1898), Ho's pseudo-second order model (Ho and McKay 1999) and Elovich model (Ho 2006) to results.

The pseudo first-order model (PFO), firstly used by Lagergren, is mathematically expressed by:

$$\frac{dq_t}{dt} = k_1(q_e - q_t) \quad (3)$$

where k_1 (h^{-1}) is the rate constant of pseudo-first order. Its non-linear form, obtained after integration and application of the boundary condition q_t=0 at t=0, is:

$$q_t = q_e(1 - e^{-k_1 t}) \quad (4)$$

The pseudo second-order (PSO) was proposed by Ho, and contrary to the previous model it usually represents the sorption evolution along full time range (Ho et al. 2000). The kinetic rate equation is expressed as:

$$\frac{dq_t}{dt} = k_2(q_e - q_t)^2 \quad (5)$$

where k_2 (g µg^{-1} h^{-1}) is pseudo-second order rate constant. The integrated form of Eq. 5, after applying the boundary conditions t=0 to t=t and q_t=0 to q_t=q_e, is:

$$q_t = \frac{q_e^2 k_2 t}{1 + q_e k_2 t} \quad (6)$$

When q/t approaches zero, the initial sorption rate (h) is expressed by the following equation:

$$h = k_2 q_e^2 \quad (7)$$

Elovich model was established by Zeldowitsch to describe kinetics of gas chemisorption onto solids (Ho 2006), but in recent years it as has also been used to describe the sorption of contaminants from aqueous solutions (Ho and McKay 2004). Elovich model is defined by the following equation:

$$\frac{dq_t}{dt} = \alpha e^{-\beta q_t} \quad (8)$$

where q_t is the quantity of Hg adsorbed during the time t, α (μg g^{-1} h^{-1}) is the Elovich initial sorption rate at zero coverage, and β (g μg^{-1}) is the Elovich desorption constant related to the extent of surface coverage and activation energy for chemisorption. Integrating Eq. 6, by using the boundary conditions of $q=0$ at $t = 0$ and $q = q$ at $t = t$, and assuming that $\alpha\beta t >> 1$, the simplify form of Elovich's equation is expressed by:

$$q_t = \frac{1}{\beta} \ln(1 + \alpha\beta t) \quad (9)$$

3.2.7.2 Statistical analysis

All statistical data analysis was performed using GraphPad 6.0. This tool was also used to plot all graphs as well as to calculate all kinetic parameters through nonlinear regression. GraphPad 6.0 uses the least-squares as fitting method and the method of Marquardt and Levenberg, which blends two other methods, the method of linear descent and the method of Gauss-Newton for adjusting the variables. Coefficient of determination (R^2) and standard deviation of residuals ($S_{y.x}$) were analysed in order to evaluate the goodness of fit and to compare models. The relative error (Er) between experimental and predicted values of q_e was also calculated. These statistical parameters can be mathematically defined by:

$$R^2 = 1 - \frac{\sum (y_i - \hat{y})^2}{\sum (y_i - \bar{y})^2} \quad (10)$$

$$S_{y.x} = \sqrt{\frac{(y_i - \hat{y})^2}{df}} \quad (11)$$

$$E_r = \frac{|y_i - \hat{y}|}{y_1} \times 100 \quad (12)$$

where y_i are the experimental data values, $\hat{y}$ are the predicted values, $\bar{y}$ is the mean of the experimental data and *df* is the number of the degrees of freedom (equal to number of data points minus the number of parameters fit).

3.3 Results and discussion

3.3.1 Mercury uptake by three macroalgae species

Figure 3.1A, B and C shows the uptake/accumulation of Hg by living *U. lactuca*, *G. gracilis* and *F. vesiculosus* over time, when exposed to Hg spiked seawater, at different concentrations, namely, 10, 50 and 100 µg L-1. Values of q_t were obtained through mass balance using Eq. (1). Baseline concentrations of Hg in macroalgae, obtained through analysis of their biomass after collection in the field were: *U. lactuca*, 0.029 ±0.002 µg g^{-1}; *G. gracilis*, 0.047 ±0.003 µg g^{-1}; *F. vesiculosus*, 0.045 ±0.002 µg g^{-1}. These values are typical of macroalgae growing on no-polluted sites (Coelho et al. 2005,Eisler 2010).

In general, accumulation profiles for the three macroalgae present similarities, i.e. initially for all concentrations there was a sudden and noteworthy uptake of Hg, followed by a phase where the rate of accumulation decreased until a plateau is reached and Hg content in biomass remained invariable. The remarkable uptake observed in the first hours of contact is explained by the high driving force at the beginning of the sorption process, since at that time macroalgae surface was Hg-free. Thus, at this stage uptake process is essentially passive (metabolic-independent), of physical-chemical nature. Afterwards, external bonding and internal accumulation (metabolic-dependent) of Hg occur simultaneously (Andrade et al. 2006,Chojnacka 2007) until the steady-state is achieved, corresponding to low concentrations of Hg in solution (Figure 3.1D, E and F).

For all macroalgae, initial concentration of Hg in solution had an effect on Hg accumulation. For a given time, greater amount of Hg was always sorbed with the increase on initial concentration of Hg in solution (dose-dependent accumulation). This enhanced uptake is attributed to the fact that higher concentration provides great driving force to overcome all mass transfer resistances of Hg between solution and macroalgae (Rocha et al. 2013). In addition, none of the macroalgae species seemed to prevent or restrict accumulation. So, the amount of Hg stored by algae is closely related to its initial concentration in solution ($0.998<R^2<0.999$; $P<0.001$). It should be noted that no adverse effects caused by the accumulation of Hg, such as sporulation or marked loss of color which indicates algae decay (Han and Choi 2005) were observed for the studied concentrations during the trials (lasting 6 days).

Bioconcentration factors (BCF), defined as the relation between the concentration of Hg in macroalgae biomass at the end of exposure and the initial concentration of Hg in solution, varied between 1870 and 2267 for all seaweeds and Hg concentrations. These results are indicative of the great affinity existing between these seaweeds and Hg, and are in agreement with those studies which support the use of macroalgae as biomonitors of metal contamination in coastal waters (Wang and Dei 1999, Barreiro et al. 2002, El-Said 2012). A few field studies reported that metal concentrations in algae tissues react faithfully to gradients of metal concentration in the surrounding environment (Coelho et al. 2005, Varma et al. 2011). In fact, metal content in a good biomonitor should be directly proportional to the bioavailable metal concentration in water column (Wang and Dei 1999), requirement which was fulfilled by the three macroalgae here studied.

However, some differences on Hg uptake rate were observed among macroalgae species. For the lowest Hg concentration, there were significant differences in the Hg removal pattern, along contact time, between the green macroalga and the other two species (ANOVA, Wilcoxon matched-pairs signed rank test, two-tailled; $P<0.01$). After a period of contact of just 6 h, Hg level in *U. lactuca* was 11.3 µg g^{-1}, corresponding to 48% of removal (6 µg L^{-1} of Hg remained in solution). At the same time, *G. gracilis* removed c.a. 30% and *F. vesiculosus* just 23%, which correspond to accumulations of 6.6 and 5.3 µg Hg g^{-1}, respectively. After 72h of exposure, the percentages of removal for *F. vesiculosus* and *G. gracilis* were, respectively, 88% (q_t= 20.0 µg g^{-1}) and 87% (q_t= 19.9 µg g^{-1}), whereas *U. lactuca* achieved similar removal efficiency after just 48h. The differences on Hg accumulation between the three algae vanished after 96h, mainly because Hg concentrations in solution were already very low and the removal process was close to reach a point of equilibrium (0.31 µg L^{-1} < C_e< 0.84 µg L^{-1}).

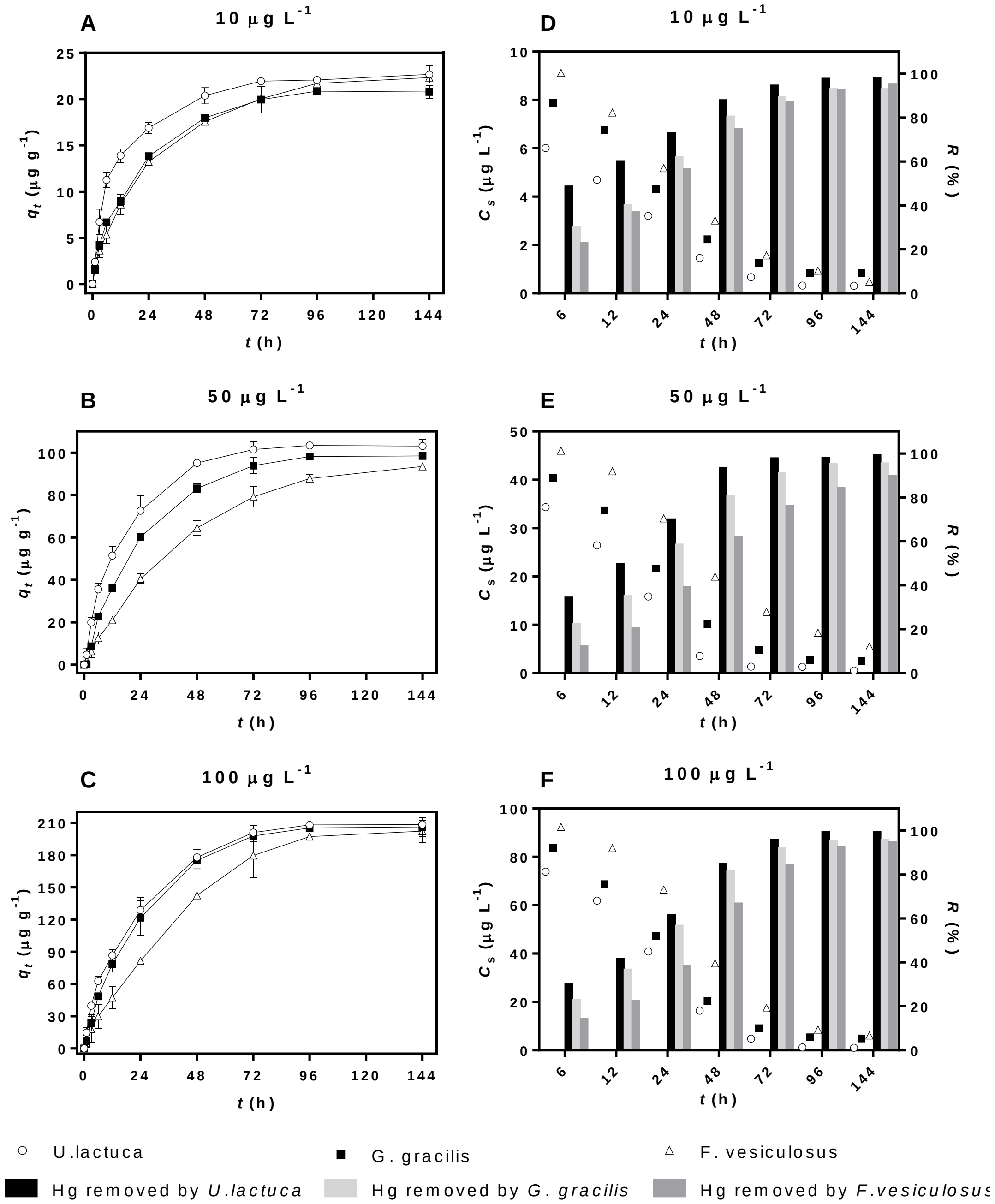

Figure 3.1: Hg accumulation (q_t, expressed in µg g^{-1}, dry weight) by three living algae (○ – *U. lactuca*; ■ - *G. gracilis*; Δ -*F. vesiculosus*) over time (t, h), when in contact with spiked seawater (A, B and C). The results are expressed as the mean ± standard deviation (n=3). The variation of the concentration of Hg in seawater (C_S, expressed in µg L^{-1}) as well the percentage of Hg removed between 6 and 144h of contact are also presented in D, E and F (for clarity the error bars were omitted).

The removal kinetic behaviour was also significantly different between the three species for 50 µg L^{-1} (ANOVA, Wilcoxon matched-pairs signed rank test, two-tailled; P<0.01). About 70, 58 and 38% of Hg was removed after 24 hours of contact time with green, red and brown algae, respectively, leading to Hg concentrations in solution of 15.8, 21.7 and 32.0 µg L^{-1}, and accumulations of 72.6, 60.2 and 40.5 µg Hg g^{-1}, respectively. 72h was the period of time required for *U. lactuca* to incorporate 98% of the all Hg originally in solution (q_t= 101.5 µg g^{-1}, C_t=1.3 µg L^{-1}). After the same period of time *G. gracilis* removed 91% (q_t=93.9 µg g^{-1}, C_t=4.8 µg L^{-1}), while the removal efficiency for *F. vesiculosus* was limited to 76% (q_t= 79.2 µg g^{-1}, C_t=12.7 µg L^{-1}).

For the highest initial concentration, despite visual similarities on Hg uptake profiles, over the entire range of time, between *U. lactuca* and *G. gracilis*, Wilcoxon matched-pairs signed rank test indicates significant differences among these two macroalgae (P=0.0039). Removal percentages after 24h of contact time were between 56% and 61% (q_t (*G. gracilis*)=121.6 µg g^{-1}; q_t (*U. lactuca*)= 128.8 µg g^{-1}) reaching equilibrium values (at 96h) of 95% (q_t = 205.2 µg g^{-1}, C_t=5.0 µg L^{-1}) and 99% (q_t = 208.1 µg g^{-1}, C_t =1.1 µg L^{-1}) for *G. gracilis* and *U. lactuca*, respectively. Once again *F. vesiculosus* was the slowest in incorporate Hg from the solution, whose removal efficiencies and accumulations after 24h and 96h of exposure were, respectively, 32%, 81.5 µg g^{-1} and 92%, 197.3 µg g^{-1}.

Overall, bioaccumulation capacities of Hg (q_e) for the three living macroalgae, determined at the end of the trials, are in the range of 20.8-22.7, 93.5-103.2 and 202.3-208.5 µg g^{-1}, for initial Hg concentrations of 10, 50 and 100 µg L^{-1}, respectively. These results may be of great value for studies concerning transference and bioconcentration of Hg along a coastal trophic web.

In all cases, *U. lactuca* proved to be faster in removing Hg from the solution, which can be attributed to its specific characteristics such as metabolism and surface area. In fact, this green macroalga is known to possess very high growth rates (Villares et al. 2001, Costa et al. 2011, Nikolaisen 2011), suggesting that *U. lactuca* will be faster to assimilate essential elements as well as toxic metals from the surrounding medium (Torres et al. 2008). So, *U. lactuca* will reflect punctual changes in environmental contamination faster than perennial, slow growing macroalgae such as *G. gracilis* and *F. vesiculosus* (Coelho et al. 2005). In addition, its thin and sheet-like thallus translates into a large surface area, with structurally uniform and physiologically active cells acting as “biofilters”. Sorption area to volume ratio also explains why living *G. gracilis* performed better on removing Hg than living *F. vesiculosus*, despite some studies on metal sorption by dead algae biomass report that brown algae have higher sorption capacities than any other algae (mainly due to the presence of alginates in their cell wall) (Davis et al. 2003, Romera et al. 2007). *F. vesiculosus* have a more compact structure (less surface area) than *G. gracilis*, whose thin thallus with several growing cylindrical branches covers a higher volume of solution (less mass transfer resistance).

Besides environmental monitoring aspects, results may be important for elaborating wastewater treatment techniques based on the use of live macroalgae. From this point of view, *U. lactuca* stands out, not only because of the faster Hg removal, but also it was the only one who, for all experimental conditions, led to residual concentrations of Hg in solution equal or lower than 1 µg L^{-1}, which is the guideline value for drinking water quality (Council Directive 98/83/ EC). Moreover, this green alga proved to be the easiest to maintain in laboratory (being more tolerable to changes in temperature and luminosity). Cultivation of *U. lactuca* is well documented (Nielsen et al. 2012), being that of the macroalgae studied so far, it has the highest annual yield (up to 45 tons dry matter per hectare were reported by Nikolaisen (2011)). Another huge advantage for adopting this species is its high assimilation of CO_2 (production of 1 ton of algae takes up about 1.5 ton of CO_2) (Nikolaisen 2011), which contributes to compliance of mandatory CO_2 reductions.

On this basis following studies were focused on *U. lactuca*, evaluating the perspectives of using live seaweed (bioaccumulation) instead of its dead biomass (biosorption) to promote Hg removal from saline waters.

3.3.2 *Ulva lactuca*: comparison between biosorption and bioaccumulation of Hg

Figure 3.2 depicts the kinetic curves (C_t *vs.* t) for different initial concentrations of Hg in seawater: 0, 10, 50 and 100 µg L^{-1}, when in contact with dead biomass or with live biomass of *U. lactuca*. Data regarding controls are also presented, revealing that for all concentrations, Hg losses were minimal and were under control over the full time range. Results for 0 µg L^{-1}, which corresponds to Blanks, confirms that macroalgae (dead or alive) did not constitute a Hg source to solutions, and prove that care taken throughout the study, to reduce uncertainties associated with potential contamination issues, took effect.

For the remaining concentrations, regardless of the removal process, kinetic profiles are characterized by a rapid and marked decrease in the concentration of Hg, C_t, in the initial period (C_0-dependent), which represents a rapid metal biosorption/bioaccumulation by macroalgae. This step was followed by a subtle decrease in C_t until equilibrium was established. After a contact time of 6 hours between dead macroalgae and contaminated seawater, initial concentrations of Hg of 10, 50 and 100 µg L^{-1} were reduced to 2.9, 26.2 and 64.5 µg L^{-1}, respectively. For the same range of initial concentrations and equal period of time, living *U. lactuca* led to concentrations of Hg in solution of 6.0, 34.3 and 77.2 µg L^{-1}. Removal efficiencies for dead and living macroalgae after 12h were, respectively, 80 and 60% $C_{0,Hg}$ of 10 µg L^{-1}, 60 and 48% for $C_{0,Hg}$ of 50 µg L^{-1} and 45 and 37% for $C_{0,Hg}$ of 100 µg L^{-1}.

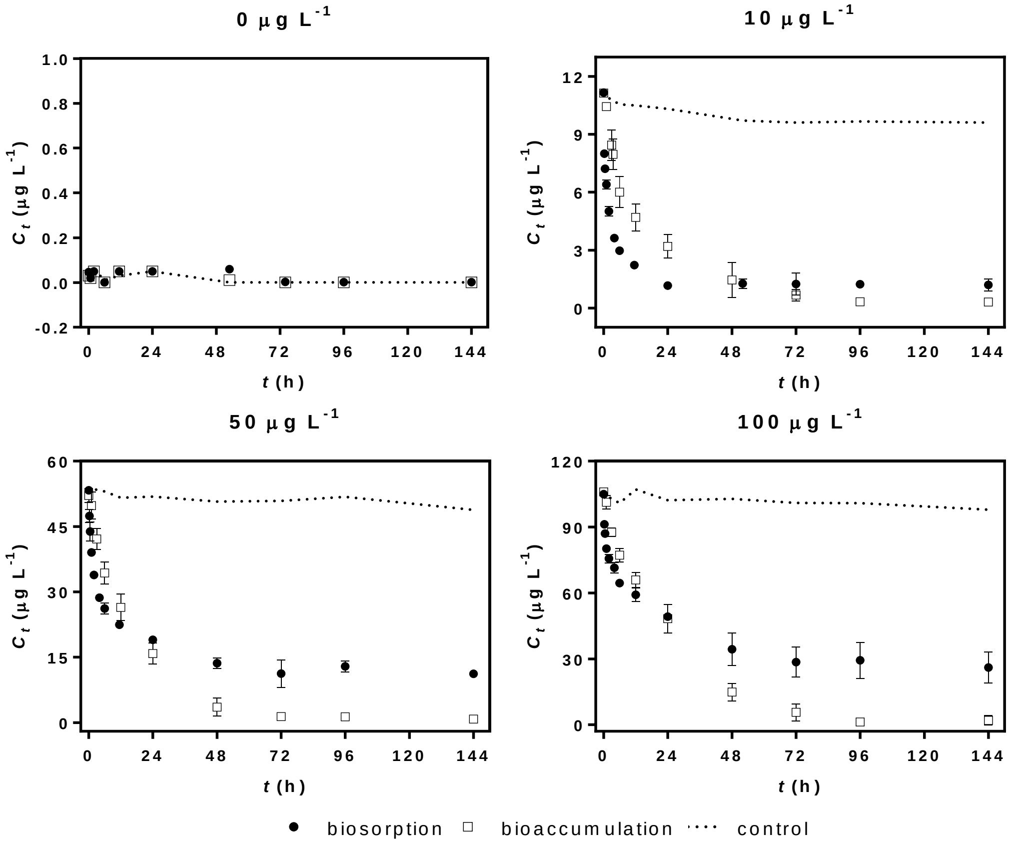

Figure 3.2: Hg concentration (C_S, expressed in µg L^{-1}) in spiked seawater during the contact with living *U. lactuca* (□ – bioaccumulation) and with its dead biomass (● – biosorption). The results are expressed as the mean ± standard deviation (n=3). The dashed line (…) corresponds to the control, i.e. spiked seawater without algae.

Biosorption is therefore a quicker process, which may be explained by the fact that only physicochemical mechanisms are involved on Hg uptake (Chojnacka 2010), whereas some biological efflux mechanisms may occur in bioaccumulation (Vasconcelos and Leal 2001, Velasquez and Dussan 2009). Additionally, the use of dead *U. lactuca* in granular form, which increases its surface area (Lopes et al. 2014), provided a greater number of available sorption sites, at the beginning of the sorption process, in relation to bioaccumulation (Figure 3.2).

Steady state was also reached more rapidly in biosorption trials (Figure 3.3A), although it has resulted on higher residual levels of Hg in solution (Figure 3.3B). The period of time needed to attain equilibrium, t_e, was dependent on the initial concentration of Hg in solution, ranging between 24 to 72h for Hg concentrations of 10 to 100 µg L^{-1}, respectively. It should be noted that equilibrium does not mean that all binding sites on macroalgae surface were occupied, since increasing initial Hg concentration resulted in higher values of q_e (Figure 3.3C). In fact, as biosorption occurs surface charge will increase and electrostatic interactions become more intense, leading to an equilibrium between q_t and C_t (Rocha et al. 2013).

The removal process relying on the use of live *U. lactuca* lasted longer (between 72 to 96h until reach equilibrium), nevertheless with a better performance than biosorption, since for all experimental conditions, Hg levels were reduced by 97-99% (Figure 3.3D). With dead macroalgae, only for the lowest Hg concentration it was accomplished a removal efficiency above 90%, corresponding to a final concentration of Hg in solution of 1.2 µg L^{-1}, which is very close to the limit value set by the European Union for waters for human consumption, 1 µg L^{-1} (Directive 98/83/EC). The superior performance of bioaccumulation against biosorption is due to its additional stage of internal accumulation. Mercury bound on the surface was actively transported into the cells through helper proteins (Velasquez and Dussan 2009) under the same mechanism by which essential metals are transported across membranes (Sunda and Huntsman 1998, Torres et al. 2008). As consequence, binding sites on the surface are released (regeneration) and equilibrium shifts towards lower concentrations of Hg in solution. Organism growth, i.e. cellular multiplication, leads to an additional rise on the number of available binding sites for Hg, also contributing to greater metal ion binding capacity, q_e (Chojnacka 2010). Similar assumptions were made by Chojnacka (2007) when comparing the biosorption and bioaccumulation of Cr(III) by blue-green alga *Spirulina* sp.. According to the author, the higher bioaccumulation capacity in relation to biosorption was justified by the transfer of metal from the outside into the inside of cells and by microbial growth.

On the contrary, Kadukova and Vircikova (2005) pointed to some disadvantages of the application of living algal cells (*Chlorella kessleri*) to promote Cu removal from solution. Authors reported that Cu significantly damaged the surface of living cells, which resulted in partial loss of cell-binding abilities and release of accumulated copper back into solution. Results obtained by Flouty and Estephane (2012) also showed higher metal removal efficiencies for dead cells of *Chlamydomonas reinhardtii* than for living cells, and thus it was concluded that biosorption was the most advantageous process in that case.

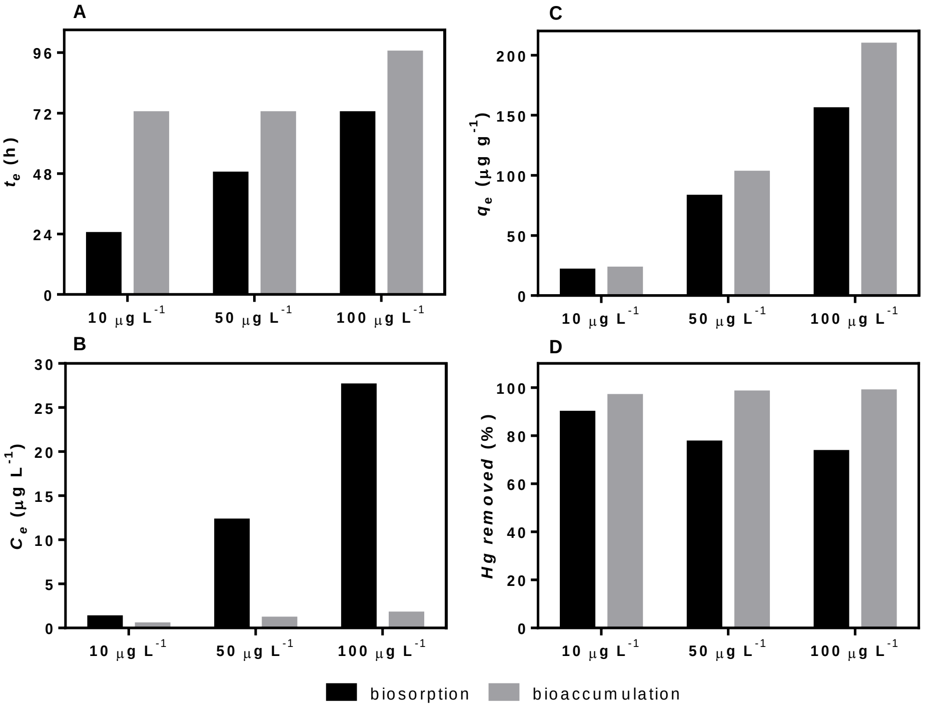

Figure 3.3: Equilibrium data on Hg uptake by dead (biosorption) and living (bioaccumulation) biomass of *U. lactuca*: percentage of Hg removed (A), amount of Hg sorbed per unit of alga biomass (B), time elapsed until the equilibrium state was reached (C) and residual concentration of Hg in seawater (D). Experimental conditions: initial concentration of Hg in seawater 10, 50 and 100 µg L^{-1}; amount of algae used ≈ 0.5 g L^{-1}, dry weight.

In fact, bioaccumulation as a full remediation process brings great advantages but only if the contaminated water fulfils the criteria of minimal growth medium and exerts no critical toxic effect to cells (at least until removal process is finished and organism is harvested), which was verified for *U. lactuca* and for the full range of experimental conditions studied. It should be highlighted that the concentrations of Hg used in this work were chosen to be representative of real scenarios, aiming that results can be translated into the real world. For the same reason, real seawater was used in the experiments (not synthetic water), having been added only the contaminant. Indeed, a failure existing in the literature regarding metal sorption is the focus on initial concentrations extremely larger than those that may be found in effluents or aquatic systems affected to wastewater discharges (Rocha et al. 2013). Although high removal percentages (> 90%) are reported, the final levels of contaminant in solution still are, in most cases, higher than that allowed by law, thus missing the quality criteria. For the same reasons, the comparison between removal performances of living *U. lactuca* and other commonly used biosorbents is not an easy and always feasible task.

For effluents with larger load of Hg, which eventually cause cell disruption, living *U. lactuca* may be used in the final stage of treatment, i.e. as polishing step after application of traditional methods (Chojnacka 2010), which are not capable (or become prohibitively expensive) of removing Hg at low concentrations (Farooq et al. 2010), or at levels required by the law.

3.3.2.1 Kinetic modelling

The fittings of experimental data, on Hg uptake/accumulation along time by dead biomass and by live biomass of *U. lactuca*, to PFO, PSO and Elovich models are presented in Figure 3.4A-F. The estimated values for the different kinetic parameters as well as the goodness of fit and experimental q_e are summarized in Table 3.1.

Of the three kinetic models studied, PFO model presents the poorest performance on describing the biosorption of Hg, over the whole period of time and range of concentrations studied (Figure 3.4A-C), which is corroborated by the lowest values of R^2 (0.868-0.935) and the highest values of $S_{y.x}$ (2.05-20.8). This may be indicative that more than one sorption mechanism (e.g ion-exchange, surface precipitation, redox reaction) is involved in the Hg uptake process (Cheung et al. 2000, Chojnacka 2010). Furthermore, the amount of Hg sorbed by the dead biomass of *U. lactuca* at equilibrium, q_e, is always underestimated by this model, with relative errors of 5.7, 7.1 and 9.0%, for initial Hg concentrations of 10, 50 and 100 µg L^{-1}, respectively. On the contrary, PSO model accurately predicts all values of q_e, with a maximum relative error of 1.9%. In addition, it provides the best fit to the results obtained for 10 µg L^{-1}.

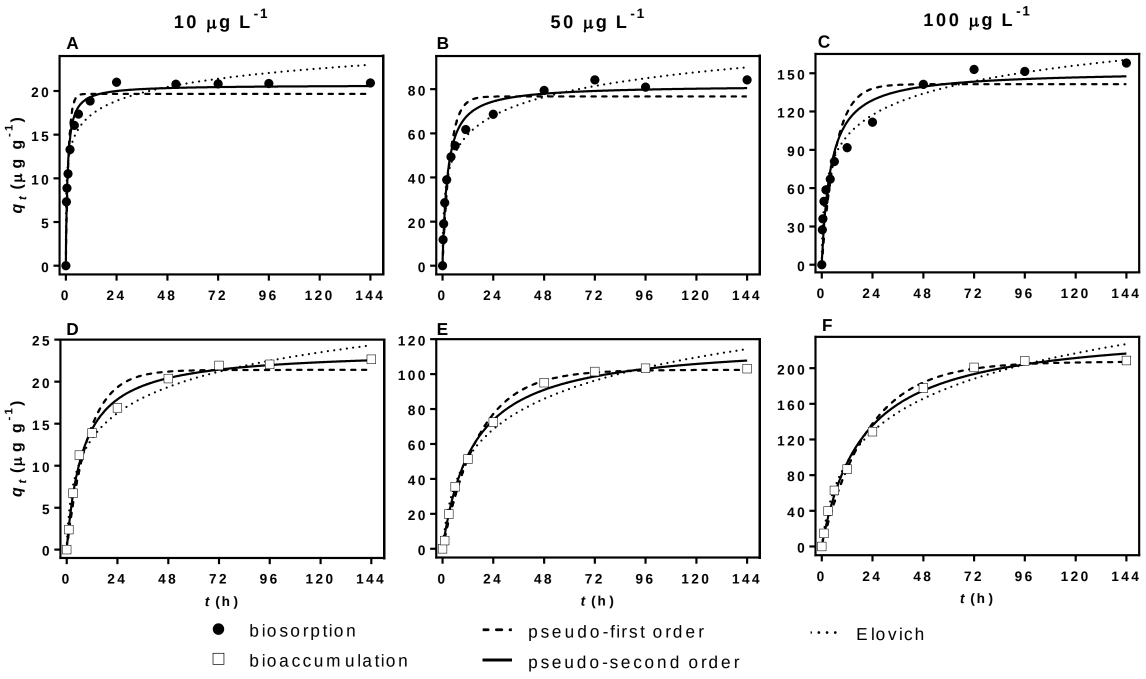

Figure 3.4: Kinetic modelling of the experimental data regarding the uptake of Hg by non-living (●) and living (□) biomass of U. lactuca during the contact (t, h) with spiked seawater. The following three kinetic models were applied: pseudo-first order (---), pseudo-second order (▬) and Elovich (…).Experimental conditions: initial concentration of Hg in seawater 10, 50 and 100 µg L^{-1}; amount of algae used ≈ 0.5 g L^{-1}, dry weight.

For the highest initial concentrations of Hg, a better agreement is observed between the biosorption results and the fittings accomplished by the Elovich model (R^2=0.987), which reinforces a heterogeneous sorption mechanism (Cheung et al. 2000). Overall, results of kinetic modelling assume a chemical sorption or chemisorption, i.e. suggest that the rate-limiting step in the Hg biosorption is a chemical interaction between Hg ions and superficial functional groups of the biosorbent (Bulgariu and Bulgariu 2014), which involves valence forces through sharing or exchange of electrons, complexation, coordination and/or chelation, rather than physisorption (Ho and McKay 1999). Similar behaviour and assumptions have been reported for the dead *U. lactuca* applied to the removal of Cd, Pb, Zn and Co (Sari and Tuzen 2008, Bulgariu and Bulgariu 2014) as well as for various types of algae used as biosorbents (Freitas et al. 2008, He and Chen 2014).

For bioaccumulation, all kinetic models adequately fit the experimental results over the entire course of experiments, although PSO model has slightly better performances, as shown by the goodness of fit in Table 3.1. However, the theoretical values of q_e, predicted by the PFO model are closer to the experimental q_e values (maximum relative error of 4.5%)

than those overestimated by PSO model (relative errors between 5.5 and 15%). The kinetic constants of the PFO (k_1) and PSO (k_2) kinetic models (Table 3.1) are in accordance with the considerably faster Hg removal observed early in the biosorption process compared to the process of bioaccumulation. The values of initial sorption rate, *h*, respectively 24.8, 33.5 and 46.2 µg g^{-1} h^{-1} for biosorption and 2.8, 8.4 and 12.1 µg g^{-1} h^{-1} for bioaccumulation, corresponding respectively to initial concentrations of Hg in solution of 10, 50 and 100 µg L^{-1}, also corroborate that observation.

In the literature, unlike what is observed for biosorption, there is a scarcity of systematic studies concerning the bioaccumulation of metals, which include mathematical modelling of the kinetics (Chojnacka 2007), especially in experiments involving live macroalgae. An exception is the work of Vasconcelos and Leal (2001) on the accumulation of Cu, Pb, Cd and Hg by macroalgae *Porphyra spp.* and *Enteromorpha spp.*, although exposure experiments lasted only up to 24h. The authors assumed that metal sorption by the macroalgae followed PFO rates, and identified three linear segments in the modelled curves with different (decreasing) slopes, corresponding to different uptake rates (Vasconcelos and Leal 2001).

Overall, the present kinetic results are in accordance with the idea that mechanisms controlling the Hg bioaccumulation rate by living macroalgae shall be the same which are involved in the biosorption of Hg by the dead macroalgae (Vasconcelos and Leal 2001, Chojnacka 2007), with the addition of intrinsic metabolic mechanisms. As stated earlier, initial rapid uptake will correspond to chemical sorption on cell surface, and eventually to simple diffusion into cells or intercellular spaces. The slow uptake will correspond to metabolism-dependent incorporation into cells (and eventually continuous or occasional excretion of Hg) (Vasconcelos and Leal 2001). Literature reports that cellular membrane is virtually impermeable to charged or highly polar neutral species, and thus metal ions bind to specialized membrane proteins and then either dissociate back into the medium or are transported and released into the cytoplasm (Sunda and Huntsman 1998). However, some neutrally charged, non-polar complexes, such as $HgCl_2$, can diffuse directly across cell's lipid bilayer without the help of those membrane proteins, in a simple and metabolic-passive diffusion process. Once inside the cell, $HgCl_2$ may react with biological ligands, such as sulfhydryls and be internally fixed (Sunda and Huntsman 1998, Vasconcelos and Leal 2001). Studies have demonstrated that for seawater, chlorocomplexes of Hg dominates the Hg speciation in the medium (Turner 1987, Mason et al. 1996). Yet, simulations performed in Visual MinteQ showed that for the experimental conditions used, most of the mercury was in the form of negatively charged chlorocomplexes (about 98%), $HgCl_3^{-1}$ and $HgCl_4^{-2}$. Thus, simple diffusion of neutral species of Hg, such as $HgCl_2$, only accounts for a small part (up to 2%) of the all Hg transported into cells.

Table 3.1: Kinetic constants obtained by fitting the experimental data on Hg uptake by non-living and living biomass of *U. lactuca* to the following models: Pseudo-first order, Pseudo-second order and Elovich.

			Non-living biomass of *U. lactuca*			Living biomass of *U. lactuca*		
		Initial [Hg] in seawater	10 µg L^{-1}	50 µg L^{-1}	100 µg L^{-1}	10 µg L^{-1}	50 µg L^{-1}	100 µg L^{-1}
		q_e exp ± SD (µg g^{-1})	20.9 ± 0.08	82.2 ± 1.57	155± 3.37	22.5 ± 0.353	102 ± 0.679	209 ± 0.808
Pseudo-first order	Best-fit values	$q_{e\,1}$ ± SD (µg g^{-1})	19.7 ± 0.753	76.4 ± 3.27	141 ± 9.18	21.4 ± 0.676	102 ± 1.65	207 ± 5.10
		k_1 ± SD (h^{-1})	0.785 ± 0.152	0.290 ± 0.051	0.154 ± 0.039	0.097 ± 0.012	0.058 ± 0.003	0.046 ± 0.004
	Goodness of Fit	R^2	0.914	0.935	0.868	0.977	0.996	0.992
		Sy.x	2.05	7.74	20.8	1.36	2.90	8.00
Pseudo-second order	Best-fit values	$qe_2 \pm SD$ (µg g^{-1})	20.7 ± 0.465	81.8 ± 2.06	152 ± 8.28	23.8 ± 0.397	119 ± 2.54	246 ± 6.75
		k_2 ± SD (g µg^{-1} h^{-1})	0.058 ± 0.008	0.005 ± 0.0007	0.002 ± 0.0004	0.005 ± 0.0004	0.0006 ± 0.00005	0.0002 ± 0.00005
	Goodness of Fit	R^2	0.975	0.981	0.929	0.996	0.996	0.995
		Sy.x	0.638	4.13	14.6	0.571	2.79	6.29
Elovich	Best-fit values	$\beta \pm SD$ (g µg^{-1})	0.434 ± 0.038	0.081 ± 0.004	0.041 ± 0.002	0.219 ± 0.016	0.038 ± 0.004	0.017 ± 0.001
		$\alpha \pm SD$ (µg g^{-1} h^{-1})	348± 197	129 ± 26.2	129 ± 24.5	6.55 ± 1.51	13.4 ± 3.24	19.9 ± 3.55
	Goodness of Fit	R^2	0.960	0.987	0.987	0.985	0.980	0.988
		Sy.x	1.40	3.42	6.27	1.10	6.30	9.66

Experimental conditions: initial concentration of Hg in seawater 10, 50 and 100 µg L^{-1}; amount of algae used ≈ 0.5 g L^{-1}, dry weight; Experimental *qe* exp and the goodness of fit were also presented in order to assess and compare models.

3.3.3 Total mercury concentration in the living *U. lactuca*

Total Hg concentrations of the algae discs collected during bioaccumulation trials are presented in Figure 3.5A-C, along with the corresponding theoretical concentrations, deduced through mass balance analysis, using Eq. 1. Results confirm our assumption that Hg removed from seawater along time was being incorporated by macroalgae. Over the entire course of experiments and range of concentrations studied, no significant differences were observed between time profiles representing mass balance and direct analysis (ANOVA, Wilcoxon matched-pairs signed rank test, two-tailled; $P<0.01$). The occasional and slight deviations observed between the curves may be attributed to variations in luminosity and growth rate among replicates, factors known to influence the bioaccumulation behaviour (Wang and Dei 1999).

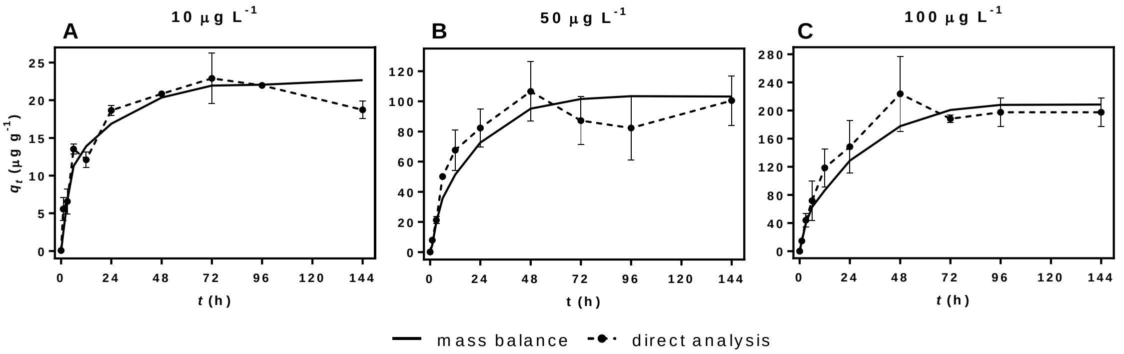

Figure 3.5: Concentration of Hg in the living *U. lactuca* (µg g^{-1}) along the contact time with spiked seawater: 1) deduced by mass balance using Eq. 1 (— mass balance); 2) analytically quantified through direct analysis of the biomass using LECO AMA-254 © (-●- direct analysis). The results are expressed as the mean ± standard deviation (n=3); Experimental conditions: initial concentration of Hg in seawater 10 (A), 50 (B) and 100 (C) µg L^{-1}; amount of algae used ≈ 0.5 g L^{-1}, dry weight.

At the end of exposure trials, contents of Hg determined in macroalgae discs were 18.7 ± 1.14, 100.5 ± 16.4 and 197.7 ± 20.2 µg g^{-1} for initial concentrations of 10, 50 and 100 µg L^{-1}, respectively. These values nearly match with those obtained through mass balance, with the exception of the value achieved for 10 µg L^{-1} (Figure 3.5A), which was slightly lower (relative error of 17%). This may be explained by a "dilution effect" due to the increase of biomass (with growth), which was not followed by uptake of Hg, since after 72h the concentration of Hg remaining in solution was almost zero (Figure 3.2). Similar behaviour was already observed for the bioaccumulation of Hg by *U. lactuca* at static conditions: after an initial fast accumulation, a decreasing phase occurred (Costa et al. 2011). For the initial Hg concentrations of 50 and 100 µg L^{-1}, this effect was not so apparent (Figure 3.5B-C) because Hg contents in the biomass were 5 to 10 times larger than those measured in macroalgae discs after exposure to 10 µg L^{-1}.

The good agreement between theoretical and experimental q_t values with time reinforces the great potential of these algae for environmental biomonitoring of Hg contamination. Results also point out the feasibility of using *U. lactuca* as "chemical reagent", in processes of pre-concentration for analytical chemistry (Romero-Gonzalez et al. 2000). These processes are particularly advantageous in situations where the determination of Hg in natural water samples is difficult, either because its concentration is very low or the preservation of samples is not possible. Once accumulated by *U. lactuca*, Hg will remain stable, strongly bounded in their tissues (as proved by results showed in Figure 3.2 and Figure 3.5), and its quantification is simple: direct analysis of biomass by thermal decomposition atomic absorption spectrometry.

Results of total mercury analysis in macroalgae biomass allow to exclude oxidation-reduction phenomena of the main mechanisms contributing for the Hg removal from water. Is it well known that photochemical and biological reduction of Hg(II) to Hg(0) may occur in aquatic systems, leading to substantial decreases in its toxicity and biological uptake (Sunda and Huntsman 1998). Elemental mercury is unreactive toward complex formation, highly volatile, and if formed intracellularly it will readily diffuse out of the cells (Sunda and Huntsman 1998). In that case Hg levels in algal biomass would be much lower than those measured.

Among the different processes that may be involved in the mechanism of metals removal by biosorbents (Farooq et al. 2010), ion-exchange and chemical coordination/complexation with functional groups on the cell surface are generally pointed as the dominant processes in studies involving dead or living algae (particularly in the rapid stage of metal uptake, which is identical to biosorption) (Gupta and Rastogi 2008, Flouty and Estephane 2012, Bulgariu and Bulgariu 2014, He and Chen 2014). Recently, Michalak and co-authors (2014) studied the bioaccumulation of Zn and Cu by green freshwater macroalga *Vaucheria sessilis*. By using SEM-EDX and ICP-OES analysis, authors concluded that ion-exchange was the main mechanism in the sorption process, and that K^+ and Ca^{2+} cations played a dominating role in the process of cation exchange (Michalak et al. 2014). In the present study, SEM-EDS analysis of the surface of clean macroalgae (data not shown) revealed the presence of high relative levels of K^+, Na^+, Ca^{2+} and in greater extension Mg^{2+}. These cations could be potentially involved in the exchange with free Hg ions in solution. However, even though multi-elemental characterization of seawater after bioaccumulation of Hg by *U. lactuca* has not been performed, this is unlikely (at least as a main mechanism), since as mentioned earlier, Hg was present in solution predominantly in the form of stable chlorocomplexes.

3.3.4 FTIR spectra analysis

In order to gain more insight into the nature of bindings present on the surface of algal cells, infrared analysis of *U. lactuca* was carried out. FTIR spectra of the green marine macroalga before and after exposure to Hg (100 µg L^{-1}) are presented in Figure 3.6. Several important peaks, corresponding to essential functional groups were observed and assigned according to literature. The broad and strong band at 3250 cm^{-1} is attributed to the overlapping of O–H and N–H stretching vibrations (Bulgariu and Bulgariu 2014) and its shift to 3303 cm^{-1} indicates changes in those groups during bioaccumulation of Hg (Ghoneim et al. 2014). The peaks observed at 2931 cm^{-1} (before bioaccumulation) and 2941 cm^{-1} (after bioaccumulation) are assigned to carboxylic/phenolic stretching vibrations (Bulgariu and Bulgariu 2014), while the asymmetrical stretching band at 1627 and the weaker symmetric stretching band 1412 cm^{-1} (1629 and 1404 cm^{-1} after bioaccumulation) are associated to carboxylate groups (Trinelli et al. 2013). Peak at 1412 may be also due to HO- bonds of quinine, along with the peak at 1545 cm^{-1} (Bulgariu and Bulgariu 2014).

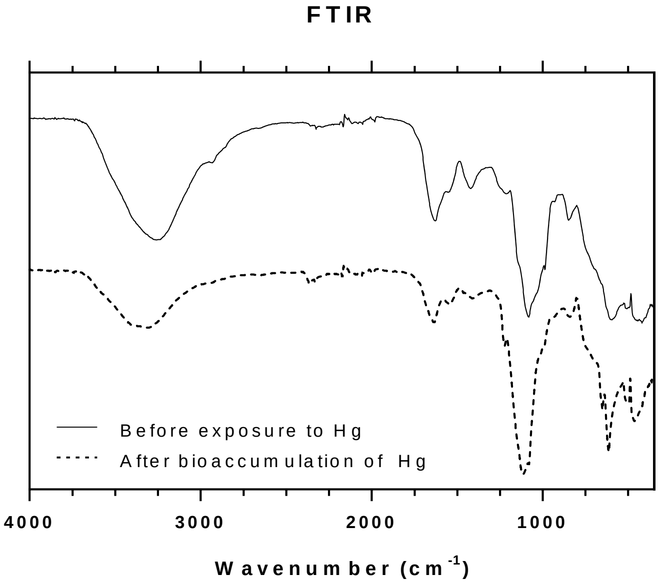

Figure 3.6: FTIR spectra of marine macroalgae *U. lactuca* before (solide line) and after contact (144h) with Hg spiked seawater (dashed line) with an initial concentration of 100 µg L^{-1}.

Ulva lactuca spectra shows a maximum absorption band at 1082 cm^{-1} (1111 cm^{-1} after bioaccumulation), which has been attributed to C-O stretching from the two main sugars, rhamnose and glucuronic acid (Trinelli et al. 2013). Peaks at 595 cm^{-1} (607 cm^{-1} after bioaccumulation) and 439 cm^{-1} (457 cm^{-1} after bioaccumulation) correspond to C–N–S due to polypeptides structure of algae cells (Bulgariu and Bulgariu 2014).

The shifts and changes in intensity observed for most of the bands after bioaccumulation of Hg by the macroalgae are indicative of the interactions between this metal and the main functional groups in the alga surface. However, it should be noted that no quantitative analysis or information about the level of affinity to Hg of the functional groups were provided by the FTIR spectra.

3.3.5 Organometallic mercury in the living *U. lactuca*

Mercury methylation in aquatic systems is generally induced by microorganisms, however production of methylated Hg and Pb by macroalgae from the genus *Fucus* has been already reported (Coelho et al. 2005).

In order to assess the potential methylation of Hg during the removal process carried by the *U. lactuca*, levels of organo-metallic Hg (as total organo-metallic Hg - OrgHg) in macroalga tissues, after collection in the field (reference value), and after bioaccumulation trials were quantified and compared. Results are presented in Table 3.2. As expected, “natural” OrgHg content in reference seaweed was low, 0.004 µg g^{-1}, corresponding to a percentage of 13.8% in relation to the respective total Hg concentration. These values are in line with those observed by Coelho et al. (2005), as well as with those reported early by Neff (2002). After contact with Hg spiked seawater, absolute values of OrgHg in macroalgae increased with the rise of the initial concentration of Hg in solution (Table 3.2). Still, the overall OrgHg concentrations are very low and in the range of those reported for macroalgae worldwide (up to 1.95 µg g^{-1} (Neff 2002)). These values correspond to low conversion rates of inorganic Hg to OrgHg (0.02-0.05%), which diminish with the increase of initial Hg content in solution. Similar findings were reported by Coelho et al. (2005) in their field study concerning macroalgae response to a mercury contamination gradient. Besides the decrease in OrgHg concentrations in all macroalgae, increasing the distance to source of Hg contamination, the authors observed a similar pattern, i.e. OrgHg fractions tended to be higher in low contamination sites (Coelho et al. 2005).

Table 3.2: Organo-metallic Hg concentrations (mean value, n=3) in *U. lactuca* tissues before (reference) and after exposure to Hg spiked seawater, with initial concentrations of 10, 50 and 100 µg L^{-1}.

	Reference	After exposure to 10 µg L^{-1}	After exposure to 50 µg L^{-1}	After exposure to 100 µg L^{-1}
[Hg]org (µg g^{-1})	0.004	0.010	0.029	0.043
Hg org/total (%)	13.8	0.05	0.03	0.02

Results suggest that methylation of Hg is a slow process, whose rate will not be directly controlled by the total concentration of Hg in solution, but by other factors such as metabolism (Coelho et al. 2005). Further studies are needed in order to obtain a better insight on both uptake of OrgHg and methylation of Hg by macroalgae.

According to the results, one can conclude that seawater decontamination achieved by the use of live macroalgae did not resulted in a significant increase of the risks associated with exposure to OrgHg, under the experimental conditions used.

3.4 Conclusions

The uptake/accumulation of Hg by three marine macroalgae, widely abundant temperate coasts – *U. lactuca*, *G. gracilis* and *F. vesiculosus* – was studied and compared, under environmental realistic conditions. All living seaweeds showed huge bioaccumulation capabilities, reaching up to 209 µg of Hg per gram of macroalgae (d.w.), which corresponds to 99% of Hg removed from the contaminated seawater. The accumulation was dose-dependent, with bioconcentration factors ranging from 1870 to 2267. *U. lactuca* was the fastest to accumulate Hg, and under the experimental conditions studied, allowed to reach final levels of Hg in solution equal or lower than 1 µg L^{-1}, thus fulfilling the legal criteria for drinking water quality (Council Directive 98/83/ EC). The better performance demonstrated by this green macroalga in relation to the other two species was justified by its high growth rate, combined with its uniform structure and larger surface area. The removal efficiencies obtained for biosorption and for bioaccumulation processes were compared and the results showed that, for all experimental conditions tested, the use of living *U. lactuca* instead of its dead biomass is more advantageous, since internal accumulation, together with the organism growth allowed to obtain lower residual concentrations of Hg in seawater. Biosorption and bioaccumulation kinetic data fitted to the pseudo-second-order and Elovich models, suggesting chemisorption as the rate limiting step in the sorption of Hg. Analysis of the FTIR spectra confirmed the presence of some important functional groups in the surface of *U. lactuca*, as well as their chemical interaction with Hg ions. Results on total Hg contents in algal biomass over time proved that all Hg removed from seawater is incorporated by the organism, and thus hypothetical reduction of Hg(II) to Hg(0) and consequent volatilization were not considered as responsible for the decrease of Hg levels in solution. During decontamination of seawater carried out by the living *U. lactuca*, there was a very small conversion of inorganic mercury to organometallic Hg (0.02-0.05%), corresponding to very low concentrations of OrgHg in macroalga tissues, which were within the range reported for macroalgae collected in the field throughout the world. It was assumed that methylation is a slow process, but further studies are needed to clarify the role of macroalgae.

Overall, results evidenced the tremendous potential of the studied macroalgae to bioaccumulate Hg from contaminated seawater, which may be useful and very important not only for studies on Hg transfer and bioaccumulation along food chain, but also for the development of new water treatment technologies. Marine macroalgae, particularly living ones, could be the basis of a remediation biotechnology for saline waters contaminated with metals, more efficient and with lower costs than the traditional treatment methods.

3.5 References

Andrade, S., L. Contreras, J. W. Moffett and J. A. Correa (2006). "Kinetics of copper accumulation in Lessonia nigrescens (Phaeophyceae) under conditions of environmental oxidative stress." Aquatic Toxicology **78**(4): 398-401.

Barreiro, R., L. Picado and C. Real (2002). "Biomonitoring heavy metals in estuaries: a field comparison of two brown algae species inhabiting upper estuarine reaches." Environ Monit Assess **75**(2): 121-134.

Bulgariu, L. and D. Bulgariu (2014). "Enhancing Biosorption Characteristics of Marine Green Algae (Ulva lactuca) for Heavy Metals Removal by Alkaline Treatment." Bioprocessing & Biotechniques **4**(1).

Cheung, C. W., J. F. Porter and G. McKay (2000). "Sorption kinetics for the removal of copper and zinc from effluents using bone char." Separation and Purification Technology **19**(1–2): 55-64.

Chojnacka, K. (2007). "Bioaccumulation of Cr(III) ions by Blue-Green alga *Spirulina sp.* Part I. A Comparison with Biosorption." American Journal of Agricultural and Biological Sciences **2**(4): 218-223.

Chojnacka, K. (2010). "Biosorption and bioaccumulation - the prospects for practical applications." Environment International **36**(3): 299-307.

Chojnacka, K. and M. Mikulewicz (2014). Bioaccumulation. Encyclopedia of Toxicology (Third Edition). P. Wexler. Oxford, Academic Press**:** 456-460.

Clarkson, T. W. and L. Magos (2006). "The toxicology of mercury and its chemical compounds." Critical Reviews in Toxicology **36**(8): 609-662.

Coelho, J. P., M. E. Pereira, A. Duarte and M. A. Pardal (2005). "Macroalgae response to a mercury contamination gradient in a temperate coastal lagoon (Ria de Aveiro, Portugal)." Estuarine, Coastal and Shelf Science **65**(3): 492-500.

Costa, S., D. Crespo, B. M. G. Henriques, E. Pereira, A. C. Duarte and M. A. Pardal (2011). "Kinetics of Mercury Accumulation and Its Effects on Ulva lactuca Growth Rate at Two Salinities and Exposure Conditions." Water Air and Soil Pollution **217**(1-4): 689-699.

Costley, C. T., K. F. Mossop, J. R. Dean, L. M. Garden, J. Marshall and J. Carroll (2000). "Determination of mercury in environmental and biological samples using pyrolysis atomic absorption spectrometry with gold amalgamation." Analytica Chimica Acta **405**(1-2): 179-183.

Davis, T. A., B. Volesky and A. Mucci (2003). "A review of the biochemistry of heavy metal biosorption by brown algae." Water Research **37**(18): 4311-4330.

Decision No 2455/2001/EC (2001). Decision No 2455/2001/EC of the European Parliament and of the Council of 20 November 2001 establishing the list of priority substances in the field of water policy and amending Directive 2000/60/EC Official Journal of the European Union. **311:** 1-5.

Directive 84/156/EEC (1984). Council Directive 84/156/EEC of 8 March 1984 on limit values and quality objectives for mercury discharges by sectors other than the chlor-alkali electrolysis industry, Official Journal of the European Communities**:** 74: 29-74: 29.

Directive 2013/39/EU (2013). Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy, Official Journal of the European Union. **226:** 1-17.

Eisler, R. (2010). Algae and Macrophytes. Compendium of Trace Metals and Marine Biota. Amsterdam, Elsevier**:** 7-97.

El-Khaiary, M. I. and G. F. Malash (2011). "Common data analysis errors in batch adsorption studies." Hydrometallurgy **105**(3-4): 314-320.

El-Said, G. F. (2012). "Bioaccumulation of Key Metals and Other Contaminants by Seaweeds from the Egyptian Mediterranean Sea Coast in Relation to Human Health Risk." Human and Ecological Risk Assessment: An International Journal **19**(5): 1285-1305.

Farooq, U., J. A. Kozinski, M. A. Khan and M. Athar (2010). "Biosorption of heavy metal ions using wheat based biosorbents - A review of the recent literature." Bioresource Technology **101**(14): 5043-5053.

Flouty, R. and G. Estephane (2012). "Bioaccumulation and biosorption of copper and lead by a unicellular algae Chlamydomonas reinhardtii in single and binary metal systems: A comparative study." Journal of Environmental Management **111**: 106-114.

Freitas, O. M. M., R. J. E. Martins, C. M. Delerue-Matos and R. A. R. Boaventura (2008). "Removal of Cd(II), Zn(II) and Pb(II) from aqueous solutions by brown marine macro algae: Kinetic modelling." Journal of Hazardous Materials **153**(1-2): 493-501.

Ghoneim, M. M., H. S. El-Desoky, K. M. El-Moselhy, A. Amer, E. H. Abou El-Naga, L. I. Mohamedein and A. E. Al-Prol (2014). "Removal of cadmium from aqueous solution using marine green algae, Ulva lactuca." The Egyptian Journal of Aquatic Research(0).

Gupta, V. K. and A. Rastogi (2008). "Biosorption of lead(II) from aqueous solutions by non-living algal biomass Oedogonium sp. and Nostoc sp.—A comparative study." Colloids and Surfaces B: Biointerfaces **64**(2): 170-178.

Hamdy, A. A. (2000). "Biosorption of heavy metals by marine algae." Current Microbiology **41**(4): 232-238.

Han, T. and G.-W. Choi (2005). "A novel marine algal toxicity bioassay based on sporulation inhibition in the green macroalga Ulva pertusa (Chlorophyta)." Aquatic Toxicology **75**(3): 202-212.

He, J. and J. P. Chen (2014). "A comprehensive review on biosorption of heavy metals by algal biomass: Materials, performances, chemistry, and modeling simulation tools." Bioresource Technology **160**(0): 67-78.

Henriques, B., S. M. Rodrigues, C. Coelho, N. Cruz, A. C. Duarte, P. F. A. M. Römkens and E. Pereira (2013). "Risks associated with the transfer of toxic organo-metallic mercury from soils into the terrestrial feed chain." Environment International **59**(0): 408-417.

Ho, Y.-S. (2006). "Review of second-order models for adsorption systems." Journal of Hazardous Materials **136**(3): 681-689.

Ho, Y. S. and G. McKay (1999). "Pseudo-second order model for sorption processes." Process Biochemistry **34**(5): 451-465.

Ho, Y. S. and G. McKay (2004). "Sorption of copper(II) from aqueous solution by peat." Water Air and Soil Pollution **158**(1): 77-97.

Ho, Y. S., J. C. Y. Ng and G. Mckay (2000). "Kinetics of pollutant sorption by biosorbents: Review." Separation and Purification Methods **29**(2): 189-232.

Kabata-Pendias, A., Mukherjee, Arun B. (2007). Trace Elements from Soil to Human, Springer.

Kadukova, J. and E. Vircikova (2005). "Comparison of differences between copper bioaccumulation and biosorption." Environment International **31**(2): 227-232.

Lagergren, S. (1898). "About the theory of so-called adsorption of soluble substances." Kung Sven Veten Hand **24**: 1-39.

Lopes, C. B., J. R. Oliveira, L. S. Rocha, D. S. Tavares, C. M. Silva, S. P. Silva, N. Hartog, A. C. Duarte and E. Pereira (2014). "Cork stoppers as an effective sorbent for water treatment: the removal of mercury at environmentally relevant concentrations and conditions." Environ Sci Pollut Res Int **21**(3): 2108-2121.

Mason, R. P., J. R. Reinfelder and F. M. M. Morel (1996). "Uptake, Toxicity, and Trophic Transfer of Mercury in a Coastal Diatom." Environmental Science & Technology **30**(6): 1835-1845.

Michalak, I., K. Marycz, K. Basińska and K. Chojnacka (2014). "Using SEM-EDX and ICP-OES to Investigate the Elemental Composition of Green Macroalga Vaucheria sessilis." The Scientific World Journal: 8.

Nam, D. H. and N. Basu (2011). "Rapid methods to detect organic mercury and total selenium in biological samples." Chemistry Central Journal **5**.

Neff, J. M. (2002). Chapter 6 - Mercury in the Ocean. Bioaccumulation in Marine Organisms. J. M. Neff. Oxford, Elsevier**:** 103-130.

Nielsen, M. M., A. Bruhn, M. B. Rasmussen, B. Olesen, M. M. Larsen and H. B. Moller (2012). "Cultivation of Ulva lactuca with manure for simultaneous bioremediation and biomass production." Journal of Applied Phycology **24**(3): 449-458.

Nikolaisen, L. D. J., P.; Svane Bech, K. (2011). Energy production from marine biomass (Ulva lactuca). Denmark, Danish Technological Institute 72.

Pato, P., M. Otero, M. Válega, C. B. Lopes, M. E. Pereira and A. C. Duarte (2010). "Mercury partition in the interface between a contaminated lagoon and the ocean: The role of particulate load and composition." Marine Pollution Bulletin **60**(10): 1658-1666.

Rocha, L., C. Lopes, J. A. Borges, A. C. Duarte and E. Pereira (2013). "Valuation of Unmodified Rice Husk Waste as an Eco-Friendly Sorbent to Remove Mercury: a Study Using Environmental Realistic Concentrations." Water, Air, & Soil Pollution **224**(7): 1-18.

Romera, E., F. Gonzalez, A. Ballester, M. L. Blazquez and J. A. Munoz (2007). "Comparative study of biosorption of heavy metals using different types of algae." Bioresource Technology **98**(17): 3344-3353.

Romero-Gonzalez, M. E., C. J. Williams and P. H. E. Gardiner (2000). "The application of dealginated seaweed as a cation exchanger foron-line preconcentration and chemical speciation of trace metals." Journal of Analytical Atomic Spectrometry **15**(8): 1009-1013.

Sari, A. and M. Tuzen (2008). "Biosorption of Pb(II) and Cd(II) from aqueous solution using green alga (Ulva lactuca) biomass." Journal of Hazardous Materials **152**(1): 302-308.

Singh, S., S. Pradhan and L. C. Rai (2000). "Metal removal from single and multimetallic systems by different biosorbent materials as evaluated by differential pulse anodic stripping voltammetry." Process Biochemistry **36**(1–2): 175-182.

Sunda, W. G. and S. A. Huntsman (1998). "Processes regulating cellular metal accumulation and physiological effects: Phytoplankton as model systems." Science of The Total Environment **219**(2–3): 165-181.

Torres, M. A., M. P. Barros, S. C. G. Campos, E. Pinto, S. Rajamani, R. T. Sayre and P. Colepicolo (2008). "Biochemical biomarkers in algae and marine pollution: A review." Ecotoxicology and Environmental Safety **71**(1): 1-15.

Trinelli, M. A., M. M. Areco and M. D. Afonso (2013). "Co-biosorption of copper and glyphosate by Ulva lactuca." Colloids and Surfaces B-Biointerfaces **105**: 251-258.

Turner, D. R. (1987). Speciation and Cycling of Arsenic, Cadmium, Lead and Mercury in Natural Waters. Lead, Mercury, Cadmium and Arsenic in the Environment, John Wiley & Sons, Ltd**:** 175-186.

Ullrich, S. M., M. A. Ilyushchenko, I. M. Kamberov and T. W. Tanton (2007). "Mercury contamination in the vicinity of a derelict chlor-alkali plant. Part I: Sediment and water contamination of Lake Balkyldak and the River Irtysh." Science of The Total Environment **381**(1-3): 1-16.

Válega, M., S. Abreu, P. Pato, L. Rocha, A. Gomes, M. Pereira and A. Duarte (2006). "Determination of Organic Mercury in Biota, Plants and Contaminated Sediments Using a Thermal Atomic Absorption Spectrometry Technique." Water, Air, & Soil Pollution **174**(1): 223-234.

Varma, R., A. Turner and M. T. Brown (2011). "Bioaccumulation of metals by Fucus ceranoides in estuaries of South West England." Marine Pollution Bulletin **62**(11): 2557-2562.

Vasconcelos, M. T. S. D. and M. F. C. Leal (2001). "Seasonal variability in the kinetics of Cu, Pb, Cd and Hg accumulation by macroalgae." Marine Chemistry **74**(1): 65-85.

Velasquez, L. and J. Dussan (2009). "Biosorption and bioaccumulation of heavy metals on dead and living biomass of Bacillus sphaericus." Journal of Hazardous Materials **167**(1-3): 713-716.

Villares, R., X. Puente and A. Carballeira (2001). "Ulva and Enteromorpha as indicators of heavy metal pollution." Hydrobiologia **462**(1): 221-232.

Wang, W.-X. and R. C. H. Dei (1999). "Kinetic measurements of metal accumulation in two marine macroalgae." Marine Biology **135**(1): 11-23.

Chapter 4

Developing a macroalgae-based biotechnology for water remediation: simultaneous removal of Cd, Pb and Hg by living *Ulva lactuca*

4 Developing a macroalgae-based biotechnology for water remediation: simultaneous removal of Cd, Pb and Hg by living *Ulva lactuca*

4.1 Introduction

Marine algae inhabit the oceans for more than 2 billion years, and always played an important role in human life (Simpson and Roger 2004). They are responsible for over 50% of the O_2 produced globally (Chojnacka 2009), having long been used for food, agricultural, cosmetic and pharmaceutical purposes (Caliceti et al. 2002, Kumar et al. 2013, He and Chen 2014). Recently, a strong enthusiasm is been created around macroalgae in the pursuit of "greener" technologies to be applied for environment protection (Torres et al. 2008, Sekabira et al. 2011).

It is well recognized that water availability can be compromised by human activities, such as mining, smelting, or coal burning, which mobilize and release large amounts of metals into the environment (Freitas et al. 2008). Given the awareness for the harmful effects that potential toxic elements such as Cd, Pb and Hg – the so-called toxic trio – may have on human and animal health, environmental policies are becoming more stringent every day.

Conventional water treatment procedures present major drawbacks (Farooq et al. 2010), and fail to reduce metal levels to concentrations such low as required by law, or become prohibitively expensive (He and Chen 2014). Due to their large abundance worldwide and ability to bind and retain metals from the surrounding environment (Coelho et al. 2005, Kamala-Kannan et al. 2008, Jayakumar et al. 2014), macroalgae may be the needed alternative, forming the basis of new remediation biotechnologies, more efficient and cost-effective.

In the last years, an intense investigation has focused on the application of macroalgae dried biomass to promote the removal of metals from aqueous solutions (biosorption) (Romera et al. 2007, Wang and Chen 2009, Trinelli et al. 2013), however, the use of living organisms (bioaccumulation) (Chojnacka 2010), although less studied, may be advantageous in some scenarios. Living macroalgae are able to remove simultaneously metals and nutrients (nitrates and phosphates) from aquaculture wastewaters, for example, while growing (Nielsen et al. 2012). Additionally, bioaccumulation by living cells, may lead to lower residuals levels of contaminant in solution due to additional intracellular accumulation (Chojnacka 2007). As a treatment method, bioaccumulation requires simpler installation when compared with biosorption (Chojnacka 2010), and avoid the need for additional processes, such as drying, processing and activation prior to use (Li et al. 2013), which are indispensable for biosorption.

A significant part of the laboratory studies concerning accumulation of metals by living macroalgae (Lee and Wang 2001, Masakorala et al. 2008) reports short exposure times (Wang and Dei 1999, Turner et al. 2008a) and ignores the mathematical description of the kinetic of the process. Among the metals studied less attention is given to Hg (Costa et al. 2011), and above all no study dealt with the effects of simultaneous presence of metals (mixtures) on macroalgae growth rate (Vasconcelos and Leal 2001, Han and Choi 2005, Han et al. 2008, Turner et al. 2008a). In fact, most of researchers follow an idealistic approach, assessing the performance of the removal process for monometallic solutions (Chojnacka 2009). However, real wastewaters are complex systems, containing a multiplicity of different ions, which interact and compete for binding sites, affecting removal efficiency. Moreover, extremely high concentrations of metal are often used, which does not comply with the values found in real contaminated waters bodies (Rocha et al. 2013, Lopes et al. 2014). Besides the scarcity of works conducted for multimetallic systems, literature also lacks on studies performed in saline water. Regardless of major cities are concentrated in the proximity of marine coasts (Coelho et al. 2005), with wastewaters, from both industrial and domestic sources, finding their way into coastal environments (Torres et al. 2008), most of the reported results are obtained in synthetic water or freshwater. Additionally, removal is also dependent on speciation and mobility of metal ions in water, which varies according to the matrix. For instance, in river water Cd, Hg, and Pb ions are bound to humic substances in a greater extent than in seawater (Kabata-Pendias 2007), where Cd and Hg are heavily complexed by chloride ions and Pb by carbonate ions (Sunda and Huntsman 1998).

Ulva lactuca is a green marine macroalga (*Chlorophyta*), very common at coastal areas, which owns several attractive characteristics from the viewpoint of remediation. Since it is perfectly adapted to the salinity variations occurring in estuaries due to tides, this macroalga can grow in a wide range of salinity (Yamashita et al. 2009, Costa et al. 2011). Additionally it withstands moderate pollution and eutrophic conditions. Its structure is relatively simple, with a thin (just 2 cell layers thick) and sheet-like thallus, which translates into a large surface area, containing structurally uniform and physiologically active cells (Sari and Tuzen 2008). The cell wall is constituted by cellulose along with a high percentage of proteins bonded to polysaccharides (Romera et al. 2007, Trinelli et al. 2013), which comprises several functional groups such as amino, hydroxyl, carboxyl, and sulfate, capable of acting as binding sites for metal (Romera et al. 2007). Additionally, this algae grows fast (Nikolaisen and Jensen 2013), since their photosynthetic products are quickly converted to cell growth (Easton et al. 2011).

Thus, the purpose of this work was to study and evaluate the accumulation of metals by *Ulva lactuca* from saline water. The efficiency of the removal process carried by the macroalga was assessed for: i) monometallic solutions of Hg, Cd and Pb, in a range of different environmentally relevant concentrations; ii) multi-metallic solutions containing Hg, Cd and Pb in the equal concentration, and at different concentrations. The evaluation of the influence of the chosen metals on *Ulva* growth rate, during accumulation, in both mono and multi-system, was also performed.

4.2 Materials and methods

4.2.1 Material and chemicals

All chemical reagents used in this work were of analytical reagent grade, and were used as received from the suppliers. The nitric acid 65% (suprapur) and the standard stock solutions of mercury (1001 ± 2mgL^{-1}), cadmium (1000 ± 2mgL^{-1}) and lead (1000 ± 2mgL^{-1}) nitrate were purchased from Merck. All working solutions, including standards for the calibration curves, were obtained by diluting the corresponding stock solution. All the material used in the experiments was previously washed in Derquim 5% rinsed in Milli-Q water (18 MΩ cm^{-1}), soaked in 25% HNO_3 for at least 24h and subsequently rinsed with Milli-Q water.

4.2.2 Macroalgae collection and maintenance

The green macroalgae *Ulva lactuca* was collected in the Mondego estuary (Figueira da Foz, Portugal, 40°08′N, 8°50′W) and transported to laboratory in isothermal plastic bags, containing local water. After rinse with seawater to remove debris and epibionts, a small part of the macroalgae was immediately freeze dried for latter quantification of the inherent metal concentrations (baseline), following the procedures described below. The values obtained were 0.015±0.004 µg g^{-1} for Cd, 0.300±0.025 µg g^{-1} for Pb and 0.029±0.003 µg g^{-1} for Hg, which are characteristic of macroalgae from uncontaminated areas, being below the reference limits for human consumption (Leal et al. 1997, Almela et al. 2002, Ryan et al. 2012). The remaining macroalgae was transferred to 30 L clear glass tanks equipped with air pump and filled with filtered seawater enriched with Provasoli stock solution (Costa et al. 2011). Macroalgae were maintained in the aquariums (water was weekly changed) under natural light, and at room temperature of 20±2°C, until the beginning of the experiments. Seawater for experiments and macroalgae maintenance was collected at Vagueira beach (18 km southwest of Aveiro, Portugal), filtered through 0.45 µm Millipore membrane and stored in the dark at 4°C until further use. A brief characterization of the seawater which includes pH, conductivity, salinity and major and minor elements was performed. The pH (7.9), conductivity (54.3 mS cm^{-1}) and salinity (35 g L^{-1}) were recorded on a WTW meter and the concentration of major and minor elements was obtained by inductively coupled plasma spectroscopy, using a Jobin – Yvon JY70 Plus Spectrometer. All the measured parameters were in acceptable ranges according to considered non-polluted waters. Concentrations of Cd and Pb were below the detection limit of the quantification analysis, while total Hg concentration, determined using the methodology described by Pato et al. (2010), was 3.1 ng L^{-1}, which is typical of a non-polluted water.

4.2.3 *Ulva lactuca* characterization

In order to assess the dry/fresh weight ratio of *U. lactuca*, several pieces were cut from the macroalgae thallus, weighed, and dried at 40ºC until constant weight.

FTIR spectrum of *U. lactuca* was recorded by using a Bruker optics tensor 27 spectrometer coupled to a horizontal attenuated total reflectance (ATR) cell using 256 scans at a resolution of 4 cm^{-1}.The dried sample was examined directly and data were obtained as transmittance.

4.2.4 Bioaccumulation experiments

Ulva lactuca was exposed, during 144h, to monometallic and multimetallic solutions of Cd, Pb and Hg, according to the experimental conditions presented in Table 4.1.

Table 4.1: Experimental conditions of the performed bioaccumulation experiments.

Matrix	System	Metal		Concentration (µg L^{-1})
Spiked natural seawater	Single-contaminant		Cd	10, 50, 100 and 200
			Pb	50, 100, 200 and 1000
			Hg	10, 50 and 100
	Multi-contaminant	M1	Cd	50
			Pb	50
			Hg	50
		M2	Cd	200
			Pb	1000
			Hg	50

The chosen concentrations were considered environmentally relevant, intending to mimic real cases: 50, 200 and 1000 µg L^{-1} are, respectively, the "old" limits for Hg, Cd and Pb wastewaters discharges (Directive 83/513/EEC 1983, Directive 84/156/EEC 1984, Decree-Law No. 236/98 1998). It should be remembered that European Commission has recently revised water policies, and determined new environmental quality standards (EQS) for surface waters, considering stringent levels of contaminants (Directive 2013/39/EU 2013) in the receiving water bodies, instead of limit values for effluents discharges, since emissions, losses and or discharges of Pb, Cd and Hg were restricted or prohibited. The remaining concentrations used may represent a situation of accidental discharge of untreated effluent, or metal concentrations in aquatic ecosystems after the dilution of the discharged effluent into the water body.

Bioaccumulation tests were conducted in Schott Duran® glass bottles of 1 L, where macroalgae were placed in contact with natural filtered seawater, spiked with metal, through the addition of a desired volume of metal standard solution. All the solutions were left to stabilize during 24h before the beginning of the experiments. An aliquot of each solution was collected immediately before adding the macroalgae to confirm the exact initial concentration of metals. Small discs with uniform size (10 discs of 18 mm and 5 discs of 56 mm) were cut from the healthy thallus of *U. lactuca* (Wang and Dei 1999), and added to the spiked seawater, in a total of approximately 1.6 g L^{-1} fresh weight. All algal pieces were acclimatized in clean seawater for several hours prior to metals exposure. Metal uptake by living macroalgae along time was followed by determining the concentration of each metal in solution samples (5-10 mL) collected at defined crescent periods of time. All samples were acidified to pH ≤ 2 using Suprapur HNO_3 and stored at 4°C until further analysis. All bioaccumulation trials were performed in triplicate, under natural light, at room temperature of 20±2°C. Blanks (macroalgae in clean seawater) and controls (metal spiked seawater in the absence of algae) were always running in parallel with the experiments. No pH buffer was added to the system, and no pH adjustments were made at any time, since this could interfere with the metals uptake and cellular metabolism of the organisms (Vasconcelos and Leal 2001, Velasquez and Dussan 2009). However, pH was daily monitored. At the end of the experiments, macroalgae were harvested, rinsed and freeze dried for later quantification of metal contents.

4.2.5 Metal quantification

4.2.5.1 Metal concentration in solution

Mercury quantification in water samples was performed by cold vapour atomic fluorescence spectroscopy (CV-AFS), on a PSA 10.025 Millennium Merlin Hg analyser and using $SnCl_2$ (2% m/v in HCl 10% v/v) as reducing agent. The concentration of Hg in the collected samples was determined through a calibration curve ($r^2 \geq 0.999$) using five daily prepared standard solutions ranging from 0.0 to 0.5 µg L^{-1}. In this range, detection and quantification limits, obtained through blank measurements (n = 20) were 0.007 µg L^{-1} and 0.021 µg L^{-1}, respectively. Cadmium and Pb quantifications were performed by inductively coupled plasma mass spectrometry (ICP-MS), on a Thermo ICP-MS XSeries equipped with a Burgener nebuliser. Calibration curves for Cd and Pb were obtained using standards (0.1–50 µg L^{-1}) prepared by dilution of certified standard solutions of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ in nitric acid (2 % v/v). The limits of quantification of the method were 0.1 µg L^{-1} and 0.2 µg L^{-1}, for Cd and Pb respectively, with a precision and accuracy <10 %. In order to avoid matrix interferences on metal quantification by ICP-MS, since seawater has high salinity, all samples were diluted 20-fold prior analysis, which results in “real” quantification limits of 2 and 4 µg L^{-1}, for Cd and Pb respectively.

4.2.5.2 Metals concentration in algal biomass

Total Hg concentration in macroalgae biomass was measured by thermal decomposition atomic absorption spectrometry with gold amalgamation using a LECO© AMA-254, as described by Costley et al. (2000). Analysis was performed directly in the sample (1 to 20 mg, dry weight) without digestion or pre-specific treatment, avoiding mercury losses or contamination as well as matrix interferences. At least three replicate measurements were carried out for each sample, and a maximum coefficient of variation of 10% was adopted as acceptance criteria. Detection and quantification limits were 0.01 ng Hg and 0.03 ng Hg, respectively (n=20, 99.5% confidence level). Several blanks (i.e. an empty sample nickel boat) were run before and between sample analyses to ensure that mercury was not being accumulated between samples. The quality of the results was assessed by using Certified Reference Material (CRM) BCR-60 (*Lagarosiphon major*). The CRM was analyzed every day prior to the beginning of the analysis, and repeated at the end of the day. All percentages of recovery for total Hg were within the range of 85–107% (n = 17).

For the determination of Cd and Pb contents in macroalgae, samples and certified reference material (BCR60) were previously digested, in duplicate (Monterroso et al. 2003). Briefly, about 200 mg of freeze-dried sample was accurately weighted into an acid-washed Teflon reactor; HNO_3 (conc.) was added, and reactors were placed in an oven at 60 °C for 12 h, and then at 100 °C for 1 h. Afterward, H_2O_2 was added, and reactors were heated at 80 °C for 1 h. After digestion, samples were diluted with Milli-Q water, filtered, and then analysed by ICP-MS. Percentages of recovery for Cd and Pb were, respectively, 85 and 92% (n=2).

4.2.6 Effects of metal exposure on macroalgae growth

The influence of studied contaminants on *U. lactuca* growth, along with time, was evaluated by means of relative growth rate (RGR), calculated by following equation:

$$RGR(\%\ day^{-1}) = ((\ln A_t - \ln A_0)/t) \times 100 \quad (1)$$

where A_0 is the initial algae area and A_t is the algae area at time t (Han et al. 2008).

Two algal discs were daily collected from each reaction bottle, placed in a transparent plastic sheet, and then scanned with a resolution of 200 ppi (Figure 4.1). Afterwards, discs area was determined using the software *ImageJ*. Overall, the combined mass of all discs collected along the experiment for RGR measurements is <10% of the initial total algal biomass.

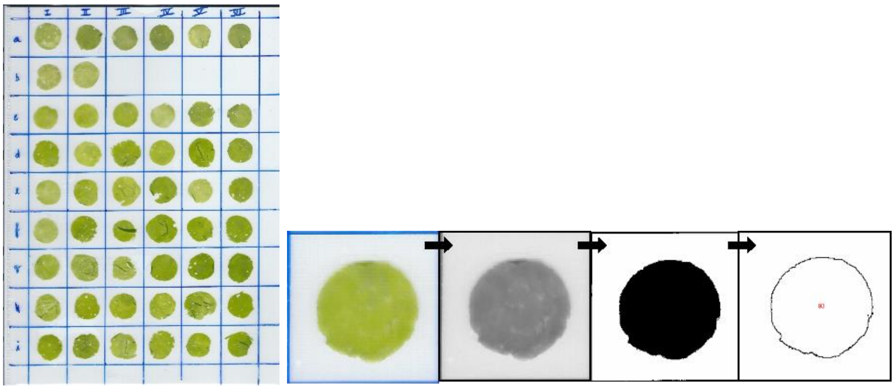

Figure 4.1: Scan of *U. lactuca* discs, and further processing by ImageJ for measurement of RGR.

4.2.7 Experimental data analysis

The exposure of *U. lactuca* to contaminated seawater leads to an increase of metals content in macroalgae biomass with the correspondent decrease on metal concentration in solution. Thus, metal accumulation by macroalgae, defined as the amount of metal bound by unit of mass at a given time t (q_t, µg g^{-1}), may be deduced from the mass balance between the initial metal concentration in the solution (C_0, µg L^{-1}) and the concentration after a particular period of contact time t (C_t, µg L^{-1}):

$$q_t = \frac{(C_0 - C_t)V}{m} \quad (2)$$

where V (L) is the volume of the solution and m (g) is the macroalgae biomass, in dry weight. When equilibrium is attained, $t=t_e$, $q_t=q_e$ and $C_t=C_e$ (residual metal concentration in solution). The performance of the removal process was also evaluated and compared using the metal removal percentage (R, %), which at time t is defined by:

$$R_t = \frac{(C_0 - C_t)}{C_0} \times 100 \quad (3)$$

4.2.7.1 Bioaccumulation kinetics

The most commonly used kinetic reaction models in metal removal batch experiments are Lagergren pseudo-first-order model, Ho's pseudo-second order model and Elovich model (Ho et al. 2000).

The pseudo-first-order model was firstly applied by Lagergren (Lagergren 1898) and is mathematically expressed by:

$$\frac{dq_t}{dt} = k_1(q_e - q_t) \quad (4)$$

where k_1 (h^{-1}) is the rate constant of pseudo-first order and q_e (µg g^{-1}) is the amount of solute sorbed per gram of sorbent at equilibrium. After integration and application of the boundary condition q_t=0 at t=0, Eq. (4) can be expressed by the following non-linear equation:

$$q_t = q_e(1 - e^{-k_1 t}) \quad (5)$$

However, it is well known that Lagergren model may not represent the sorption evolution along full time range (Ho et al. 2000).

The pseudo-second-order model was firstly described by Ho (Ho and McKay 1998) and in contrast with the previous model, usually correlates the behaviour over the whole range of sorption. The kinetic rate equation is expressed as:

$$\frac{dq_t}{dt} = k_2(q_e - q_t)^2 \quad (6)$$

where k_2 (g μg^{-1} h^{-1}) is the pseudo-second order rate constant. By applying the boundary conditions t=0 to t=t and q_t=0 to q_t=q_e, the integrated form of Eq.(6) is:

$$q_t = \frac{q_e{}^2 k_2 t}{1 + q_e k_2 t} \quad (7)$$

The Elovich model was established by Zeldowitsch to describe the kinetic law of chemisorption (Ho 2006) and it is described by the following equation:

$$\frac{dq_t}{dt} = \beta e^{-\alpha q} k_2(q_e - q_t)^2 \quad (8)$$

where, α (µg g^{-1} h^{-1}) is the initial adsorption rate, and β (g $µg^{-1}$) is the desorption constant. Integrating equation **(**8), by using the boundary conditions of *q*=0 at *t*=0 and *q*=*q* at *t*=*t*, and assuming that $\alpha\beta t$>>1, the simplify form of Elovich's equation is defined by:

$$q_t = \frac{1}{\beta}\ln(1+\alpha\beta t) \quad (9)$$

Recently, Elovich's equation has been widely used to describe the sorption of pollutants from aqueous solutions (Ho 2006).

4.2.7.2 *Statistical analysis*

All statistical analysis in this work was performed using GraphPad 6.0. This tool was also used to plot all graphs as well as to calculate all kinetic parameters through nonlinear regression. GraphPad 6.0 uses the least-squares as fitting method and the method of Marquardt and Levenberg, which blends two other methods, the method of linear descent and the method of Gauss-Newton for adjusting the variables.

The coefficient of determination (R^2) and the standard deviation of residuals ($S_{y.x}$) were analyzed in order to evaluate the goodness of fit. The relative error (E_r) between experimental and predicted values of q_e was also calculated. These statistical parameters can be mathematically defined by:

$$R^2 = 1 - \frac{\sum (y_i - \hat{y})^2}{\sum (y_i - \bar{y})^2} \quad (10)$$

$$S_{y,x} = \sqrt{\frac{(y_i - \hat{y})^2}{df}} \quad (11)$$

$$E_r = \frac{|y_i - \hat{y}|}{y_i} \times 100 \quad (12)$$

where y_i are the experimental data values, $\hat{y}$ are the predicted values, $\bar{y}$ is the mean of the experimental data and *df* is the number of the degrees of freedom (equal to number of data points minus the number of parameters fit).

$$AIC = N\ln\left(\frac{SSE}{N}\right) + 2N_p + \frac{2N_p\left(N_p+1\right)}{N-N_p-1}$$

Akaike's Information Criterion (AIC) (El-Khaiary and Malash 2011), which is based on information theory and maximum likelihood theory, was used to determine which model is more likely to be correct and how much more likely. For small sample size, AIC is calculated from the equation:

(13)

where SSE is the sum-of-squared deviations, N is the number of data points and N_p is the number of parameters in the model. AIC values can be compared using Evidence ratio (numerical value representative of the number of times that the model with a lower AIC is more likely to be correct) which is defined by:

$$\text{Evidence ratio} = \frac{1}{e^{-0.5\Delta}} \qquad (14)$$

where Δ is the absolute value of the difference in AIC between the two compared models.

4.3 Results and discussion

4.3.1 *Ulva lactuca* characterization

The dry/fresh weight ratio of *U. lactuca* ranged between 0.21 and 0.33, corresponding to an average water content of 73.9 ± 4.3% (n=20). These values are in line with those reported by Bruhn et al. (2011) for *U. lactuca* from the Danish coast (76.7 ± 2.7% of water content), and slightly lower than those found at New Zealand (79.6 ± 1.43% of water content) by Lamare and Wing (2001).

The FTIR spectrum of green macroalgae *U. lactuca* (Figure 4.2) shows bands at wave numbers of 3250, 2931, 1627, 1545, 1412, 1082, 595 and 439 cm^{-1}, which were attributed to main functional groups of the algae according to the assignments made by other authors (Trinelli et al. 2013, Bulgariu and Bulgariu 2014).

The broad and strong band at 3250 cm^{-1} is attributed to the overlapping of O–H and N–H stretching vibrations (Bulgariu and Bulgariu 2014). The peaks observed at 2931 cm^{-1} is assigned to carboxylic/phenolic stretching vibrations (Bulgariu and Bulgariu 2014), while the asymmetrical stretching band at 1627 and the weaker symmetric stretching band 1412

cm^{-1} are associated to carboxylate groups (Trinelli et al. 2013). Peak at 1412 may be also due to HO- bonds of quinine, along with the peak at 1545 cm^{-1} (Bulgariu and Bulgariu 2014).

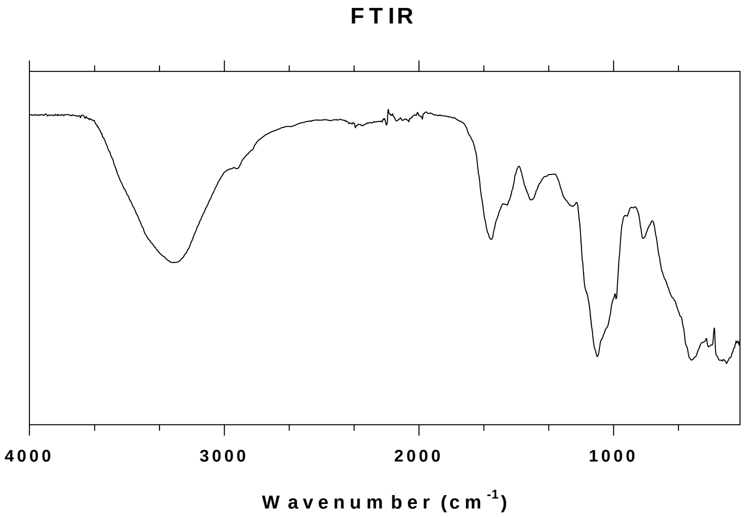

Figure 4.2: FTIR spectra of marine macroalgae *U. lactuca***.**

Ulvan spectra shows a maximum absorption band at 1082 cm^{-1}, which has been attributed to C-O stretching from the two main sugars, rhamnose and glucuronic acid (Trinelli et al. 2013). Peaks at 595 cm^{-1} and 439 cm^{-1} correspond to the C–N–S shearing due to polypeptides structure of algae cells (Bulgariu and Bulgariu 2014).

4.3.2 Bioremediation in single-contaminant systems

The capability of *U. lactuca* to remove (bioaccumulate) toxic metals, such as Pb, Cd and Hg, from saline water was initially evaluated for monometallic solutions (single-contaminant systems), with different initial concentrations, representing various contamination scenarios.

Figure 4.3A-D shows the variation of normalized concentrations of Cd in spiked seawater along time (C_t/C_0 *vs* t), for the initial concentrations of 10, 50, 100 and 200 µg L^{-1}. The results of controls (dashed line) show that levels of Cd in solution, in the absence of macroalgae, remained stable throughout the full period of time in which the assays were held. By contrary, the kinetic profiles of all solutions in contact with *U. lactuca* were characterized by a rapid and marked decrease in C_t/C_0, at the initial period of time, representing a quick biosorption/bioaccumulation of metal by the living macroalgae. After just 12 hours of contact time (by using only c.a. 500 mg, d.w., of macroalgae), levels of Cd in seawater were reduced by about 77.8, 78.4, 66.7%, respectively for initial concentrations of 10, 50, 100 µg L^{-1}, whereas for 200 µg L^{-1} a removal percentage of 83.8% was achieved after a period of time of 24h. Results suggest that for higher concentrations a longer period of time is required in order to obtain similar removal efficiencies (in terms of % of metal removed). However, it should be noted that an increase in the initial concentration of Cd always leads to higher amounts of metal accumulated by living macroalgae - dose-dependent accumulation (e.g 15.8, 66.4, 113.6 and 153.6 µg g^{-1} for 10, 50, 10 and 200 µg L^{-1}, respectively, after 12h of contact time). Additionally, overall, initial sorption rate, v_0, also increases with the rise of initial concentrations of Cd (Table 4.2), which can be attributed to the greater driving force allowing to overcome all mass transfer resistance of Cd between the liquid and macroalgae (Rocha et al. 2013).

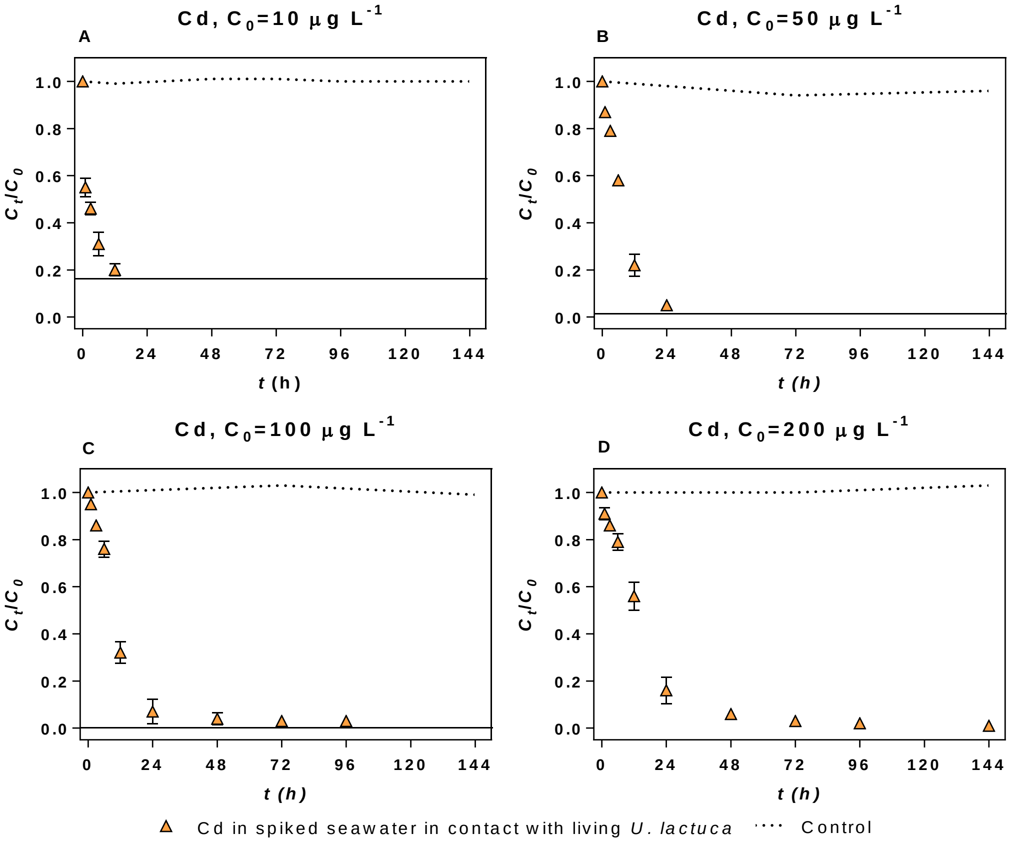

Figure 4.3: Normalized concentrations (C_t/C_0) of Cd in spiked seawater along time (*t*, h) – single-contaminant systems. The results are expressed as the mean ± standard deviation (n=3). The solid line represents the value of the limit of quantification (in C_t/C_0).

All samples collected after 12h for 10 µg L^{-1}, 24h for 50 µg L^{-1} and 96h for 100 µg L^{-1}, (Figure 4.3A-C), had concentrations of Cd below the “limit of quantification” of the method, 2 µg L^{-1}, underscoring the potential application of this seaweed in water bioremediation processes. Additionally, the kinetic pattern observed for 10 and 50 µg L^{-1} suggests that removal continued after the referred times, possibly until all metal in solution was accumulated by the living macroalgae, or until an equilibrium was established, with residual concentrations in solution near to zero. For 200 µg L^{-1}, the remarkable reduction observed in the beginning was followed by a subtle decrease in C_t/C_0, until a balance was established, corresponding to a residual value of Cd in solution of c.a. 2 µg L^{-1} (98.5% of removal).

The fittings of pseudo-first order (PFO), pseudo-second order (PSO) and Elovich models to experimental data regarding the bioaccumulation of Cd by *U. lactuca* over time are presented in Figure 4.4, while respective estimated parameters and goodness of fit are summarized in Table 4.2. Kinetic analysis was performed on a molar basis, in order to enable further comparison between metals.

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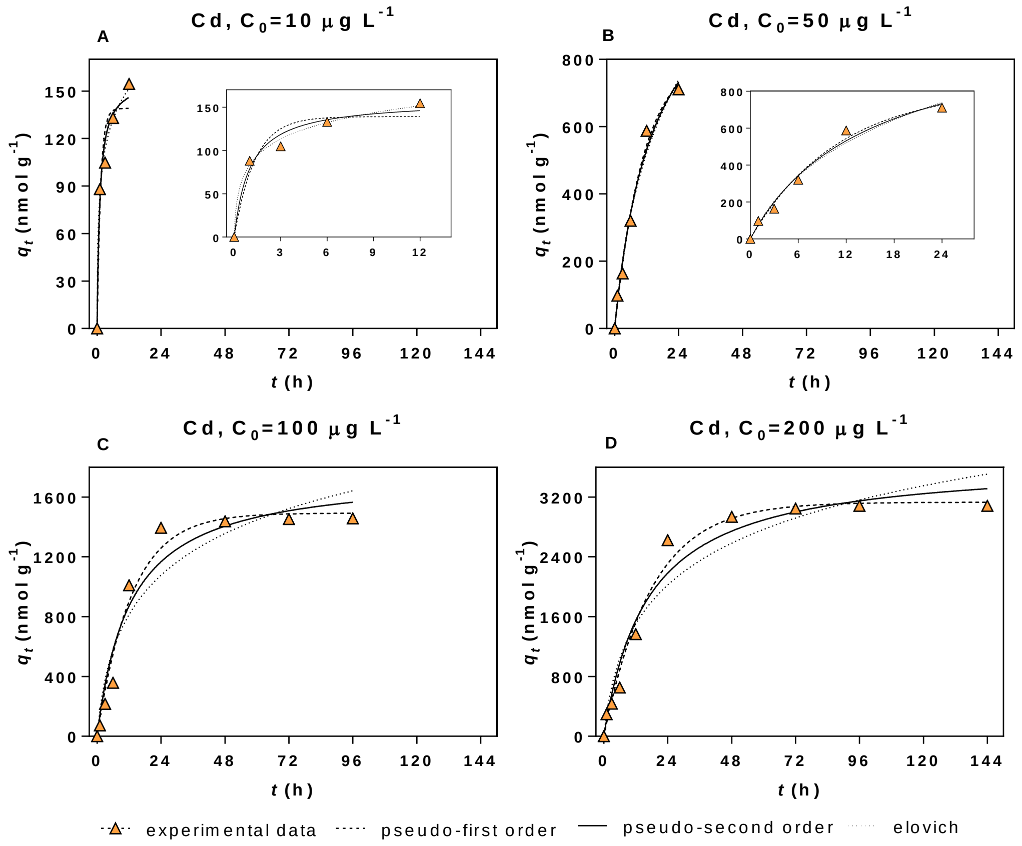


Figure 4.4: Kinetic modelling of the experimental data regarding the uptake of Cd by living *U. lactuca* (single-contaminant systems). Error bars were omitted for clarity.

All mathematical models studied adequately fit the experimental results over the entire course of experiments, which is corroborated by the values of the coefficient of correlation ($0.939<R^2<0.993$). Akaike's Information Criterion (AIC) (El-Khaiary and Malash 2011) was used to choose which model is more likely to be correct and quantify how much more likely (probability ratio) - Table 4.2.

Table 4.2: Best-fit parameters (± standard error) and goodness of the fittings of the kinetic models used to describe the bioaccumulation kinetics of Cd by living *U. lactuca* (single-contaminant systems).

C_0 (μg L^{-1})	PFO	PSO	Elovich	Preferred model*
10	**k_1:** 0.7638±0.2700	**k_2:**0.06294±0.002601	**α:** 520.3±227.2	***Elovich*** *Probability ratio*: 214.63 *Difference AIC*: 10.74
	q_e: 139.1±11.98	**q_e:** 158.2±12.20	**β:** 0.03571±0.004464	
	R^2: 0.939	**R^2:** 0.975	**R^2:** 0.993	
	$S_{y/x}$: 16.88	**$S_{y/x}$:** 10.78	**$S_{y/x}$:** 5.768	
	ν_0: 106	**ν_0:** 1575	-	
50	**k_1:** 0.09133±0.01568	**k_2:** $5.809e^{-05}\pm2.435e^{-05}$	**α:** 89.19±19.64	***PFO*** *Probability ratio*: 2.47 *Difference AIC*: 1.808
	q_e: 817.1±67.79	**q_e:** 1179±167.9	**β:** 0.0025±0.0006	
	R^2: 0.988	**R^2:** 0.985	**R^2:** 0.981	
	$S_{y/x}$: 32.17	**$S_{y/x}$:** 38.75	**$S_{y/x}$:** 43.77	
	ν_0: 75	**ν_0:** 81	-	
100	**k_1:** 0.07721±0.01174	**k_2:** $4.598e^{-05}\pm1.75e^{-05}$	**α:** 194.5±98.32	***PFO*** *Probability ratio*: 18.91 *Difference AIC*: 5.880
	q_e: 1493±65.63	**q_e:** 1766±154.0	**β:** 0.002345±0.00056	
	R^2: 0.976	**R^2:** 0.954	**R^2:** 0.920	
	$S_{y/x}$: 107.4	**$S_{y/x}$:** 148.9	**$S_{y/x}$:** 195.7	
	ν_0: 115	**ν_0:** 143	-	
200	**k_1:** 0.05569±0.005942	**k_2:**$1.628e^{-05}\pm4.588e^{-06}$	**α:** 335.7±136.8	***PFO*** *Probability ratio*: 55.30 *Difference AIC*: 8.026
	q_e: 3131±92.44	**q_e:** 3694±233.1	**β:** 0.001151±0.00021	
	R^2: 0.987	**R^2:** 0.971	**R^2:** 0.942	
	$S_{y/x}$: 159.6	**$S_{y/x}$:** 238.4	**$S_{y/x}$:** 337.5	
	ν_0: 174	**ν_0:** 222	-	

*Comparison of the two fits with the highest R^2 and lower $S_{y/x}$ values. ν_0 calculated according to: PFO $\upsilon_0 = k_1 q_e$; PSO $\upsilon_0 = k_2 q_e^2$

Generally, the best performance was attributed to PFO model (Table 4.2), which in fact described precisely q_t over the full range of time, specially for 100 and 200 µg L^{-1}, where both PSO and elovich models overestimated $q_{t,144h}$ (Figure 4.4C-D).

The notable affinity of *U. lactuca* toward Cd was previously reported by Wang and Dei (1999), although results found by the authors suggested a slower accumulation than that observed in the present study. After 47h of contact time, for 10 µg L^{-1}, Wang and Dei (1999) recorded values of Cd internalized by macroalgae between 15 and 20 µg g^{-1} (d.w.), while in our case, for an equal initial concentration of Cd, an accumulation of about 18 µg g^{-1} (d.w.) was achieved just in 12h. Furthermore, authors always observed a linear relationship between q_t and *t*, without any steady-state has been reached, which may due to the fact that exposure to metal only lasted 2 days (Wang and Dei 1999).

The profiles of the kinetic curves (C_t/C_0 *vs t*), for different initial concentrations of Pb in seawater are presented in Figure 4.5A-D. Results revealed that, as for Cd, seaweed are the main responsible for the decrease in concentrations of Pb in seawater, since metal levels in controls remained constant over time. Yet, a slightly different behaviour from Cd was observed for Pb, particularly in the initial removal period. The decrease on C_t/C_0,Pb at the beginning of experiments was less pronounced, which is corroborated by the lower values of initial bioaccumulation rate, v_0, estimated for Pb compared to those found for Cd (Tables 4.1 and 4.2). In addition, after the quick initial sorption, the drop in C_t/C_0 becomes less marked with the rise of initial concentration of Pb in solution.

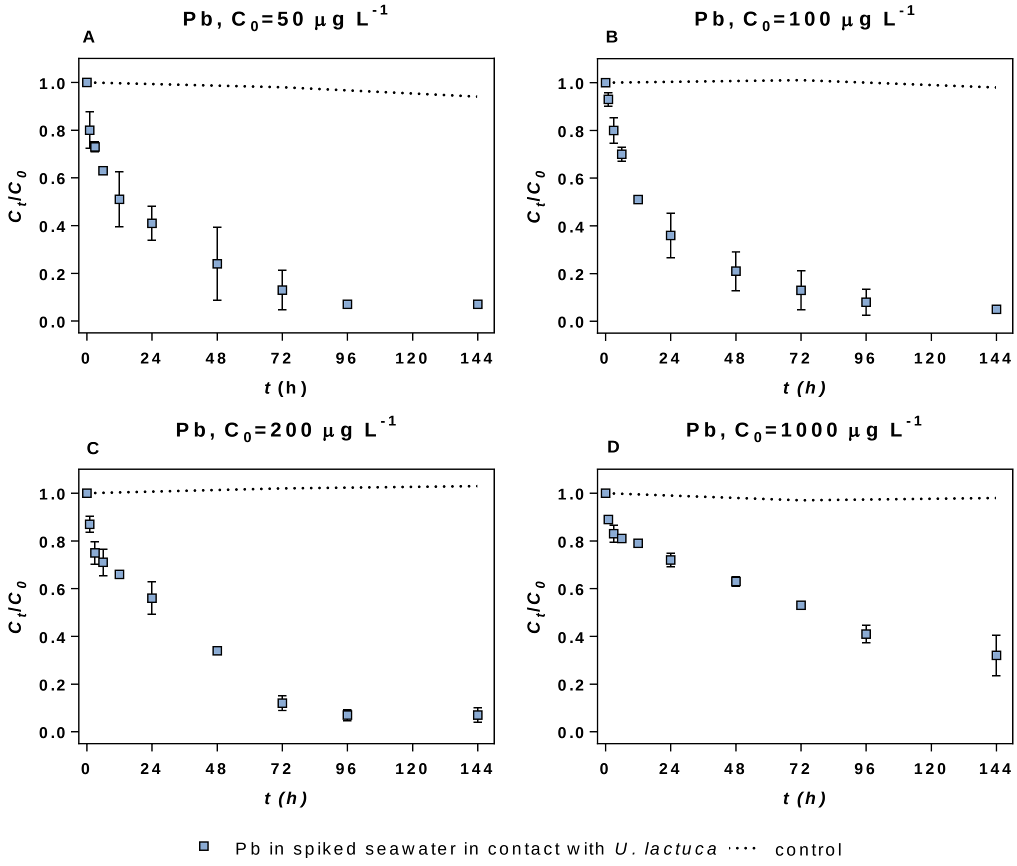


Figure 4.5: Normalized concentrations (C_t/C_0) of Pb in spiked seawater along time (*t*, h) – single-contaminant systems. The results are expressed as the mean ± standard deviation (n=3).

Nevertheless, experimental data demonstrated that living *U. lactuca* also has a remarkable capacity to remove Pb from saline waters. After a period of contact of 12h with living macroalgae, levels of Pb in seawater were reduced by 49.5, 48.5, 34.4 and 21.2% for C_0,Pb of 50, 100, 200 e 1000 µg L^{-1}, whereas increasing the contact time to 48h allowed to obtain reductions of 75.6, 78.8, 66.2 and 37%, respectively. As mentioned previously for Cd, the rise in initial metal concentrations, which represents an increase of the driving force, leads to higher initial sorption rates (Table 4.3) and larger amounts of Pb bioaccumulated, *qt*, (Table 4.5).

For initial concentrations of 50, 100 and 200 µg L^{-1}, equilibrium was reached after 96h (Figure 4.5A-C), whereas the kinetic pattern observed for 1000 µg L^{-1} indicates that a longer period of exposure time is required in order to attain a steady-state (Figure 4.5D).

The results of kinetic modelling of data on Pb uptake by living *U. lactuca*, are shown graphically in Figure 4.6A-D, while the best-fit parameters are summarized in Table 4.3.

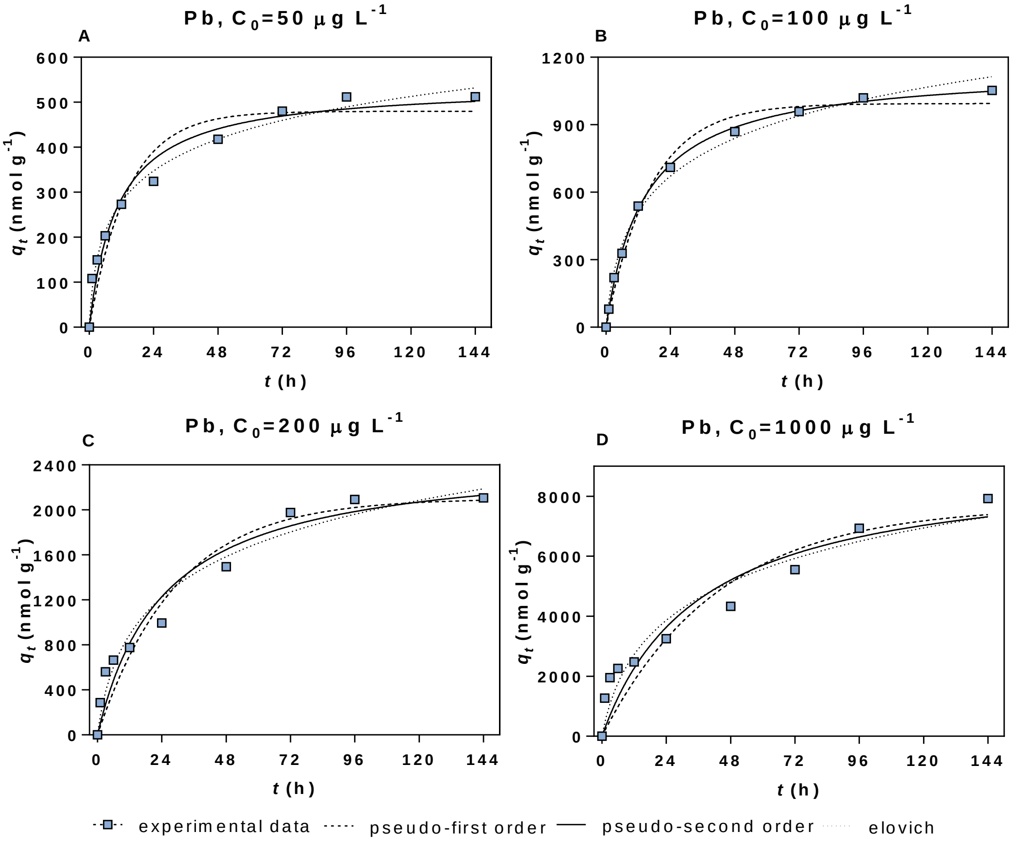


Figure 4.6: Kinetic modelling of the experimental data regarding the uptake of Pb by living *U. lactuca* (single-contaminant systems). Error bars were omitted for clarity.

Overall, there is a good agreement between experimental data and the fittings accomplished by the three kinetic models, with R^2 varying between 0.935 and 0.999, for 50 to 200 µg L^{-1}. A worst performance was recorded for 1000 µg L^{-1}, in which case equilibrium has not reached, with coefficients of correlation ranging from 0.886 to 0.939.

Comparison between models by using AIC indicates that, in most cases, Elovich model is likely to be the most suitable model to describe bioaccumulation kinetics of Pb by the green macroalgae, which points to a heterogeneous uptake/bioaccumulation mechanism (Cheung et al. 2000). Moreover, as *qt vs t* for Cd and Pb followed different kinetic (PFO vs Elovich, respectively), probably the mechanisms behind the removal of these metals by the seaweeds will not be exactly the same. In their comparative study on biosorption of Cd, Zn, and Pb from aqueous solutions by dried marine macroalgae, Freitas et al. (2008) also found that PFO was better for the system Cd/*B. bifurcate*, whereas for Pb/*B. bifurcate* PSO performed better. Differences in behaviour were justified by the different properties of metals ions in solution and different interactions between metals and biomass (Freitas et al. 2008).

Table 4.3: Best-fit parameters (± standard error) and goodness of the fittings of the kinetic models used to describe the bioaccumulation kinetics of Pb by living *U. lactuca* (single-contaminant systems).

C_0 (μg L^{-1})	PFO	PSO	Elovich	Preferred model*
50	k_1: 0.0701±0.0145	k_2: $1.732e^{-04} \pm 4.112e^{-05}$	α: 115.7±20.10	***Elovich*** *Probability ratio*: 283.95 *Difference AIC*: 11.3
	q_e: 479.8±26.21	q_e: 539.2±26.16	β: 0.00954±0.000573	
	R^2: 0.935	R^2: 0.970	R^2: 0.990	
	$S_{y/x}$: 48.96	$S_{y/x}$: 33.33	$S_{y/x}$: 18.95	
	ν_0: 34	ν_0: 50		
100	k_1: 0.05974±0.005767	k_2: $6.047e^{-05} \pm 3.056e^{-06}$	α: 136.8±19.32	***PSO*** *Probability ratio*: *Difference AIC*: -18.69
	q_e: 994.3±26.17	q_e: 1153±12.64	β: 0.00391±0.000229	
	R^2: 0.988	R^2: 0.999	R^2: 0.993	
	$S_{y/x}$: 46.40	$S_{y/x}$: 14.01	$S_{y/x}$: 35.68	
	ν_0: 59	ν_0: 80		
200	k_1: 0.03408±0.007915	k_2: $1.611e^{-05} \pm 5.949e^{-06}$	α: 179.0±50.92	***Elovich*** *Probability ratio*: 5.96 *Difference AIC*: 3.57
	q_e: 2101±157.8	q_e: 2499±227.1	β: 0.00175±0.000241	
	R^2: 0.938	R^2: 0.952	R^2: 0.966	
	$S_{y/x}$: 204.9	$S_{y/x}$: 179.8	$S_{y/x}$: 150.4	
	ν_0: 72	ν_0: 101		
1000	k_1: 0.02294±0.007838	k_2: $2.980e^{-06} \pm 1.655e^{-06}$	α: 466.4±169.3	***Elovich*** *Probability ratio*: 7.36 *Difference AIC*: 3.993
	q_e: 7669±1026	q_e: 9174±1384	β: $4.78e^{-04} \pm 9.342e^{-05}$	
	R^2: 0.886	R^2: 0.910	R^2: 0.939	
	$S_{y/x}$: 911.8	$S_{y/x}$: 811.8	$S_{y/x}$: 664.9	
	ν_0: 176	ν_0: 251		

*Comparison of the two fits with the highest R^2 and lower $S_{y/x}$ values. ν_0 calculated according to: PFO $\upsilon_0 = k_1 q_e$; PSO $\upsilon_0 = k_2 q_e^2$

Despite the best performance in mathematics description of the kinetics over the entire period of time of trials, Elovich model did not estimated accurately the values of $q_{t,144\text{h}}$ (Figure 4.6A-D). Amounts of Pb in macroalgae tissues predicted by the PSO, for t=144h, are closer to the experimental points than those under- and overestimated by PFO and Elovich models, respectively.

Figure 4.7A-C shows the time evolution of normalized concentrations of Hg in seawater, in the presence and absence of *U. lactuca*, for different initial concentrations of metal, 10, 50 and 100 µg L^{-1}.

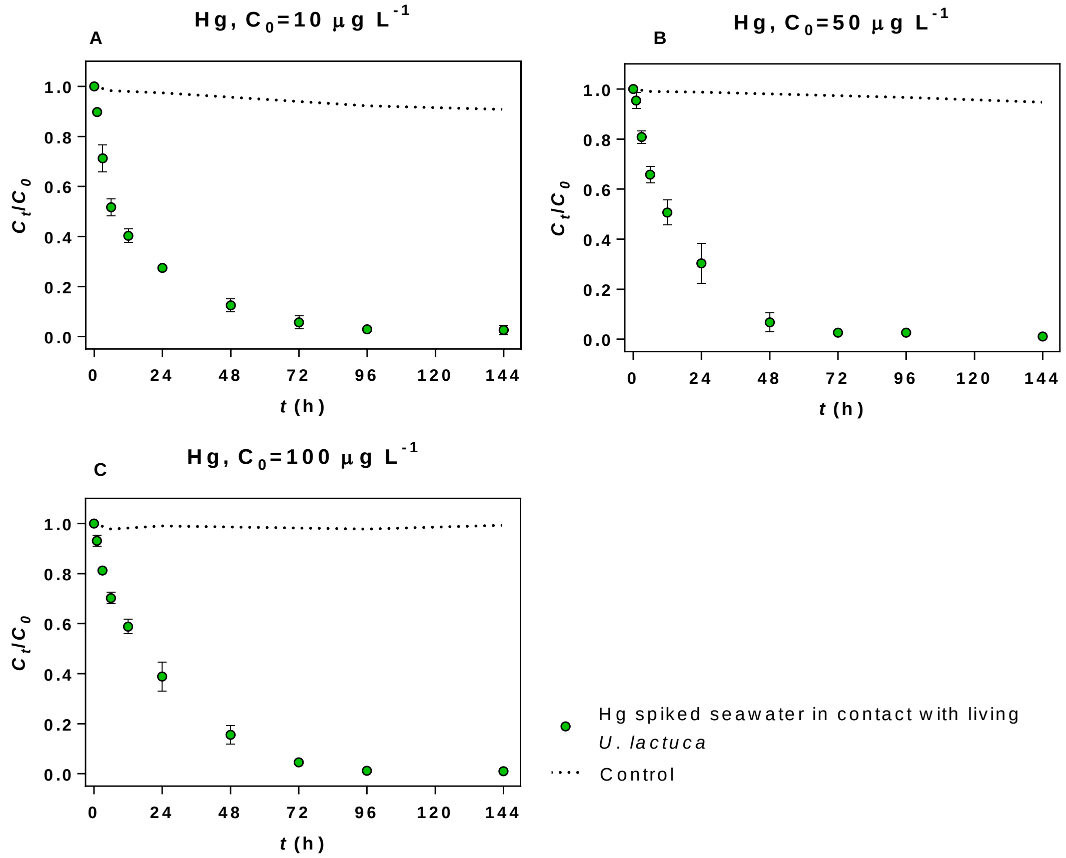


Figure 4.7: Normalized concentrations (C_t/C_0) of Hg in spiked seawater along time (t, h) – single-contaminant systems. The results are expressed as the mean ± standard deviation (n=3).

Regardless the initial metal content in seawater, kinetic profiles are characterized by a fast and noticeable decrease in C_t/C_0, in the initial period of time, followed by a subtle decrease in C_t/C_0, until equilibrium was established (at 72h for 10 µg L^{-1} and at 96h for 50 and 100 µg L^{-1}). Overall, macroalgae-free experiments show a constant metal concentration with time, proving that removal of Hg may only be attributed to interactions metal-*Ulva lactuca*.

After 12h of contact time, green macroalgae led to removal percentages of 59.7, 49.3 and 41.2%, for 10, 50, and 100 µg L^{-1}, respectively, corresponding to Hg contents in macroalgae tissues of 13.9, 51.4 and 86.7 µg g^{-1}, respectively. Like it was verified for Cd and Pb, accumulation of Hg is dose-dependent, being in accordance to those studies which claim that *U. lactuca* may serve as a good biomonitor of metal contamination in waters

(Wang and Dei 1999, Coelho et al. 2005, Kamala-Kannan et al. 2008). A 10-fold increase in the initial concentration of Hg in solution resulted in a rise on the initial bioaccumulation rate of 3.6 to 4.6-fold (Table 4.4), which is explained by the higher driving forces, at the beginning of experiments, as mentioned previously.

Fittings of experimental data on Hg uptake/accumulation along time by the macroalgae are expressed in Figure 4.8. Goodness of fit as well as models parameters are presented in Table 4.6.

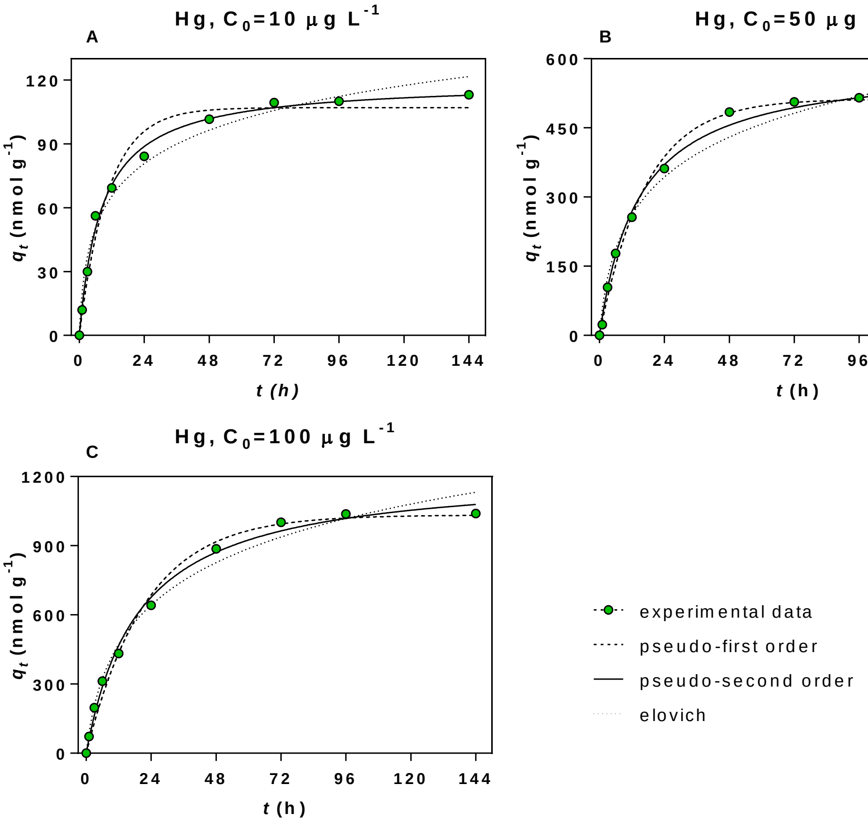


Figure 4.8: Kinetic modelling of the experimental data regarding the uptake of Hg by living *U. lactuca* (single-contaminant systems). Error bars were omitted for clarity.

All kinetic models showed high performances in the description of results, in most cases with better correlations ($0.975<R^2<0.996$) than those recorded for Cd and Pb. Although values of q_t at 144h, for 50 and 100 µg L^{-1}, were better estimated by PFO model (Figure 4.8B-C), AIC suggests that PSO is the model more likely to be correct for all concentrations (higher R^2 and lower Sy/x). Thus, kinetic analysis suggests a Hg sorption of chemical nature, relying on chemical interactions between Hg ions and superficial functional groups of macroalgae surface (Bulgariu and Bulgariu 2014), such as ion-exchange and chemical adsorption. Along with those interactions, metabolism-dependent incorporation into cells should control bioaccumulation rates (Vasconcelos and Leal 2001, Chojnacka 2007).

A summary of results obtained at the end of the assays of bioaccumulation, conducted in saline water, for monometallic solutions of Cd, Pb and Hg in contact with *U. lactuca* is presented in Table 4.5.

Results show that, in percentage terms, green macroalgae have a similar and impressive ability to remove different metals from monometallic solutions, since in general removal percentages were between 93% and 99%. However, comparing the removal efficiencies of *U. lactuca* toward the metals studied, in terms of time of contact required, it is possible to conclude that Cd removal is more efficient, since in most cases, it was more rapid (Figure 4.3, 4.5 and 4.7).

The content of Cd in macroalgae, measured after digestion, for the highest initial concentration ($C_{0,\mathrm{Cd}}$=200 µg L^{-1}; $q_{t,\mathrm{144h.exp}}$=2876±93.98 nmol g^{-1}) was very close to the value calculated ($q_{t,\mathrm{144h.theor}}$=3083 nmol g^{-1}) by mass balance between metal concentrations in solution at the beginning and end of the trial (relative error of 6%). This indicates that all Cd removed from the solution is actually bound in the seaweed tissue. In 1999, Wang and Dei studied the uptake/accumulation of Cd by *U. lactuca* and *Gracilaria. blodgetti*, and examined metal depuration by the algae in clean seawater. Researchers reported that *U. lactuca* retained Cd very efficiently in their tissues, as no noticeable loss of Cd to water was measured (Wang and Dei 1999). Furthermore, chemical quantification of Cd in the algae biomass, allowed to confirm the assumption that for concentrations of 10-100 µg L^{-1}, removal have continued beyond the point where it was possible to quantify this metal in solution, reaching virtually 100% of removal. The outstanding capability of *U. lactuca* to accumulate and retain Cd, gives it a high potential for application not only in processes of wastewater treatment, but also in processes of pre-concentration for analytical chemistry (Romero-Gonzalez et al. 2000).

Table 4.4: Best-fit parameters (± standard error) and goodness of the fittings of the kinetic models used to describe the bioaccumulation kinetics of Hg by living *U. lactuca* (single-contaminant systems).

C_0 (µg L^{-1})	PFO	PSO	Elovich	Preferred model*
10	k_1: 0.09435±0.01130	k_2: $1.015e^{-03}$±$9.059e^{-05}$	α: 30.46±7.48	***PSO*** *Probability ratio*: 1222 *Difference AIC*: -14.22
	q_e: 107.0±3.220	q_e: 119.3±2.069	β: 0.04317±0.003518	
	R^2: 0.975	R^2: 0.996	R^2: 0.983	
	$S_{y/x}$: 6.459	$S_{y/x}$: 2.924	$S_{y/x}$: 5.953	
	ν_0: 10	ν_0: 14		
50	k_1: 0.05871±0.003593	k_2: $1.167e^{-04}$±$1.312e^{-05}$	α: 68.39±17.31	***PSO*** *Probability ratio*: 1.74 *Difference AIC*: 1.104
	q_e: 512.5±8.582	q_e: 592.8±14.48	β: 0.00757±0.000803	
	R^2: 0.995	R^2: 0.996	R^2: 0.978	
	$S_{y/x}$: 15.98	$S_{y/x}$: 15.12	$S_{y/x}$: 32.97	
	ν_0: 30	ν_0: 41		
100	k_1: 0.04553±0.003841	k_2: $4.201e^{-05}$±$4.978e^{-06}$	α: 99,55±17.71	***PSO*** *Probability ratio*: 11.11 *Difference AIC*: -4.816
	q_e: 1033±25.41	q_e: 1224±33.67	β: 0.00347±0.000291	
	R^2: 0.992	R^2: 0.995	R^2: 0.988	
	$S_{y/x}$: 39.90	$S_{y/x}$: 31.36	$S_{y/x}$: 48.15	
	ν_0: 47	ν_0: 63		

*Comparison of the two fits with the highest R^2 and lower $S_{y/x}$ values. ν_0 calculated according to: PFO $\upsilon_0 = k_1 q_e$; PSO $\upsilon_0 = k_2 q_e^2$

For Pb, results showed to be different, since only 46-58% of metal removed from the solution was really accumulated by the macroalgae, which suggests that other mechanisms may intervene in the purification of water, such as precipitation (Chen and Wang 2007). As concentrations remained stable in controls, precipitation of Pb had to be induced by the macroalgae, indirectly by raising the pH of solution (there was a gradual ascent along the experience until 9.3±0.3), or directly through microprecipitation on alga surface (Chojnacka 2009) and/or by production of exudates capable of complexing metals (Coelho et al. 2005, Torres et al. 2008). Further studies are required in order to understand those mechanisms.

As seen for Cd, determination of Hg levels in macroalgae biomass, at 144h, confirmed that all metal removed from seawater along time was being incorporated by macroalgae (Table 4.5). Thus, Hg-reduction phenomena and subsequent H_0 volatilization (Sunda and Huntsman 1998) may be excluded. The largest relative error between theoretical and experimental $q_{t,144\text{h}}$ (17%) was recorded for the lowest initial concentration, 10 µg L^{-1}, which may be explained by a "dilution effect", due to fast growth of macroalgae, not balanced with accumulation of Hg (Costa et al. 2011).

Table 4.5: Effect of initial metal concentrations on the amount of metal bioaccumulated by the *U. lactuca* ($q_{t,144h}$ – theoretical and experimental), removal percentage and residual concentration of metal in solution ($C_{t,144h}$).

Metal	C_0, µg L^{-1} (nmol L^{-1})	$q_{t,144h}$, theoretical [1] nmol g^{-1}	$q_{t,144h}$, experimental[2] nmol g^{-1}	Removal[3]$_{144h}$, %	$C_{t,144h}$, µg L^{-1}
Cd	10 (89)	>154.5	193.3±15.78	>78.9 (100)	<2.0
	50 (445)	>710.9	821.8±119.6	>94.9 (100)	<2.0
	100 (890)	>1457	1464±136.1	>97.5 (98)	<2.0
	200 (1779)	3083	2876±93.98	98.5	2.4
Pb	50 (241)	512.0	266.1±79.15	92.6	4.0
	100 (483)	1053	561.0±131.9	95.4	4.9
	200 (965)	2106	977.3±129.8	93.3	16
	1000 (4826)	7922	4631±35.81	67.7	379
Hg	10 (50)	113.0	93.22±5.680	97.4	0.31
	50 (249)	514.2	501.0±81.75	98.9	0.84
	100 (499)	1039	985.5±100.7	99.0	1.0

[1] – obtained through mass balance using eq. 1; [2] - obtained by chemical quantification in seaweed; [3]- obtained by using eq. 2 (obtained considering the amount of metal available initially in solution and the amount of metal in macroalgae biomass at 144h, determined by chemical quantification in seaweed).

Overall, data demonstrate the effectiveness of *U. lactuca* in removing metals from salt water. Less than half-gram per litter of this green macroalgae allowed to reduce all concentrations of Hg in solution to values equal or below the limit set by the European Union for waters for human consumption, 1 µg L^{-1} (Council Directive 98/83/EC 1998). Legal criteria were also met for Cd, since all residual levels of metal in solution were below the maximum permissible values (MPV) for drinking water, 5 µg L^{-1} (Decree-Law No. 103/2010 2010), and bellow or very close to the maximum allowable concentration (MAC) of EQS for Cd in surface waters (Directive 2013/39/EU 2013).

As for Pb and concentrations of 50 and 100 µg L^{-1}, levels of metal in solution at the end of the process were below 10 and 7.2 µg L^{-1}, which are respectively MAC values for safe drinking water, according to Portuguese law (Decree-Law No. 103/2010 2010) and annual average (AA) of EQS for Pb in surface waters (Directive 2013/39/EU 2013). For 1000 µg L^{-1}, final concentration of Pb in seawater exceeds the allowed values by about 40 times. However, it is important to note that for this concentration, no steady-state was reached within 144h, and if exposure time was prolonged, macroalgae would have improved water quality even more.

Bioconcentration factors (BCF) in *U. lactuca*, defined as the ratio between metal concentrations in the organisms to metal concentration in seawater, ranged between 1616-2169 for Cd, 960-1160 for Pb and 1855-2010 for Hg. Overall, BCF varied inversely with the concentration gradient in seawater (Wang and Dei 1999, Coelho et al. 2005).

4.3.3 Bioremediation in multi-contaminant systems

After evaluating the ability of *U. lactuca* to remove Cd, Pb and Hg individually, metal uptake/accumulation by this green macroalgae was also assessed for multi-contaminant systems, comprising simultaneously those three metals. Two different contamination scenarios were studied: Mixture M1 – Cd, Pb and Hg were in equal mass concentration in seawater, 50 µg L^{-1}; Misture M2 – Cd, Pb and Hg were in seawater, in concentrations of 200, 1000 and 50 µg L^{-1}, respectively.

The variation of normalized concentrations of Hg, Cd and Pb (C_t/C_0 *vs t*) in ternary solutions, M1 and M2, are presented in Figure 4.9A-B.

Results show that *U. lactuca* is capable of remove simultaneously Cd, Pb and Hg, yet with a removal efficiency for each metal depending greatly on the exposure condition. In M1, the profiles of curves C_t/C_0 were similar among metals, and removal percentages at the end of assays were also comparable, c.a. 96%, suggesting an equal affinity of green macroalgae toward different metals. However, it should be noted that initial molar concentration of Cd was ca. 1.8 folds the molar concentrations of Pb and Hg, and initial bioaccumulation rates varied among metals, according to Pb>Hg>Cd. This sequence is in agreement with the values of the kinetic constants of PFO (k_1) and PSO (k_2) models (Table 4.6), and follows an inverse trend of the initial metal concentrations ($C_{0,Cd}>C_{0,Hg}>C_{0,Pb}$). The kinetic pattern presented in M2 was very different among the three metals, with levels of Hg being reduced very fast, reaching up to 99.7% of removal at 144h, while removal efficiencies for Cd and Pb were respectively 57.1 and 33.8%, with no equilibrium achieved. Although lower efficiencies were recorded for the metals with high initial concentration, an inverse situation was observed for the amount of metal incorporated by the macroalgae (Table 4.7).

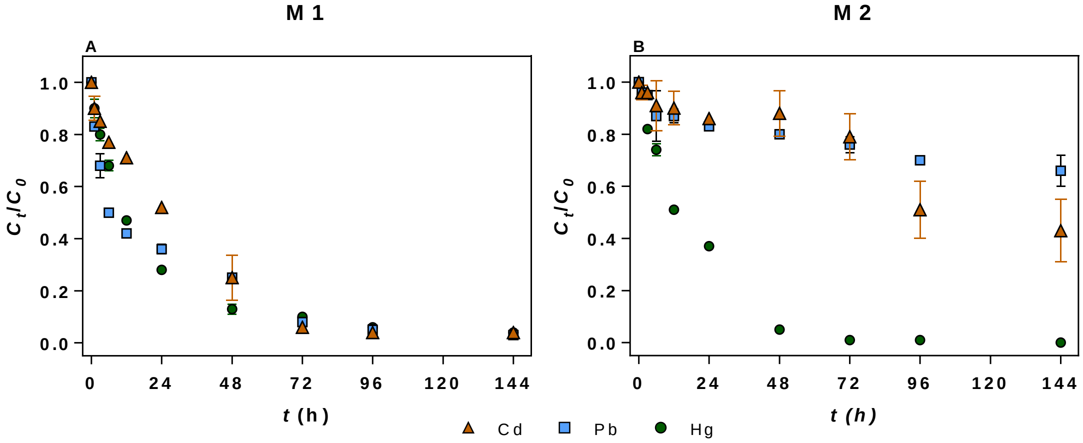


Figure 4.9: Normalized concentrations (C_t/C_0) of Cd, Pb and Hg, simultaneously present in seawater along time (t, h), when in contact with living *U. lactuca*, under two different contamination scenarios, M1 and M2. The results are expressed as the mean ± standard deviation (n=3).

The selectivity of *U. lactuca* toward the different metals may be quantified by the ratio between the distribution coefficients (K), which in turn are given by $K=q_e/C_e$ for each metal at equilibrium (Lopes et al. 2014). Assuming the experimental values of $qt,_{144h}$, the pseudo-equilibrium selectivity found for the living macroalgae toward the target metals followed the order Cd>Hg>Pb in M1, whereas for M2 the order of selectivity was Hg>Cd>Pb. It seems that relative initial concentrations play a fundamental role in selectivity: for the low contamination scenario, selectivity follows the trend of molar concentrations, while for a scenario of severe contamination the behavior is inverse. Kamala-Kannan et al. (2008) also found that the concentrations of metal in *U. lactuca* from Pulicat Lake (with levels of metal in water of 0.04-3.4 µg L^{-1}) decreased in the order: Cd > Pb, similar to that of surrounding environment. Different results were obtained by Sari and Tuzen (2008) using non-living biomass of *U. lactuca*. The authors concluded that the ability for Pb biosorption was higher than the biosorption capacity for Cd. In fact, although it is widely accepted that the major mechanisms involved in metal biosorption by dead algae are the same as occur on the surface of the living seaweed, there are differences related to the metabolism of the organism.

Comparing the time evolution of normalized concentrations of each metal in mono- and multi-metallic solutions (Figure 4.10A-F) it is possible to conclude that Hg was barely affect by the presence of Pb and Cd in both exposure conditions studied. However, after 48h the reduction in Hg concentration was slightly slower at M1, compared to Hg removal rate in the absence of competing metals, or at M2 (Figure 4.10E-F). The amount of Hg bound per unit of macroalgae mass was very similar between M1 and M2, respectively, 539.4 and 536.1 nmol g^{-1}.

Unlike what was observed for Hg, the profile of Cd removal was altered by the presence of competing metal ions, and differences were more noteworthy at M2. Initial bioaccumulation rates were lower than those recorded in the absence of Hg and Pb, and extension of process was also negatively affected. After 144h of contact, living *U. lactuca* accumulated 1022 and 2323 nmol Cd g^{-1}, respectively for M1 and M2, being required a longer time to achieve an equilibrium in M2.

In the case of Pb, the kinetics of removal was affected differently depending on the contamination scenario. In M1, as verified for Hg, removal patterns in the presence and absence of competing metal ions in solution were very similar: initially, the process of removal of Pb was very fast, followed by a subtle decrease in C_t/C_0, until a balance is established, which corresponds to an bioaccumulation of 498.4 nmol g^{-1}. In the exposure condition M2, change in kinetic profile due to the presence of other metals was evident, with a less pronounced decrease on Pb content in seawater, over time. The removal process was far from reaching equilibrium at 144h, although the algae had already incorporated an impressive amount of Pb, 3539 nmol g^{-1}.

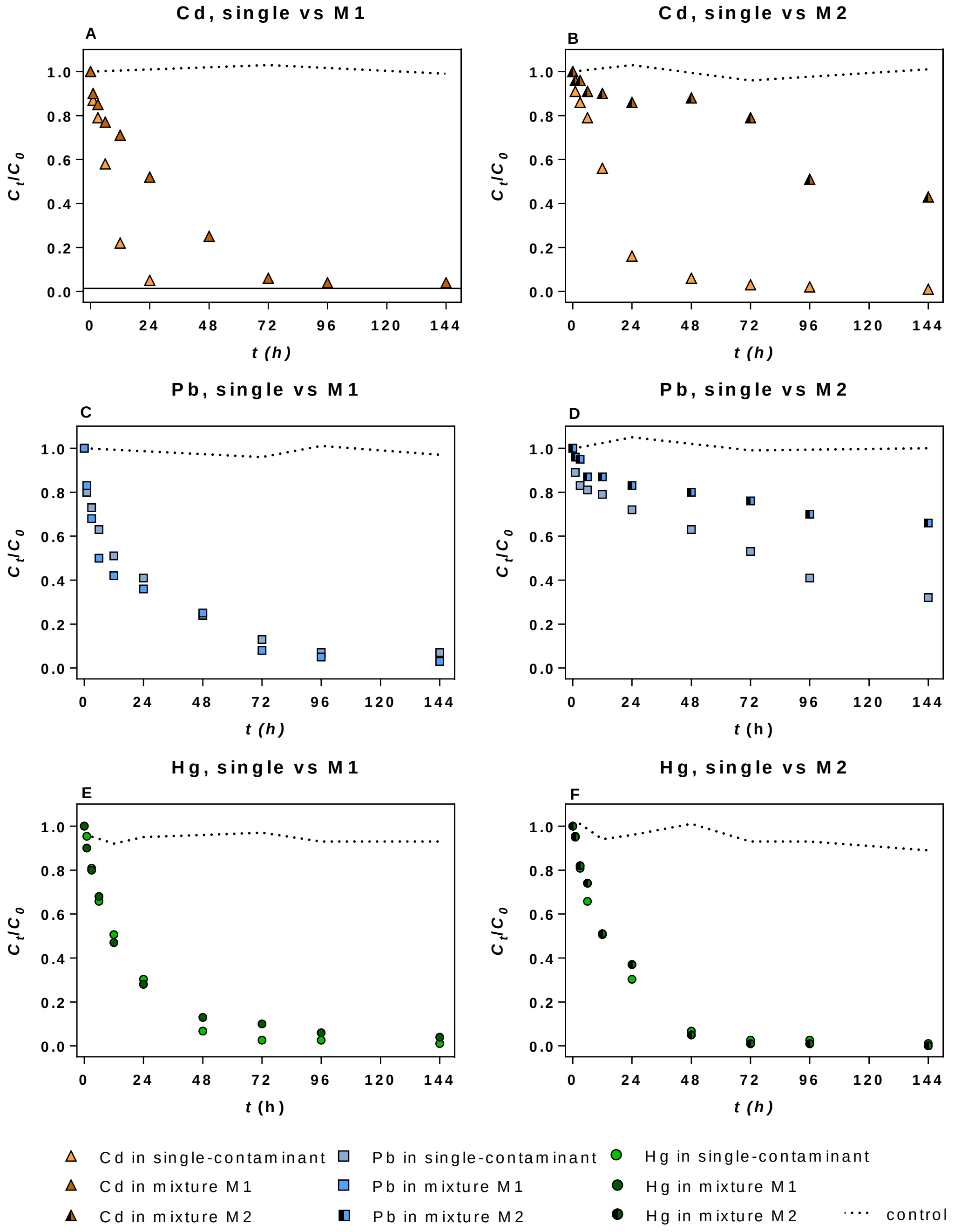


Figure 4.10: Normalized concentrations (C_t/C_0) of Cd, Pb and Hg in spiked seawater along time (*t*, h), when in contact with *U. lactuca* (single vs. multi-contaminant systems). Error bars were omitted for clarity.

The comparison between q_t values at equilibrium, obtained in mixtures and in the same conditions but in the monometallic solution, enable to assess the effect of the presence of foreign metals on the bioaccumulation of a specific metal. If $q_e^{\text{mixture}}/q_e^{\text{single}}<1$, the bioaccumulation is inhibited by the presence other metals; if $q_e^{\text{mixture}}/q_e^{\text{single}} =1$, there is no observable interaction; and if $q_e^{\text{mixture}}/q_e^{\text{single}} >1$, the bioaccumulation is favoured by the presence of co-existing metals. For Hg, values found were 1.05 and 1.04, respectively for M1 and M2, which indicates that bioaccumulation of Hg was neither favoured nor inhibited in both systems. The bioaccumulation of Pb was not affected by the presence of Hg and Cd in M1 (1.03), whereas for M2 it was highly inhibited (0.45). As for Cd, bioaccumulation was slightly inhibited in the case of low contamination scenario (0.94), and somehow inhibited by the presence of Hg and Pb, in the severe contamination scenario (0.75).

The fitting of experimental data, on metal removal in ternary systems, to PFO, PSO and Elovich models are presented in Figure 4.11A-F. Table 4.6 summarises the results of fits.

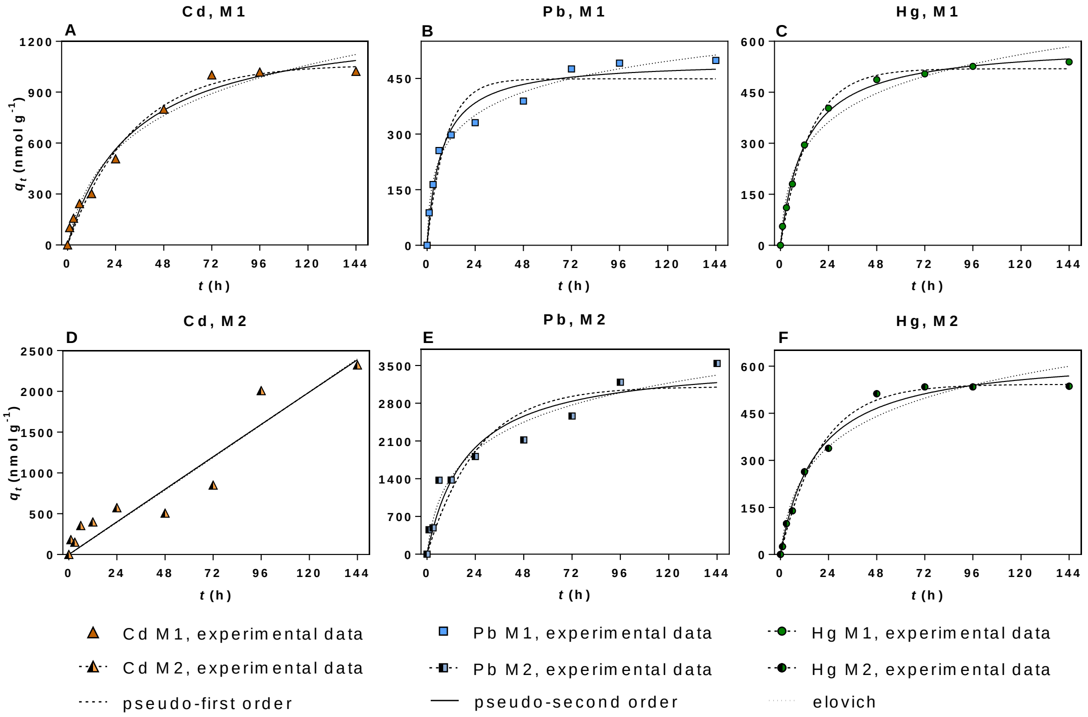


Figure 4.11: Kinetic modelling of the experimental data regarding the uptake of Cd, Pb and Hg by living *U. lactuca* in multi-contaminant systems, at two different contamination scenarios, M1 and M2. Error bars were omitted for clarity.

Overall, there was a good agreement between experimental data obtained in M1 and models applied ($0.968<R^2<0.998$). For each metal, the best fit model is consistent with the observations in monometallic solutions: Cd (PFO); Pb (Elovich); Hg (PSO). In M2, the fits showed a worst performance, with exception of Hg ($0.973<R^2<0.993$). Pseudo-first order and PSO models failed to describe the bioaccumulation of Cd (ambiguous), while for Pb coefficients of correlation ranged between 0.896 and 0.965.

Table 4.6: Best-fit parameters (± standard error) and goodness of the fittings of the kinetic models used to describe the bioaccumulation kinetics of Cd, Pb and Hg by living *U. lactuca* in multi-contaminant systems, under two different contamination scenarios, M1 and M2.

C_0 (μg L^{-1})	**PFO**	**PSO**	**Elovich**	**Preferred model***
Cd in M1	**k_1:** 0.03079±0.003608	**k_2:** $2.292e^{-05}$±$5.713e^{-06}$	**α:** 56.13±12.82	***PFO*** *Probability ratio*: 3.14 *Difference AIC*: 2.288
	q_e: 1063±42.12	**q_e:** 1333±87.68	**β:** 0.00283±0.000389	
	R^2: 0.987	**R^2:** 0.983	**R^2:** 0.977	
	$S_{y/x}$: 50.29	**$S_{y/x}$:** 56.38	**$S_{y/x}$:** 66.37	
Cd in M2	**k_1:** Ambiguous	**k_2:** Ambiguous	**α:** 16.37±4.461	***Elovich*** *Probability ratio*: - *Difference AIC*: -
	q_e: Ambiguous	**q_e:** Ambiguous	**β:** $-1.523e^{-05}$±$2.74e^{-04}$	
	R^2: -	**R^2:** -	**R^2:** 0.903	
	$S_{y/x}$: -	**$S_{y/x}$:** -	**$S_{y/x}$:** 263.2	
Pb in M1	**k_1:** 0.1072±0.02438	**k_2:** 0.0014±0.0003	**α:** 172,2±34.74	***Elovich*** *Probability ratio*: 197.74 *Difference AIC*: 10.57
	q_e: 448.8±25.10	**q_e:** 498.3±22.12	**β:** 0.01094±0.000673	
	R^2: 0.922	**R^2:** 0.968	**R^2:** 0.989	
	$S_{y/x}$: 51.59	**$S_{y/x}$:** 32.90	**$S_{y/x}$:** 19.39	
Pb in M2	**k_1:** 0.03961±0.01173	**k_2:** $1.383e^{-05}$±$5.726e^{-06}$	**α:** 320.2±96.81	***Elovich*** *Probability ratio*: 20.9 *Difference AIC*: 6.080
	q_e: 3108±280.8	**q_e:** 3624±350.1	**β:** 0.00122±0.000167	
	R^2: 0.896	**R^2:** 0.935	**R^2:** 0.965	
	$S_{y/x}$: 405.8	**$S_{y/x}$:** 321.0	**$S_{y/x}$:** 236.9	
Hg in M1	**k_1:** 0.06841±0.003913	**k_2:** $1.367e^{-04}$±$8.851e^{-06}$	**α:** 86.36±20.28	***PSO*** *Probability ratio*: 61.72 *Difference AIC*: -8.245
	q_e: 519.4±7.889	**q_e:** 595.1±8.097	**β:** 0.00786±0.000721	
	R^2: 0.996	**R^2:** 0.998	**R^2:** 0.982	
	$S_{y/x}$: 14.63	**$S_{y/x}$:** 9.688	**$S_{y/x}$:** 30.17	
Hg in M2	**k_1:** 0.04963±0.003788	**k_2:** $8.720e^{-05}$±$1.549e^{-05}$	**α:** 55.14±15.40	***PFO*** *Probability ratio*: 14.98 *Difference AIC*: 5.413
	q_e: 542.2±11.78	**q_e:** 639.5±25.88	**β:** 0.00665±0.000857	
	R^2: 0.993	**R^2:** 0.989	**R^2:** 0.972	
	$S_{y/x}$: 19.32	**$S_{y/x}$:** 25.33	**$S_{y/x}$:** 39.76	

*Comparison of the two fits with the highest R^2 and lower $S_{y/x}$ values.

A summary of the data acquired at the end of bioaccumulation trials, conducted in saline water, for mixtures of Cd, Pb and Hg, M1 and M2, in contact with living *U. lactuca* is presented in Table 4.7.

Table 4.7: Summary of results obtained for mixtures, M1 and M2, in contact with living *U. lactuca*: initial bioaccumulation rate (v_0) and amount of metal bioaccumulated ($q_{t,144h}$ – theoretical and experimental), removal percentage and residual concentration of each metal in solution ($C_{t,\ 144h}$) at the end of trials.

Mixture	***Metal: C_0,*** **nmol L^{-1}**	***$q_{t,144h}$*** **theoritecal[1] nmol g^{-1}**	***$q_{t,144h}$*** **experimental[2] nmol g^{-1}**	***Rem.,$_{144h}$*[3] %**	***$Ct,_{144h}$*** **µg L^{-1}**
M1	Cd: 445	1022	1008±206.9	96.3	2.0
	Pb: 241	498.4	248.5±12.17	96.7	2.0
	Hg: 249	539.4	518.4±10.79	96.4	2.0
M2	Cd: 1779	2323	2463±63.34	57.1	77.3
	Pb: 4826	3539	2519±249.1	33.8	718
	Hg: 249	536.1	525.3±12.37	99.7	0.15

* calculated through the derivative of the function $q_t= f(t)$, when $t=0$; [1] – obtained through mass balance using eq. 1; [2] - obtained by chemical quantification in seaweed; [3]- obtained through variation of metal concentration in solution using eq. 2 (obtained considering the amount of metal available initially in solution and the amount of metal in macroalgae biomass at 144h, determined by chemical quantification in seaweed).

The measurement of metal content in macroalgal biomass revealed, once again, that all Cd and Hg removed from the seawater was fixed in the organism tissues, since experimental values of $q_{t,144h}$ matched perfectly with theoretical values of $q_{t,144h}$ ($1.4\%<E_r<5.7\%$). By contrary, only 49% and 71% of all Pb removed from the dissolved phase of seawater, in M1 and M2 respectively, showed to be bioaccumulated by the marine macroalgae. Results follow the trend observed in the experiments of exposure to single-contaminant, and are in accordance with the findings of Turner et al. (2008b), who conducted short-term experiments in synthetic seawater, in order to study the effect of humic substances and salinity in the uptake of Pd, Cd, Hg and Pb by *U. lactuca*. Researchers also operationally discriminated intracellular uptake and surface adsorption (by washing macroalgae in 3 mM EDTA), and found that internalization follow the sequence: Hg>Pd>Cd>Pb, being that Hg fraction internalized was c.a. 100% and Pb fraction internalized was below 30% (Turner et al. 2008b).

Bioconcentration factors in M1 were also in line with those obtained in monometallic solutions, respectively, 2266, 1032 and 2078 for Cd, Pb and Hg, whereas for M2, BCF for Cd and Pb were slightly lower, respectively 1384 and 522, and similar for Hg, 2106.

Analysing the efficiency of the removal process in terms of compliance with legal criteria on water quality, we can conclude that, for the lowest contamination scenario, MPVs for water for human consumption were achieved for Cd and Pb (Decree-Law No. 103/2010 2010), as well as EQS for Pb in surface waters (Directive 2013/39/EU 2013). The residual concentration of Hg in M1 was only slightly above the legal limits for drinking waters, however, in M2 the level of Hg was much lower than that allowable limit, due to synergistic effect of Cd and Pb. None of the legal criteria was accomplished for Cd and Pb in the highest contamination scenario, however it is important to remember that time profiles (Figure 4.10B and D) indicate that better water quality would be attained if the period of time was extended.

Based on all results, it was concluded that living *U. lactuca* may effectively be the basis of an alternative and cost-effective biotechnology for the simultaneous removal of Cd, Hg and Pb from wastewaters and contaminated salt waters. It should be noted that experimental conditions used in the present work intended to mimic real situations of contamination in water. Natural seawater was used as matrix along with environmental relevant concentrations of metal.

4.3.4 Effects of metals on macroalgae growth

The presence of toxic metals in the culture medium may affect organisms growth rate, and eventually lead to death, either by direct toxicity (Costa et al. 2011), or by nutrient deficiency, induced by metal competition for binding sites and transport proteins (Sunda and Huntsman 1998). The macroalgal growth rate is closely linked to the removal efficiency, since a fast growth will lead to a rapid increase of their biomass, and consequently to the multiplication of specific sites for metal binding (Chojnacka 2010). By other hand, macroalgae decay during the removal process would make unfeasible its application on water remediation, since all metal accumulated would be immediately released back into the water. Thus, the influence of Cd, Pb and Hg in *Ulva lactuca* growth rate was evaluated along the bioaccumulation experiments, for single and multi-contaminant systems. It should be highlighted that for the full range of concentration used, no sporulation or marked loss of color, which indicates algae decay (Han and Choi 2005) was observed during the 6 days of trials.

Figure 4.12 shows the relative growth rate (RGR) of *Ulva lactuca*, expressed in % day^{-1}, at defined periods of exposure time to monometallic solutions of the studied metals. After 24 hours of contact time, the RGR of reference (0 µg L^{-1}, macroalgae in clean seawater) was 10.6±3.3 % day^{-1}, which is in line with the values recorded by Han et al. (2008) for two macroalgae of the genus *Ulva* (c.a. 12%). Costa et al. (2011) documented higher RGR for *Ulva lactuca* (18±1 % day^{-1}) in seawater, at 24h, however, researchers added nutrients to the culture medium. In this study, no nutrient medium was used in the bioaccumulation trials, since preliminary tests showed that the availability of metals in solution was very affected.

On the whole, different growth patterns were observed between reference and the various contaminated solutions, however, with significant differences (two-way ANOVA, Tukey post hoc test, $\rho<0.05$) only for shorter exposure time (24h).

Cadmium (Figure 4.12A) seems to enhance the cellular metabolism of the organism, causing a significant increase of RGR, at 24h, in 10 and 50 µg L^{-1} when compared with reference (Tukey test, $Q=4.172$ and $Q=4.572$, respectively; $\rho<0.05$). The reasons for that unexpected faster growing are not clear, and may be related to a defense mechanism of the macroalgae against high levels of metal in cells, since increasing its biomass a “dilution effect” occurs (Sunda and Huntsman 1998, Costa et al. 2011), and internal concentration of metal diminishes.

With the increase of contact time, differences between RGR of reference and Cd spiked seawaters become smaller, with almost none differences at 144 hours. A similar behavior has been reported by Costa et al. (2011) when exposed *U. lactuca* to different concentrations of Hg in seawater. The authors explained this behavior by the fact that the metal concentration in solution decreases over time, but also by a possible physiological adaptation of the macroalgae to the presence of metal.

On the other hand, there was a trend towards decrease of *Ulva lactuca* RGR along time, i.e., in all cases macroalgae grew (RGR is positive) but at a progressively slower rate. This pattern was also verified for Pb and Hg, and may be attributed to the increasingly lower availability of nutrients and CO_2, which were consumed as algae grew.

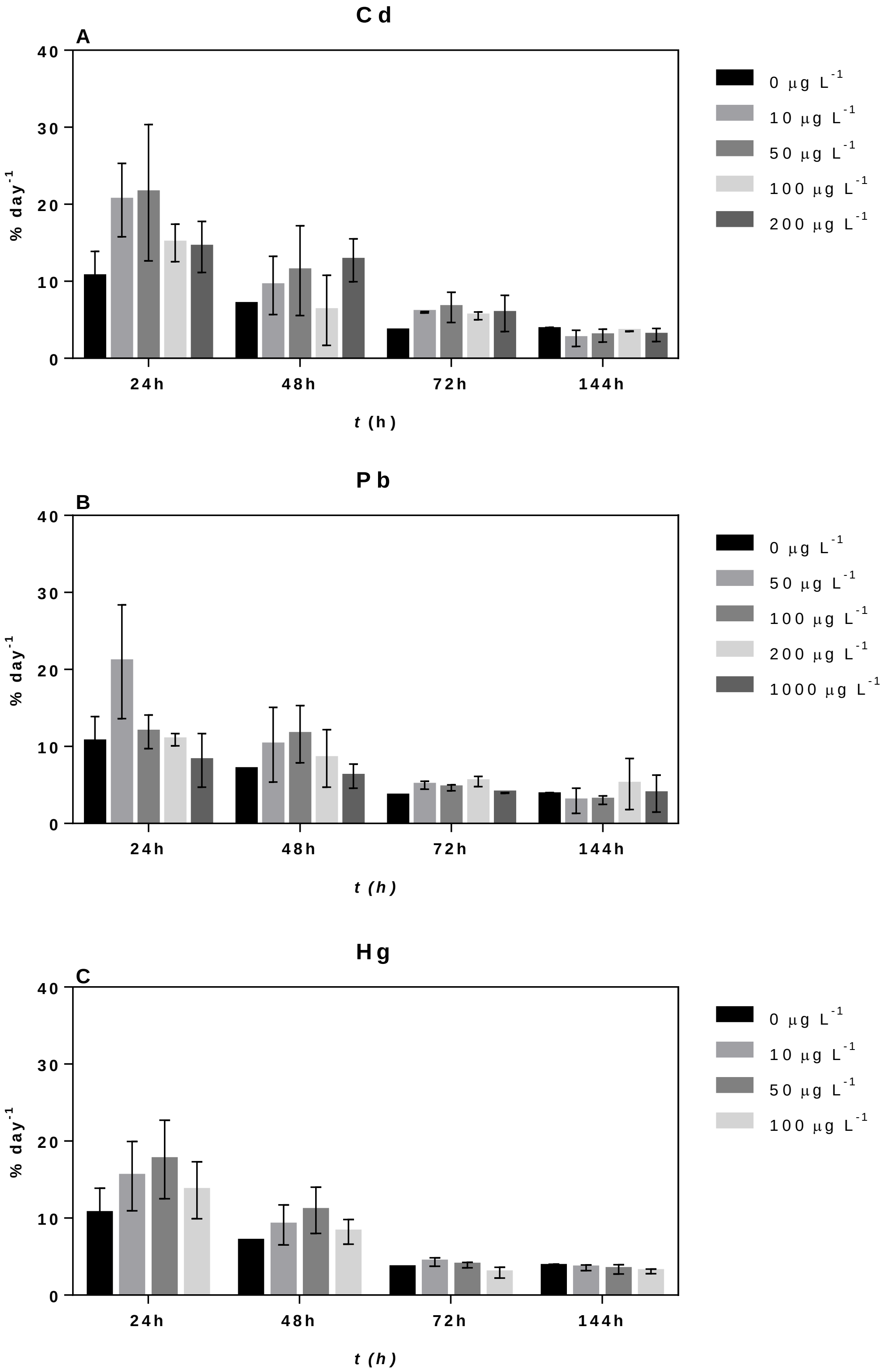


Figure 4.12: Relative growth rate of *U. lactuca* in clean seawater (0 µg L^{-1}), and when exposed to monometallic solutions of Cd (A), Pb (B) and Hg (C), in different concentrations, at 24, 48, 72 and 144h. Results are expressed as mean and errors bars represent standard deviations (n=6).

At 24 hours (Figure 4.12B), only the RGR associated to Pb,C_0=50 µg L^{-1} was considerably higher (21.0±7.3 % day^{-1}) that RGR of reference (Tukey test, Q=4.744; $\rho<0.05$). Additionally, for the highest concentration of Pb, 1000 µg L^{-1}, a decrease on RGR was observed (8.2±3.2 % day^{-1}) when compared to the reference, however with no significance (two-way ANOVA, $\rho>0.05$).

Exposure to Hg (Figure 4.12C), affected growth rate positively ($RGR_{Pb}/RGR_{reference}>1$) but two-way ANOVA test only indicated significant differences between reference and 50 µg L^{-1}. As observed for Cd and Pb, RGRs of macroalgae exposed to Hg decreased over time for all concentrations, which can be explained by the reduced availability of nutrients and CO_2 in solution, capable of sustain growth rates similar to those observed in the beginning.

The evolution of *U. lactuca* RGR over time, when exposed to mixtures of Cd, Pb and Hg, under two different scenarios, M1 (equal mass concentrations of metals, C_0=50 µg L^{-1}) and M2 (Cd,C_0=200 µg L^{-1}, Pb,C_0=1000 µg L^{-1} and Hg,C_0=50 µg L^{-1}) is presented in Figure 4.13.

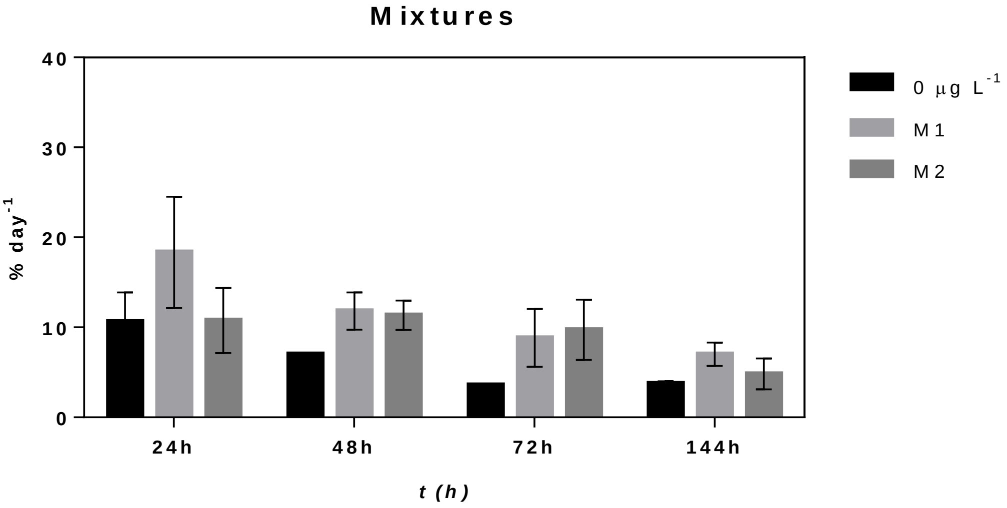


Figure 4.13: Relative growth rate of *U. lactuca* in clean seawater (0 µg L^{-1}), and in contact with spiked multimetallic solutions of Cd, Pb and Hg in equal mass concentrations (M1) and in different concentrations of the three elements (M2).

Along the experimental trials, RGRs of macroalgae in contact with multimetallic solutions were in most cases higher than the correspondent RGR of reference. Significant differences were only observed at 24h, between control and M1 (Tukey test, Q=4.175; $\rho<0.05$), which suggests that, for the concentrations tested, growth is not adversely influenced by the simultaneous presence of metals in solution. In general, the growth pattern was very similar for the two contamination scenarios, with exception of RGRs at 24h, where M1 RGR (18.4±6.1 % day^{-1}) was significantly higher than that of M2 (10.7±3.6 % day^{-1}). Once again, macroalgae RGRs decreased with time in all cases.

4.3.5 Variation on pH

During the experiments, the pH of solutions was daily monitored in order to evaluate the influence of macroalgae metabolism in that parameter, and to verify if there was a correlation with metallic contamination. As already mentioned, initial pH of seawater was 7.9, and the addition of metal stock solution leads to a slightly decrease of seawater pH (generally less than 0.1). The major decrease was observed when the highest concentration of Pb, 1000 µg L^{-1}, was prepared (pH=7.3).

Over the full period of time, pH of all controls (metal spiked seawater in the absence of *U. lactuca*) remained unchanged. By contrary, there was a gradually increasing of pH along time, in all solutions containing macroalgae (including clean seawater with *U. lactuca*), until a maximum value of 8.9-9.6 was reached at 144h. This increment on pH was attributed to the natural metabolism of the organism (Velasquez and Dussan 2009). The uptake of CO_2 by the macroalgae during photosynthesis, reduces HCO_3^- levels in seawater and increases the concentration of OH^- ions, leading to a rise of pH. Since experimental vessels were always sealed with Parafilm (to minimize eventual contamination or loss of metal) gas exchanges between culture medium and surrounding atmosphere were limited, as consequently CO_2 replenishment. Collecting several solution samples, and leaving them in open vials for one day, lead to a reduction of pH values close to original, supporting our hypothesis. As explained previously, no buffer was added to solutions and no pH adjustments were made because that may interfere with organism metabolism (Vasconcelos and Leal 2001, Velasquez and Dussan 2009).

4.4 Conclusions

The effectiveness of living *U. lactuca* in the removal of metals from saline water was confirmed under different contamination scenarios, mimicking those found in the field. Fast-growing marine macroalgae were able to bioconcentrate Pb, Cd and Hg in their tissues 500 to 2200-fold the initial concentrations of solution, leading to high metal removal percentages. Initial bioaccumulation rates, and final amount of metal bound in macroalgae biomass were positively correlated with initial concentrations of metal in solution.

For monometallic solutions of Cd and Hg, removal efficiencies were always above 97%, representing final concentrations of metal in solution below, or very close to both surface and drinking water legal limits. Water quality criteria were also met for the lowest concentrations of Pb studied, while for highest concentrations a longer contact time would be required for improved results, since no steady-state was attained in 144h.

Under metal competition (ternary solutions), living *U. lactuca* was able to simultaneously remove Pb, Cd and Hg, although removal efficiencies were dependent of exposure condition. For the lowest contamination scenario, all removal percentages were above 96%, whereas for the system comprising the three metals, in concentration equal to the respective level previously allowed for industrial effluents, removal efficiencies were c.a. 99% for Hg, 57% for Cd and 33% for Pb. Thus, increasing level of contamination, a synergistic effect was observed for Hg, while the presence of other metals affected the removal of Cd and Pb. Under those conditions *U. lactuca* showed significant different affinities toward studied metals: Hg>Cd>Pb.

The bioaccumulation kinetic data was well described by the PFO, PSO and Elovich models, although in most cases the best fit model was different for each metal-macroalgae system.

Metal quantification in macroalgae biomass, after bioaccumulation, demonstrated that indeed all Cd and Hg removed from the aqueous solution was incorporated by the macroalgae, while only c.a. 50% of Pb proved to be fixed in macroalgae cells. This suggests that another mechanism, such as precipitation, occurred concurrently, leading to decreased levels of this metal in the solution over time.

It is noteworthy that in none of bioaccumulation assays occurred death of the organism. As regards to seaweed growth rate at the end of the tests, and for the full range of concentration studied, it was concluded that RGR was not negatively affected by the presence of Cd, Pb and Hg, even under multi-stressor exposure. However, at 24h RGR was generally higher in the presence of metals, which may represent a defense mechanism of seaweed, in order to dilute the internalized metal concentration.

Overall, results highlighted the remarkable affinity of living *U. lactuca* to metals, which gives it a huge potential application in the treatment of saline waters. In fact, macroalgae-based biotechnologies may be the need alternative, for water remediation, environmentally friendly and cost-effective.

4.5 References

Almela, C., S. Algora, V. Benito, M. J. Clemente, V. Devesa, M. A. Suner, D. Velez and R. Montoro (2002). "Heavy metal, total arsenic, and inorganic arsenic contents of algae food products." J Agric Food Chem **50**(4): 918-923.

Bruhn, A., J. Dahl, H. B. Nielsen, L. Nikolaisen, M. B. Rasmussen, S. Markager, B. Olesen, C. Arias and P. D. Jensen (2011). "Bioenergy potential of Ulva lactuca: Biomass yield, methane production and combustion." Bioresource Technology **102**(3): 2595-2604.

Bulgariu, L. and D. Bulgariu (2014). "Enhancing Biosorption Characteristics of Marine Green Algae (Ulva lactuca) for Heavy Metals Removal by Alkaline Treatment." Bioprocessing & Biotechniques **4**(1).

Caliceti, M., E. Argese, A. Sfriso and B. Pavoni (2002). "Heavy metal contamination in the seaweeds of the Venice lagoon." Chemosphere **47**(4): 443-454.

Chen, C. and J. Wang (2007). "Influence of metal ionic characteristics on their biosorption capacity by Saccharomyces cerevisiae." Applied Microbiology and Biotechnology **74**(4): 911-917.

Cheung, C. W., J. F. Porter and G. McKay (2000). "Sorption kinetics for the removal of copper and zinc from effluents using bone char." Separation and Purification Technology **19**(1–2): 55-64.

Chojnacka, K. (2007). "Bioaccumulation of Cr(III) ions by Blue-Green alga *Spirulina sp.* Part I. A Comparison with Biosorption." American Journal of Agricultural and Biological Sciences **2**(4): 218-223.

Chojnacka, K. (2009). Biosorption and bioaccumulation in practice. New York, Nova Science Publishers, Inc.

Chojnacka, K. (2010). "Biosorption and bioaccumulation - the prospects for practical applications." Environment International **36**(3): 299-307.

Coelho, J. P., M. E. Pereira, A. Duarte and M. A. Pardal (2005). "Macroalgae response to a mercury contamination gradient in a temperate coastal lagoon (Ria de Aveiro, Portugal)." Estuarine, Coastal and Shelf Science **65**(3): 492-500.

Costa, S., D. Crespo, B. M. G. Henriques, E. Pereira, A. C. Duarte and M. A. Pardal (2011). "Kinetics of Mercury Accumulation and Its Effects on Ulva lactuca Growth Rate at Two Salinities and Exposure Conditions." Water Air and Soil Pollution **217**(1-4): 689-699.

Costley, C. T., K. F. Mossop, J. R. Dean, L. M. Garden, J. Marshall and J. Carroll (2000). "Determination of mercury in environmental and biological samples using pyrolysis atomic absorption spectrometry with gold amalgamation." Analytica Chimica Acta **405**(1-2): 179-183.

Council Directive 98/83/EC (1998). "Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption." Journal of the European Communities.

Decree-Law No. 103/2010 (2010). Decree-Law No. 103/2010 of the Portuguese Ministry of Environment and Spatial Planning of 24 September on pollution reduction, Diário da República : I Série. **187:** 4289-4298.

Decree-Law No. 236/98 (1998). Decree-Law No. 236/98 of the Portuguese Ministry of the Environment of 1 August establishing water quality standards, Diário da República : I Série. **176:** 3676-3722.

Directive 83/513/EEC (1983). Council Directive 83/513/EEC of 26 September 1983 on limit values and quality objectives for cadmium discharges (Daughter to 2006/11/EC), Official Journal of the European Communities. **291:** 1-8.

Directive 84/156/EEC (1984). Council Directive 84/156/EEC of 8 March 1984 on limit values and quality objectives for mercury discharges by sectors other than the chlor-alkali electrolysis industry, Official Journal of the European Communities**:** 74: 29-74: 29.

Directive 2013/39/EU (2013). Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy, Official Journal of the European Union. **226:** 1-17.

Easton, C., A. Turner and G. Sewell (2011). "An evaluation of the toxicity and bioaccumulation of cisplatin in the marine environment using the macroalga, Ulva lactuca." Environmental Pollution **159**(12): 3504-3508.

El-Khaiary, M. I. and G. F. Malash (2011). "Common data analysis errors in batch adsorption studies." Hydrometallurgy **105**(3-4): 314-320.

Farooq, U., J. A. Kozinski, M. A. Khan and M. Athar (2010). "Biosorption of heavy metal ions using wheat based biosorbents - A review of the recent literature." Bioresource Technology **101**(14): 5043-5053.

Freitas, O. M. M., R. J. E. Martins, C. M. Delerue-Matos and R. A. R. Boaventura (2008). "Removal of Cd(II), Zn(II) and Pb(II) from aqueous solutions by brown marine macro algae: Kinetic modelling." Journal of Hazardous Materials **153**(1-2): 493-501.

Han, T. and G.-W. Choi (2005). "A novel marine algal toxicity bioassay based on sporulation inhibition in the green macroalga Ulva pertusa (Chlorophyta)." Aquatic Toxicology **75**(3): 202-212.

Han, T., S.-H. Kang, J.-S. Park, H.-K. Lee and M. T. Brown (2008). "Physiological responses of Ulva pertusa and U. armoricana to copper exposure." Aquatic Toxicology **86**(2): 176-184.

He, J. and J. P. Chen (2014). "A comprehensive review on biosorption of heavy metals by algal biomass: Materials, performances, chemistry, and modeling simulation tools." Bioresource Technology **160**(0): 67-78.

Ho, Y.-S. (2006). "Review of second-order models for adsorption systems." Journal of Hazardous Materials **136**(3): 681-689.

Ho, Y. S. and G. McKay (1998). "A comparison of chemisorption kinetic models applied to pollutant removal on various sorbents." Process Safety and Environmental Protection **76**(B4): 332-340.

Ho, Y. S., J. C. Y. Ng and G. Mckay (2000). "Kinetics of pollutant sorption by biosorbents: Review." Separation and Purification Methods **29**(2): 189-232.

Jayakumar, R., M. Rajasimman and C. Karthikeyan (2014). "Sorption of hexavalent chromium from aqueous solution using marine green algae Halimeda gracilis: Optimization, equilibrium, kinetic, thermodynamic and desorption studies." Journal of Environmental Chemical Engineering **2**(3): 1261-1274.

Kabata-Pendias, A., Mukherjee, Arun B. (2007). Trace Elements from Soil to Human, Springer.

Kamala-Kannan, S., B. Prabhu Dass Batvari, K. J. Lee, N. Kannan, R. Krishnamoorthy, K. Shanthi and M. Jayaprakash (2008). "Assessment of heavy metals (Cd, Cr and Pb) in water, sediment and seaweed (Ulva lactuca) in the Pulicat Lake, South East India." Chemosphere **71**(7): 1233-1240.

Kumar, P., M. Govindaraju, S. Senthamilselvi and K. Premkumar (2013). "Photocatalytic degradation of methyl orange dye using silver (Ag) nanoparticles synthesized from Ulva lactuca." Colloids and Surfaces B: Biointerfaces **103**(0): 658-661.

Lagergren, S. (1898). "About the theory of so-called adsorption of soluble substances." Kung Sven Veten Hand **24**: 1-39.

Lamare, M. D. and S. R. Wing (2001). "Calorific content of New Zealand marine macrophytes." New Zealand Journal of Marine and Freshwater Research **35**(2): 335-341.

Leal, M. C. F., M. T. Vasconcelos, I. Sousa-pinto and J. P. S. Cabral (1997). "Biomonitoring with benthic macroalgae and direct assay of heavy metals in seawater of the Oporto coast (northwest Portugal)." Marine Pollution Bulletin **34**(12): 1006-1015.

Lee, W.-Y. and W.-X. Wang (2001). "Metal accumulation in the green macroalga Ulva fasciata: effects of nitrate, ammonium and phosphate." Science of The Total Environment **278**(1–3): 11-22.

Li, C., Y. Xu, W. Jiang, X. Dong, D. Wang and B. Liu (2013). "Effect of NaCl on the heavy metal tolerance and bioaccumulation of Zygosaccharomyces rouxii and Saccharomyces cerevisiae." Bioresource Technology **143**(0): 46-52.

Lopes, C. B., J. R. Oliveira, L. S. Rocha, D. S. Tavares, C. M. Silva, S. P. Silva, N. Hartog, A. C. Duarte and E. Pereira (2014). "Cork stoppers as an effective sorbent for water treatment: the removal of mercury at environmentally relevant concentrations and conditions." Environ Sci Pollut Res Int **21**(3): 2108-2121.

Masakorala, K., A. Turner and M. T. Brown (2008). "Influence of synthetic surfactants on the uptake of Pd, Cd and Pb by the marine macroalga, Ulva lactuca." Environmental Pollution **156**(3): 897-904.

Monterroso, P., S. N. Abreu, E. Pereira, C. Vale and A. C. Duarte (2003). "Estimation of Cu, Cd and Hg transported by plankton from a contaminated area (Ria de Aveiro)." Acta Oecologica **24, Supplement 1**(0): S351-S357.

Nielsen, M. M., A. Bruhn, M. B. Rasmussen, B. Olesen, M. M. Larsen and H. B. Moller (2012). "Cultivation of Ulva lactuca with manure for simultaneous bioremediation and biomass production." Journal of Applied Phycology **24**(3): 449-458.

Nikolaisen, L. S. and P. D. Jensen (2013). 3 - Biomass feedstocks: categorisation and preparation for combustion and gasification. Biomass Combustion Science, Technology and Engineering. L. Rosendahl, Woodhead Publishing**:** 36-57.

Pato, P., M. Otero, M. Válega, C. B. Lopes, M. E. Pereira and A. C. Duarte (2010). "Mercury partition in the interface between a contaminated lagoon and the ocean: The role of particulate load and composition." Marine Pollution Bulletin **60**(10): 1658-1666.

Rocha, L., C. Lopes, J. A. Borges, A. C. Duarte and E. Pereira (2013). "Valuation of Unmodified Rice Husk Waste as an Eco-Friendly Sorbent to Remove Mercury: a Study Using Environmental Realistic Concentrations." Water, Air, & Soil Pollution **224**(7): 1-18.

Romera, E., F. Gonzalez, A. Ballester, M. L. Blazquez and J. A. Munoz (2007). "Comparative study of biosorption of heavy metals using different types of algae." Bioresource Technology **98**(17): 3344-3353.

Romero-Gonzalez, M. E., C. J. Williams and P. H. E. Gardiner (2000). "The application of dealginated seaweed as a cation exchanger foron-line preconcentration and chemical speciation of trace metals." Journal of Analytical Atomic Spectrometry **15**(8): 1009-1013.

Ryan, S., P. McLoughlin and O. O'Donovan (2012). "A comprehensive study of metal distribution in three main classes of seaweed." Environmental Pollution **167**(0): 171-177.

Sari, A. and M. Tuzen (2008). "Biosorption of Pb(II) and Cd(II) from aqueous solution using green alga (Ulva lactuca) biomass." Journal of Hazardous Materials **152**(1): 302-308.

Sekabira, K., H. O. Origa, T. A. Basamba, G. Mutumba and E. Kakudidi (2011). "Application of algae in biomonitoring and phytoextraction of heavy metals contamination in urban stream water." International Journal of Environmental Science and Technology **8**(1): 115-128.

Simpson, A. G. B. and A. J. Roger (2004). "The real 'kingdoms' of eukaryotes." Current Biology **14**(17): R693-R696.

Sunda, W. G. and S. A. Huntsman (1998). "Processes regulating cellular metal accumulation and physiological effects: Phytoplankton as model systems." Science of The Total Environment **219**(2–3): 165-181.

Torres, M. A., M. P. Barros, S. C. G. Campos, E. Pinto, S. Rajamani, R. T. Sayre and P. Colepicolo (2008). "Biochemical biomarkers in algae and marine pollution: A review." Ecotoxicology and Environmental Safety **71**(1): 1-15.

Trinelli, M. A., M. M. Areco and M. D. Afonso (2013). "Co-biosorption of copper and glyphosate by Ulva lactuca." Colloids and Surfaces B-Biointerfaces **105**: 251-258.

Turner, A., S. S. Pedroso and M. T. Brown (2008a). "Influence of salinity and humic substances on the uptake of trace metals by the marine macroalga, Ulva lactuca: Experimental observations and modelling using WHAM." Marine Chemistry **110**(3-4): 176-184.

Turner, A., S. S. Pedroso and M. T. Brown (2008b). "Influence of salinity and humic substances on the uptake of trace metals by the marine macroalga, Ulva lactuca: Experimental observations and modelling using WHAM." Marine Chemistry **110**(3–4): 176-184.

Vasconcelos, M. T. S. D. and M. F. C. Leal (2001). "Seasonal variability in the kinetics of Cu, Pb, Cd and Hg accumulation by macroalgae." Marine Chemistry **74**(1): 65-85.

Velasquez, L. and J. Dussan (2009). "Biosorption and bioaccumulation of heavy metals on dead and living biomass of Bacillus sphaericus." Journal of Hazardous Materials **167**(1-3): 713-716.

Wang, J. L. and C. Chen (2009). "Biosorbents for heavy metals removal and their future." Biotechnology Advances **27**(2): 195-226.

Wang, W.-X. and R. C. H. Dei (1999). "Kinetic measurements of metal accumulation in two marine macroalgae." Marine Biology **135**(1): 11-23.

Yamashita, M., K. Tomita-Yokotani, H. Hashimoto, N. Sawaki and M. Notoya (2009). "Sodium and potassium uptake of Ulva – Application of marine macro-algae for space agriculture." Advances in Space Research **43**(8): 1220-1223.

Chapter 5

Bioaccumulation of Hg, Cd and Pb by *Fucus vesiculosus* in seawater: study on single and multi-metal systems

5 Bioaccumulation of Hg, Cd and Pb by *Fucus vesiculosus* in seawater: study on single and multi-metal systems

5.1 Introduction

Nowadays, water is no longer regarded as an inexhaustible resource, and there is a general awareness about the need to safeguard its quality and integrity from the deleterious effects of contaminants (Chojnacka 2009). Metals, particularly the so-called toxic-trio, Cd, Hg and Pb, are among the most common, and hazardous contaminants, being widely recognized that they cause adverse effects even when released at low concentrations into aquatic environments (He and Chen 2014). Anthropogenic activities, such as coal burning, metal plating, battery manufacture and mining, among others, are the main responsibles for the increased mobilization and bioavailability of Cd, Hg and Pb in water bodies (Freitas et al. 2008). As metals are not biodegradable, they persist indefinitely, being successively accumulated by the aquatic organisms along the trophic chain (Coelho et al. 2005). Eventually they reach human beings through diet, primarily from fish consumption, and often exceed the toxic levels before produce visible effects on health (Srivastava and Goyal 2010). Even those metals, that deposit in marine or estuarine sediments for years, getting immobilized, remain a hazard since, at any time, they may be released to the water column (Kabata-Pendias 2007).

Currently, European Union classifies Cd and Hg as “priority hazardous substances” in the field of water policy (Directive 2013/39/EU 2013), imposing that their emissions, discharges and losses cease or be phase out by 2021. Lead is grouped with the substances with detrimental effect, whose discharges are conditioned to prior authorization, and emission values set (Directive 2013/39/EU 2013). Strict limits, designated by “Environmental Quality Standards” are included in the text, referring to the concentration of these metals not in effluents but in the receiving surface water.

Taking into account those limits and the need for alternatives to conventional methods of treatment – which have major disadvantages (Farooq et al. 2010, Fu and Wang 2011), processes such as biosorption and bioaccumulation have been intensively studied (Chojnacka 2010). In fact, methods based on the use of biological materials widely available and inexpensive, or even biowaste, promise to be an environmentally friendly, and cost-effective way to remove metals from solutions.

Biosorption uses not living (dried) biomasses, of most varied origins (Lesmana et al. 2009, Tuzen and Sari 2009, Vijayaraghavan et al. 2011, Rocha et al. 2013, Lopes et al. 2014), including algae, which have proven to be of the best biosorbents (He and Chen 2014). The outperformance of algae is due to the diversity and quantity of functional groups present on their cell walls, representing up to 80% of their total dry weight (Mata et al. 2008). Among the studied seaweeds, brown algae stand out (Davis et al. 2003, Mata et al. 2009). Their cell walls are characterized by presence of cellulose, acting as structural support, alginic acid, a polymer of mannuronic and guluronic acids (Lesmana et al. 2009), having two adjacent carboxylic groups at the right distance for metal bonding (Romera et al. 2007), and sulphated polysaccharides.

In bioaccumulation, beyond the bonding to surface (similar to biosorption), metals are also metabolically accumulated inside the cells, which potentially leads to higher removal efficiencies (Chojnacka 2010). From the industrial scale-up viewpoint, the application of living macroalgae, for the remediation of wastewaters (and contaminated estuarine waters), may be simpler and cheaper than biosorption, because complex systems of stirring and separation of biomass from treated water (filtration), which can represent up to 60% of the total cost (Mata et al. 2009), are dismissed. Still, metal bioaccumulation studies have been focused on microalgae and bacteria (Kadukova and Vircikova 2005, Chojnacka 2007) although biomonitoring works often reports high contents of metal in macroalgal biomasses (Giusti 2001, Coelho et al. 2005). Conducting complementary studies, under controlled conditions, with arrays that mimic the actual waters (including seawater), using environmentally relevant concentrations of contaminants (Rocha et al. 2013, Lopes et al. 2014), is required in order to obtain more knowledge about potential application of certain macroalgae in remediation. Furthermore, research should include mathematical description of the kinetic of bioaccumulation (Chojnacka 2007), which is a major gap in literature, and refer to longer exposure times than those usually studied (Wang and Dei 1999), in order to evaluate the potential release of metals back to the solution. Additionally, in the literature there is a scarcity on studies focusing on the simultaneous removal of Cd, Hg and Pb by living macroalgae, as well as of their effects on growth rate (Costa et al. 2011).

Thus the main objective of this study was to evaluate the bioremoval of metal contaminants in seawater by living macroalgae *Fucus vesiculosus*. The efficiency of the removal process was assessed not only for monometallic solutions of Pb, Cd and Hg but also for mixtures of those metals, using environmentally realistic concentrations. Further objectives relate to the study of the influence of these metals on *Fucus* growth, and the assessment of the ability of this species to debug the accumulated metals.

Fucus vesiculosus is a brown seaweed (*Phaeophyta*), cosmopolitan, which can be found in habitats with a rocky substrates (Coyer et al. 2011). It belongs to the same lineage of *Fucus spiralis, F. radicans, F. ceranoides* and *F. virsoides*, being a key species in diverse habitats ranging from the Atlantic Ocean to the Pacific Ocean. It is commonly used as a nutritional supplement, animal feed and agricultural fertilizers (Mata et al. 2008). In the last decade, the application of dried seaweeds of genus *Fucus* as biosorbents of metals and dyes has generated a growing interest (Freitas et al. 2008, Mata et al. 2008, Mata et al. 2009, Cobas et al. 2014) although the application of living organisms has been unexplored.

5.2 Materials and methods

5.2.1 Material and chemicals

All chemical reagents used in this work were of analytical reagent grade and were used as received from the suppliers. The nitric acid 65% (suprapur) and the standard stock solutions of mercury (1001 ± 2mgL^{-1}), cadmium (1000 ± 2mgL^{-1}) and lead (1000 ± 2mgL^{-1}) nitrate were purchased from Merck. All working solutions, including standards for the calibration curves, were obtained by diluting the corresponding stock solution. All the material used in the experiments was previously washed in Derquim 5% rinsed in Milli-Q water (18 MΩ cm^{-1}), soaked in 25% HNO_3 for at least 24h and subsequently rinsed with Milli-Q water. All glass vessels used in the experiments as reaction vessels or in the storage of the water samples were additionally soaked in concentrated HNO_3 (65%) for at least 24h, before reuse.

5.2.2 Macroalgae collection and maintenance

The brown macroalga *F. vesiculosus* (Phaeophyta) was collected in the Mondego estuary (Figueira da Foz, Portugal, 40°08′N, 8°50′W) and transported to the laboratory in isothermal plastic bags containing some local water. After rinse with seawater to remove debris and epibionts, a small part of the algae was immediately freeze dried for further quantification of “natural” concentrations of metals, following the procedures described below. The remaining algae was transferred to 30 L clear glass tanks equipped with air pump and filled with filtered seawater enriched with Provasoli stock solution (Costa et al. 2011). Macroalgae were maintained in the aquariums (water was weekly changed) under natural light (approximately 12L:12D photoperiod), at room temperature of 20±2°C, until the beginning of the experiments. Seawater used for macroalgae maintenance and for the bioaccumulation experiments was collected at Vagueira beach (18 km southwest of Aveiro, Portugal), filtered through 0.45 µm Millipore membrane and stored in the dark at 4°C until further use. A brief characterization of the seawater which includes pH, conductivity, salinity and major and minor elements was performed. The pH (7.9), conductivity (54.3 mS cm^{-1}) and salinity (35 g L^{-1}) were recorded on a WTW meter and the concentration of major and minor elements was obtained by inductively coupled plasma spectroscopy, using a Jobin – Yvon JY70 Plus Spectrometer. All the measured parameters were in acceptable ranges according to considered non-polluted waters.

5.2.3 *Fucus vesiculosus* characterization

In order to assess the dry/fresh weight ratio of *F. vesiculosus* several pieces were cut from the macroalgae apical tips were cut, weighed, and dried at 40ºC until constant weight.

FTIR spectrum of *F. vesiculosus* was recorded by using a Bruker optics tensor 27 spectrometer coupled to a horizontal attenuated total reflectance (ATR) cell using 256 scans at a resolution of 4 cm^{-1}.The dried sample was examined directly and data were obtained as transmittance.

5.2.4 Bioaccumulation experiments

Bioaccumulation assays were conducted in Schott Duran® glass bottles of 1 L (reaction vessels), where *F. vesiculosus* was exposed to natural seawater spiked with known concentrations of metal. Studies were performed for single- and multi-contaminant systems, according to experimental conditions summarized in Table 5.1.

With the range of conditions used, we intend to simulate different contamination scenarios, and the concentrations used may be considered environmental realistic. 50, 200 and 1000 µg L^{-1} correspond, respectively, to the “old” limits for Hg, Cd and Pb wastewaters discharges (Directive 83/513/EEC 1983, Directive 84/156/EEC 1984, Decree-Law No. 236/98 1998). Remaining concentrations may represent a gradient of metal levels in a surface water, after dilution of the effluent in the water body.

Table 5.1: Experimental conditions of bioaccumulation experiments.

Matrix	System		Metal	Concentration (µg L^{-1})
Spiked natural seawater	Single-contaminant		Cd	10, 50, 100 and 200
			Pb	50, 100, 200 and 1000
			Hg	10, 50 and 100
	Multi-contaminant	M1	Cd	50
			Pb	50
			Hg	50
		M2	Cd	200
			Pb	1000
			Hg	50

The spiked solutions were obtained by adding the desired volume of the metal standard solution to filtered seawater. All the solutions were left to pre-equilibrate during 24h before the beginning of the experiments. An aliquot of each solution was collected immediately before the addition of macroalgae, to confirm the exact initial concentration of metal. Approximately 1.7 g L^{-1} (fresh weight) of macroalgae was used in each assay. Small pieces of the apical tips were cut (Hemmi et al. 2005) and acclimatized in clean seawater for several hours prior to metal exposure. The uptake of metals by the living macroalgae was followed during 7 days by determining the concentration of each metal in solution samples (5-10 mL) collected at defined crescent periods of time. All samples were acidified to pH ≤ 2 using Suprapur HNO_3 and stored at 4°C until further analysis. All bioaccumulation trials were performed in triplicate, under natural light at room temperature of 20±2°C. Blanks (macroalgae in clean seawater) and controls (metal spiked seawater in the absence of macroalgae) were always running in parallel with the experiments. No pH buffer was added to the system and no pH adjustments were made at any time, since this could interfere with the metals uptake and cellular metabolism of the seaweeds (Vasconcelos and Leal 2001, Velasquez and Dussan 2009). However, pH was daily monitored. At the end of the experiments, macroalgae were harvested, rinsed and freeze dried for later quantification of Hg, Cd and Pb.

5.2.5 Depuration experiments

Living macroalgae may present a variety of biochemical strategies to reduce the toxicity of accumulated metals, such as internal safe sequestration of metals by metallothioneins or their release back to the solution (Torres et al. 2008). In order to evaluate the capacity of *F. vesiculosus* to retain accumulated metal, thus avoiding its transference again to the seawater, depuration experiments were also conducted in this work. Immediately following 7-day of exposure, macroalgae were harvested and placed in contact with clean seawater, for a period of 7 days (Wang and Dei 1999). At pre-determined intervals of time, aliquots of seawater were collected for further analysis of metals content. All samples were acidified to $pH \leq 2$ using Suprapur HNO_3 and stored at 4°C after collected.

5.2.6 Metal quantification

5.2.6.1 Metal concentration in solution

Mercury quantification in water samples was performed by cold vapour atomic fluorescence spectroscopy (CV-AFS), on a PSA 10.025 Millennium Merlin Hg analyser and using $SnCl_2$ (2% m/v in HCl 10% v/v) as reducing agent. The concentration of Hg in the collected samples was determined through a calibration curve ($r^2 \geq 0.999$) using five daily prepared standard solutions ranging from 0.0 to 0.5 µg L^{-1}. In this range, the detection and quantification limits, obtained through blank measurements (n = 20) were 0.007 µg L^{-1} and 0.021 µg L^{-1}, respectively. Total Hg concentration in clean seawater was determined after addition of 500 µL of a saturated solution of potassium persulfate to 50 mL of filtered seawater and irradiation by a UV lamp (1000 W) for 30 min. Following irradiation, the excess of oxidant was reduced with 37.5 µL of hydroxylamine solution 12% (w/v) (Pato et al. 2010). The measurement was then performed by CV-AFS, using a calibration curve from 0 to 60 ng L^{-1}, with detection and quantification limits of 1.6 and 3.0 ng L-1, respectively (n=15, 99.5% confidence level). The value obtained was 3.1 ng L^{-1}, which is a typical value of a non-polluted water

Cadmium and Pb quantification in aqueous solution was performed by inductively coupled plasma mass spectrometry (ICP-MS), on a Thermo ICP-MS XSeries equipped with a Burgener nebuliser. The calibration curve for Cd and Pb quantification was obtained through standards (0.1–50 µg L^{-1}), prepared by dilution of the certified standard solution of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ in nitric acid solution (2 % v/v). Quantification limits of the method were 0.1 µg L^{-1} and 0.2 µg L^{-1}, for Cd and Pb respectively, with a precision and accuracy <10 %. Since water samples had high salinity, in order to avoid matrix interferences, a 20-fold dilution with HNO3 (2%) was always performed prior the measurements by ICP-MS. Therefore the "real" quantification limits for Cd and Pb were 20-fold higher than values referred previously. The concentrations of Cd and Pb in clean seawater were below the detection limit.

5.2.6.2 Metal concentration in macroalgal biomass

Total mercury concentration in macroalgae biomass was measured by thermal decomposition atomic absorption spectrometry with gold amalgamation using a LECO© AMA-254, as described by Costley et al. (2000). Analysis was performed directly in the sample (1 to 20 mg, dry weight) without digestion or pre-specific treatment, avoiding mercury losses or contamination as well as matrix interferences. At least three replicate measurements were carried out for each sample, and a maximum coefficient of variation of 10% was adopted as acceptance criteria. Detection and quantification limits were 0.01 ng Hg and 0.03 ng Hg, respectively (n=20, 99.5% confidence level). Several blanks (i.e. an empty sample nickel boat) were run before and between sample analyses to ensure that mercury was not being accumulated between samples. The quality of the results was assessed by using Certified Reference Material (CRM) BCR-60 (*Lagarosiphon major*, Total Hg 0.34 ± 0.04 µg g^{-1}). The CRM was analyzed every day prior to the beginning of the analysis and repeated at the end of the day. All percentages of recovery for total Hg were within the range of 85–107% (n = 17).

Cadmium and Pb contents in macroalgae were determined by ICP-MS, after sample digestion, as described by Monterroso et al. (2003). Briefly, about 200 mg of freeze-dried sample was accurately weighted into acid-washed Teflon reactor and HNO_3 (conc.) was added. Reactors were placed in an oven at 60 °C for 12h and then at 100 °C for 1h. Afterwards, H_2O_2 was added, and reactor was heated at 80 °C for 1 h. After digestion, samples were diluted with Milli-Q water and filtered. Percentages of recovery for Cd and Pb were, respectively, 102 and 87% (n=4).

The baseline concentrations of Hg, Cd and Pb in *F. vesiculosus* were 0.045±0.002, 0.29±0.044 and 4.1±0.29 µg g^{-1}, respectively. These values are characteristic of macroalgae growing in uncontaminated areas (Giusti 2001, Coelho et al. 2005).

5.2.7 Effects of metal bioaccumulation on macroalgae growth

The influence of exposure to metal on *F. vesiculosus* growth, for mono and multimetallic solutions, was evaluated by means of the relative growth rate (RGR), using the equation:

$$RGR(\%\ day^{-1}) = ((\ln A_t - \ln A_0)/t) \times 100 \qquad (1)$$

where A_0 is the initial macroalgae area and A_t is the macroalgae area at time t (Han et al. 2008). Areas, at the beginning and end of the exposure (7 days), were determined using *ImageJ* software after scanning, with a resolution of 200 ppi (Figure 5.1A-B).

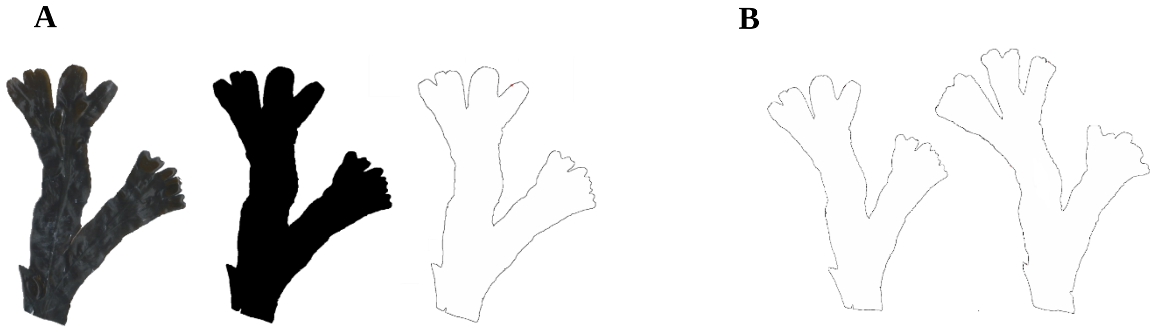


Figure 5.1: Example of image treatment for determination of growth area (A) and *F. vesiculosus* growth evolution over a period of 15 days (B).

5.2.8 Experimental data analysis

The uptake of metals from contaminated seawater leads to an increase of metals content in macroalgae tissues, with the correspondent decrease in solution. Thus, metal bioaccumulation, in terms of amount of metal bound by unit of macroalgae mass, at a given time *t* (q_t, µg g^{-1}), was deduced from the mass balance between the initial metal concentration in the solution (C_0, µg L^{-1}) and the concentration after a particular period of contact time *t* (C_t, µg L^{-1}):

$$q_t = \frac{(C_0 - C_t)V}{m} \quad (2)$$

where *V* (L) is the volume of the solution and *m* (g) is the macroalga biomass, in dry weight. When equilibrium is attained, $t=t_e$, $q_t=q_e$ and $C_t=C_e$ (residual concentration of metal in solution). The performance of the removal process was also evaluated and compared using the removal percentage (*R*, %), which at time *t* is defined by:

$$R_t = \frac{(C_0 - C_t)}{C_0} \times 100 \quad (3)$$

5.2.8.1 Bioaccumulation kinetic models

In this study, three of the most widely accepted and commonly used kinetic models were applied to experimental data, in order to describe the kinetic of the metal uptake process: the Lagergren pseudo-first-order model (Lagergren 1898), the Ho's pseudo-second order model (Ho and McKay 1998) and the Elovich model (Ho 2006). Models were used in their non-linear forms (El-Khaiary and Malash 2011):

$$q_t = q_e(1 - e^{-k_1 t}) \quad (4)$$

$$q_t = \frac{q_e^2 k_2 t}{1 + q_e k_2 t} \quad (5)$$

$$q_t = \frac{1}{\beta}\ln(1 + \alpha\beta t) \quad (6)$$

where k_1 (h^{-1}) is the rate constant of pseudo-first order, k_2 (g µg^{-1} h^{-1}) is the pseudo-second order rate constant, *α is* the initial adsorption rate (µg g^{-1} h^{-1}), and β is the desorption constant (g µg^{-1}).

5.2.8.2 Statistical analysis

All statistical data were analysed using GraphPad 6.0. This tool was also used to plot all graphs as well as to calculate all kinetic parameters through nonlinear regression. GraphPad 6.0 uses the least-squares as fitting method and the method of Marquardt and Levenberg, which blends two other methods, the method of linear descent and the method of Gauss-Newton for adjusting the variables. The goodness of fit was evaluated by the analysis of coefficient of determination (R^2) and the standard deviation of residuals ($S_{y.x}$). The relative error (E_r) between experimental and predicted values of q_e was also calculated. These statistical parameters can be mathematically defined by:

$$R^2 = 1 - \frac{\sum (y_i - \hat{y})^2}{\sum (y_i - \bar{y})^2} \qquad (7)$$

$$S_{y,x} = \sqrt{\frac{(y_i - \hat{y})^2}{df}} \qquad (8)$$

$$E_r = \frac{|y_i - \hat{y}|}{y_i} \times 100 \qquad (9)$$

where y_i are the experimental data values, $\hat{y}$ are the predicted values, $\bar{y}$ is the mean of the experimental data and df is the number of the degrees of freedom (equal to number of data points minus the number of parameters fit).

Akaike's Information Criterion (AIC) (El-Khaiary and Malash 2011), which is based on information theory and maximum likelihood theory, was used to determine which model is more likely to be correct and how much more likely. For small sample size, AIC is calculated from the equation:

$$AIC = N \ln\left(\frac{SSE}{N}\right) + 2N_p + \frac{2N_p\left(N_p + 1\right)}{N - N_p - 1} \qquad (10)$$

where SSE is the sum-of-squared deviations, N is the number of data points and N_p is the number of parameters in the model. AIC values can be compared using Evidence ratio (numerical value representative of the number of times that the model with a lower AIC is more likely to be correct) which is defined by:

$$\text{Evidence ratio} = \frac{1}{e^{-0.5\Delta}} \quad (11)$$

where Δ is the absolute value of the difference in AIC between the two compared models.

5.3 Results and discussion

5.3.1 *Fucus vesiculosus* characterization

The dry/fresh weight ratio and the water content (in %) of the algae were evaluated for all algae sample used in the essays. The dry/fresh weight ratio ranged between 0.20 and 0.32 and the average water content was 74.6±4.2% (*n*=22), which is in agreement with the value reported by Back et al. (1992) for *F. vesiculosus*.

FTIR spectrum of *F. vesiculosus* is presented in Figure 5.2. The most specific peaks were attributed to functional groups of the macroalgae according to the assignments made by other authors (Pereira et al. 2003, Sheng et al. 2004, Mata et al. 2009).

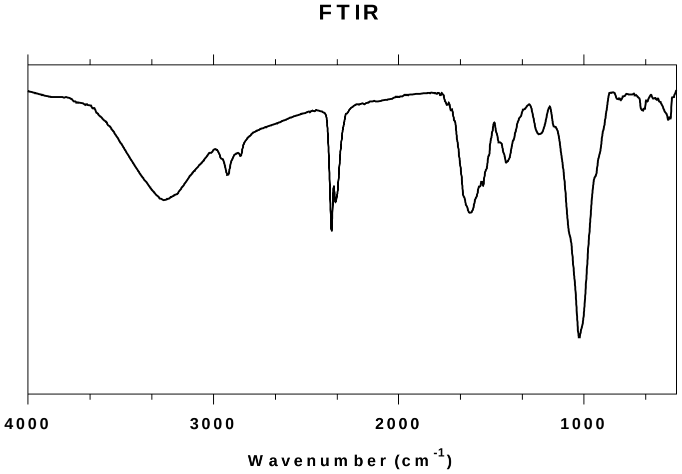


Figure 5.2: FTIR spectra of marine macroalgae *F. vesiculosus*.

The broad band at 3264 cm^{-1} represents bonded –OH and –NH groups, whereas asymmetric bands at 2919, 2848 and 1614 cm^{-1} are associated with –CH stretch (Sheng et al. 2004, Mata et al. 2009). The weak band at 1540 cm^{-1} and the strong asymmetrical peak at 1417 cm^{-1} were attributed to COO^- deformation (Radwan et al. 1996, Sheng et al. 2004, Mata et al. 2009). Finally, symmetrical peak at 1243 cm-1 and intense band at 1023 cm-1 are associated with C-O-C and C-OH stretching modes (Pereira et al. 2003, Mata et al. 2009).

5.3.2 *Bioremediation in single-metal contamination scenarios*

The capacity of *F. vesiculosus* to bioremediate contaminated salt waters was evaluated for monometallic solutions (single-contaminant systems) of Pb, Cd and Hg, in a range of concentrations, representing various contamination scenarios.

The bioremoval curves of Pb at four contamination scenarios (C_0 = 50, 100, 200 and 1000 µg L^{-1}) are shown in Figure 5.3a-*d*, in terms of normalized concentration *vs* time, *i.e.* C_t/C_0 *vs t*.

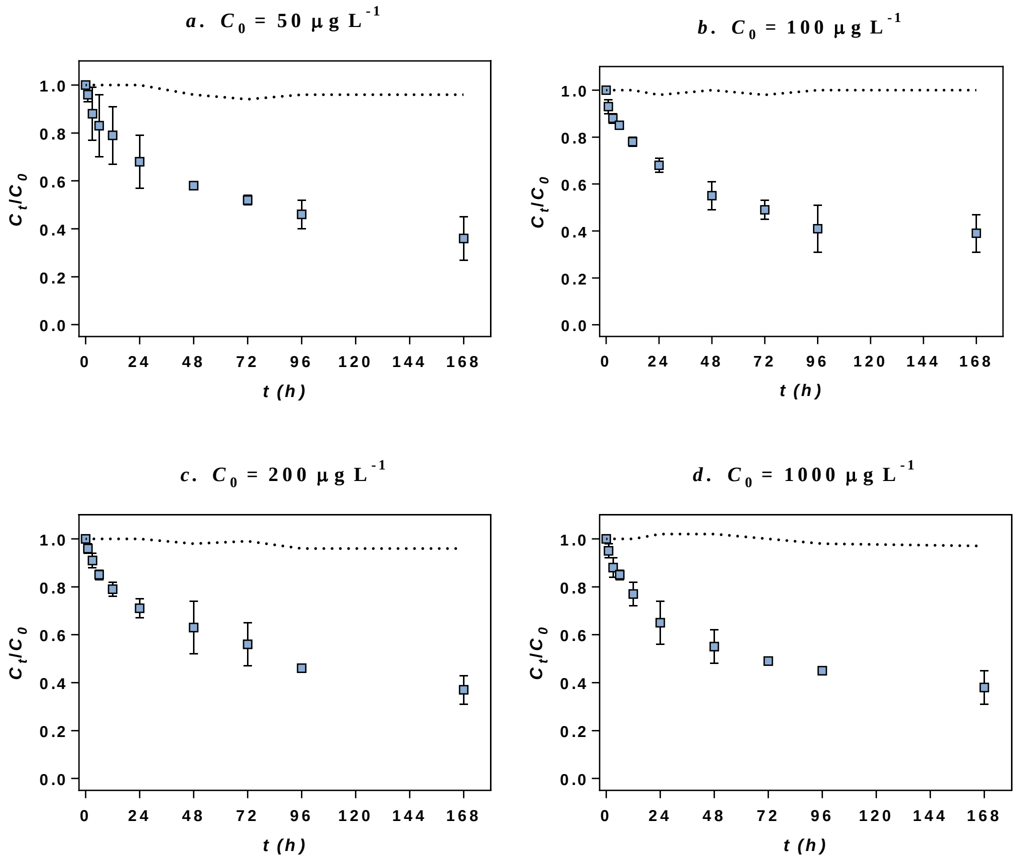


Figure 5.3: Normalized concentrations (C_t/C_0) of Pb in solution with time: circles – sample essay: Pb solution + *F. vesiculosus*; dash line – control essay: Pb solution.

The results showed that c.a. 450 mg of *F. vesiculosus* (d.w.) can bioaccumulate a considerably amount of Pb and consequently decreased extensively its concentration in the water (1L). The reduction of Pb concentration in solution is attributed to the interactions metal-algae since data from macroalgae-free experiments reveal a constant metal concentration with time (Figure 5.3). Moreover, for all levels of contamination, the Pb uptake by the macroalgae is faster at the beginning of the experiments, slowing down with time without the attainment of a plateau value on C_t, during the period of the essay. This fact suggests that *F. vesiculosus* will continue to uptake lead from solution, promoting a better water quality.

Moreover, under the experimental conditions tested, the efficiency of the bioremediation process (in terms of removal %) seems to be blind to the contamination level. For all initial Pb concentrations, the C_t/C_0 kinetic profiles have an almost coincident pattern, corresponding to similar removal degrees. At the end of the essays the removal percentages were 64.4, 61.5, 62.5 and 66.5, respectively for C_0 of 50, 100, 200 and 1000 µg L^{-1}.

However, the initial metal concentration provides an important driving force to overcome all mass transfer resistance of Pb between the liquid and solid phases. Consequently, an increase of C_0 from 50 to 1000 µg L^{-1} increased the initial removal rate (estimated from the first derivative of $C_t=f(t)$ at $t=0$) from 2.65 to 43.9 µg L^{-1} h^{-1}, and the amount of Pb bioaccumulated by the *F. vesiculosus* from *ca.* 82 to approximately 1500 µg g^{-1}, as observed in Table 5.2.

Table 5.2: The effect of initial metal concentrations on the amount of metal bioaccumulated by the *F. vesiculosus* (q_t) and the initial removal rate (ν_0).

Metal	C_0 (µg L^{-1})	$q_{t,168h}$ (µg g^{-1})	ν_0 (µg L^{-1} h^{-1})*
Pb	50	81.8	2.65
	100	143	4.20
	200	231	5.87
	1000	1504	43.9
Cd	10	17.7	0.22
	50	53.8	1.64
	100	98.0	1.27
	200	122	0.80
Hg	10	23.1	0.54
	50	108	1.63
	100	215	3.70

*Estimated from the first derivative of $Ct=f(t)$ at $t=0$.

The experimental amount of Pb bioaccumulated by the algae with time, q_t *vs t*, for the different contamination levels are shown in Figure 5.4a-d, together with the fittings of the pseudo-first- order(PFO), pseudo-second-order (PSO) and Elovich models.

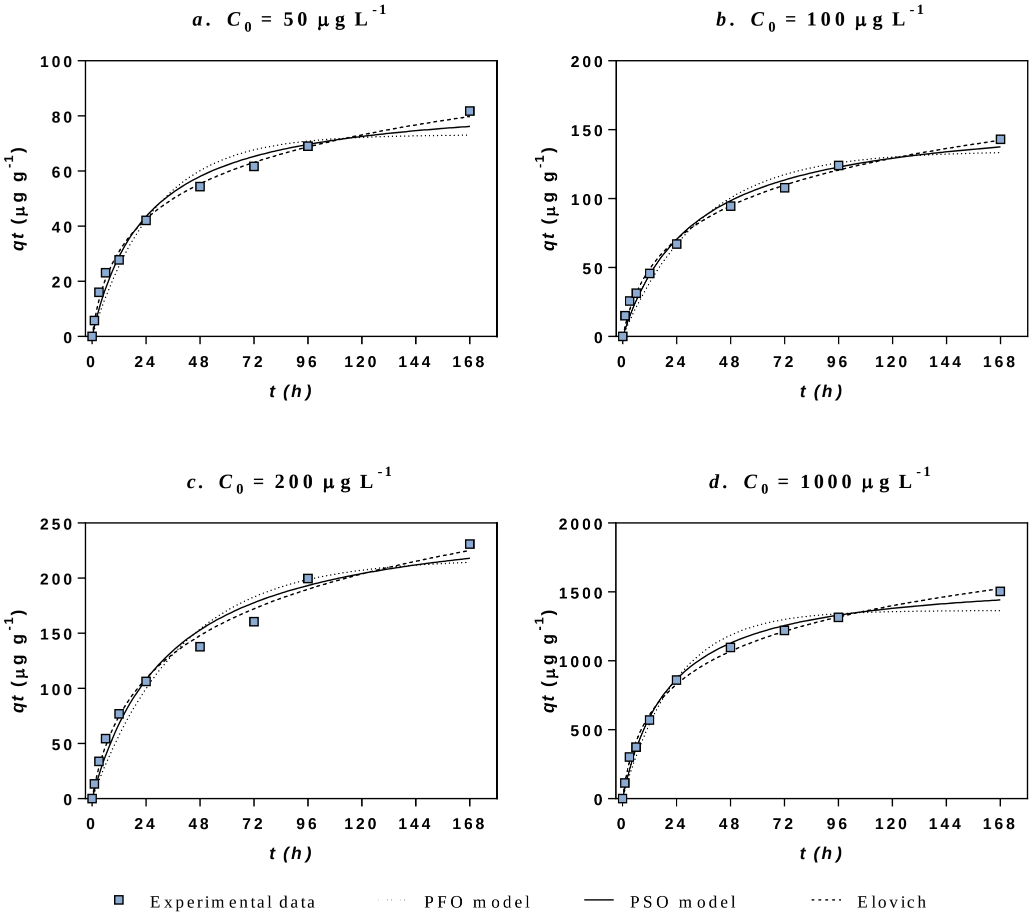


Figure 5.4: Experimental and modelling results for the amount of Pb bioaccumulated by the algae *F. vesiculosus*. (Error bars were omitted for clarity).

The results show a good agreement between experimental data and the fittings accomplished by the models adopted in this work. The coefficient of determination, R^2, obtained for the different models was always higher than 0.95 (PFO: 0.955-0.976; PSO: 0.978-0.993; Elovich: 0.992-0.997). The goodness of the fits was also compared by the Akaike's Information Criterion (AIC) (El-Khaiary and Malash 2011) (Table 5.3), which confirm that the Elovich model is likely to be the most appropriated model to describe the bioaccumulation kinetics of Pb by *F. vesiculosus* (lower AIC), corroborating the initial conclusion achieved from R^2 comparison. Moreover, for almost all contamination scenarios, the Elovich model was able to fit the data in the transition between the steep ascendant part of each curve and the horizontal branch, where the kinetic profile is usually difficult to fit or simulate, while both the pseudo-first- and pseudo-second-order models underestimated the equilibrium q_t values.

Table 5.3: Best-fit parameters (± standard error) and goodness of the fittings of the kinetic models used to describe the bioaccumulation kinetics of Pb by *F. vesiculosus* algae.

C_0 (µg L^{-1})	PFO	PSO	Elovich	Preferred model*
50	**k_1:** 0.035±0.007	**k_2:**4.9e^{-04}±1.1e^{-04}	**α:** 6.06±0.63	***Elovich*** *Probability*:99.9%
	$q_{t,e}$: 73.2±4.6	**$q_{t,e}$:** 87.1±4.9	**β:** 0.049±0.002	
	R^2: 0.955	**R^2:** 0.980	**R^2**: 0.996	
	$S_{y/x}$: 6.29	**$S_{y/x}$:** 4.18	**$S_{y/x}$**: 1.98	
100	**k_1:** 0.029±0.005	**k_2:** 1.9e^{-04}±4.4e^{-05}	**α:** 8.03±0.97	***Elovich*** *Probability:98.8%*
	$q_{t,e}$: 135±8	**$q_{t,e}$:** 163±10	**β:** 0.025±0.002	
	R^2: 0.968	**R^2:** 0.984	**R^2**: 0.993	
	$S_{y/x}$: 9.45	**$S_{y/x}$:** 6.69	**$S_{y/x}$**: 4.30	
200	**k_1:** 0.026±0.005	**k_2:** 1.1e^{-04}±3.0e^{-05}	**α:** 11.7±1.6	***Elovich*** *Probability:99.3%*
	$q_{t,e}$: 217±16	**$q_{t,e}$:** 263±19	**β:** 0.015±0.001	
	R^2: 0.958	**R^2:** 0.978	**R^2**: 0.992	
	$S_{y/x}$: 17.3	**$S_{y/x}$:** 12.5	**$S_{y/x}$**: 7.63	
1000	**k_1:** 0.042±0.006	**k_2:**2.9e^{-05}±4.0e^{-06}	**α:** 127±10	***Elovich*** *Probability:99.3%*
	$q_{t,e}$: 1365±58	**$q_{t,e}$:** 1622±52	**β:** 0.0027±0.0001	
	R^2: 0.976	**R^2:** 0.993	**R^2**: 0.997	
	$S_{y/x}$: 88.5	**$S_{y/x}$:** 47.8	**$S_{y/x}$**: 29.3	

*Comparison of the two fits with the highest R^2 and lower $S_{y/x}$ values; Probability reported by Graphad6® after model comparison using Akaike's Information Criterion.

The bioremoval of cadmium by the algae *F. vesiculosus* is illustrated in Figure 5.5a-*d*, in terms of normalized concentration *vs* time, *i.e.*C_t/C_0 *vs* t, for the contamination scenarios selected (C_0 = 10, 50, 100 and 200 µg L^{-1}).

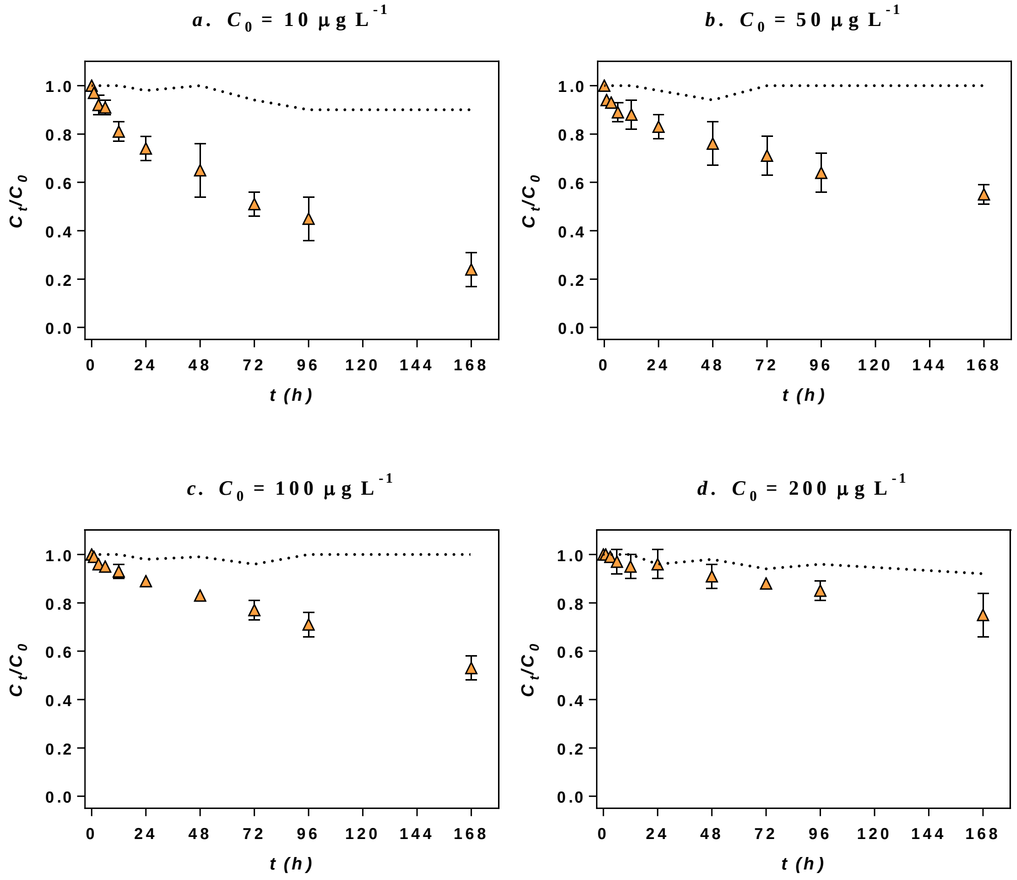


Figure 5.5: Normalized concentrations (C_t/C_0) of Cd in solution with time: diamonds – sample essay: Cd solution + *F. vesiculosus*; dash line – control essay: Cd solution.

The results show that *F. vesiculosus* is able to uptake Cd from saltwaters, however the bioaccumulation capacity and kinetics depend on the contamination level. For the lowest concentrations studied (10 and 50 µg L^{-1}), the kinetic profile of the bioaccumulation curves for Cd are characterized by a speedy decrease on Cd concentration in solution, but as long as C_0 increases the uptake process becomes slower, as confirmed by the kinetic constants estimated by the PFO and PSO models (Table 5.2). Although, the equilibrium was not attained due to the continuous metal uptake by the macroalgae, at the end of the essays the removal percentages were 76.3, 45.4, 46.2 and 25.1, respectively for C_0 of 10, 50, 100 and 200 µg L^{-1}, reflecting the effect of the contamination level on the bioremoval process.

However, as mentioned previously, the initial metal concentration provides an important driving force to overcome all mass transfer resistance of the metal between the liquid and solid phases, and consequently, an increase of C_0 from 10 to 200 µg L^{-1}, increased the amount of Cd bioaccumulated by the *F. vesiculosus* from *ca.* 18 to *ca.* 122 µg g^{-1} (Table 5.2).

The fittings of the PFO, PSO and Elovich models for the amount of Cd bioaccumulated by the macroalgae with time, and the q_t values evaluated experimentally are shown in Figure 5.6a-d.

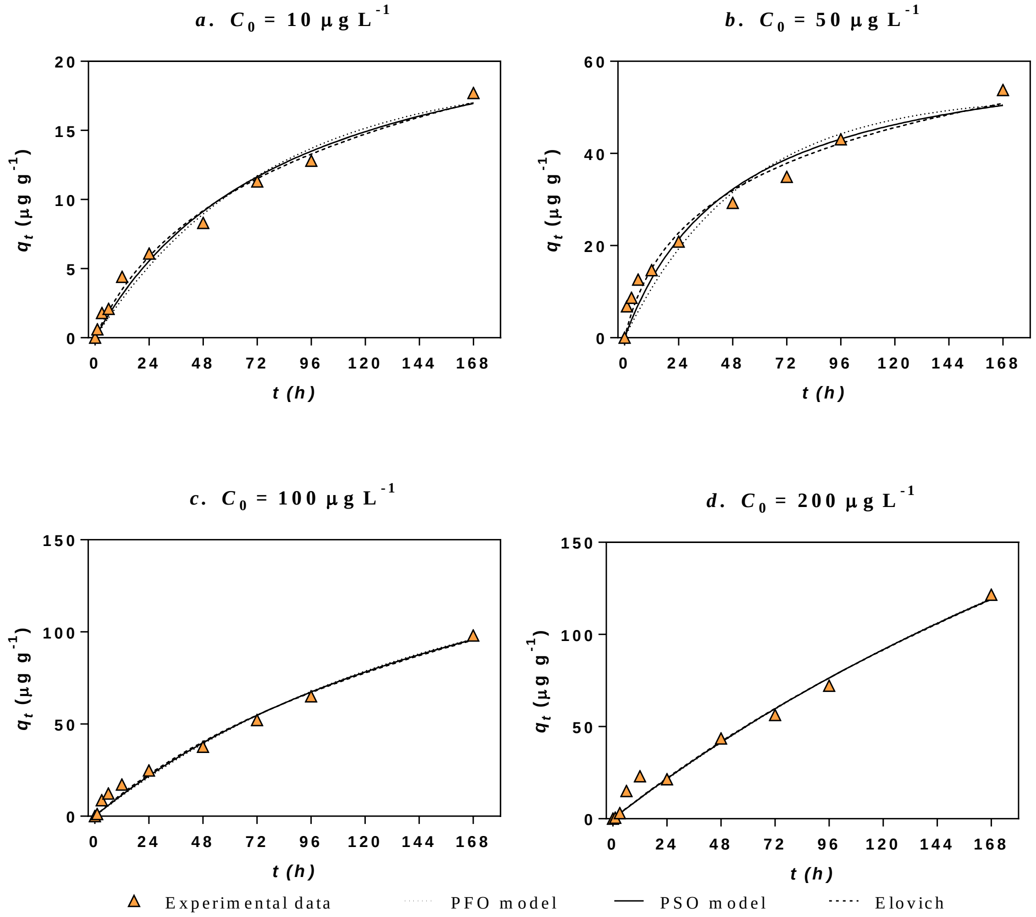


Figure 5.6: Experimental and modelling results for the amount of Cd bioaccumulated by the algae *F. vesiculosus*. (Error bars were omitted for clarity).

For all contamination scenarios there is a good agreement between the experimental data and the fittings accomplished by the kinetic models. Indeed, the fittings given by the models adopted in this work are very similar and the comparison of goodness of the fits by the Akaike's Information Criterion (AIC) indicates that in most of the cases the Elovich model is likely to be the most appropriated model to describe the bioaccumulation kinetics of Cd by *F. vesiculosus* (lower AIC) but the ratio of probability is small (Table 5.4).

Table 5.4: Best-fit parameters (± standard error) and goodness of the fittings of the kinetic models used to describe the bioaccumulation kinetics of Cd by *F. vesiculosus* algae.

C_0 (µg L^{-1})	**PFO**	**PSO**	**Elovich**	**Preferred model***
10	**k_1:** 0.013±0.002	**k_2:** $4.5e^{-04}\pm1.4e^{-04}$	**α:** 0.367±0.046	***Elovich*** *Probability:91.4%*
	$q_{t,e}$: 19.1±1.8	**$q_{t,e}$:** 25.7±2.7	**β:** 0.129±0.016	
	R^2: 0.978	**R^2:** 0.984	**R^2**: 0.990	
	$S_{y/x}$: 0.934	**$S_{y/x}$:** 0.790	**$S_{y/x}$**: 0.624	
50	**k_1:** 0.019±0.005	**k_2:** $3.1e^{-04}\pm4.4e^{-05}$	**α:** 2.04±0.48	***Elovich*** *Probability:91.6%*
	$q_{t,e}$: 52.8±5.7	**$q_{t,e}$:** 65.2±7.9	**β:** 0.061±0.009	
	R^2: 0.938	**R^2:** 0.954	**R^2**: 0.972	
	$S_{y/x}$: 4.60	**$S_{y/x}$:** 3.93	**$S_{y/x}$**: 3.10	
100	**k_1:** 0.007±0.002	**k_2:** $2.1e^{-05}\pm1.0e^{-05}$	**α:** 1.10±0.13	***Elovich*** *Probability:63.9%*
	$q_{t,e}$: 142±24	**$q_{t,e}$:** 219±43	**β:** 0.012±0.003	
	R^2: 0.984	**R^2:** 0.985	**R^2**: 0.987	
	$S_{y/x}$: 4.30	**$S_{y/x}$:** 4.11	**$S_{y/x}$**: 3.88	
200	**k_1:** 0.003±0.002	**k_2:**$4.2e^{-06}\pm4.3e^{-06}$	**α:** 0.972±0.125	***Elovich*** *Probability:52.5%*
	$q_{t,e}$: 277±117	**$q_{t,e}$:** 473±216	**β:** 0.005±0.003	
	R^2: 0.980	**R^2:** 0.980	**R^2**: 0.981	
	$S_{y/x}$: 5.85	**$S_{y/x}$:** 5.80	**$S_{y/x}$**: 5.74	

*Comparison of the two fits with the highest R^2 and lower $S_{y/x}$ values; Probability reported by Graphad6® after model comparison using Akaike's Information Criterion..

For Hg, the bioremoval curves, C_t/C_0 *vs t*, for three contamination levels (C_0=10, 50 and 100 µg L^{-1}) are shown in Figure 5.7a-c.

Like for lead and cadmium, the decrease on Hg concentration in solution may only be attributed to the interactions metal-algae since data from macroalgae-free experiments show a constant metal concentration with time. Moreover, for the contamination scenarios selected, the Hg uptake by the macroalgae was very faster at the beginning of the experiments, slowing down with time. Although the equilibrium was not achieved, at the end of the essay it was possible to reach an almost totally Hg-free solution, with the removal efficiencies ranging from 93 to 97%.

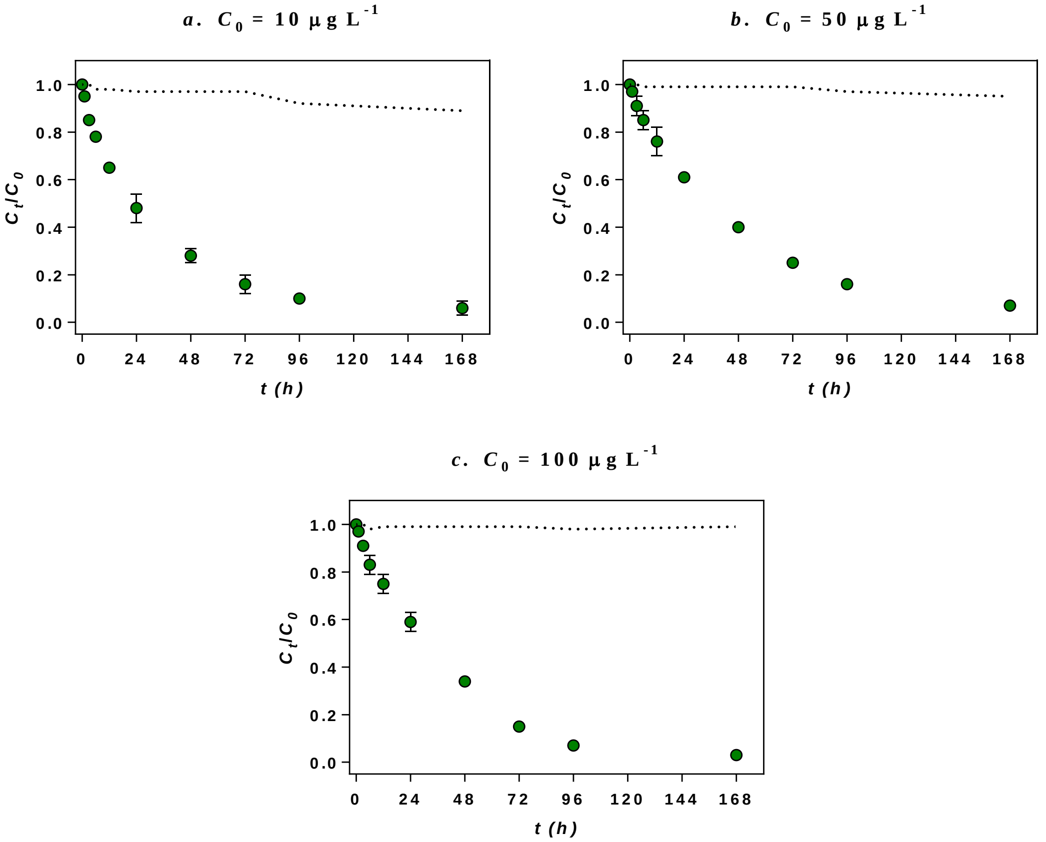


Figure 5.7: Normalized concentrations (C_t/C_0) of Hg in solution with time: squares – sample essay: Hg solution + *F. vesiculosus*; dash line – control essay: Hg solution.

Like for the others contaminants an increase on the initial Hg concentration resulted on an increase of the amount of Hg bioaccumulated by the algae and of the initial bioaccumulation rate as a consequence of a higher driving force. In the particular case of an increase on C_0 from 10 to 100 µg L^{-1}, the q_t values raised from *ca*. 23 to 215 µg g^{-1} while the initial removal rate changed from 0.54 to 3.70 µg L^{-1} h^{-1} (Table 5.2).

The time evolution of the q_t values for the bioaccumulation of Hg by *F. vesiculosus* and the fittings obtained using the PFO, PSO and Elovich models are shown in Figure 5.8*a*-*c*. For all experimental conditions there is a good agreement between experimental data and the fittings accomplished by the kinetic models used (R^2 >0.98). However, the model that has displayed higher R^2 and lower $S_{y/x}$ was the PSO (Table 5.5).

Table 5.5: Best-fit parameters (± standard error) and goodness of the fittings of the kinetic models used to describe the biosorption kinetics of Hg by *F. vesiculosus* macroalgae.

C_0 (µg L^{-1})	PFO	PSO	Elovich	Preferred model*
10	**k_1:** 0.037±0.003	**k_2:** 1.5e^{-03}±1.4e^{-04}	**α:** 1.69±0.25	***PSO*** *Probability:99.1%*
	$q_{t,e}$: 22.6±0.6	**$q_{t,e}$:** 27.1±0.6	**β:** 0.153±0.012	
	R^2: 0.992	**R^2:** 0.997	**R^2:** 0.991	
	$S_{y/x}$: 0.832	**$S_{y/x}$:** 0.522	**$S_{y/x}$:** 0.901	
50	**k_1:** 0.022±0.001	**k_2:** 1.5e^{-04}±1.3e^{-05}	**α:** 3.87±0.38	***PSO*** *Probability:93.8%*
	$q_{t,e}$: 110±3	**$q_{t,e}$:** 141±4	**β:** 0.0252±0.002	
	R^2: 0.997	**R^2:** 0.998	**R^2:** 0.995	
	$S_{y/x}$: 2.39	**$S_{y/x}$:** 1.82	**$S_{y/x}$:** 2.97	
100	**k_1:** 0.023±0.002	**k_2:** 7.5e^{-05}±1.4e^{-05}	**α:** 7.96±1.38	***PFO*** *Probability:96.8%*
	$q_{t,e}$: 223±6	**$q_{t,e}$:** 288±15	**β:** 0.012±0.002	
	R^2: 0.996	**R^2:** 0.993	**R^2:** 0.986	
	$S_{y/x}$: 5.45	**$S_{y/x}$:** 7.68	**$S_{y/x}$:** 10.8	

*Comparison of the two fits with the highest R^2 and lower $S_{y/x}$ values; Probability reported by Graphad6® after model comparison using Akaike's Information Criterion.

Comparing the bioaccumulation efficiency of the macroalgae *F. vesiculosus* toward the metals studied it is possible to conclude that the highest removal percentages were record for Hg and the lowest for Cd. Indeed, in the case of Hg it was possible to reach an almost totally metal-free solution, even in a complex matrix like saltwater. The fact, that the equilibrium was not achieved in any system suggests that the algae *F. vesiculosus* still has capacity to uptake more metal and consequently improve even more the water quality, highlighting the great potential of the algae for water decontamination. Moreover, in the case of Pb and Hg the level of contaminations did not have a strong effect on the bioremoval efficiency (%), contrary to Cd which removal from water decrease *ca*. 67% with the increase of C_0 from 10 to 200 µg L^{-1}.

In an analogous studied performed with the macroalgae *U. lactuca* (personal data), the lowest bioaccumulation efficiency was record for Pb suggesting that each macroalgae has its specificities and the chemical composition of the macroalgae play a vital role on the biosorption/bioaccumulation of metals.

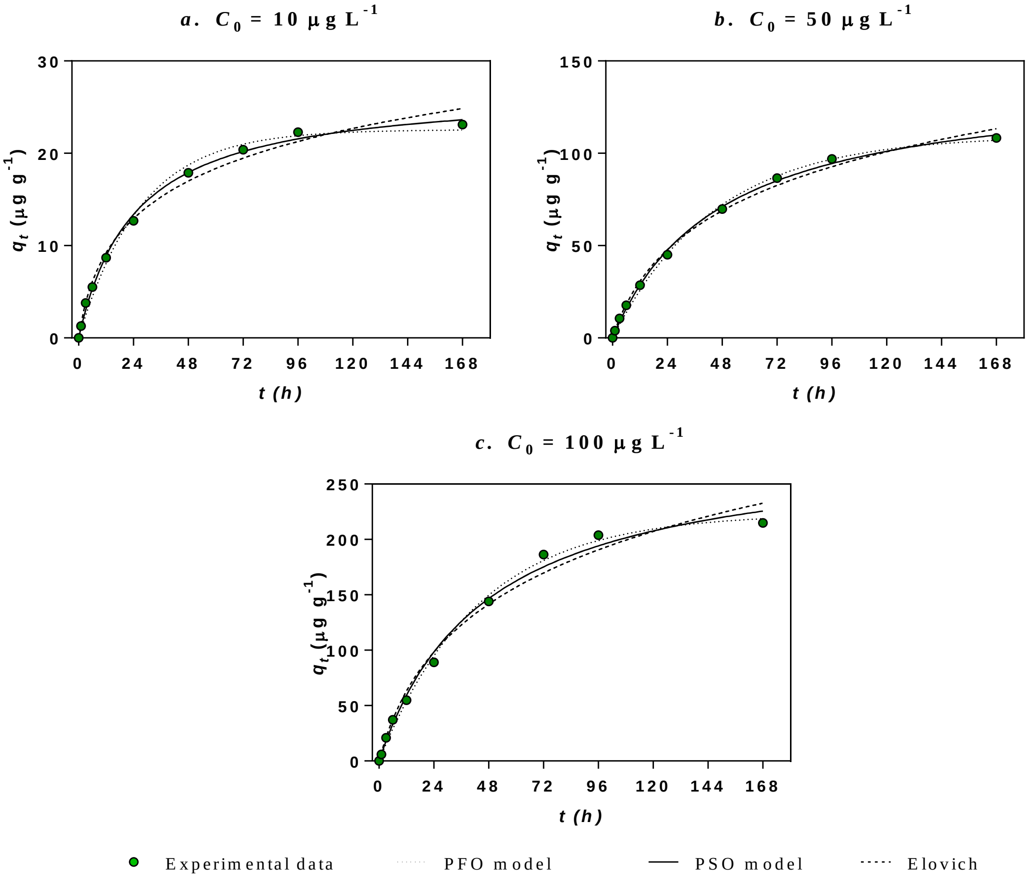


Figure 5.8: Experimental and modelling results for the amount of Hg bioaccumulated by the macroalgae *F. vesiculosus*. (Error bars were omitted for clarity).

5.3.3 *Bioremediation in multi-metal contamination scenarios and algae selectivity*

The bioaccumulation capacity of *F. vesiculosus* toward metals such as Pb, Cd and Hg was evaluated under metal competition (ternary solutions) for two contamination scenarios, designated by mixture 1 ($C_{Pb,0}$=$C_{Cd,0}$=$C_{Hg,0}$=50 µg L^{-1}) and mixture 2 ($C_{Pb,0}$=1000 µg L^{-1}; $C_{Cd,0}$=200 µg L^{-1}; $C_{Hg,0}$=50 µg L^{-1}).

The curves of the metal removal by *F. vesiculosus* from solution are shown in Figure 5.9, in terms of normalized concentration C_t/C_0 *vs* *t* for both mixtures.

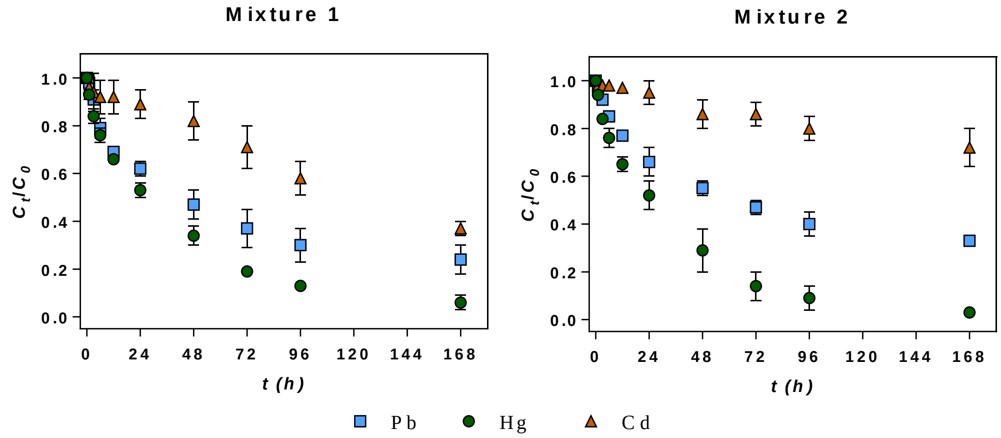


Figure 5.9: Normalized concentrations (C_t/C_0) of Pb, Cd and Hg in solution with time for different contamination scenarios: mixture 1 ($C_{Pb,0}$=$C_{Cd,0}$=$C_{Hg,0}$=50 µg L^{-1}); mixture 2 ($C_{Pb,0}$=1000 µg L^{-1}; $C_{Cd,0}$=200 µg L^{-1}; $C_{Hg,0}$=50 µg L^{-1}).

The data shows that in ternary metal solutions the macroalgae *F. vesiculosus* is still able to bioaccumulate the three metals. However, the extension of the bioaccumulation depends on the metal and/or on the contamination level. As it may be seen, the residual concentration of Hg in solution in both situations was remarkably low, despite the presence of the other metals, even when their concentration is in large excess. In mixture 1, the values of the metal removal percentage followed the order Hg>Pb>Cd, and the values were respectively, 94, 76 and 63%. However, it must be highlighted that the initial molar concentration of Cd was *ca.* 1.8 folds the molar concentrations of Hg and Pb. Please remember, that the relative atomic masses of Hg, Pb and Cd are 200.6, 207.2 and 112.4 g mol^{-1}, respectively. This fact, is reflected in terms of the amount of metal that is bioaccumulated by the macroalgae: 0.605 µmol g^{-1} of Hg, 0.596 µmol g^{-1} of Cd and 0.397 µmol g^{-1} of Pb. In mixture 2, the values of metal removal kept the same order, Hg>Pb>Cd, but with a decrease on the magnitude of the value for the metals whose the initial concentration was increased. Note, that the initial concentrations of Hg, Cd and Pb used, corresponded to the value of the old maximum legal limit allowed for wastewater discharges (respectively, 50, 200 and 1000 µg L^{-1}). The removal values achieved for this contamination scenario were 97% for Hg, 68% for Pb and 28% for Cd. In terms of the amount of metal bioaccumulated by the macroalgae an inverse situation was observed, i.e. the values increased with an increase on C_0, respectively 0.605 µmol g^{-1} for Hg, 1.02 µmol g^{-1} for Cd and 6.85 µmol g^{-1} for Pb.

The selectivity of the macroalgae toward the different metals (*S*) is given by the ratio between the distribution coefficients (*K*) of the metals at equilibrium. Where, the distribution coefficient is defined as $q_{t,e}/C_{t,e}$. Considering the experimental values of q_t at 168 hours, the *pseudo-equilibrium* selectivity found for the *F. vesiculosus* toward the target metals followed the order Hg>Pb>Cd, independently of the initial metal concentrations. This order is in agreement with the results achieved by Mata et al. (2008) in their study of metal biosorption by dried biomass of *F. vesiculosus*. According to the equilibrium Langmuir isotherm model, authors concluded that affinity of metals for the biomass followed this order: Pb > Cu > Cd. Similar findings were reported for the biosorption of Pb and Cd by different types of algae (Romera et al. 2007). Both studies did not included Hg. A plausible explanation for the different affinity of brown algae toward divalent cations is described in the work of Davis et al. (2003). The authors argue that the variation in affinity for metals are related to the molecular conformation between two homopolymeric blocks (mannuronic and guluronic acid) in alginate (a linear polysaccharide present in brown algae), in a description known as the ‘‘egg-box’’ model and intrinsic metal properties, such as ionic radii and electronegativity (Davis et al. 2003).

Relevant differences were observed on the kinetics of the simultaneous bioaccumulation of Hg, Pb and Cd by the *F. vesiculosus*. The uptake of Hg and Pb by the macroalgae was very faster and similar at the beginning of the experiments, as confirmed by the kinetic constants (k_1 and k_2, Table 5.6) estimated by the PFO and PSO models and slowed down with the approach of the plateau value on C_t, that independently of the metal was not attained during the conduction of the essays. The Cd bioaccumulation was much slower than for Pb and Hg, in particular for the highest level of contamination (Figure 5.9).

Like for the monometallic systems, the modelling of the kinetic results in the mixtures showed that there is a good agreement between the experimental data and the fittings accomplished by the models (Figure 5.10): PFO model (R^2: 0.976 – 0.986); PSO model (R^2: 0.985 – 0.996) and the Elovich (R^2: 0.976 – 0.998).

Table 5.6: Best-fit parameters (± standard error) and goodness of the fittings of the kinetic models used to describe the biosorption kinetics of metals by *F. vesiculosus* algae in ternary solutions.

	PFO	PSO	Elovich	Preferred model*
		Mixture 1		
Pb	**k_1:** 0.0355±0.00530	**k_2:** 0.0903±0.0146	**α:** 0.0274±0.00305	***Elovich*** *Probability:94.1%*
	$q_{t,e}$: 0.372±0.018	**$q_{t,e}$:** 0.445±0.0176	**β:** 9.262±0.522	
	R^2: 0.976	**R^2:** 0.991	**R^2:** 0.995	
	$S_{y/x}$: 0.0241	**$S_{y/x}$:** 0.0146	**$S_{y/x}$:** 0.0110	
Cd	**k_1:** 0.00331±0.0189	**k_2:** 7.67e^{-04}±8.84e^{-04}	**α:** 4.75e^{-03}±6.59e^{-04}	***Elovich*** *Probability:50.4%*
	$q_{t,e}$: 1.40±0.644	**$q_{t,e}$:** 2.47±1.28	**β:** 0.928±0.538	
	R^2: 0.976	**R^2:** 0.976	**R^2:** 0.976	
	$S_{y/x}$: 0.0313	**$S_{y/x}$:** 0.0313	**$S_{y/x}$:** 0.0313	
Hg	**k_1:** 0.0329±0.00439	**k_2:** 0.0527±0.00844	**α:** 0.0403±0.00468	***Elovich*** *Probability:88.1%*
	$q_{t,e}$: 0.582±0.0255	**$q_{t,e}$:** 0.700±0.0279	**β:** 5.901±0.354	
	R^2: 0.980	**R^2:** 0.991	**R^2:** 0.994	
	$S_{y/x}$: 0.0331	**$S_{y/x}$:** 0.0219	**$S_{y/x}$:** 0.0179	
		Mixture 2		
Pb	**k_1:** 0.0308±0.00375	**k_2:** 4.19e^{-03}±4.91e^{-04}	**α:** 0.389±0.0273	***Elovich*** *Probability:96.7%*
	$q_{t,e}$: 6.53±0.268	**$q_{t,e}$:** 7.94±0.238	**β:** 0.499±0.0194	
	R^2: 0.984	**R^2:** 0.996	**R^2:** 0.998	
	$S_{y/x}$: 0.332	**$S_{y/x}$:** 0.173	**$S_{y/x}$:** 0.124	
Cd	**k_1:** 0.00710±0.00174	**k_2:** 1.98e^{-03}±1.00e^{-03}	**α:** 0.0113±0.00148	***PSO*** *Probability:50.8%*
	$q_{t,e}$: 1.46±0.234	**$q_{t,e}$:** 2.338±0.475	**β:** 1.10±0.288	
	R^2: 0.985	**R^2:** 0.985	**R^2:** 0.985	
	$S_{y/x}$: 0.0460	**$S_{y/x}$:** 0.0459	**$S_{y/x}$:** 0.0461	
Hg	**k_1:** 0.0328±0.00368	**k_2:** 0.0505±0.00735	**a:** 0.0392±0.00547	***PSO*** *Probability:71.8%*
	$q_{t,e}$: 0.591±0.0218	**$q_{t,e}$:** 0.714±0.0261	**β:** 5.703±0.419	
	R^2: 0.986	**R^2:** 0.993	**R^2:** 0.992	
	$S_{y/x}$: 0.0282	**$S_{y/x}$:** 0.0201	**$S_{y/x}$:** 0.0220	

*Comparison of the two fits with the highest R2 and lower Sy/x values; Probability reported by Graphad6® after model comparison using Akaike's Information Criterion.

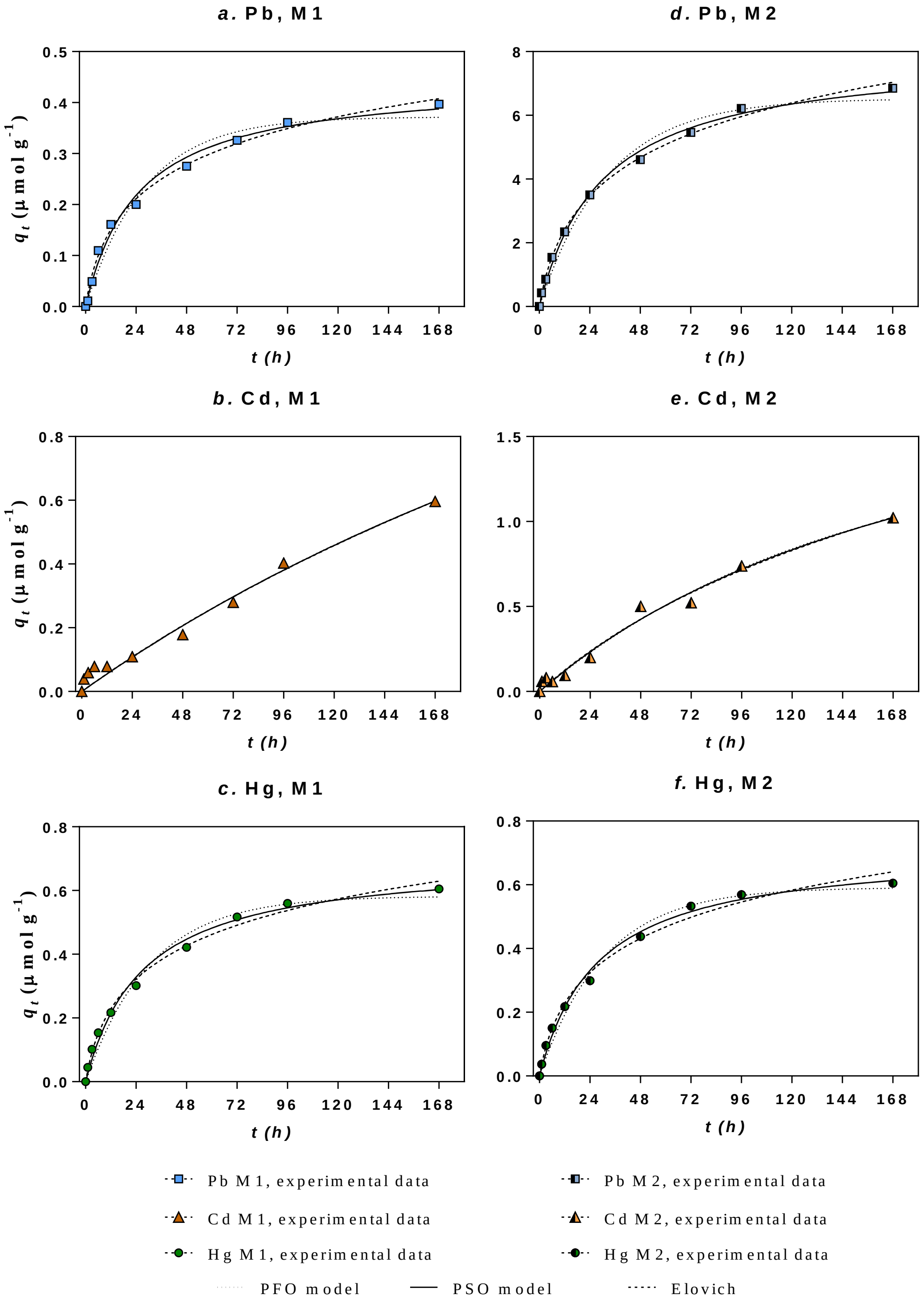


Figure 5.10: Experimental and modelling results for the amount of Pb, Cd and Hg bioaccumulated by the macroalgae *F. vesiculosus* for different contamination scenarios: mixture 1 ($C_{Pb,0}$=$C_{Cd,0}$=$C_{Hg,0}$=50 μg L^{-1}); mixture 2 ($C_{Pb,0}$=1000 μg L^{-1}; $C_{Cd,0}$=200 μg L^{-1}; $C_{Hg,0}$=50 μg L^{-1}) (Error bars were omitted for clarity).

Comparing the accumulation performance of *F. vesiculosus* for each metal in mono- and multi-metallic solutions it is possible to conclude that Hg was barely affected by the presence of Pb and Cd. Only a small increase on the initial bioaccumulation rate, from 0.008

to 0.015 µmol L^{-1} h^{-1}, and on the percentage of removal from 93 to 97% was observed from monometallic systems to the mixtures. Moreover, by comparing the $q_{t,e}$ value for Hg in the mixtures with the one obtained previously in the same conditions but in the monometallic solution, it is possible to evaluate the effect of the presence of the metals on the Hg bioaccumulation. If, $q_{t,e}^{mixture}/q_{t,e}^{mono}$ <1, the bioaccumulation of Hg is inhibited by the presence of Cd and/or Pb; if $q_{t,e}^{mixture}/q_{t,e}^{mono}$ =1, there is no observable interaction; and if $q_{t,e}^{mixture}/q_{t,e}^{mono}$ >1, the bioaccumulation is favored by the presence of the other metals. The values found were 1.12 for both mixture 1 and 2, indicating that in both systems the bioaccumulation of Hg by the macroalgae *F. vesiculosus* was slightly favored by the presence of Cd and Pb, probably due to an increase of the ionic strength of the solution.

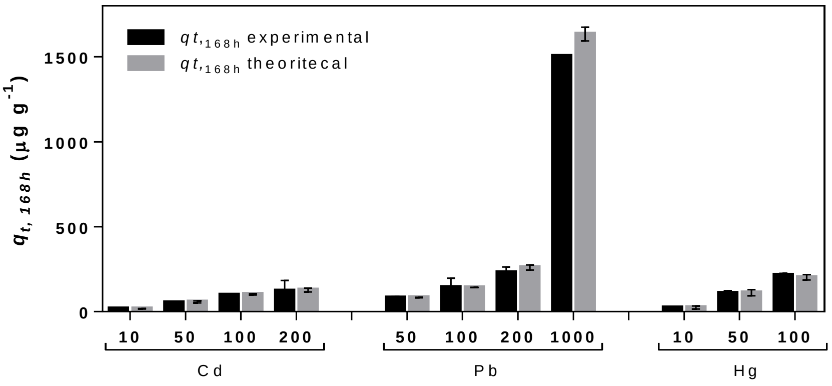
In the case of the Pb bioaccumulation, it was observed a decrease on the initial removal rates in the mixtures in comparison with the respective monometallic solutions (from 0.012 to 0.009 µmol L^{-1} h^{-1} for C_0 of 50 µg L^{-1}, and from 0.207 to 0.163 µmol L^{-1} h^{-1} for C_0 of 1000 µg L^{-1}), and an increase on the final degree of metal removal from solution. The value found for the $q_{t,e}^{mixture}/q_{t,e}^{mono}$ coefficient was 1.00 for mixture 1 and 0.94 for mixture 2, suggesting none or little influence of the other metals on Pb bioaccumulation.

For Cd it was observed a small increase on the metal removal percentage in the mixtures in comparison with the respective monometallic solution. For an initial concentration of 50 µg L^{-1}, the Cd bioaccumulation was favored by the presence of other metals ($q_{t,e}^{mixture}/qt_{,e}^{mono}$ coefficient of 1.24), while for the more contaminated scenario occurred a small inhibition of the Cd bioaccumulation by the macroalgae ($q_{t,e}^{mixture}/q_{t,e}^{mono}$ coefficient of 0.96).

5.3.4 *Metal contents in F. vesiculosus and bioconcentration factors*

Metal contents in *F. vesiculosus* biomass, immediately after bioaccumulation trials, are presented in Figure 5.11, along with the corresponding theoretical concentrations, obtained through mass balance analysis between the initial concentration of metal in solution and the concentration at the end of experiments. Results confirm our assumption that all metal removed from seawater along time was incorporated by macroalgae, since for the full range of contamination scenarios studied, experimental and theoretical values of $qt_{,168h}$ perfectly matched (maximum relative error of 7.9% for Pb, 1000 µg L^{-1}).

a. single-contaminant



b. mixtures

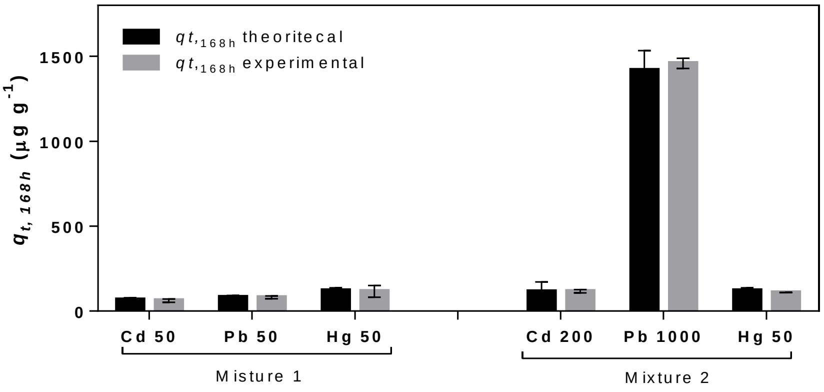


Figure 5.11: Metal contents in *F. vesiculosus* after exposure of 168h to single contaminant (a) and mixture (b) systems, determined through mass balance using Eq. 1 ($q_{t,168h}$ theoritecal) and obtained by chemical quantification in seaweed ($q_{t,168h}$, experimental).

The good agreement between theoretical and experimental values of q_t , even in mixtures, reinforces the great potential of this brown macroalgae as biomonitor of environmental contamination (Varma et al. 2011), as well as a useful tool in processes of analytical chemistry (Romero-Gonzalez et al. 2000, Vidotti 2004). The ability of *F. vesiculosus* concentrate metals on tissues, along with the characterization of the bioaccumulation kinetics, may allow to estimate the concentrations of these metals in natural waters at very low levels, which determination may be difficult for the usual analytical techniques. By other hand, from the viewpoint of application in wastewater remediation, a biotechnology based in the use of this macroalgae assures the safe removal of metal from solution, i.e. none of the metal removed from the solution remain in the system, for example as precipitates, after macroalgae are harvest from the medium.

As previously mentioned, the amount of metal bound by macroalgae was positively related with the initial metal concentration in solution (Figure 5.11a), and thus highest metal contents in biomass were observed for Pb, 1000 µg L^{-1}. However, maximum bioconcentration factors (BCF), defined as the ratio between the concentration of metal in macroalgae biomass at the end of exposure and the initial concentration of metal in solution, were recorded for Hg (2147 to 2308 in monometallic solutions and c.a. 2400 for mixtures). In single contaminant, an increase of initial concentration of Cd from 10 to 200 µg L^{-1} resulted in a decrease of BCF from 1772 to 608, while for Pb, BCFs were not strongly affected by the initial concentration. Under metallic competition, BCF of Cd and Pb were respectively 1340 and 1645 for the lowest contamination scenario, while for mixture 2, BCFs were 574 and 1419 for Cd and Pb respectively.

5.3.5 *Depuration experiments*

The results presented in the previous section demonstrated that all metal removed from the solution was bound to the seaweed tissues. However, in order to evaluate the detoxifying ability of the seaweed, i.e. the capacity to release the accumulated metals back to a clean solution, additional experiments were conducted. After bioaccumulation trials, the living seaweed was immediately placed in clean seawater, and the concentrations of metals in solution were quantified over 7 days. Results are presented in Figure 5.12 as percentage of metal retained by the brown seaweed over time.

Cleary, there was no loss of Hg during the 7 days of contact with clean seawater, demonstrating the irreversibility of Hg binding in *F. vesiculosus*. Even when the biomass of the living macroalgae was loaded with the three metals (Mixtures 1 and 2), no Hg was released back to solution, which highlights the strong interactions Hg-algae.

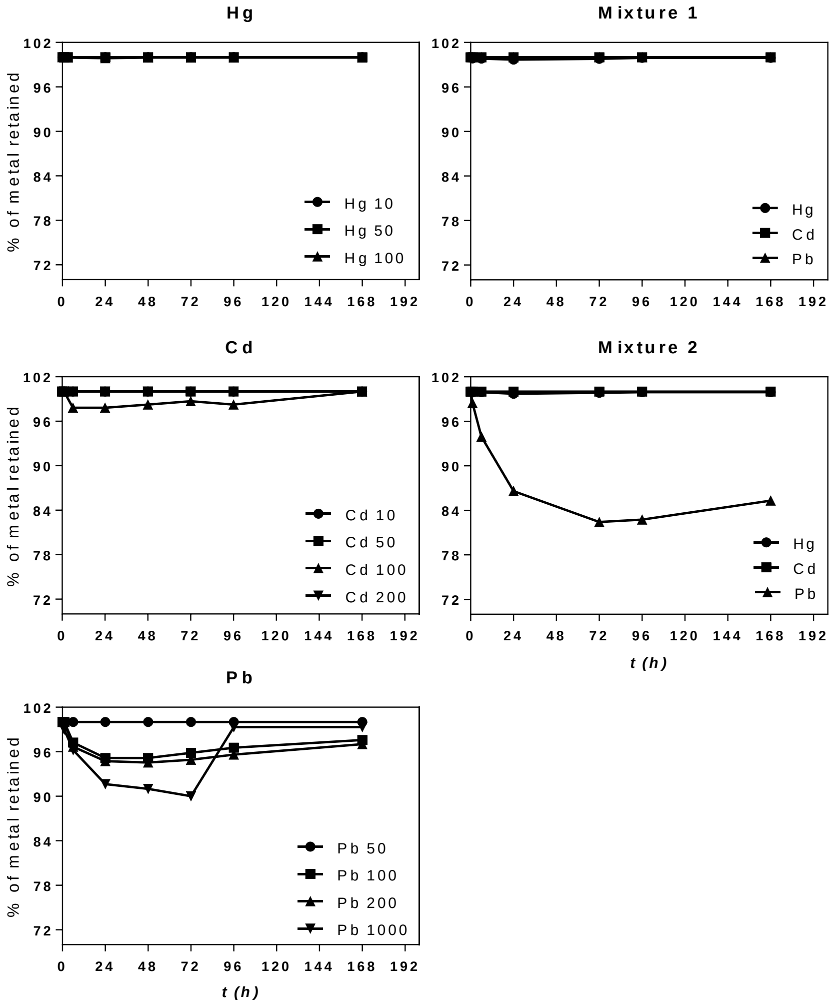


Figure 5.12: Metal retention in algae for different metals concentrations, in both monometallic and multimetallic solutions.

Like Hg, none of Cd bioaccumulated was released back to solution, with exception of macroalgae exposed to the highest concentrations of Cd (only for single-contaminant), where a negligible loss of metal (less than 2%) was recorded in the first 12h. Moreover, until the end of experiments, *F. vesiculosus* bioaccumulated again that metal, leading to residual concentrations of Cd in solution below the limit of quantification. These results are in accordance with the findings of Boisson et al. (1997), who reported a very efficient retention of metals by this brown seaweed. The efflux rate constant for Cd determined by the authors was not significantly different from zero (Boisson et al. 1997).

A different behaviour from that of Hg and Cd, was observed for Pb, which was always released to the solution in the beginning of depuration experiments, with the exception of macroalgae exposed to the lowest concentration, 50 µg L^{-1} (in both single- and multi-contaminant systems). The initial loss of metal increased with the growing degree of contamination that macroalgae were exposed. Thus, the highest percentage of release (of about 10%) was recorded at the highest concentration of Pb (1000 µg L^{-1}). This tiny fraction of Pb was probably associated with the apparent free space in macroalgae (Wang and Dei 1999), or was weakly bound to extracellular polymers produced by the macroalgae as a defense mechanism (Andrade et al. 2010). However, the pattern observed for Pb indicates a re-uptake of Pb over time, which eventually would lead to metal concentrations in solution of almost zero.

Overall, *F. vesiculosus* does not seem to be able to debug, to eliminate the metals that have been accumulated.

5.3.6 *Effects of metals on macroalgae growth*

The effect of Cd, Pb and Hg on *F. vesiculosus* growth was evaluated after 7-day period exposure to mono and multimetallic solutions, with different concentrations. For this purpose, relative growth rates (RGR) were determined by measuring macroalgae area at the beginning and at the end of each assay. The RGR of blank (macroalgae in seawater in the absence of contaminant), which was always run in parallel with the bioaccumulation assays was used as reference.

In the absence of any contaminant, the RGR of *F. vesiculosus* varied between 0.5 and 1.7 % day^{-1} (n=10), which is in agreement with the values obtained by (Lehvo et al. 2001) for *F. vesiculosus* growing on Baltic sea (less than 0.3 % day^{-1} at winter and 0.7 % day^{-1} at summer).

In order to compare the effect on growth rate among metals, all data were normalized considering the correspondent RGR of blank ($RGR_{normalized}=RGR_{metal}/RGR_{blank}$). The normalized values are presented in Figure 5.13.

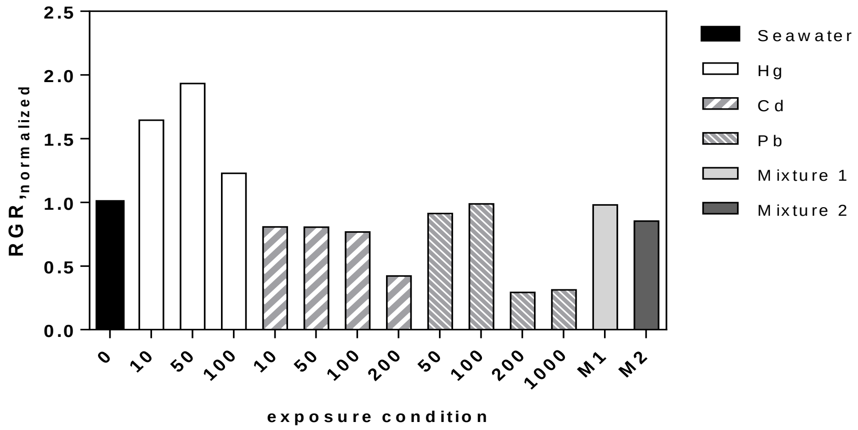


Figure 5.13: Relative growth rate (normalized) of *F. vesiculosus* in seawater and when exposed for 7 days to mono and multimetallic solutions of Hg, Cd and Pb.

Results shows an enhancement of macroalgae growth rate exposed to different monometallic solutions of Hg (RGR 1.2 to 1.9 times higher than reference). This apparently positive effect on growth may in fact represent a defense mechanism of the seaweed against toxic metal. *F. vesiculosus* may increase its cellular metabolism, growing faster, in order to reduce the concentration of internalized Hg, by a rise in its biomass area - dilution effect (Sunda and Huntsman 1998, Costa et al. 2011). A similar behavior was verified for green macroalgae *U. lactuca* when exposed to Hg, Cd and Pb, under the same experimental conditions (personal data). However, contrary to observed in that case, in the present study only for Hg occurred an enhancement of RGR, which is nonetheless intriguing and even contradictory. Further studies are needed in order to clarify the mechanisms of Hg-acting on the cellular metabolism of macroalgae and their strategies for response to toxicity.

In the case of Cd, levels of 10, 50 and 100 µg L^{-1} in solution induced a slightly decrease in macroalgae RGR (RGR was 80 % of blank RGR), while the highest concentration (200 µg L^{-1}) caused a more pronounced negative effect on RGR (42 % of blank RGR). Indeed, the amount of Cd bioaccumulated by the brown seaweed, at 200 µg L^{-}1, was 2 to 7-fold that recorded for the lowest concentrations.

Lead presented two distinct behaviours according to level of contamination. For lower concentrations (50 and 100 µg L^{-1}) there was almost no influence of contaminant in the macroalgae growth. However, for higher concentrations (200 and 1000 µg L^{-1}), a strong deleterious effect on RGR was verified, with a reduction of about 70% compared to blank RGR.

RGRs of brown macroalgae in multimetallic solutions were not significantly affected by the simultaneous presence of Cd, Hg and Pb, even in the scenario of severe contamination (Mixture 2).

Overall, results of RGR, particularly those achieved in mixtures, together with the fact that no sporulation or marked loss of color, which indicates algae decay (Han and Choi 2005), was observed, are a good evidence of the potential application of these algae in the remediation of real aquatic systems (at least for contamination levels similar to that studied).

5.4 Conclusions

The high capacity to accumulate metals by the brown seaweed *F. vesiculosus* was demonstrated (bioconcentration factors varied between 600 and 2300), in single and multi-contaminant systems, pointing to its great potential in the removal of metals from saline waters. Its application would be particularly advantageous when a large volume of contaminated salt water, with relative low concentrations of metals needs to be remediated, as for example a confined estuarine or aquaculture system. Moreover, its high availability, would render the biotechnology also very effective economically.

In all cases, higher removal percentages were achieved for Hg, followed by Pb and then Cd. Yet the highest amount of metal bioaccumulated was recorded for Pb, as metal uptake reflects its occurrence in solution. The kinetics of the removal process was accurately described by the reaction kinetic models studied, and the highest bioaccumulation rates were observed for Hg.

Results on metal contents on macroalgae biomass, after bioaccumulation, and the depuration experiments, revealed that macroalgae strongly bound all metal removed from the solution. This suggests that seaweeds could act as a protecting agent against hazard effects of metals in aquatic ecosystems, since removing metals from the dissolved phase and keeping them “safes” internally, avoid the bioaccumulation by key-species organisms, more sensitive to metal toxicity.

Finally, it may be concluded that metal bioaccumulation did not affect significantly the relative growth rate of the brown macroalge, during the 7 days of exposure, with the exception of Cd, 200 µg L^{-1}, and Pb, 200 and 1000 µg L^{-1}. Nevertheless, further studies are needed to explain de different behaviour of Hg in *F. vesiculosus* metabolism, in relation to Cd and Pb.

5.5 References

Andrade, L. R., R. N. Leal, M. Noseda, M. E. R. Duarte, M. S. Pereira, P. A. S. Mourão, M. Farina and G. M. Amado Filho (2010). "Brown algae overproduce cell wall polysaccharides as a protection mechanism against the heavy metal toxicity." Marine Pollution Bulletin 60(9): 1482-1488.

Back, S., J. C. Collins and G. Russel (1992). "Comparative ecophysiology of Baltic and Atlantic Fucus vesiculosus." Marine Ecology Progress Series 84: 71-82.

Boisson, F., D. A. Hutchins, S. W. Fowler, N. S. Fisher and J. L. Teyssie (1997). "Influence of temperature on the accumulation and retention of 11 radionuclides by the marine alga Fucus vesiculosus (L.)." Marine Pollution Bulletin 35(7–12): 313-321.

Chojnacka, K. (2007). "Bioaccumulation of Cr(III) ions by Blue-Green alga Spirulina sp. Part I. A Comparison with Biosorption." American Journal of Agricultural and Biological Sciences 2(4): 218-223.

Chojnacka, K. (2009). Biosorption and bioaccumulation in practice. New York, Nova Science Publishers, Inc.

Chojnacka, K. (2010). "Biosorption and bioaccumulation - the prospects for practical applications." Environment International 36(3): 299-307.

Cobas, M., M. A. Sanromán and M. Pazos (2014). "Box–Behnken methodology for Cr (VI) and leather dyes removal by an eco-friendly biosorbent: F. vesiculosus." Bioresource Technology 160(0): 166-174.

Coelho, J. P., M. E. Pereira, A. Duarte and M. A. Pardal (2005). "Macroalgae response to a mercury contamination gradient in a temperate coastal lagoon (Ria de Aveiro, Portugal)." Estuarine, Coastal and Shelf Science 65(3): 492-500.

Costa, S., D. Crespo, B. M. G. Henriques, E. Pereira, A. C. Duarte and M. A. Pardal (2011). "Kinetics of Mercury Accumulation and Its Effects on Ulva lactuca Growth Rate at Two Salinities and Exposure Conditions." Water Air and Soil Pollution 217(1-4): 689-699.

Costley, C. T., K. F. Mossop, J. R. Dean, L. M. Garden, J. Marshall and J. Carroll (2000). "Determination of mercury in environmental and biological samples using pyrolysis atomic absorption spectrometry with gold amalgamation." Analytica Chimica Acta 405(1-2): 179-183.

Coyer, J. A., G. Hoarau, J. F. Costa, B. Hogerdijk, E. A. Serrão, E. Billard, M. Valero, G. A. Pearson and J. L. Olsen (2011). "Evolution and diversification within the intertidal brown macroalgae Fucus spiralis/F. vesiculosus species complex in the North Atlantic." Molecular Phylogenetics and Evolution 58(2): 283-296.

Davis, T. A., B. Volesky and A. Mucci (2003). "A review of the biochemistry of heavy metal biosorption by brown algae." Water Research 37(18): 4311-4330.

Decree-Law No. 236/98 (1998). Decree-Law No. 236/98 of the Portuguese Ministry of the Environment of 1 August establishing water quality standards, Diário da República : I Série. 176: 3676-3722.

Directive 83/513/EEC (1983). Council Directive 83/513/EEC of 26 September 1983 on limit values and quality objectives for cadmium discharges (Daughter to 2006/11/EC), Official Journal of the European Communities. 291: 1-8.

Directive 84/156/EEC (1984). Council Directive 84/156/EEC of 8 March 1984 on limit values and quality objectives for mercury discharges by sectors other than the chlor-alkali electrolysis industry, Official Journal of the European Communities: 74: 29-74: 29.

Directive 2013/39/EU (2013). Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy, Official Journal of the European Union. 226: 1-17.

El-Khaiary, M. I. and G. F. Malash (2011). "Common data analysis errors in batch adsorption studies." Hydrometallurgy 105(3-4): 314-320.

Farooq, U., J. A. Kozinski, M. A. Khan and M. Athar (2010). "Biosorption of heavy metal ions using wheat based biosorbents - A review of the recent literature." Bioresource Technology 101(14): 5043-5053.

Freitas, O. M. M., R. J. E. Martins, C. M. Delerue-Matos and R. A. R. Boaventura (2008). "Removal of Cd(II), Zn(II) and Pb(II) from aqueous solutions by brown marine macro algae: Kinetic modelling." Journal of Hazardous Materials 153(1-2): 493-501.

Fu, F. and Q. Wang (2011). "Removal of heavy metal ions from wastewaters: A review." Journal of Environmental Management 92(3): 407-418.

Giusti, L. (2001). "Heavy metal contamination of brown seaweed and sediments from the UK coastline between the Wear river and the Tees river." Environment International 26(4): 275-286.

Han, T. and G.-W. Choi (2005). "A novel marine algal toxicity bioassay based on sporulation inhibition in the green macroalga Ulva pertusa (Chlorophyta)." Aquatic Toxicology 75(3): 202-212.

Han, T., S.-H. Kang, J.-S. Park, H.-K. Lee and M. T. Brown (2008). "Physiological responses of Ulva pertusa and U. armoricana to copper exposure." Aquatic Toxicology 86(2): 176-184.

He, J. and J. P. Chen (2014). "A comprehensive review on biosorption of heavy metals by algal biomass: Materials, performances, chemistry, and modeling simulation tools." Bioresource Technology 160(0): 67-78.

Hemmi, A., A. Mäkinen, V. Jormalainen and T. Honkanen (2005). "Responses of growth and phlorotannins in Fucus vesiculosus to nutrient enrichment and herbivory." Aquatic Ecology 39(2): 201-211.

Ho, Y.-S. (2006). "Review of second-order models for adsorption systems." Journal of Hazardous Materials 136(3): 681-689.

Ho, Y. S. and G. McKay (1998). "A comparison of chemisorption kinetic models applied to pollutant removal on various sorbents." Process Safety and Environmental Protection 76(B4): 332-340.

Kabata-Pendias, A., Mukherjee, Arun B. (2007). Trace Elements from Soil to Human, Springer.

Kadukova, J. and E. Vircikova (2005). "Comparison of differences between copper bioaccumulation and biosorption." Environment International 31(2): 227-232.

Lagergren, S. (1898). "About the theory of so-called adsorption of soluble substances." Kung Sven Veten Hand 24: 1-39.

Lehvo, A., S. Bäck and M. Kiirikki (2001). "Growth of Fucus vesiculosus L. (Phaeophyta) in the Northern Baltic proper: Energy and nitrogen storage in a seasonal environment." Botanica Marina 44: 345-350.

Lesmana, S. O., N. Febriana, F. E. Soetaredjo, J. Sunarso and S. Ismadji (2009). "Studies on potential applications of biomass for the separation of heavy metals from water and wastewater." Biochemical Engineering Journal 44(1): 19-41.

Lopes, C. B., J. R. Oliveira, L. S. Rocha, D. S. Tavares, C. M. Silva, S. P. Silva, N. Hartog, A. C. Duarte and E. Pereira (2014). "Cork stoppers as an effective sorbent for water treatment: the removal of mercury at environmentally relevant concentrations and conditions." Environ Sci Pollut Res Int 21(3): 2108-2121.

Mata, Y. N., M. L. Blázquez, A. Ballester, F. González and J. A. Muñoz (2008). "Characterization of the biosorption of cadmium, lead and copper with the brown alga Fucus vesiculosus." Journal of Hazardous Materials 158(2-3): 316-323.

Mata, Y. N., M. L. Blázquez, A. Ballester, F. González and J. A. Muñoz (2009). "Biosorption of cadmium, lead and copper with calcium alginate xerogels and immobilized Fucus vesiculosus." Journal of Hazardous Materials 163(2-3): 555-562.

Monterroso, P., S. N. Abreu, E. Pereira, C. Vale and A. C. Duarte (2003). "Estimation of Cu, Cd and Hg transported by plankton from a contaminated area (Ria de Aveiro)." Acta Oecologica 24, Supplement 1(0): S351-S357.

Pato, P., M. Otero, M. Válega, C. B. Lopes, M. E. Pereira and A. C. Duarte (2010). "Mercury partition in the interface between a contaminated lagoon and the ocean: The role of particulate load and composition." Marine Pollution Bulletin 60(10): 1658-1666.

Pereira, L., A. Sousa, H. Coelho, A. M. Amado and P. J. A. Ribeiro-Claro (2003). "Use of FTIR, FT-Raman and 13C-NMR spectroscopy for identification of some seaweed phycocolloids." Biomolecular Engineering 20(4–6): 223-228.

Radwan, A., G. Davies, A. Fataftah, E. Ghabbour, S. Jansen and R. Willey (1996). "Isolation of humic acid from the brown algaeAscophyllum nodosum, Fucus vesiculosus, Laminaria saccharina and the marine angiospermZostera marina." Journal of Applied Phycology 8(6): 553-562.

Rocha, L., C. Lopes, J. A. Borges, A. C. Duarte and E. Pereira (2013). "Valuation of Unmodified Rice Husk Waste as an Eco-Friendly Sorbent to Remove Mercury: a Study Using Environmental Realistic Concentrations." Water, Air, & Soil Pollution 224(7): 1-18.

Romera, E., F. Gonzalez, A. Ballester, M. L. Blazquez and J. A. Munoz (2007). "Comparative study of biosorption of heavy metals using different types of algae." Bioresource Technology 98(17): 3344-3353.

Romero-Gonzalez, M. E., C. J. Williams and P. H. E. Gardiner (2000). "The application of dealginated seaweed as a cation exchanger foron-line preconcentration and chemical speciation of trace metals." Journal of Analytical Atomic Spectrometry 15(8): 1009-1013.

Sheng, P. X., Y.-P. Ting, J. P. Chen and L. Hong (2004). "Sorption of lead, copper, cadmium, zinc, and nickel by marine algal biomass: characterization of biosorptive capacity and investigation of mechanisms." Journal of Colloid and Interface Science 275(1): 131-141.

Srivastava, S. and P. Goyal (2010). Novel Biomaterials: Decontamination of Toxic Metals from Wastewater, Springer

Sunda, W. G. and S. A. Huntsman (1998). "Processes regulating cellular metal accumulation and physiological effects: Phytoplankton as model systems." Science of The Total Environment 219(2–3): 165-181.

Torres, M. A., M. P. Barros, S. C. G. Campos, E. Pinto, S. Rajamani, R. T. Sayre and P. Colepicolo (2008). "Biochemical biomarkers in algae and marine pollution: A review." Ecotoxicology and Environmental Safety 71(1): 1-15.

Tuzen, M. and A. Sari (2009). "Removal of mercury(II) from aqueous solution using moss (Drepanocladus revolvens) biomass: Equilibrium, thermodynamic and kinetic studies." Journal of Hazardous Materials 171(1-3): 500-507.

Varma, R., A. Turner and M. T. Brown (2011). "Bioaccumulation of metals by Fucus ceranoides in estuaries of South West England." Marine Pollution Bulletin 62(11): 2557-2562.

Vasconcelos, M. T. S. D. and M. F. C. Leal (2001). "Seasonal variability in the kinetics of Cu, Pb, Cd and Hg accumulation by macroalgae." Marine Chemistry 74(1): 65-85.

Velasquez, L. and J. Dussan (2009). "Biosorption and bioaccumulation of heavy metals on dead and living biomass of Bacillus sphaericus." Journal of Hazardous Materials 167(1-3): 713-716.

Vidotti, E. C., & Rollemberg, M. C. (2004). "Algas: Da economia nos ambientes aquáticos à bioremediação e à química analítica." Quimica Nova 27: 139–145.

Vijayaraghavan, K., H. Y. N. Winnie and R. Balasubramanian (2011). "Biosorption characteristics of crab shell particles for the removal of manganese(II) and zinc(II) from aqueous solutions." Desalination 266(1–3): 195-200.

Wang, W.-X. and R. C. H. Dei (1999). "Kinetic measurements of metal accumulation in two marine macroalgae." Marine Biology 135(1): 11-23.

Chapter 6

Comparative study on metal biosorption by two macroalgae species in saline waters: mono and multi-metal systems

6 Comparative study on metal biosorption by two macroalgae species in saline waters: mono and multi-metal systems

6.1 Introduction

Biosorption is singled out as the most promising alternative to the traditional physicochemical methods of water treatment (e.g. chemical precipitation, membrane filtration, electrochemical techniques) (Freitas et al. 2008), which present severe disadvantages in removing metals from aqueous solutions (Lo et al. 2014). Formation of toxic by-products, high generation of sludge requiring a proper disposal, and high initial and running costs, are some of the weaknesses that jeopardize the viability of such conventional methods (Farooq et al. 2010). Additionally, they do not allow compliance with the increasingly strict criteria imposed by environmental legislation in the field of water (Chojnacka 2010). For example, emissions, discharges and losses of Cd and Hg were recently prohibited by the European Union, who imposed concentration levels of these metals in surface waters in the order of few µg L^{-1} (Directive 2013/39/EU 2013). Lead is also a metal with special relevance from the environmental point of view, owning very tight limits of concentration in the water resources (Directive 2013/39/EU 2013). The concern of governmental entities toward these elements relates to their temporal persistence, toxicity, and ease of exposition, as they may be easily bioaccumulated and bioamplified along the food chain (Coelho et al. 2005).

As biosorption relies on the use of abundant biological material, which features cell walls rich in functional groups with high affinity to metal ions (Davis et al. 2003), this technology offers remarkable removal efficiencies, being environmentally friendly and cost-effective (Farooq et al. 2010).

A growing number of scientific studies, carried out in recent years, has shown that dried algae possess a high capacity to bind metals (Romera et al. 2006, Mata et al. 2008), presenting recoveries higher than those achieved by activated carbon and natural zeolites, and comparable to those obtained with synthetic ion exchange resins (El-Sikaily et al. 2007, Romera et al. 2007). Some authors even stated that algal cells are able to concentrate metals 2–3 orders of magnitude as compared to other organisms (Chojnacka and Mikulewicz 2014).

Some studies have tackled the enhancement of the biosorptive capacity of macroalgae biomass, through chemical pretreatments (Bulgariu and Bulgariu 2014), however those modifications on cell surface will not be profitable at large scale (Park et al. 2010).

The affinity toward specific metals, and consequently removal efficiencies are related to the chemical state of the algal surface, amount and diversity of existent functional groups (Davis et al. 2003). Thus is important to explore and investigate different species of algae, seeking to better understand the mechanisms behind the sorption process and selectivity, which are still poorly understood (Park et al. 2010). As metal speciation in solution and ionic competition also play a fundamental role in biosorption, research should go ahead of the usual idealistic approach (using a single contaminant and synthetic water) and focus on the simultaneous removal of metals (Pagnanelli et al. 2002) by using complex systems mimicking real waters and wastewaters. Additionally, studies should be performed for metal concentrations with environmental relevance (Rocha et al. 2013, Lopes et al. 2014), with values lower than those extremely high often found in the literature, which do not allow the findings to be translated into the real world.

The development of treatment technologies to be applied for saline waters, although very desirable, since estuaries and coastal environments are the main receiver of industrial effluents (Torres et al. 2008), has been neglected to date. So research should also point to that path.

Ulva lactuca (Chlorophyta) and *Fucus vesiculosus* (Phaeophyta) are two marine macroalgae, respectively green and brown species, very common worldwide. Besides their abundance, these macroalgae possess several specific characteristics very attractive for biosorption application. *Ulva* structure is relatively simple, with a thin and sheet-like thallus, which translates into a large surface area, containing structurally uniform and physiologically cells (Sari and Tuzen 2008). The cell wall is constituted by cellulose along with a high percentage of proteins bonded to polysaccharides (Romera et al. 2007, Trinelli et al. 2013), containing several functional groups such as amino, hydroxyl, carboxyl, and sulfate, capable of acting as binding sites for metal (Romera et al. 2007). Additionally, this algae grows quickly (Nikolaisen and Jensen 2013), since their photosynthetic products are quickly converted to cell growth (Easton et al. 2011).

Although slower growing, seaweeds of genus *Fucus* as biosorbents of metals and dyes have generated a growing interest in the last decades (Freitas et al. 2008, Mata et al. 2008, Mata et al. 2009, Cobas et al. 2014). Some studies have pointed to the superior performance of brown algae in the removal of metals due to their chemical constitution (Davis et al. 2003, Mata et al. 2009). The cell walls of brown macroalgae are characterized by the presence of cellulose, acting as structural support, sulphated polysaccharides and alginic acid, a polymer of mannuronic and guluronic acids (Lesmana et al. 2009), which have two adjacent carboxylic groups just at the right distance for metal bonding (Romera et al. 2007).

The main objective of this work was to assess and compare the biosorption capabilitiy of two very abundant macroalgae, in respect of three metals: Cd, Hg and Pb, using non-treated dried biomass and environmentally relevant concentrations of these metals in seawater (single and multi-contaminant solutions). Ultimately, the approach followed aimed to evaluate the feasibility of using these macroalgae as a low-cost treatment method, to be applied to effluents rich in salts, or in contaminated estuarine environments.

6.2 Materials and methods

6.2.1 Material and chemicals

All chemicals were of analytical reagent grade and were used as received from the suppliers. The nitric acid 65% (suprapur) and the standard stock solutions of mercury (1001 ± 2mgL^{-1}), cadmium (1000 ± 2mgL^{-1}) and lead (1000 ± 2mgL^{-1}) nitrate were purchased from Merck. All working solutions, including standards for the calibration curves, were obtained by diluting the corresponding stock solution. All glassware material used in the experiments was acid-washed prior to use.

6.2.2 Biosorbent material

Green marine macroalga *Ulva lactuca* and brown marine macroalgae *Fucus vesiculosus* used in this work were collected in the Mondego estuary (Figueira da Foz, Portugal, 40°08′N, 8°50′W). The algae biomass was used without any specific pre-treatment. Algae were simply washed with distilled water, air-dried during several days, dried at 60°C for 24h and milled using a domestic coffee grinder. The milled material was sieved using a mechanical sieve shaker and only particles with size <200 µm were used. Algae biomass was stored at room temperature in plastic containers until further use. Total Hg concentration in both macroalgae was evaluated by pyrolysis atomic absorption spectrometry with gold amalgamation (LECO® model AMA-254) with no sample digestion. For Cd and Pb content, macroalgae were analysed by inductively coupled plasma spectroscopy (Jobin – Yvon JY70 Plus Spectrometer), after digestion following the procedure described below. Results indicate that both brown and green macroalgae have a low metal content (0.040 µg g^{-1} and 0.030 µg g^{-1}, respectively for Hg, 0.015 µg g^{-1} and 0.29 µg g^{-1}, respectively for Cd, and 0.30 µg g^{-1} and 4.1 µg g^{-1}, respectively for Pb).

6.2.3 Seawater sampling and characterization

Seawater used for biosorption experiments was collected at Vagueira beach (Aveiro, Portugal) using polyethylene bottles that were rinsed with surface water before the filling. Seawater was then filtered through 0.45 µm Millipore membrane and stored in the dark at 4°C until further use. A brief characterization of the seawater which included pH, conductivity, salinity and major and minor elements was performed. The pH (7.9), conductivity (54.3 mS cm^{-1}) and salinity (35 g L^{-1}) were recorded on a WTW meter and the concentration of major and minor elements was obtained by inductively coupled plasma spectroscopy, using a Jobin – Yvon JY70 Plus Spectrometer. All the measured parameters were in line with those reported by Lopes et al. (2014), which are in ranges to be considered non-polluted waters. The concentration of Cd and Pb were below the detection limit of the quantification method. Hg concentration was determined using the methodology described by Pato et al. (2010) and the value found (3.1 ng L^{-1}) is typical of a non-polluted water.

6.2.4 Batch biosorption studies

Batch sorption kinetic experiments were performed at room temperature (20±2 °C) by contacting 500 mg of macroalgae dried biomass (particle size < 200 µm) with a known concentration of metal solution. Schott Duran® glass flasks (1 L) were used as reaction vessels, where the macroalgae biomass was placed in contact with natural seawater spiked with metal, under constant stirring (800 rpm).

Biosorption experiments were conducted for single and multi-contaminants systems, simulating different contaminations scenarios. A summary of the experimental concentrations of metal used is presented in Table 6.1. Concentrations were considered environmentally realistic, intending to mimic real cases: 50, 200 and 1000 µg L^{-1} are, respectively, the “old” limits for Hg, Cd and Pb wastewater discharges (Directive

83/513/EEC 1983, Directive 84/156/EEC 1984, Decree-Law No. 236/98 1998). The remaining concentrations may represent a situation of accidental discharge of untreated effluent, or metal concentrations in aquatic medium after dilution of the discharged effluent into the water body.

Table 6.1: Experimental conditions used in the biosorption studies.

Matrix	System		Metal	Concentration (µg L^{-1})
Spiked natural seawater	Single-contaminant		Cd	10, 50, 100 and 200
			Pb	50, 100, 200 and 1000
			Hg	10, 50 and 100
	Multi-contaminant	M1	Cd	50
			Pb	50
			Hg	50
		M2	Cd	200
			Pb	1000
			Hg	50

All experiments were carried out at the intrinsic pH of seawater (7.9) and no pH adjustments were made at any time. All the experiments were performed in duplicate and the results are always expressed as the mean value obtained. Controls, defined as metal spiked seawater in the absence of macroalgae biomass, were always run together with all experiments, in order to assess the loss of metals to glass vessels or in the samples filtration process. Metals solutions were prepared through the adequate dilution of specific standard stock solution to the desired concentration in seawater. Before the kinetic batch sorption begin an aliquot (*ca.* 10 mL) of each solution was collected to confirm the real initial concentration of metals. After adding macroalgae to the solution, samples (*ca.* 10 mL) were collected at crescent periods of time, filtered through a pre-acid washed 0.45 µm Millipore membrane and then acidified to pH<2 with nitric acid. Afterwards, samples were stored at 4 °C until being analysed.

6.2.5 Metals quantification

Mercury analysis was performed by cold vapour atomic fluorescence spectroscopy (CV-AFS), on a PSA cold vapour generator, model 10.023, using $SnCl_2$ as reducing agent. The Hg concentration was quantified through a calibration curve of five standards prepared in a nitric acid solution (2% v/v), by dilution from the certified standard solution of mercury(II) nitrate, whose concentrations ranged from 0.0 to 0.5 µg L^{-1}. In this range, the limit of detection of the method is 0.02 µg L^{-1}, and the precision and accuracy are <5%.

The quantification of Cd and Pb was performed by inductively coupled plasma mass spectrometry (ICP-MS), on a Thermo ICP-MS X Series equipped with a Burgener nebulizer. The calibration curve for Cd and Pb quantification was obtained using standards (0.1 – 50 µg L^{-1}) prepared by dilution of the certified standard solution of cadmium nitrate or lead nitrate in a nitric acid solution (2% v/v). Due to the high salinity of samples, they were diluted 20-fold prior to measurement in ICP-MS, in order to avoid matrix interferences. Quantification limits of the method (before dilution) were 0.1 µg L^{-1} and 0.2 µg L^{-1}, for Cd and Pb respectively, with a precision and accuracy <10 %.

6.2.6 Data evaluation

6.2.7 *Metal removal efficiency*

Metal uptake by algae, defined as the amount of metal bound by unit of mass at a given time t (q_t, µg g^{-1}), was deduced from the mass balance between the initial metal concentration in the solution (C_0, µg L^{-1}) and the concentration after a particular period of contact time t (C_t, µg L^{-1}):

$$q_t = \frac{(C_0 - C_t)\mathrm{V}}{m} \quad (1)$$

where V (L) is the volume of the solution and m (g) is the dry weight of alga biomass. When the equilibrium is attained, $t=t_e$, $q_t=q_e$ and $C_t=C_e$ (residual metal concentration in solution). The performance of the removal process was also evaluated using the metal removal percentage (R, %), which at time t is defined by:

$$R_t(\%) = \frac{(C_0 - C_t)}{C_0} \times 100 \quad (2)$$

6.2.8 *Sorption kinetic models*

Kinetic modelling may provide a better understanding on the interactions between the sorbent and the sorbate, giving detailed information on the rate at which these interactions occur. Hence, it is an important characteristic in assessing how rates are affected by sorption capacity or by the character of the sorbent, i.e., in comparing the efficiency of a sorption process (Ho et al. 2000, Park et al. 2010).

In this work, the sorption kinetics was studied using three different kinetic models, namely the pseudo-first-order model (or Lagrergren model), the pseudo-second-order model (Ho model) and the Elovich model (Table 6.2).

Table 6.2: Sorption kinetic models.

Kinetic model	Equation	Notes
Pseudo-first-order (Lagergren)	$q_t = q_e(1-e^{-k_1 t})$	Adsorption capacity
Pseudo-second-order (Ho)	$q_t = \frac{q_e^2 k_2 t}{1+q_e k_2 t}$	Adsorption capacity
Elovich	$q_t = \frac{1}{\beta}\ln\ (1+\alpha\beta t)$	Chemisorption

Legend to symbols:

q_t – amount of solute sorbed per gram of sorbent at time t (mg g^{-1});
q_e – amount of solute sorbed per gram of sorbent at equilibrium (mg g^{-1});
k_1 – rate constant of pseudo-first order (h^{-1});
k_2 – rate constant of pseudo-second order (g mg^{-1} h^{-1});
α – initial sorption rate (mg g^{-1} h^{-1});
β – desorption constant (g mg^{-1});
Bt – function of F;

6.2.9 *Error analysis*

All kinetic reaction model parameters were calculated through nonlinear regression using GraphPad Prism 6 software program. The coefficient of determination (R^2) and the standard deviation of residuals ($S_{y.x}$) were analyzed in order to evaluate the goodness of fit and to compare models with equal degrees of freedom. The relative error (E_r) between experimental and predicted values of q_e was also calculated. These statistical parameters can be mathematically defined by:

$$R^2 = 1 - \frac{\sum (y_i - \hat{y})^2}{\sum (y_i - \bar{y})^2} \quad (3)$$

$$S_{y,x} = \sqrt{\frac{(y_i - \hat{y})^2}{df}} \qquad (4)$$

$$E_r = \frac{|y_i - \hat{y}|}{y_i} \times 100 \qquad (5)$$

where y_i are the experimental data values, $\hat{y}$ are the predicted values, $\bar{y}$ is the mean of the experimental data and *df* is the number of the degrees of freedom (equal to number of data points minus the number of parameters fit).

In order to compare the goodness of the different models applied, the Akaike's Information Criterion (AIC) (El-Khaiary and Malash 2011) was used. AIC is based on information theory and maximum likelihood theory, and as such, it determines which model is more likely to be correct and quantifies how much more likely. For small sample size, AIC is calculated from the equation:

$$AIC = N \ln\left(\frac{SSE}{N}\right) + 2N_p + \frac{2N_p\left(N_p + 1\right)}{N - N_p - 1} \qquad (6)$$

where SSE is the sum-of-squared deviations, N is the number of data points and N_p is the number of parameters in the model.

AIC values can be compared using Evidence ratio (numerical value representative of the number of times that the model with a lower AIC is more likely to be correct) which is defined by:

$$\text{Evidence ratio} = \frac{1}{e^{-0.5\Delta}} \qquad (7)$$

where Δ is the absolute value of the difference in AIC between the two compared models.

6.3 Results and discussion

6.3.1 Sorption kinetics of Hg, Pb and Cd in single-contaminant systems

The biosorption capability of green and brown macroalgae powders toward the metals Hg, Pb and Cd was evaluated in monometallic solutions, for several contamination scenarios.

Figure 6.1 shows the kinetic curves corresponding to the biosorption of Hg onto green and brown macroalgae, expressed as normalized concentration *vs* time, *i.e.*, C_t/C_0 *vs* t for the three levels of contamination (C_0 = 10, 50 and 100 µg L^{-1}).

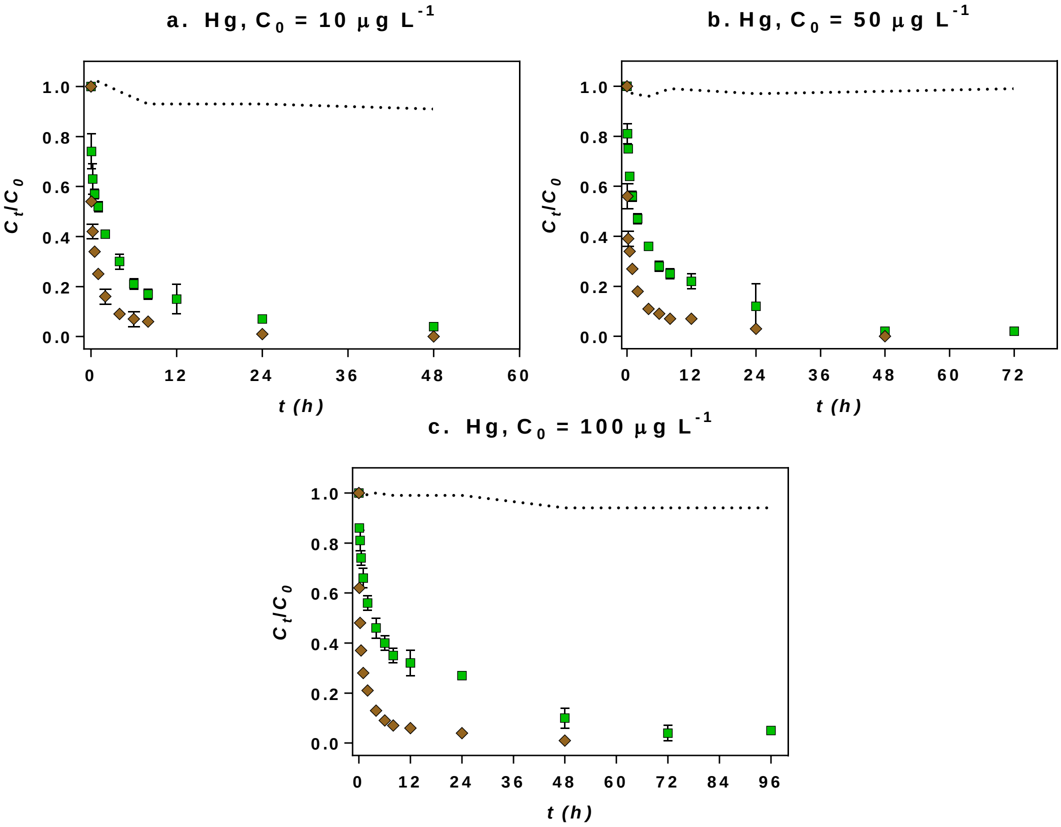


Figure 6.1: Normalized concentrations (C_t/C_0) of Hg in solution with time: green squares – sample essay: Hg solution + *U. lactuca*; brown diamonds – sample essay: Hg solution + *F. vesiculosus*; dash line – control essay: Hg solution.

The results clearly show that both macroalgae are very effective at sorbing Hg from solution. Indeed, the green macroalgae was able to decrease the concentration of Hg in solution to values lower than the guideline value for drinking water quality (1 µg L^{-1}) (Council Directive 98/83/EC 1998), for two contamination levels (10 and 50 µg L^{-1}), while the brown one was able to reach that value for all contamination scenarios studied. Independently of the type of algae, the kinetic profile show that the uptake is very fast at the beginning of the process and then slows down with the attainment of equilibrium. This is explained by the high driving forces to mass transfer that prevailed at the beginning since the macroalgae were initially free of metal.

Independently of the initial Hg concentration, the sorption of this metal by the brown alga was always faster than by the green one, as can be observed by the initial slope of the curves (Figure 6.1), and confirmed by the initial sorption rates estimated from the first derivative of C=f(t) at t=0 (ν_B and ν_G, where the subscript letters denote the algae, respectively brown and green) and displayed in Table 6.3. The higher initial sorption rates for the systems Hg:brown algae helped Hg to approach more quickly the equilibrium in these systems, which was most notable at the highest concentration. However both macroalgae presented similar equilibrium uptakes of Hg (Table 6.3).

The increase of the initial metal concentration provides an important driving force to overcome all mass transfer resistance of Hg between the liquid and solid phases. Consequently, the rise of C_0 from 10 to 100 µg L^{-1} increased not only the initial biosorption rate but also the amount of Hg biosorbed by the macroalgae from *ca.* 0.1 µmol g^{-1} to approximately 1 µmol g^{-1}, as observed in Table 6.3. Nevertheless, the increase of the contamination level also resulted in the necessity of having a higher contact time to reach equilibrium. For the brown macroalgae the equilibrium time change from 24 to 48 hours, while for the green algae the equilibrium was set after 24 hours for the lowest contamination scenario and after 72 hours for the highest one. In brief, it was also observed that the relative difference between the initial sorption rates for both algae increased with the level of contamination.

Moreover, it was recorded that in the case of seawater contamination with Hg, both green and brown macroalgae have shown good capacity to sorb the metal, achieving removal efficiencies between 96 and 99% (Table 6.3). Furthermore, the removal of Hg from spiked seawater can only be related to its interaction with the algae since the corresponding controls did not show such behavior. The efficiency of *U. lactuca* as biosorbent for Hg removal was already demonstrated by Zeroual et al. (2003) (removal >90%), although experiments have been conducted in distilled water, and the algal biomass has been pre-treated with 0.3 M H_2SO_4. More recently, Vijayaraghavan and Joshi (2012) also investigated the interaction of Hg ions with brown (*Sargassum* sp. and *Turbinaria conoides*) and green (*Ulva* sp.) macroalgae biomasses, reporting removal efficiencies up to 58%. However, contrary to the findings of the present study, no significant differences were observed between the rates of Hg biosorption by the three seaweed, and higher equilibrium uptakes were recorded for brown algae (Vijayaraghavan and Joshi 2012).

Table 6.3: Estimated initial sorption rates (v_0, µg L^{-1} h^{-1}), removal percentages (R, %) and amount of metal sorbed per unit of algae biomass (q_t, µmol g^{-1}), at different times (8h and at equilibrium), for monometallic mercury solutions.

	Brown algae *F. vesiculosus*				**Green algae *U. lactuca***			
C_0 (µg L^{-1})	v_0 (µg L^{-1} h^{-1})	t (h)	R (%)	q_t (µmol g^{-1})	v_0 (µg L^{-1} h^{-1})	t (h)	R (%)	q_t (µmol g^{-1})
10	85	8	94	0.108	53	8	83	0.096
		eq(24h)	99	0.114		eq(24h)	93	0.107
50	383	8	93	0.508	177	8	75	0.400
		eq(48h)	99	0.545		eq(48h)	98	0.527
100	674	8	93	1.052	281	8	65	0.716
		eq(48h)	99	1.120		eq(72h)	96	1.060

The experimental amount of Hg biosorbed by both macroalgae with time, q_t *vs t*, for different contamination levels are shown in Figure 6.2, together with the fittings of the pseudo-first- order (PFO), pseudo-second-order (PSO) and Elovich models.

In general, the data set obtained with green macroalgae show a better agreement with the fittings accomplished by the models adopted in this work than the data set obtained with the brown algae. Moreover, for both macroalgae the poorest fitting was always the one obtained with the PFO model, while the best was the one obtained with the Elovich model (Table 6.4). Comparison of the fits goodness by the Akaike's Information Criterion (AIC) (El-Khaiary and Malash 2011), indicates that the Elovich model is likely to be by far the most appropriated model to describe the biosorption kinetics of Hg onto *U. lactuca*, (probability always higher than 99.99%), while in the case of *F. vesiculosus*, the probability of the Elovich to be better than the PSO model is 78.5, 99.4 and 85.9%, respectively for C_0 of 10, 50 and 100 µg L^{-1} (Table 6.4). This fact may suggest the Hg biosorption mechanism may be slightly different from macroalgae to macroalgae. Additionally, the estimated kinetic parameters of the models corroborate that the Hg biosorption was much faster onto *F. vesiculosus* than onto *U. lactuca* (Table 6.4).

Moreover, for almost all systems studied, the Elovich model was able to fit the data in the transition between the steep ascendant part of each curve and the horizontal branch, where most of the times the kinetic profile is usually difficult to fit or simulate, and both pseudo-first- and pseudo-second-order models were not able to accurately estimate the equilibrium q_e values, underestimating them.

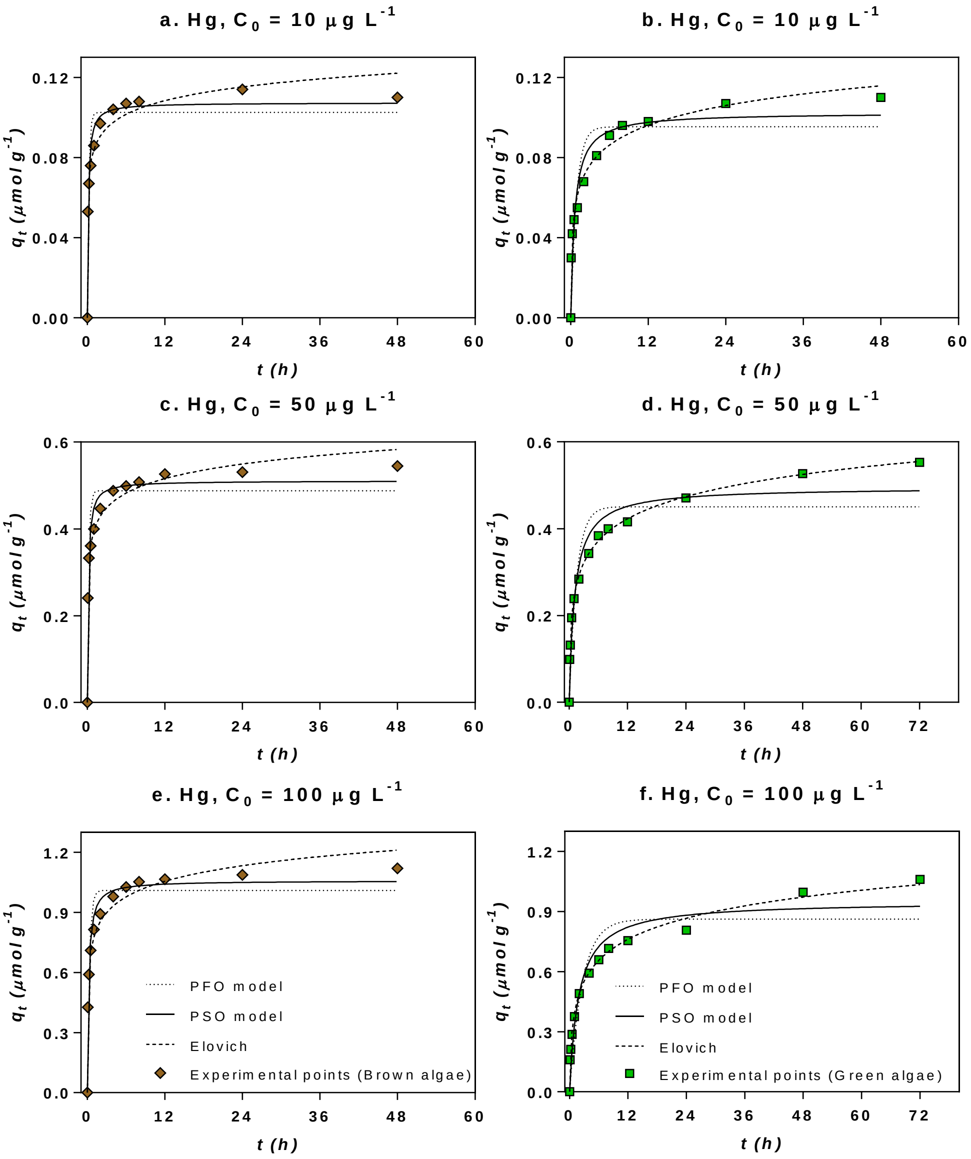


Figure 6.2: Experimental and modelling results for the amount of Hg biosorbed by the macroalgae *F. vesiculosus* (brown diamonds) and by *U. lactuca* (green squares). (Error bars were omitted for clarity).

Table 6.4: Best-fit parameters (± standard error) and goodness of the fittings of the kinetic models used to describe the biosorption kinetics of Hg by *F. vesiculosus* and *U. lactuca* macroalgae.

C_0 (µg L^{-1})	PFO	PSO	Elovich	Preferred model*
		F. vesiculosus		
10	**k_1**: 4.565±1.134	**k_2**: 69.17±11.49	**α**: 49.09±42.29	***Elovich***
	q_e: 0.1025±0.004441	**q_e**: 0.1073±0.002907	**β**: 101.4±10.19	
	R^2: 0.893	**R^2**: 0.965	**R^2**: 0.972	*Probability*: 78.5%
	$S_{y/x}$: 0.01175	**$S_{y/x}$**: 0.006734	**$S_{y/x}$**: 0.005987	

50	**k_1:** 4.675±1.131	**k_2:** 14.21±2.869	**α:** 174.4±101.5	***Elovich***
	q_e: 0.4879±0.01931	**q_e:** 0.5106±0.01272	**β:** 20.70±1.434	*Probability*: 99.4%
	R^2: 0.892	**R^2:** 0.964	**R^2:** 0.984	
	$S_{y/x}$: 0.05474	**$S_{y/x}$:** 0.03175	**$S_{y/x}$:** 0.02087	
100	**k_1:** 3.188±0.6730	**k_2:** 4.773±0.8272	**α:** 114.0±59.68	***Elovich***
	q_e: 1.009±0.03847	**q_e:** 1.058±0.02460	**β:** 8.919±0.6498	*Probability*: 85.9%
	R^2: 0.911	**R^2:** 0.972	**R^2:** 0.979	
	$S_{y/x}$: 0.1054	**$S_{y/x}$:** 0.05872	**$S_{y/x}$:** 0.05051	
U. lactuca				
10	**k_1:** 1.129±0.3030	**k_2:** 16.44±4.573	**α:** 1.013±0.2365	***Elovich***
	q_e: 0.09536±0.005507	**q_e:** 0.1024±0.004701	**β:** 70.25±3.429	*Probability*: >99.99%
	R^2: 0.859	**R^2:** 0.933	**R^2:** 0.989	
	$S_{y/x}$: 0.01351	**$S_{y/x}$:** 0.009301	**$S_{y/x}$:** 0.003864	
50	**k_1:** 0.6596±0.1799	**k_2:** 1.742±0.4612	**α:** 1.880±0.1713	***Elovich***
	q_e: 0.4505±0.02662	**q_e:** 0.4950±0.02280	**β:** 13.54±0.2893	*Probability*: >99.99%
	R^2: 0.867	**R^2:** 0.941	**R^2:** 0.998	
	$S_{y/x}$: 0.06492	**$S_{y/x}$:** 0.04334	**$S_{y/x}$:** 0.008823	
100	**k_1:** 0.3822±0.1037	**k_2:** 0.5656±0.1590	**α:** 1.840±0.2580	***Elovich***
	q_e: 0.8617±0.05800	**q_e:** 0.9495±0.05065	**β:** 6.545±0.2463	*Probability*: >99.99%
	R^2: 0.870	**R^2:** 0.939	**R^2:** 0.994	
	$S_{y/x}$: 0.1231	**$S_{y/x}$:** 0.08463	**$S_{y/x}$:** 0.02827	

*Comparison of the two fits with the highest R^2 and lower $S_{y/x}$ values. Probability reported by Graphad6® after model comparison using Akaike's Information Criterion.

The sorption curves of Pb at four contamination scenarios (C_0 = 50, 100, 200 and 1000 µg L^{-1}) onto *F. vesiculosus* and *U. lactuca* algae are shown in Figure 6.3, in terms of normalized concentration *vs* time, *i.e.* C_t/C_0 *vs* t, and are characterized by a strong and quick decrease of C_t/C_0 values with time. This decrease can only be related to the presence of the macroalgae since the controls remained constant over time.

In the case of Pb contamination, both macroalgae were able to decrease Pb concentration in seawater, but the removal was not complete for any of the considered levels of contamination. For all systems, the brown macroalgae always showed a better performance than the green one, reflected in higher removal percentages and in higher initial sorption rates (Table 6.5). Contrarily to Hg, the higher initial sorption rates observed for the systems Pb:brown macroalgae did not led to a faster equilibrium, since steady-state was attained at same time (8 hours) for the two macroalgae.

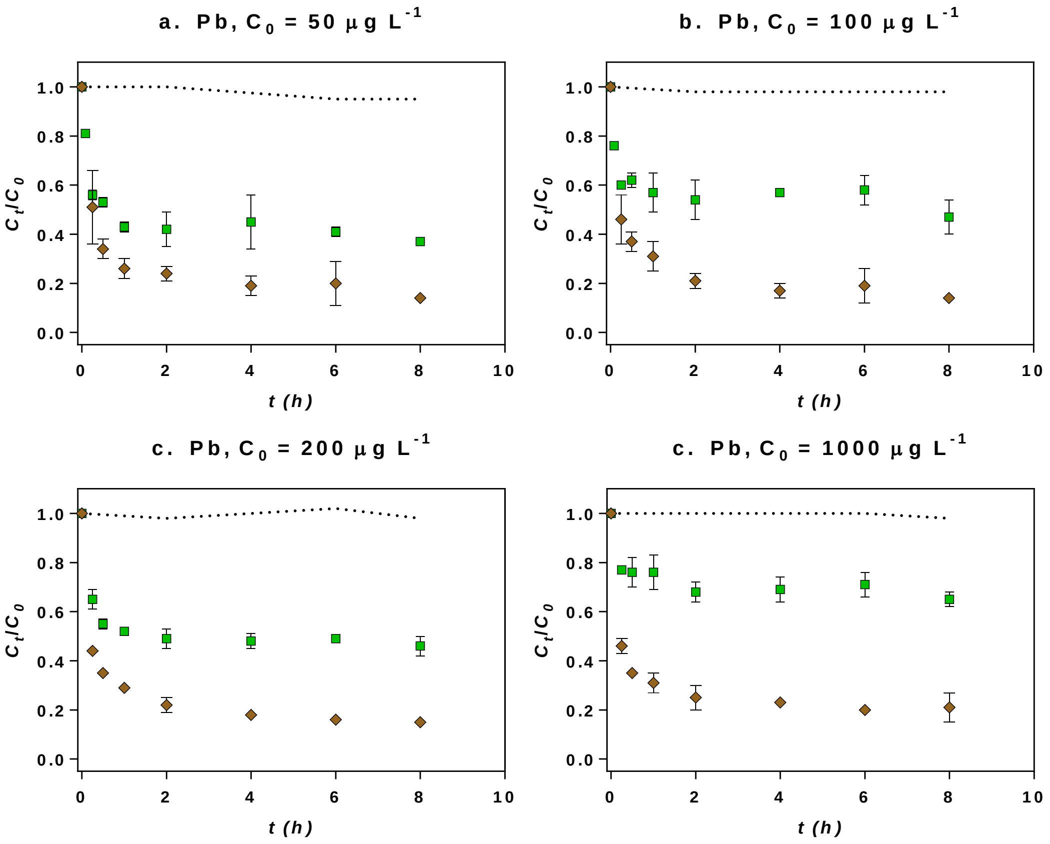


Figure 6.3: Normalized concentrations (C_t/C_0) of Pb in solution with time: green squares – sample essay: Pb solution + *U. lactuca*; brown diamonds – sample essay: Pb solution + *F. vesiculosus*; dash line – control essay: Pb solution.

The rise of the contamination level from 50 to 1000 µg L^{-1} resulted in a strong increase on the initial removal rates from *ca*. 100 µg L^{-1} h^{-1} to 3200 µg L^{-1} h^{-1} in the case of brown macroalgae or to 1490 µg L^{-1} h^{-1} in the case of the green macroalgae (Table 6.5). Also, the amount of Pb biosorbed by the macroalgae increased considerably with the rise of the initial metal concentration (around 23 folds for the brown macroalgae and 10 folds for the green one) (Table 6.5), without reaching a constant value, which indicates that both algae were not saturated in the tested conditions. On the other hand, it was noted a slight decrease on the removal efficiency for the system Pb:brown algae (86 to 79%) and a strong decrease for the system Pb:green algae (63 to 32%), which clearly highlights the higher sorption capacity and affinity of the brown macroalgae toward Pb than the green macroalgae. Moreover, for the lowest contamination level (50 µg L^{-1}), the brown alga was able to reduce the lead concentration in water to values *ca*. 5.6 µg L^{-1}, which is below the Portuguese environmental quality standard for surface waters (7.2 µg L^{-1}) (Decree-Law No. 103/2010 2010) and about a half of the guideline value for drinking water quality (10 µg L^{-1}) (Council Directive 98/83/EC 1998). Furthermore, for the contamination scenario of 100 µg L^{-1} the residual concentration obtained (11 µg L^{-1}) stayed much closed to those guideline values. The suitability of using brown algae (*Laminaria hyperborean*, *Bifurcaria bifurcate*, *Sargassum muticum* and *Fucus spiralis*) for the removal of Pb from diluted wastewaters streams was already demonstrated by Freitas et al. (2008). In their comparative study of biosorption of metals by different algae, Romera et al. (2007) also achieved lower concentrations of Pb in solution using brown algae (*Fucus spiralis*). The superior performance of brown seaweeds over green ones in the uptake of Pb was justified by the presence of alginates in their compositon (Romera et al. 2006). Indeed, alginic acids, which comprise different proportions of mannuronic and guluronic acid, are rich in carboxyl groups with high affinity to divalent metals (Davis et al. 2003).

Table 6.5: Estimated initial sorption rates (v_0, µg L^{-1} h^{-1}), removal percentages (R, %) and amount of metal sorbed per unit of algae biomass (q_t, µmol g^{-1}), at different times (2h and at equilibrium), for monometallic lead solutions.

	Brown algae				**Green algae**			
C_0 (µg L^{-1})	v_0 (µg L^{-1} h^{-1})	t (h)	R (%)	q_t (µmol g^{-1})	v_0 (µg L^{-1} h^{-1})	t (h)	R (%)	q_t (µmol g^{-1})
50	112	2	76	0.292	109	2	58	0.238
		eq(8h)	86	0.331		eq(8h)	63	0.260
100	289	2	79	0.637	214	2	46	0.385
		eq(8h)	86	0.696		eq(8h)	53	0.443
200	600	2	78	1.242	385	2	52	0.895
		eq(8h)	85	1.357		eq(8h)	54	0.934
1000	3200	2	75	6.744	1490	2	32	2.985
		eq(8h)	79	7.148		eq(8h)	32	2.985

The modelling (PFO, PSO and Elovich models) of the amount of Pb biosorbed by *F. vesiculosus* and *U. lactuca* with time and the q_t values obtained experimentally, for all contamination levels studied are shown in Figure 6.4.

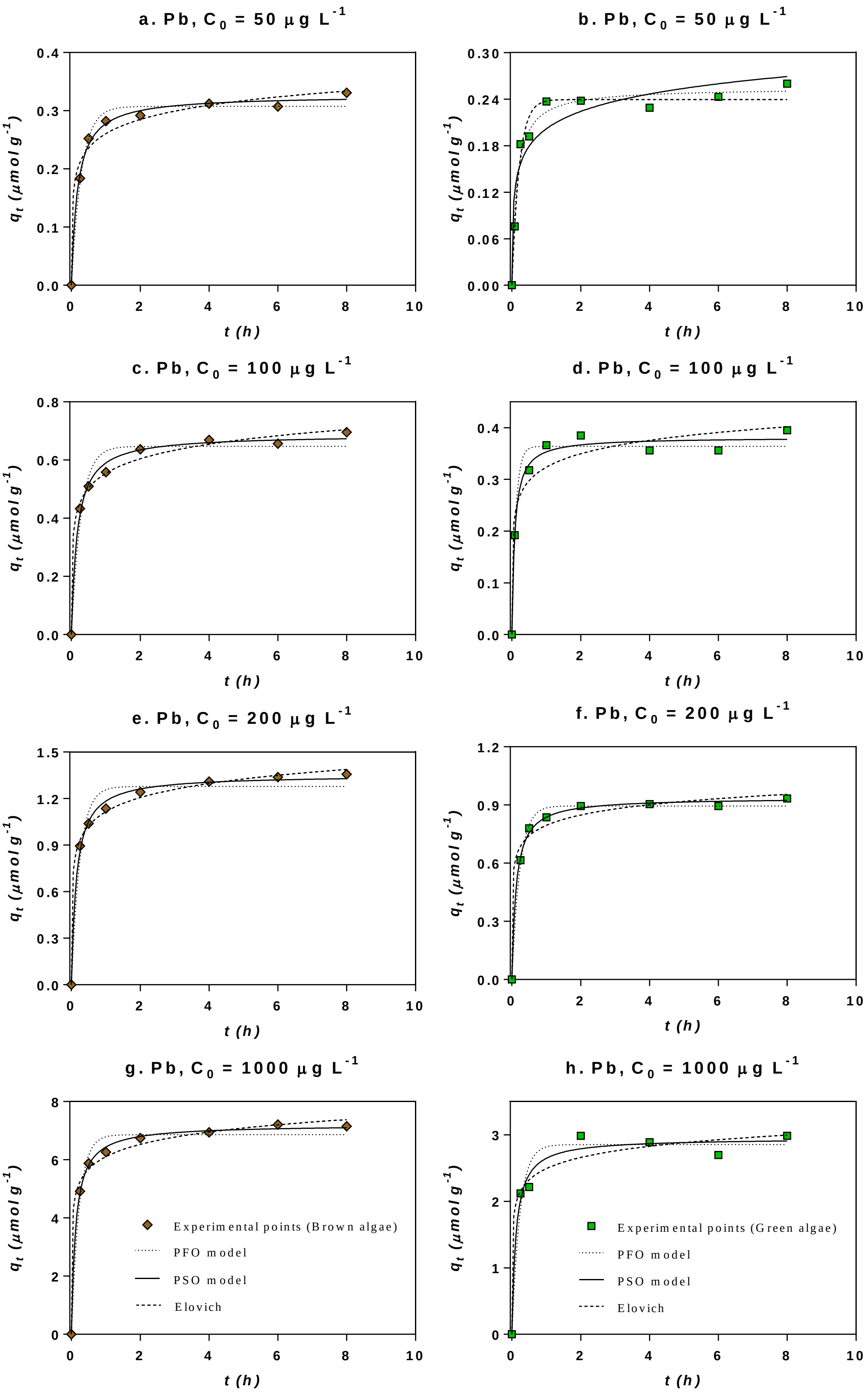


Figure 6.4: Experimental and modelling results for the amount of Pb biosorbed by the algae *F. vesiculosus* (brown diamonds) and by *U. lactuca* (green squares). (Error bars were omitted for clarity).

In the case of Pb biosorption, the data set obtained with both macroalgae show, in most of the cases, a better agreement with the fitting accomplished by the pseudo-second-order model. Moreover, according with the Akaike's Information Criterion (AIC) (El-Khaiary and Malash 2011) this model is likely to be the most appropriated model to describe the biosorption kinetics of Pb onto *U. lactuca* for all contamination scenarios, while in the case of *F. vesiculosus* for C_0 of 200 µg L^{-1} the Elovich model has a probability slightly higher (58.9%) than the PSO model (Table 6.6). Besides, the PSO model was able to estimate accurately the equilibrium values of the amount of Pb biosorbed by both macroalgae. For the brown macroalgae the relative error ranged from 0.3 to 10% and for the green algae ranged from 0.3 to 14%.

Table 6.6: Best-fit parameters (± standard error) and goodness of the fittings of the kinetic models used to describe the biosorption kinetics of Pb by *F. vesiculosus* and *U. lactuca* algae.

$C_{A,0}$ (µg L^{-1})	PFO	PSO	Elovich	Preferred model*
		F. vesiculosus		
50	k_1: 3.466±0.3556	k_2: 17.43±2.123	a: 57.05±62.66	***PSO*** *Probability*: 96.1%
	q_e: 0.3074±0.006313	q_e: 0.3265±0.005410	**β**: 28.37±4.418	
	R^2: 0.987	R^2: 0.994	R^2: 0.978	
	$S_{y/x}$: 0.01348	$S_{y/x}$: 0.009038	$S_{y/x}$: 0.01744	
100	k_1: 3.661±0.5877	k_2: 8.843±1.102	a: 153.1±101.7	***PSO*** *Probability*: 67.2%
	q_e: 0.6468±0.02028	q_e: 0.6869±0.01132	**β**: 13.82±1.254	
	R^2: 0.969	R^2: 0.994	R^2: 0.993	
	$S_{y/x}$: 0.04365	$S_{y/x}$: 0.01909	$S_{y/x}$: 0.02088	
200	k_1: 4.098±0.6315	k_2: 5.205±0.5285	a: 658.0±359.1	***Elovich*** *Probability*: 58.9%
	q_e: 1.278±0.03640	q_e: 1.352±0.01693	**β**: 7.640±0.5105	
	R^2: 0.973	R^2: 0.996	R^2: 0.997	
	$S_{y/x}$: 0.07950	$S_{y/x}$: 0.02916	$S_{y/x}$: 0.02788	
1000	k_1: 4.557±0.5632	k_2: 1.161±0.08370	a: 12874±13601	***PSO*** *Probability*: 99.7%
	$q_{A,e}$: 6.861±0.1477	$q_{A,e}$: 7.207±0.05867	**β**: 1.631±0.1798	
	R^2: 0.984	R^2: 0.998	R^2: 0.993	
	$S_{y/x}$: 0.3270	$S_{y/x}$: 0.1032	$S_{y/x}$: 0.2158	
		U. lactuca		
50	k_1: 4.767±0.6397	k_2: 28.35±5.525	a: 16.29±17.96	***PSO*** *Probability*: 61.7%
	q_e: 0.2394±0.006336	q_e: 0.2544±0.007322	**β**: 30.85±6.038	
	R^2: 0.977	R^2: 0.980	R^2: 0.922	
	$S_{y/x}$: 0.01426	$S_{y/x}$: 0.01352	$S_{y/x}$: 0.02641	

100	**k_1:** 8.935±1.692	**k_2:** 32.98±6.300	**a:** 210.4±381.2	***PSO***
	q_e: 0.3640±0.009758	**q_e:** 0.3813±0.008230	**β:** 26.67±5.916	
	R^2: 0.974	**R^2:** 0.987	**R^2:** 0.950	*Probability*: 94.2%
	$S_{y/x}$: 0.02353	**$S_{y/x}$:** 0.01661	**$S_{y/x}$:** 0.03284	
200	**k_1:** 4.415±0.3429	**k_2:** 8.912±0.8067	**a:** 2371±4396	***PSO***
	q_e: 0.8949±0.01234	**q_e:** 0.9371±0.009590	**β:** 13.00±2.420	
	R^2: 0.994	**R^2:** 0.998	**R^2:** 0.982	*Probability*: 97.9%
	$S_{y/x}$: 0.02720	**$S_{y/x}$:** 0.01686	**$S_{y/x}$:** 0.04570	
1000	**k_1:** 4.405±0.8113	**k_2:** 2.970±0.8261	**a:** 6963±17136	***PSO***
	q_e: 2.853±0.1010	**q_e:** 2.949±0.09259	**β:** 4.120±1.015	
	R^2: 0.969	**R^2:** 0.981	**R^2:** 0.974	*Probability*: 75.7%
	$S_{y/x}$: 0.2052	**$S_{y/x}$:** 0.1594	**$S_{y/x}$:** 0.1875	

(cont.) *Comparison of the two fits with the highest R^2 and lower $S_{y/x}$ values.

Comparing the modelling of Hg and Pb biosorption onto the macroalgae it is possible to state that in the case of Hg the Elovich is the better model to describe the sorption kinetics, while in the case of Pb is the pseudo-second-order model. This fact may suggest that the interaction metal-algae may depend of the nature of the metal and/or of the macroalgae (Freitas et al. 2008, Elrefaii et al. 2012).

The sorption of Cd onto green and brown macroalgae was also evaluated in monometallic conditions for four contamination scenarios (10, 50, 100 and 200 µg L^{-1}). The results obtained were very distinct from the ones obtained for Hg and Pb, since under the experimental conditions studied, Cd was barely removed from solution (data not shown). For all contamination scenarios, only a slight decrease of metal concentration in solution was observed in the first minutes, in the case of green macroalgae, while for the brown algae were observed fluctuations on Cd concentration around the initial value, with no significant removal perceived. The sorption efficiencies were always lower than 20% for both macroalgae and for all the considered contamination scenarios. The obtained results allow us to conclude that under the current experimental conditions, neither the green nor the brown macroalgae are effective biosorbents to remove Cd from saltwater.

6.3.2 Sorption kinetics of Hg, Pb and Cd in multi-contaminant systems

The simultaneous sorption of Hg, Pb and Cd onto green and brown macroalgae was evaluated for two contamination scenarios, named as M1 (mixture 1) and M2 (mixture 2). The scenario M1 corresponds to a mixture of all metals in equivalent mass concentrations that equal the old guideline value established in the Directive 84/156/EEC for maximum value allowed for Hg discharges in wastewaters (50 µg L^{-1}). The scenario M2 corresponds to mixtures of metals in concentrations that equal their old allowable limits in wastewater discharges (Hg, 50 µg L^{-1}; Cd, 200 µg L^{-1}; Pb, 1000 µg L^{-1}).

The kinetic curves of the simultaneous biosorption of Hg, Pb and Cd onto brown and green macroalgae for both multi-contamination scenarios are shown in Figure 6.5. The controls experiments were omitted from the graphs for clarity, but results indicate that in the absence of macroalgae, metal concentrations in solution remained constant along time.

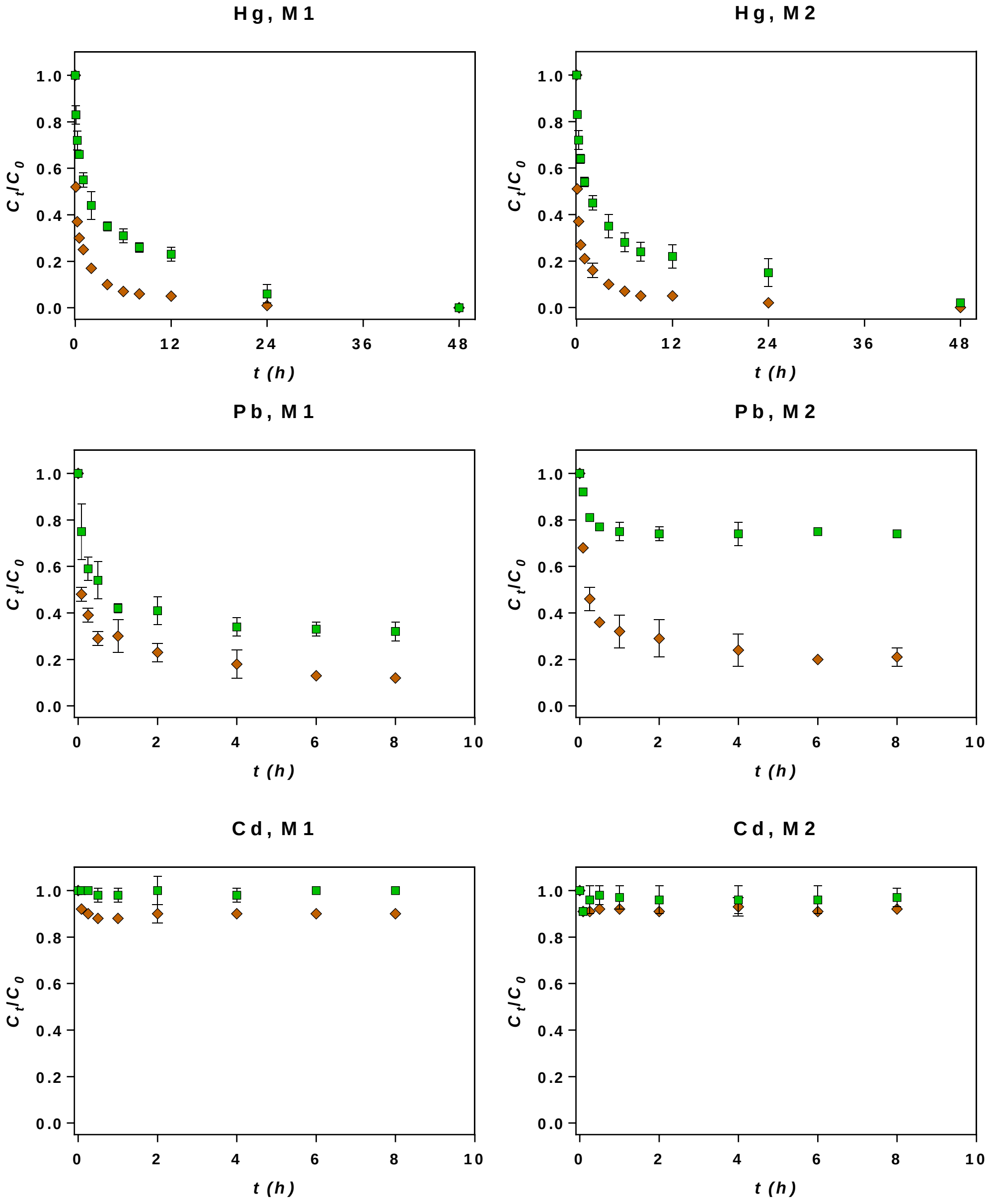


Figure 6.5: Normalized metal concentrations (C_t/C_0) in ternary solutions with time for the contamination scenarios M1 and M2: brown diamonds – brown macroalgae; green squares – green macroalgae.

The simultaneous biosorption of Hg, Pb and Cd with time showed the same pattern observed in the monometallic solutions, i.e. the biosorption of Hg and Pb was initially fast, slowing down until reach equilibrium, while the biosorption of Cd was very low, occurring only in the first minutes. After that, concentrations of Cd in solution remained almost constant.

In the contamination scenario M1, both macroalgae were able to effectively reduce Hg concentration in solution, reaching removal efficiencies up to 99% and residual concentrations lower than the guideline value for drinking water quality (1 µg L^{-1}). However, the biosorption of Hg by the brown macroalgae was much faster than onto the green one, as observed by the initial slope of the kinetic curves and corroborated by the estimated initial sorption rates (brown algae: ν_{Hg} = 431 µg L^{-1} h^{-1}; green algae: ν_{Hg} = 166 µg L^{-1} h^{-1}). This fact also resulted in different equilibrium times. While brown macroalgae reached a steady-state after 24 hours, the green macroalgae needed 48 hours to achieve the equilibrium state.

However, it is worth to highlight that the considerable increase of the presence of other divalent metals, like Cd and Pb in the solution (M2) did not originated relevant differences neither on the kinetics neither on the equilibrium of Hg uptake by both macroalgae, as can be observed by the sorption profile displayed in Figure 6.6. Despite the increase of 4- and 20-fold on initial Cd and Pb concentrations, the estimated initial Hg sorption rates in M2 scenario (ν_B = 447 µg L^{-1} h^{-1}; ν_G = 153 µg L^{-1} h^{-1}) were very similar to the ones estimated for M1, as well as the equilibration times (respectively, around 24 hours for brown algae and 48 to the green one). In terms of removal performance at equilibrium, Hg removal was higher than 98% in the highest contamination scenario, corresponding to an amount of Hg sorbed by the macroalgae of c.a. 0.5 µmol g^{-1} (Table 6.7).

Moreover, the comparison of the q_e value for Hg in the mixtures with the one obtained previously in the same conditions but in the monometallic solution, it is possible to evaluate the effect of the presence of the divalent metals on the sorption of Hg by the algae. If, $q_e^{mixture}/q_e^{mono}$ <1, the sorption of Hg is inhibited by the presence of Cd and/or Pb; if $q_e^{mixture}/q_e^{mono}$ =1, there is no observable interaction; and if $q_e^{mixture}/q_e^{mono}$ >1, the sorption is favored by the presence of the metals. The values found ranged from 1.00 and 1.09 for both macroalgae and mixtures, indicating that for the studied systems the biosorption of Hg by the algae *F. vesiculosus* and *U. lactuca* was not inhibited by the presence of Cd and Pb.

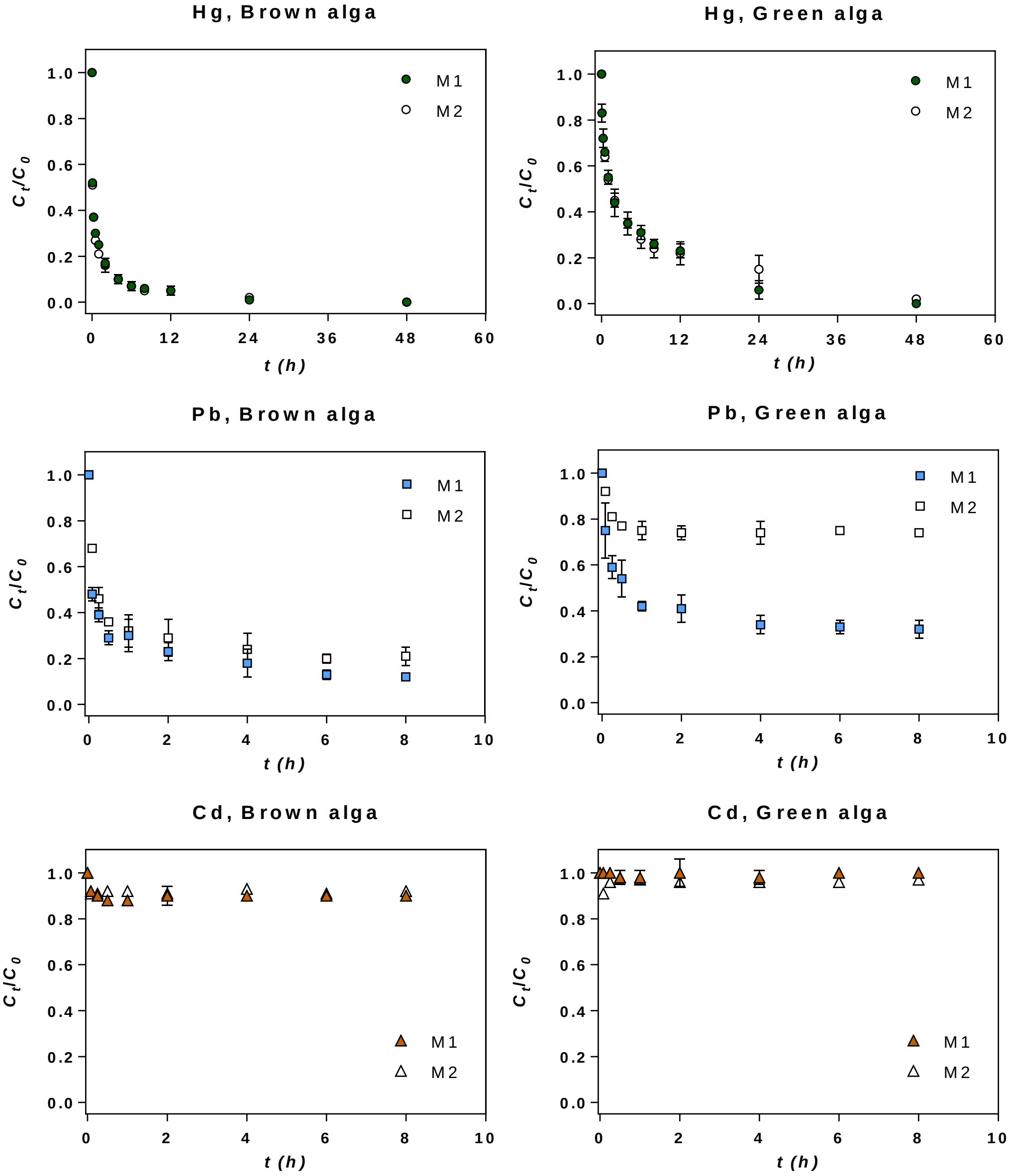


Figure 6.6: Normalized metal concentrations (C_t/C_0) in ternary solutions with time for the brown and green macroalgae: filled symbols – mixture 1; open symbols – mixture 2.

Like for Hg, the removal of Pb by both macroalgae is characterized by a strong and rapid decrease of metal concentration in solution (more pronounced in brown macroalgae) (Figure 6.5), as confirmed by the estimated initial sorption rates (ν_B = 400 µg L^{-1} h^{-1}; ν_G = 185 µg L^{-1} h^{-1} in M1 and ν_B = 3443 µg L^{-1} h^{-1}; ν_G = 1103 µg L^{-1} h^{-1} in M2), followed by a slower stage. However, contrary to Hg the equilibration time was almost the same for both macroalgae and was attained in less than 8 hours. In both contamination scenarios, the brown macroalgae was more efficient at removing Pb from solution that the green one, reaching removal efficiencies that range from ca. 80 to 88% depending on the level of initial contamination (Table 6.7).

Table 6.7: Estimated initial sorption rates (ν_0, µg L^{-1} h^{-1}), removal percentages (R, %) and amount of metal sorbed per unit of algae biomass (q, µmol g^{-1}), for multimetallic solutions.

			Brown algae				**Green algae**			
Solution	Metal	C_0 (µg L^{-1})	ν_0 (µg L^{-1} h^{-1})	t (h)	R (%)	q (µmol g^{-1})	ν_0 (µg L^{-1} h^{-1})	t (h)	R (%)	q (µmol g^{-1})
M1	Hg	50	431	48	99.9	0.597	166	48	99.8	0.587
	Cd	50		8	9.7	0.053		8	0.0	0.0
	Pb	50	400	8	87.8	0.417	185	8	67.8	0.287
M2	Hg	50	447	48	99.7	0.585	153	48	98.4	0.558
	Cd	200		8	7.6	0.284		8	3.0	0.107
	Pb	1000	3443	8	79.5	7.801	1103	8	26.0	2.531

In the contamination scenario M2, where the initial Pb concentration was much higher than in M1, it was noted a strong decrease on Pb uptake by the green macroalgae but remarkably, the brown macroalgae was almost insensitive to the increase of metal concentration (Figure 6.6). However, the rise of the initial metal concentration provides an important driving force to overcome all mass transfer resistance of Pb between the liquid and solid phases. Thus, despite the decrease on the removal percentage, an rise of C_0 from 50 (M1) to 1000 µg L^{-1} (M2) increased the initial sorption rate (as mentioned previously), and the amount of Pb biosorbed by the *F. vesiculosus* from *ca.* 0.42 to 7.8 µmol g^{-1} and by the *U. lactuca* from 0.29 to 2.5 µmol g^{-1}, as displayed in Table 6.7.

Comparing the q_e values for Pb in single and in ternary solutions it is possible to conclude that for the majority of the systems studied, the value of the coefficient $q_e^{mixture}/q_e^{mono}$ was >1, indicating that the sorption of Pb is favored by the presence of other metals. The only exception was recorded for the green macroalgae and for the highest level of contamination (M2), where $q_e^{mixture}/q_e^{mono}$ was equal to 0.83.

Like in single contamination conditions, neither the brown neither the green macroalgae were able to effectively reduce Cd contamination in seawater. Only, a small decrease on Cd concentration in solution was observed for the brown algae at the very beginning of the essays followed by no variation in Cd concentration until the end of the experiments.

In both ternary contamination scenarios the efficiency of the macroalgae in terms of removal efficiency was Hg > Pb >> Cd (Table 6.7). However, in terms of amount of metal sorbed per gram of biomass (q_t) (Table 6.7) the sequence was not the same for both scenarios due to the different initial metal concentrations: for M1 the sequence was Hg > Pb >> Cd while for M2 it was Pb >> Hg > Cd. So, besides the Hg has been almost totally removed from solution, there was more Pb sorbed than Hg by the algae. These results are corroborated by the selectivity of the macroalgae toward the different metals (S), which is given by the ratio between the distribution coefficients (K) of the metals at equilibrium, and the distribution coefficient that is defined as q_e/C_e. The equilibrium selectivity found for the *F. vesiculosus* and *U. lactuca* toward the target metals followed the order Hg>Pb>Cd, independently of the level of contamination. Romera et al. (2007) also found that the affinity of different algal biomasses for metal was higher for Pb than for Cd (Hg was not included in the study). Authors pointed to intrinsic metal properties, such as ionic radii and electronegativity of atoms, as hypothesis for different affinity toward metals (Romera et al. 2007). In fact, Freitas et al. (2008) reported that the ionic character of the binding forces decreased in the same order of the sorption capacity of different algal biosorbents with metals: Pb>Cd. The same order was previously verified by Brady and Tobin (1995), regarding the metal uptake capacity by biomass of *Rhizopus arrhizus*, which was correlated with covalent index of the metal ions (the greater the covalent index value of a metal ion, the greater its potential to form covalent bonds with biological ligands) (Brady and Tobin 1995).

Comparing both macroalgae, it is possible to conclude that in general the brown macroalgae is more efficient than the green macroalgae at sorbing Hg and Pb from saltwater both in single and ternary saltwater conditions. *F. vesiculosus* displayed higher initial sorption rates, higher removal percentages and higher amounts of metals sorbed than *U. lactuca*.

The kinetics of the sorption of Hg and Pb by *F. vesiculosus* and *U. lactuca* was also investigated using common kinetic models such as PFO, PSO and Elovich models (Figure 6.7). In the case of Hg, sorption kinetics onto both macroalgae is well described by the Elovich model (Table 6.8), while for Pb several models are able to describe the sorption process. These results are in accordance with the modelling results achieved for the monometallic solutions.

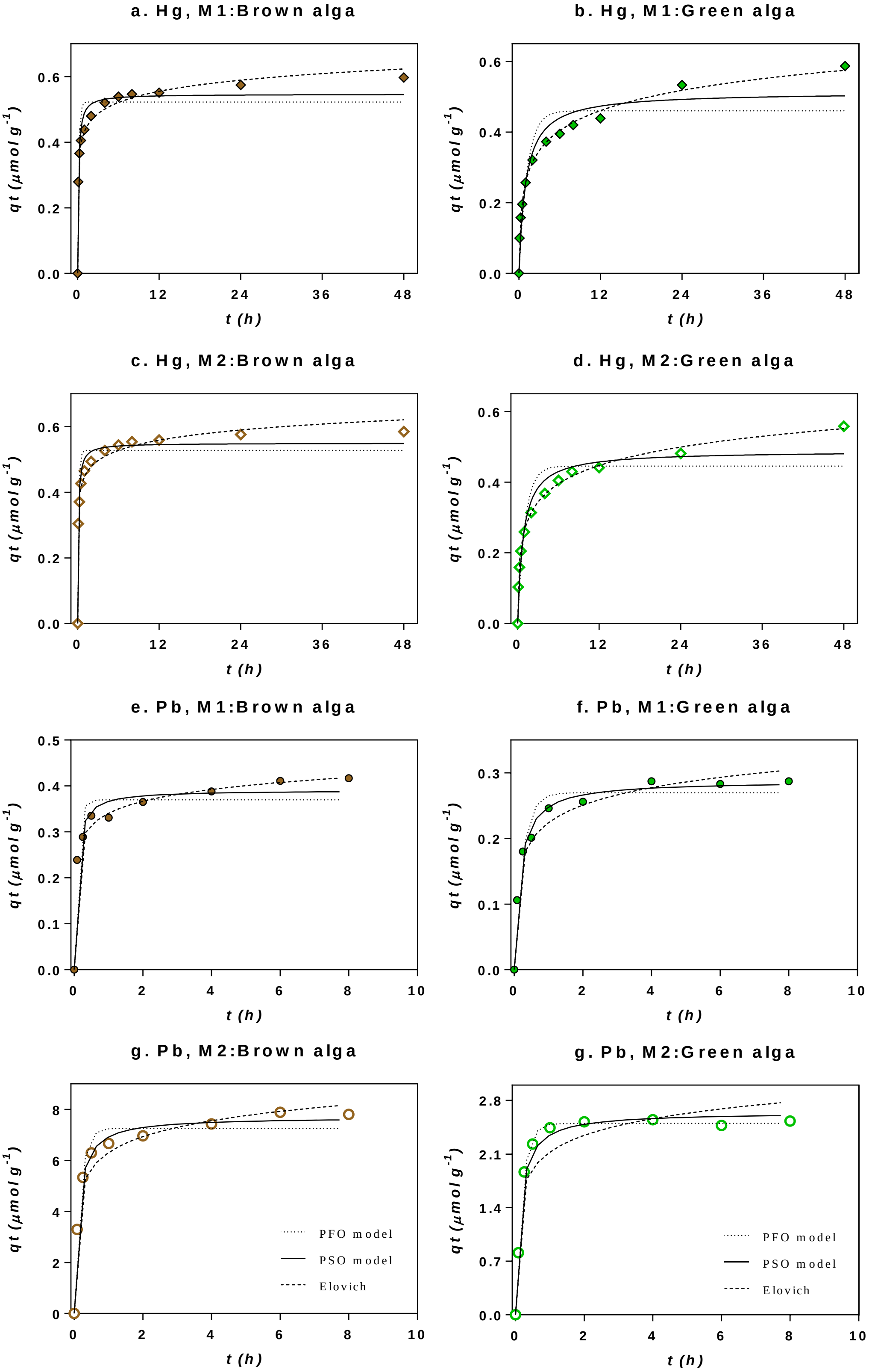


Figure 6.7: Experimental and modelling results for the amount of Hg (diamonds) and Pb (circles) biosorbed by the algae *F. vesiculosus* (brown color) and by *U. lactuca* (green color), for two contamination scenarios M1 (filled symbols) and M2 (open symbols). (Error bars were omitted for clarity).

Table 6.8: Best-fit parameters (± standard error) and goodness of the fittings of the kinetic models used to describe the biosorption kinetics of metals by *F. vesiculosus* and *U. lactuca* algae in ternary solutions.

Algae	Metal	PFO	PSO	Elovich	Preferred model*
			Mixture 1		
Brown algae	Hg	**k_1:** 5.608±1.417	**k_2:** 15.71±3.388	**a:** 325.1±160.8	***Elovich*** *Probability:* *99.99%*
		q_e: 0.5223±0.02047	**q_e:** 0.5462±0.01394	**β:** 20.33±1.118	
		R^2: 0.888	**R^2:** 0.960	**R^2:** 0.991	
		$S_{y/x}$: 0.05894	**$S_{y/x}$:** 0.03542	**$S_{y/x}$:** 0.01691	
	Pb	**k_1:** 10.32±2.846	**k_2:** 38.62±9.761	**a:** 338.2±163.3	***Elovich*** *Probability*: 99.98%
		q_e: 0.3697±0.01570	**q_e:** 0.3908±0.01164	**β:** 26.75±1.534	
		R^2: 0.9205	**R^2:** 0.970	**R^2:** 0.996	
		$S_{y/x}$: 0.03893	**$S_{y/x}$:** 0.02405	**$S_{y/x}$:** 0.009198	
Green algae	Hg	**k_1:** 0.8230±0.2331	**k_2:** 1.947±0.5781	**a:** 1.817±0.2103	***Elovich*** *Probability*: >99.99%
		q_e: 0.4602±0.02925	**q_e:** 0.5131±0.02748	**β:** 12.11±0.3628	
		R^2: 0.8651	**R^2:** 0.934	**R^2:** 0.996	
		$S_{y/x}$: 0.06825	**$S_{y/x}$:** 0.04759	**$S_{y/x}$:** 0.01180	
	Pb	**k_1:** 4.060±0.7349	**k_2:** 21.61±2.651	**a:** 12.92±6.431	***PSO*** *Probability*: 98.01%
		q_e: 0.2698±0.01012	**q_e:** 0.2878±0.005415	**β:** 25.95±2.457	
		R^2: 0.955	**R^2:** 0.991	**R^2:** 0.979	
		$S_{y/x}$: 0.02226	**$S_{y/x}$:** 0.009722	**$S_{y/x}$:** 0.01499	
			Mixture 2		
Brown algae	Hg	**k_1:** 6.528±1.565	**k_2:** 18.84±3.648	**a:** 957.4±692.6	***Elovich*** *Probability*: 99.47%
		q_e: 0.5276±0.01873	**q_e:** 0.5496±0.01196	**β:** 22.30±1.591	
		R^2: 0.902	**R^2:** 0.969	**R^2:** 0.987	
		$S_{y/x}$: 0.05464	**$S_{y/x}$:** 0.03097	**$S_{y/x}$:** 0.02003	
	Pb	**k_1:** 5.806±0.9657	**k_2:** 1.152±0.1234	**a:** 979.1±701.2	***PSO*** *Probability*: 99.75%
		q_e: 7.257±0.2234	**q_e:** 7.700±0.1152	**β:** 1.109±0.1248	
		R^2: 0.965	**R^2:** 0.994	**R^2:** 0.976	
		$S_{y/x}$: 0.5161	**$S_{y/x}$:** 0.2206	**$S_{y/x}$:** 0.4296	
Green algae	Hg	**k_1:** 0.9290±0.2350	**k_2:** 2.504±0.6362	**a:** 2.374±0.2391	***Elovich*** *Probability*: >99.99%
		q_e: 0.4454±0.02487	**q_e:** 0.4883±0.02161	**β:** 13.28±0.3236	
		R^2: 0.887	**R^2:** 0.950	**R^2:** 0.997	
		$S_{y/x}$: 0.05927	**$S_{y/x}$:** 0.03958	**$S_{y/x}$:** 0.009265	
	Pb	**k_1:** 5.098±0.2266	**k_2:** 2.994±0.5063	**a:** 265.6±373.4	***PFO*** *Probability*: 99.97%
		q_e: 2.501±0.02147	**q_e:** 2.645±0.06441	**β:** 3.170±0.7284	
		R^2: 0.998	**R^2:** 0.985	**R^2:** 0.903	
		$S_{y/x}$: 0.04875	**$S_{y/x}$:** 0.1210	**$S_{y/x}$:** 0.3054	

*Comparison of the two fits with the highest R^2 and lower $S_{y/x}$ values.

Overall, both PFO and PSO models underestimated the amount of metal bound to brown and green seaweeds biomass at equilibrium. Yet, in most cases the relative errors associated with predicted q_e were lower for PSO model.

6.4 Conclusions

The effectiveness of *U. lactuca* and *F. vesiculosus* non-treated powders in the removal of metals such as Hg, Pb and Cd from saline waters was demonstrated under different contamination scenarios.

In single-contaminant systems, both macroalgae were able to reduce initial Hg levels to values below or very close to the legal limit for waters for human consumption. Moreover, in mixtures, the biosorption of Hg by brown and green algae was not inhibited by the presence of Cd and Pb, even when the coexisting metals were present in higher concentrations in solution.

Both macroalgae showed large uptake capacities toward Pb, although the removal was not complete for any of the considered levels of contamination. In addition, a strong decrease in the removal efficiency of Pb by the green macroalgae was recorded in the scenario of higher contamination, M2.

Contrary to Hg and Pb, in all contamination scenarios, Cd was barely removed from solution by both brown and green algae (maximum removal of 20%).

Overall, dried *F. vesiculosus* proved to be more effective in removing metals from seawater than *U. lactuca*, since brown algae displayed higher initial sorption rates, higher removal percentages and higher amounts of metals biosorbed. The superior performance of brown macroalgae was attributed to the presence of alginate in their composition, which features several carboxyl groups with high affinity toward divalent metals. However, the equilibrium selectivity observed for the *F. vesiculosus* and *U. lactuca* toward the target metals followed the same order: Hg>Pb>Cd. The biosorption kinetics was well described by the models considered, over the whole period of time and full range of concentrations.

In general, the results suggests that the two macroalgae studied may be effectively used as alternative method for metal removal from contaminated seawater, more cost-effective and environmentally friendly than conventional methods.

6.5 References

Brady, J. M. and J. M. Tobin (1995). "Binding of hard and soft metal ions to Rhizopus arrhizus biomass." Enzyme and Microbial Technology **17**(9): 791-796.

Bulgariu, L. and D. Bulgariu (2014). "Enhancing Biosorption Characteristics of Marine Green Algae (Ulva lactuca) for Heavy Metals Removal by Alkaline Treatment." Bioprocessing & Biotechniques **4**(1).

Chojnacka, K. (2010). "Biosorption and bioaccumulation - the prospects for practical applications." Environment International **36**(3): 299-307.

Chojnacka, K. and M. Mikulewicz (2014). Bioaccumulation. Encyclopedia of Toxicology (Third Edition). P. Wexler. Oxford, Academic Press**:** 456-460.

Cobas, M., M. A. Sanromán and M. Pazos (2014). "Box–Behnken methodology for Cr (VI) and leather dyes removal by an eco-friendly biosorbent: F. vesiculosus." Bioresource Technology **160**(0): 166-174.

Coelho, J. P., M. E. Pereira, A. Duarte and M. A. Pardal (2005). "Macroalgae response to a mercury contamination gradient in a temperate coastal lagoon (Ria de Aveiro, Portugal)." Estuarine, Coastal and Shelf Science **65**(3): 492-500.

Council Directive 98/83/EC (1998). "Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption." Journal of the European Communities.

Davis, T. A., B. Volesky and A. Mucci (2003). "A review of the biochemistry of heavy metal biosorption by brown algae." Water Research **37**(18): 4311-4330.

Decree-Law No. 103/2010 (2010). Decree-Law No. 103/2010 of the Portuguese Ministry of Environment and Spatial Planning of 24 September on pollution reduction, Diário da República : I Série. **187:** 4289-4298.

Decree-Law No. 236/98 (1998). Decree-Law No. 236/98 of the Portuguese Ministry of the Environment of 1 August establishing water quality standards, Diário da República : I Série. **176:** 3676-3722.

Directive 83/513/EEC (1983). Council Directive 83/513/EEC of 26 September 1983 on limit values and quality objectives for cadmium discharges (Daughter to 2006/11/EC), Official Journal of the European Communities. **291:** 1-8.

Directive 84/156/EEC (1984). Council Directive 84/156/EEC of 8 March 1984 on limit values and quality objectives for mercury discharges by sectors other than the chlor-alkali electrolysis industry, Official Journal of the European Communities**:** 74: 29-74: 29.

Directive 2013/39/EU (2013). Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy, Official Journal of the European Union. **226:** 1-17.

Easton, C., A. Turner and G. Sewell (2011). "An evaluation of the toxicity and bioaccumulation of cisplatin in the marine environment using the macroalga, Ulva lactuca." Environmental Pollution **159**(12): 3504-3508.

El-Khaiary, M. I. and G. F. Malash (2011). "Common data analysis errors in batch adsorption studies." Hydrometallurgy **105**(3-4): 314-320.

El-Sikaily, A., A. E. Nemr, A. Khaled and O. Abdelwehab (2007). "Removal of toxic chromium from wastewater using green alga Ulva lactuca and its activated carbon." Journal of Hazardous Materials **148**(1-2): 216-228.

Elrefaii, A. H., L. A. Sallam, A. A. Hamdy and E. F. Ahmed (2012). "Optimization of Some Heavy Metals Biosorption by Representative Egyptian Marine Algae." Journal of Phycology **48**(2): 471-474.

Farooq, U., J. A. Kozinski, M. A. Khan and M. Athar (2010). "Biosorption of heavy metal ions using wheat based biosorbents - A review of the recent literature." Bioresource Technology **101**(14): 5043-5053.

Freitas, O. M. M., R. J. E. Martins, C. M. Delerue-Matos and R. A. R. Boaventura (2008). "Removal of Cd(II), Zn(II) and Pb(II) from aqueous solutions by brown marine macro algae: Kinetic modelling." Journal of Hazardous Materials **153**(1-2): 493-501.

Ho, Y.-S. (2006). "Review of second-order models for adsorption systems." Journal of Hazardous Materials **136**(3): 681-689.

Ho, Y. S. and G. McKay (1998). "A comparison of chemisorption kinetic models applied to pollutant removal on various sorbents." Process Safety and Environmental Protection **76**(B4): 332-340.

Ho, Y. S., J. C. Y. Ng and G. Mckay (2000). "Kinetics of pollutant sorption by biosorbents: Review." Separation and Purification Methods **29**(2): 189-232.

Lagergren, S. (1898). "About the theory of so-called adsorption of soluble substances." Kung Sven Veten Hand **24**: 1-39.

Lesmana, S. O., N. Febriana, F. E. Soetaredjo, J. Sunarso and S. Ismadji (2009). "Studies on potential applications of biomass for the separation of heavy metals from water and wastewater." Biochemical Engineering Journal **44**(1): 19-41.

Lo, Y.-C., C.-L. Cheng, Y.-L. Han, B.-Y. Chen and J.-S. Chang (2014). "Recovery of high-value metals from geothermal sites by biosorption and bioaccumulation." Bioresource Technology **160**(0): 182-190.

Lopes, C. B., J. R. Oliveira, L. S. Rocha, D. S. Tavares, C. M. Silva, S. P. Silva, N. Hartog, A. C. Duarte and E. Pereira (2014). "Cork stoppers as an effective sorbent for water treatment: the removal of mercury at environmentally relevant concentrations and conditions." Environ Sci Pollut Res Int **21**(3): 2108-2121.

Mata, Y. N., M. L. Blázquez, A. Ballester, F. González and J. A. Muñoz (2008). "Characterization of the biosorption of cadmium, lead and copper with the brown alga Fucus vesiculosus." Journal of Hazardous Materials **158**(2-3): 316-323.

Mata, Y. N., M. L. Blázquez, A. Ballester, F. González and J. A. Muñoz (2009). "Biosorption of cadmium, lead and copper with calcium alginate xerogels and immobilized Fucus vesiculosus." Journal of Hazardous Materials **163**(2-3): 555-562.

Nikolaisen, L. S. and P. D. Jensen (2013). 3 - Biomass feedstocks: categorisation and preparation for combustion and gasification. Biomass Combustion Science, Technology and Engineering. L. Rosendahl, Woodhead Publishing**:** 36-57.

Pagnanelli, F., A. Esposito and F. Veglio (2002). "Multi-metallic modelling for biosorption of binary systems." Water Research **36**(16): 4095-4105.

Park, D., Y.-S. Yun and J. Park (2010). "The past, present, and future trends of biosorption." Biotechnology and Bioprocess Engineering **15**(1): 86-102.

Pato, P., M. Otero, M. Válega, C. B. Lopes, M. E. Pereira and A. C. Duarte (2010). "Mercury partition in the interface between a contaminated lagoon and the ocean: The role of particulate load and composition." Marine Pollution Bulletin **60**(10): 1658-1666.

Rocha, L., C. Lopes, J. A. Borges, A. C. Duarte and E. Pereira (2013). "Valuation of Unmodified Rice Husk Waste as an Eco-Friendly Sorbent to Remove Mercury: a Study Using Environmental Realistic Concentrations." Water, Air, & Soil Pollution **224**(7): 1-18.

Romera, E., F. Gonzalez, A. Ballester, M. L. Blazquez and J. A. Munoz (2006). "Biosorption with algae: A statistical review." Critical Reviews in Biotechnology **26**(4): 223-235.

Romera, E., F. Gonzalez, A. Ballester, M. L. Blazquez and J. A. Munoz (2007). "Comparative study of biosorption of heavy metals using different types of algae." Bioresource Technology **98**(17): 3344-3353.

Sari, A. and M. Tuzen (2008). "Biosorption of Pb(II) and Cd(II) from aqueous solution using green alga (Ulva lactuca) biomass." Journal of Hazardous Materials **152**(1): 302-308.

Torres, M. A., M. P. Barros, S. C. G. Campos, E. Pinto, S. Rajamani, R. T. Sayre and P. Colepicolo (2008). "Biochemical biomarkers in algae and marine pollution: A review." Ecotoxicology and Environmental Safety **71**(1): 1-15.

Trinelli, M. A., M. M. Areco and M. D. Afonso (2013). "Co-biosorption of copper and glyphosate by Ulva lactuca." Colloids and Surfaces B-Biointerfaces **105**: 251-258.

Vijayaraghavan, K. and U. M. Joshi (2012). "Interaction of Mercuric Ions with Different Marine Algal Species." Bioremediation Journal **16**(4): 225-234.

Vijayaraghavan, K. and Y. S. Yun (2008). "Bacterial biosorbents and biosorption." Biotechnology Advances **26**(3): 266-291.

Zeroual, Y., A. Moutaouakkil, F. Zohra Dzairi, M. Talbi, P. Ung Chung, K. Lee and M. Blaghen (2003). "Biosorption of mercury from aqueous solution by Ulva lactuca biomass." Bioresource Technology **90**(3): 349-351.

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Chapter 7

Final considerations

7 Final considerations

Overall, the findings of the present PhD project highlight the importance of studying the transfer and accumulation of metals by living plants and macroalgae in contaminated systems.

Terrestrial plants *Lolium perenne* and *Brassica Juncea* growing in agricultural fields near an industrial area, showed to be able to uptake considerable amounts of Hg from soil. Although bioconcentration factors (BCF) of inorganic Hg had been smaller than 1, estimates revealed that, in some cases, daily intake of IHg were above the acceptable daily intake for both cow and sheep (in terms of kidney concentration). Moreover, the estimated levels of total mercury in animal organs pointed out the potential risk to human health derived from the consumption of offal from animals that graze in that area.

Organo-metallic Hg (OrgHg) contents in both terrestrial plants were in general below 1% of total Hg concentrations, yet above the respective OrgHg concentration in soil, which indicates that bioaccumulation of organometallic species (the most toxic species of Hg) also occurs in terrestrial systems. In addition, results suggested that there is no barrier to the translocation of OrgHg from roots to shoots of *L. perenne* as observed for IHg. Hence, the inclusion of limits for OrgHg in feed quality and food safety legislation was advised.

The following study dealt with the assessment of Hg bioaccumulation by three different marine macroalgae (*U. lactuca*, *G. gracilis* and *F. vesiculosus*) in a different saline matrix: Hg contaminated seawater. Experiments conducted in laboratory, with experimental conditions aiming to mimick those found in real aquatic ecosystems, emphasized the already recognized affinity between algae and metals (almost 99% of all Hg was removed from solution by the seaweeds). Bioconcentration factors were much higher than those found for terrestrial plants (c.a. 2000), which in part may be attributed to the higher bioavailability of Hg in the medium, in relation to soil, but also to the absence of any restriction mechanism to the accumulation by the algae.

OrgHg contents in *U. lactuca* biomass were very low, and below that recorded for *L. perenne* and *B. Juncea*, suggesting that methylation is a slow process.

Overall, the amount of Hg bioaccumulated by the seaweeds was positively correlated with the initial concentration of metal in solution, which is in agreement with field studies supporting their use in environmental biomonitoring. In addition, the huge capability to remove Hg from water pointed to the enormous potential of these marine macroalgae as a basis for a new remediation biotechnology. Since macroalgae represent a largely available resource, the new treatment methodology will be more cost-effective than conventional methods, besides being more environmentally friendly. In this context, of the macroalgae studied, *U. lactuca* stood out, since the removal carried out by this macroalgae was faster, and more extensive than that observed for the other two species. The better performance of *U. lactuca* was mainly attributed to its high growth rate and high surface area. Moreover, the residual concentrations of Hg in solution showed compliance with legal criteria for drinking water quality.

Despite most of works found in the literature, regarding metal removal and recovery, are conducted using dried biomass of macroalgae (biosorption), a comparative study between biosorption and bioaccumulation processes, demonstrated that the use of living organisms may be more advantageous. For the experimental conditions used, living *U. lactuca* allowed to achieve lower residual concentrations of Hg than its dried biomass. The growth of living macroalgae and the additional internalization of the sorbed metal were the appointed reasons for the best performance. Additionally, the process of separating the biosorbent from the treated solution was simpler in the bioaccumulation, since no filtration was required.

The ability of *U. lactuca* to bioaccumulate metals was also evaluated in monometallic and multimetallic solutions of Cd, Pb and Hg (which are closer to the real aquatic systems), in different levels of contamination. This study was also conducted for *F. vesiculosus*, and effects of metal exposure on both macroalgae growth were assessed. Again, the macroalgae showed an extraordinary capability for remove toxic metals from salt waters, even when the contaminants were present simultaneously. Like observed previously for Hg, the highest removal efficiencies for Pb and Cd were achieved by the green macroalgae. Furthermore, the green macroalgae showed to be tolerant to the different contamination scenarios studied, whereas the relative growth rate of *F. vesiculosus* was negatively affected by the presence of Cd and Pb in high concentrations. Nevertheless, it should be pointed out that none of the exposure conditions tested caused death or organism decay.

The green and brown macroalges presented different sequences of selectivity toward metals (*U. lactuca*: Hg>Cd>Pb; *F. vesiculosus*: Hg>Pb>Cd), which reveals that each macroalgae has its specificities, and the chemical composition of the macroalgae play a vital role on the biosorption/bioaccumulation of metals.

The results obtained from living algae seem to contradict the trend revealed by studies involving non-living algae, which indicate that the brown algae are those that have higher affinity to metals. As such, in the work that gave rise to the last chapter of this doctoral thesis, the biosorption capacity of the dried biomass of *U. lactuca* and *F. vesiculosu*s towards toxic metals studied was evaluated and compared. In order to obtain maximum performance of removal only the powder fraction (particles with size <200 µm) was used, since it is well known that biosorbent surface area is a vital parameter in biosorption. Results actually supported the assumption that brown algae have a better performance, since higher removal percentages and higher amounts of metals sorbed were achieved by the *F. vesiculosu*s. Overall, the behavioral difference recorded between bioaccumulation and biosorption experiments, reinforces the idea that the algae metabolism plays a fundamental role in metal removal. In fact, the results of biosorption clearly showed that both macroalgae biomasses were also very effective at sorbing Hg and Pb from solution. However, despite the increased surface area/solution ratio, neither the green nor the brown algae biosorbents were effective to remove Cd from saltwater, case where the application of the living ones proved to be more advantageous.

Thus, the development and implementation of a remediation biotechnology based on the use of these macroalgae, should take into account the composition of the solution to be treated, or the metal to be removed. Yet, the time required, the associated cost and the simplicity of the process are major factors when it came to choose between bioaccumulation, biosorption, and a hybrid technology.

The description of the kinetics of the removal process is critical in the development of the treatment technology, which has been successfully accomplished using kinetic reaction model, widely accepted by researchers. However, the effect of the amount or dose of algae (biomass/solution ratio) in the efficiency of removal is also very important and should be evaluated in the future.

During the studies, some questions emerged indicating future potential research directions emerged. The role of plants and macroalgae in the methylation of Hg are not clear and should be investigated. Future studies should also focus on the mechanisms of metal binding to macroalgae cell walls and intracellular accumulation. Additionally it would be interesting to assess the potential application of the studied macroalgae in the removal and recovery of elements of high economic value, as well as of emerging pollutants, such as metal nanoparticles.