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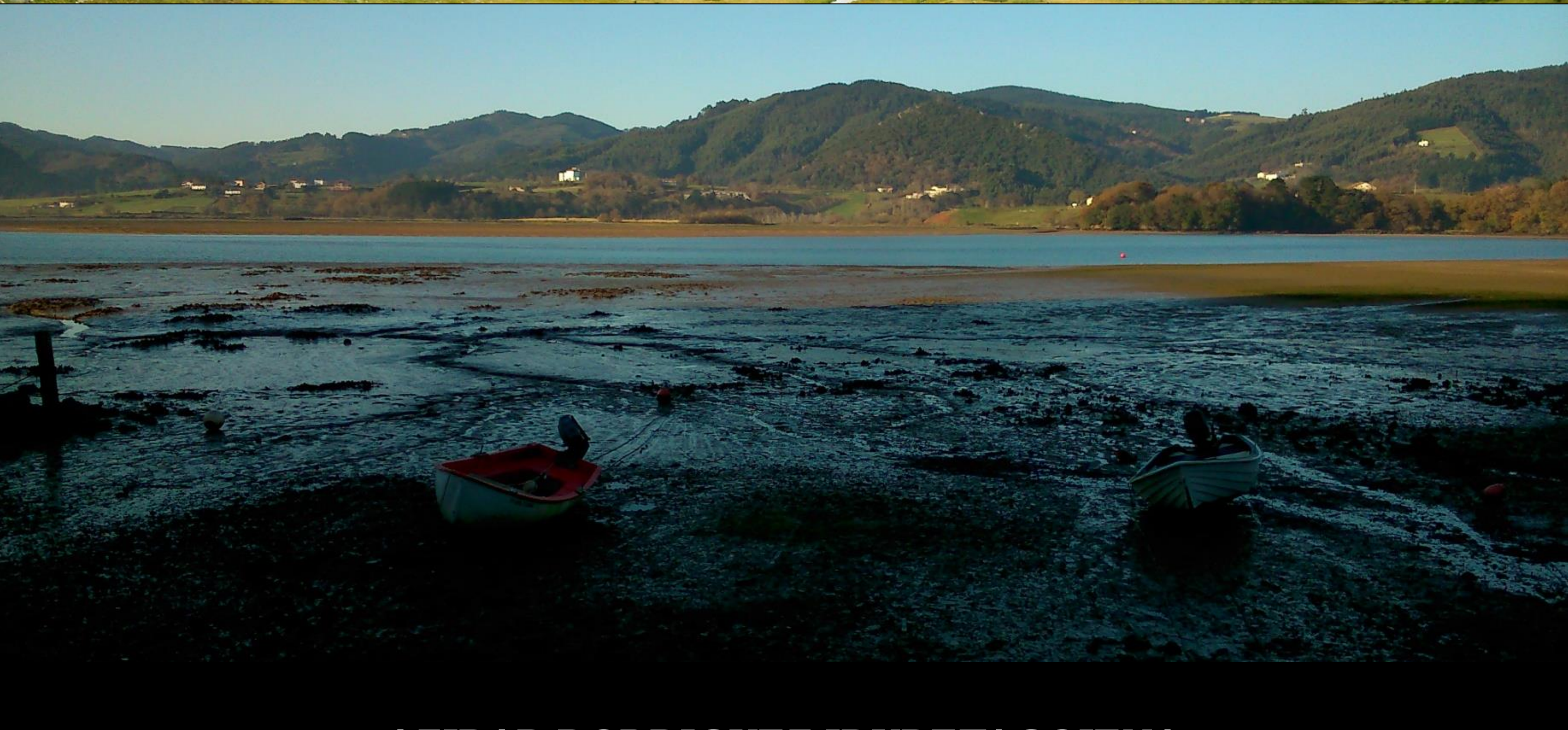

AZIBAR RODRIGUEZ IRURETAGOIENA

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METAL POLLUTION IN ESTUARIES AND HIGH ALTITUDE MOUNTAINS: GEOGRAPHICAL DISTRIBUTION, EVOLUTION IN TIME AND TOXICOLOGICAL IMPLICATIONS

This PhD Thesis has been developed in the Department of Analytical Chemistry from the University of the Basque Country (Faculty of Science and Technology, Leioa, Spain)

Azibar Rodriguez Iruretagoiena

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ESKERRAK

Lau urtetan zehar egindako lana laburbiltzen duen tesia entregatzeko ordu batzuk besterik ez dira falta. Nire kasuan, hilabete batzuk gehiago behar izan ditut tesi liburua amaitzeko, baina badirudi heldu dela azkenean eguna. Iritsi da, eta orain, ondo pentsatuta, atzera begiratu eta denbora zeinen azkar pasatzen den konturatzen zara. Lau urte luze hauetan denetarik gertatu dela esan dezaket eta orri apur hauek ez dira dena laburbiltzeko nahikoa izango.

Gertatutakoak aparte utzita, (beste liburu bat idatzi beharko nukeelako pasatako momentuak kontatzeko) lau urte hauetan ia egunero ikusi ditudan lankideez arituko naiz. Ziurrenik baten bat ahaztuko dut, baina denok betirako gogoan izango zaituztedala jakin dezazuen nahi dut, nahiz eta lerro hauetan ez agertu.

Lehenik ea behin nire tesi zuzendari izandakoekin hasiko naiz, Alberto eta Silviarekin hain zuzen. Silvia, egia esan beti izan ditugu gure hika mikak, askotan elkarrekin haserretzera eraman gaituztenak, biok nahiko burugogorrak baikara. Hika mika horietako batean, zure bulegoan inoiz ahaztuko ez dudan esaldia esan zenidan: "*¡Parece que predico al desierto!* ". Bina zuk ondo dakizunez, oso momentu alaiak ere izan ditugu, bai laginketetan zein egunerokoan, oso pertsona zarelako eta hori nabaritzen zaizu. Alberto, zurekin ere denetarik pasa dut, baina egia esan, askoz ugariagoak izan dira momentu oso onak txarrak baino. Hori bai, momentu txarra, bakarra izan arren, betirako geratuko zait markatuta, jende askori kontatu izan diodan bezala. Mintegiko nominazioak zirela eta, hori gertatu eta pare bat egun beranduago zure bulegora deitu ninduzun. Zure aurrean jesarri nintzen eta begirada batez esan zenidan "hau ez dadila berriro gertatu, hurrengoan ni arduratuko naiz zutaz eta". Nahikoa izan nuen horrekin. Baina elkarrekin pasatako egunak hortxe, nire oroitzapenean betirako egongo direla ziur nago. Asko eta asko dira momentu horiek: Pirinioetan Iratiko basoan eta lakuetan egindako laginketak, zenbait egun dutxatu gabe pasata autokarabanan lo egindako gauak, Aridaneren etxean ohe berean lo egin genuen gau hura, perretxikotan pasatako uneak edota jatetxe onetan afaltzen edo bazkaltzen janariaz disfrutatzen igarotakoak… esan bezala momentu eder asko zurekin Alberto.

Leioako mintegira pasako naiz orain. Leioako mintegian pasatako eta inoiz ahaztuko ez dudan asteetako bat nominazio aste hura izan zen. Ez dut ondo gogoratzen noiz izan ziren, baina bai Josean eta biok mintegian bata bestearen aurrean jesarrita geundela. Jendeak ezin zuela lanik egin esan eta Luis Angelek mintegiko norbait botatzea proposatu zuen. Nominazio aste horren ondorioz, Sandra eta ni kanporatu gintuzten. Egia esan, horrek mintegiko egoera baretzeko balio izan zuen, nahiz eta aste pare batez tentsio handiko egunak pasatu genituen.

Mintegiko jendearen txanda orain. Asko izan zarete lau urte hauetan egunero ikusi zaituztedanak. Bekili, Mireli, Arnatza eta Itsaski, zuekin egin dut barre gehien eta beti horrela izango dela ziur nago. Hala ez badirudi ere, oso garrantzitsuak izan zarete niretzako unibertsitatean, egunero bertara joatea eramangarriago egiten zenutelako, elkarrekin oso momentu onak pasa ditugulako. Ainara, zuk ere asko lagundu didazu urte hauetan eta elkarrekin denetarik pasa dugu: laginketa egunak, nire kotxean kantaldiak, askotan zirikatu izan zaitut baita ere, laborategiko elkarlana… eskerrik asko zuri ere hor egoteagatik. Julene eta Leticia, zuekin ere momentu oso on asko ditut, hirugarren mailako ingurumen zientziak ikasten elkartu ginenetik gainera, urte asko elkarrekin eta oraindik ere oso gustura zuekin beti. Nikole, Ane, Oscar, Leire Kortazar eta Mijangos, Haizea, Laura, Olaia, Nerea, Oier, Pati, Oihana, Naiara, Maitane, Ailette, Jone, Asier, Josu, Ibone, Nagore, Olivia, Maite eta Hector, zuei ere mila esker. Lau urte hauetako egun asko konpartitu ditugu eta momentu oso onak pasa ere. Esandakoa, norbait ez badut aipatu barkatu nazala, izena agian bai, baina berekin bizitakoa ez dut sekula ahaztuko.

Eta orain los "VIP-s"!!! "toca callar un momento porque viene gorda". Nos lo hemos pasado terrible no, lo siguiente!!! Me acuerdo que el primero que vino a hablarme uno de mis primeros día en el laboratorio fue Jousi. No hizo más que llegar, no nos conocíamos de nada y ya se lio ya, de risas desde el primer momento. Qué casualidad, que además salgo ese finde, le veo en el Galeón y se armó la que se armó que perdí hasta la txapela que me acuerdo que iba disfrazado de Olentzero, ahí ya se vio que íbamos a hacer gordas juntos Jous. No voy a dar la chapita aquí pero podemos escribir uno, dos o tres libros. Los otros dos, Arriskatu y Julianzas, grandes desde el primer momento aunque a ti Julianzas te costó bastante más, menos mal que empezamos a hacer woks rápido y se fue soltando la cosa. Con el paso de los días ibas a casa hasta a las 00:00 de la noche, el Julen que conocí empezó a cambiar y ya no cogía ni pronto el autobuya en Moyus. Arriskatu, un grande en toda regla con el que he hablado casi más de fútbol que con mi entrenador. El pesado que siempre tiene calor eso sí. Siempre en la calle y pasando frío por tu culpa y jodiendo los woks porque un día tuviste cagalerita. Momento para la historia el día de sin camisa en el bar de Manchester que ya no me acuerdo como se llama y que alguno de nosotros (no voy a decir quién pero creo que está bastante claro Josean) salió volando ayudado por el segurata. Hemos hecho grandes viajes juntos la verdad, que me encantaría que siguiéramos haciendo por lo menos una vez al año, porque está amistad que empezó en la Universidad no la vamos a perder nunca. También es verdad que hemos tenido momento malos en los que incluso no queríais aceptarme como VIP, pero al final el río siempre vuelve a su cauce y sé que nos quedan gordas juntos.

Azkenik, eskerrik asko nire lagun asko eta askori eguna joan eta eguna etorri elkarrekin baikaude eta elkarrekin pasatako momentuak oso lagungarri dira baita lanerako ere. Askok ez duzue ideiarik ere lau urte hauetan zertan aritu naizen, baina beti esan izan duzue ez dudala ezer ere egiten eta denbora pasan nagoela unibertsitatean. Beti esan izan dizuedan bezala hitz egitea debalde da. Arduratu zaitezte zuen ingurua garbi edukitzeaz ;).

Aita eta ama, zuek ere berdin. Ez dakizue zer egiten egon naizen lau urte hauetan, baina egia dena, biok asko maite zaituztedala da egunero elkarrekin gaudelako eta sarritan "a golpes" ibili arren, zuen laguntza eta konfiantza beti daukat hortxe. Momentu txar askotatik irteten laguntzen didazue eta beti bezala, oso momentu onak pasatzen ditugu elkarrekin. Eskerrik asko bihotzez!!!

PREFACE

Environmental pollution is considered by the European Environment Agency, United States Environmental Protection Agency (US-EPA) and World Health Organization (WHO) one of the greatest problems that the world is facing nowadays. All the environmental compartments are more and more affected due to an endless increase in human activity. The life style that human beings are carrying out produces noise, light and chemical pollution that seriously threatens the quality of air, water and soil.

The uncontrolled release of chemicals to the environment results in concentrations of those chemicals significantly higher than the background values considered as “natural”. Above certain threshold limits, the presence of those chemicals provokes toxic effects in the organisms living around. The term “contamination” is related to the presence of “high” (above the natural background values) concentrations of substances in the environment, whereas the term “pollution” refers to the introduction of harmful substances (with toxicological implications) into the environment. This differentiation is not of general use in the scientific community, but it is rather widely accepted.

Amongst others, some metals and metalloids are chemicals able to cause serious problems to the environment. The anthropogenic inputs of metals and metalloids to the environment have grown constantly since the industrial revolution. Their most important features, and the main reason of being of high concern, are non-degradability and persistence in the environment. Furthermore, some metals and metalloids are highly toxic and their toxicity highly depends on their concentration and speciation in each specific compartment. In addition, the speciation of a metal or metalloid is influenced by the physical and chemical characteristics of the medium where it is present.

Natural water bodies are one of the compartments more seriously affected by metal/metalloid contamination/pollution. In words of the World Health Organisation (WHO) “water is essential for life”. The amount of fresh water in earth is limited, and its quality is under constant pressure. Preserving the quality of fresh water is important for drinking-water supply, food production and recreational use. The quality of continental (lakes, rivers and underground water), oceanic and transitional waters is nowadays threatened by the direct or indirect release of chemicals like metals and metalloids all around the world.

Estuaries are important transitional water bodies that concentrate in their surroundings densely populated areas with important industrial, agricultural and urban activities. They constitute very productive areas which have been intensively exploited due to its high social and economic value. The ways that metals and metalloids of anthropogenic origin have to enter the estuary are multiples. Once in the estuary, they are subject of a wide variety of processes which extent depends on the highly changing characteristics of water. If they are not expelled to the ocean, metals and metalloids get stored in the bed sediments of the low part of the estuary in a more or less stable chemical form. Sediments have been frequently used to monitor the concentration of metals and metalloids in estuaries.

At the same time, estuaries are extremely valuable from an ecological and biological point of view. They are necessary for the survival of many species of vegetation and animals like fishes, shellfishes and birds. The metal and metalloid content in organisms is proportional to the concentration levels in the water column. In addition, bioconcentration and biomagnification processes take place in the case of some hazardous materials like certain metals and metalloids. In fact, some organisms, such as molluscs and oysters, have been used as bioindicators of the metal content in water.

Metals and metalloids are also widely emitted to the atmosphere from a variety of natural and anthropogenic sources. The WHO defines air pollution as the contamination of an indoor or outdoor environment by any chemical, physical or biological agent that modifies the natural characteristics of the atmosphere. Furthermore, the atmosphere is the layer which protects the earth and makes possible to humans and other organisms to live in it. Most of the metals and metalloids are emitted to the atmosphere as particles or attached to particles. Dry or wet deposition of these particles may occur in locations far away from their original source due to atmospheric transport by wind, for example, in high altitude remote mountains. High altitude mountain ecosystems have been consequently used to study the atmospheric contamination/pollution due to its high sensitivity to variations in atmospheric conditions.

Forests are one of those ecosystems appropriate for environmental observations. Monitoring air quality in forests has been frequently attempted by sentinel organisms such as vascular and non-vascular plants. Plants spend their whole life cycle in a single place and are sensitive to contamination/pollution, reflecting the atmospheric characteristics of specific emplacements. Biomonitoring is the name given to this kind of studies, in which the quality biosphere is analysed using living organisms.

High altitude remote lakes also constitute privilege environments to investigate the quality of air and, specifically, the amount and origin of metals and metalloids present in their waters and sediments. Since anthropogenic activities are scarce in their surroundings, the occurrence of chemicals above the natural values is assumed to arise from dry or wet deposition of those chemicals present in air which, in addition, may have been transported by the dominant winds from emission points situated at very long distances. Lakes are, consequently, very appropriate ecosystems to study long-distance transport processes in which chemicals like metals and metalloids are involved.

Our research group has been investigating the occurrence of toxic chemicals in the environment for a long time. One of our most important research lines has focused in the study of metal and metalloid contamination/pollution in estuaries of the Basque Coast using sediment samples. The situation about five years ago allowed our research group to expand its scope of action and start working, first, in estuaries of other world regions and, second, in mountain ecosystems like forests and high altitude lakes. This change was motivated by a number or research projects that were granted to the group and demanded to start working on these new scenarios. Part of these works and their results has crystallized in this PhD Thesis, whose general objectives can be listed as follows:

1. To develop and apply new and already existing chemical and statistical tools to study the geographical distribution of metal and metalloid contamination/pollution in selected environments as well as its evolution in time.

2. To investigate the toxicological implications that the presence of metals and metalloids in selected environments have on the organisms living in those environments.

3. To discern between natural and anthropogenic origin of metals and metalloids in selected environments and to make hypothesis about their possible source.

4. To compare the efficiency of different indicators to monitor metal and metalloid contamination/pollution in selected environments.

5. To propose new analytical strategies for the analysis of emerging contaminants like nanoparticles in environmental samples.

In order to achieve these objectives several specific works have been carried out. Both the methodologies used and the results obtained have been summarised in this dissertation. The document starts with a general introduction (*Chapter 1*) which refers to basic information on metals and metalloids and their behaviour in aquatic and atmospheric environments. It also reviews the most widely accepted methods to measure the concentration of metals and metalloids in environmental samples. The chapter finishes with a revision of the European Legislation in force that regulates the management of water bodies and air regarding chemical and biological pollution. A general chapter follows (*Chapter 2*) which precisely describes all the materials and methods used throughout the work.

Afterwards, the dissertation is divided in two different sections; the first one is devoted to the study of atmospheric metal contamination/pollution in mountain ecosystems, and the second one summarises all the work carried out in estuaries.

The first section starts with a short introduction (*Chapter 3*) that broadly describes the mountain range of the Pyrenees and some important points regarding the occurrence and chemistry of metals and metalloids in some of their high altitude lakes and forests, and consists of two chapters. The first one (*Chapter 4*) describes the work carried out in 18 high altitude lakes of the Pyrenees, all of them above 2000 m of altitude. Surface sediments and sediment cores from these lakes were used to investigate the geographical distribution of metals and metalloids along the Pyrenees and the historical records of pollution deduced from the depth profile of metals and metalloids in the cores. In the second one (*Chapter 5*), the efficiency of mosses, lichens and beech leaves as bioindicators of atmospheric metal contamination in the Irati Forest is compared and critically discussed. In addition, the geographical distribution of metals and metalloids in the forest and their possible origin is also studied.

The second section refers to metal and metalloid occurrence in estuaries. It starts with a general introduction (*Chapter 6*) about estuaries and the behaviour of metals and metalloids in them. In *Chapter 7* the specific problematic of the estuary of the Tubarão River (Santa Catarina, Brazil), seriously affected by coal mining activities, after typical tropical strong rainfall events is investigated, in order to study the mobility of metals and metalloids along the water catchment, and between sediments and the water column. *Chapter 8* describes the geographical distribution of metals and metalloids in surface sediments of the Cávado estuary (Esposende, Portugal), a relatively clean area close to Porto in which only agricultural and recreational activities take place nowadays. In *Chapter 9*, the results obtained after four sampling campaigns in 2009, 2010, 2011 and 2014, in which surface sediments were collected at 50 points of the estuary of the Nerbioi-Ibaizabal River (Bay of Biscay, Basque Country), are summarised. The analysis of the samples to measure the concentration of metals and metalloids allowed us arriving to conclusions about their geographical distribution and the evolution in time within the estuary. A general survey on the occurrence of metals and metalloids in the estuary of the Oka River, located within the limits of the protected area of the Biosphere Reserve of Urdaibai (Bay of Biscay, Basque Country), and the toxicological implications in autochthonous oysters derived from their presence is presented in *Chapter 10*. In fact, cell and tissue level biomarkers measured in oysters are used as bioindicators of the overall health status of the estuary. To finish this section, in *Chapter 11*, surface sediments from the Hugli River, the major tributary of the Ganges River (India), were analysed to check the possible presence of silver nanoparticles in theirs sediments. The main part of this chapter is devoted to the development of a methodology to extract silver nanoparticles from sediments and to detect and characterise them using different analytical approaches, such as single particle Inductively Coupled Plasma Mass Spectrometry (spICP/MS).

The final conclusions of the work have been finally summarised in *Chapter 12*.

The next research projects have partially funded the work described in this document:

- *Oka ibaiaren itsasadarreko kutsadura metalikoaren azterketa biogeokimikoa (OKAMET, UNESCO 09/23).* Funding organisation*:* Cátedra UNESCO de "Desarrollo sostenible y Educación ambiental" de la UPV/EHU.

- *Red de observaciones atmosféricas medioambientales del Pirineo (PYNATEO, CTP 2010/P03)*. Funding organisation: Working community of the Pyrenees (CTP).

- *Impacto de la contaminación metálica difusa (atmosférica) y local (procedente de actividades mineras) sobre comunidades microbianas de lagos pirenaicos (METMIC, CTP 2012/P08)*. Funding organisation: Working community of the Pyrenees (CTP).

- *Global change and heritage (UFI 11/26)*. Funding organisation: Research and formation unit (UPV/EHU).

- *Investigación e innovación analítica (IBeA) en medio ambiente físico, urbano construido, recursos naturales y salud (grupo consolidado tipo A) (GIC12/151)*. Funding organisation: Basque Government.

- *Observatoire de recherche sur la qualité de l´environnement du Grand Sud-Ouest Européen (ORQUE SUDOE, SOE3/P2/F591/5)*. Funding organisation: the European Commision (Interreg IV B Programme).

- *Critical Evaluation of Available Toxicity due to Silver Nanoparticles (AgNP) in Indian Sundarban Mangrove Wetland, a UNESCO World Heritage Site.* Funding organisation: UKIERI, UK-India Education and Research Iniciative.

Most of the works of this PhD Thesis has been carried out in the Department of Analytical Chemistry of the Faculty of Science and Technology of the University of the Basque Country (UPV/EHU) in the framework of the Environmental Contamination and Toxicology Doctorate Programme (CTA). The stay to meet the requirements for the International PhD mention was carried out under the supervision of Helfrid Schulte-Herbrüggen and Margaret Graham, at the Institute for Infrastructure and Environment, within the School of engineering at the University of Edinburgh. Thanks are due to all the people who provided us with samples or collaborated in their collection, and to those who helped in the interpretation and discussion of certain results, especially Dr. Tedy Stoichev and Dr. Paulo Morais, from the University of Porto (Portugal), Dr. Luis Felipe Oliveira Silva, from the Centro Universitário La Salle (Brasil), Dr. Santosh Kumar Sarkar, from the University of Calcutta (India), Dr. Jesus Miguel Santamaria and Dr. David Elustondo, from the University of Navarra (Basque Country), David Amouroux, from the Université de Pau et des Pays del'Adour (France), Dr. Luis Camarero, from the Consejo Superior de Investigaciones Científicas (Spain), Dr. Jean Christophe Auguet, from the Université de Montpellier (France), Dr. Aridane Gonzalez, nowadays in the Université de Brest (France), and Beñat Zaldibar and Ainara Gredilla, from the University of the Basque Country (Basque Country). The author wants finally to thank the University of the Basque Country (UPV/EHU) for his pre-doctoral fellowships.

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SECTION 1

SECTION 2

Chapter 1

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

1. Metals, metalloids and heavy metals

A metal is a chemical element which loses electrons easily, creating positive ions (cations) and in which there are metallic bonds between atoms. Metals are one of the four basic element groups, the other three being noble gases, metalloids and non-metals (Figure 1). Metals' main characteristics include their hardness, shine, thermal conductivity and electrical conductivity. Metals' other characteristics include malleability, fusibility and considerable ductility (Prasad, 2001).

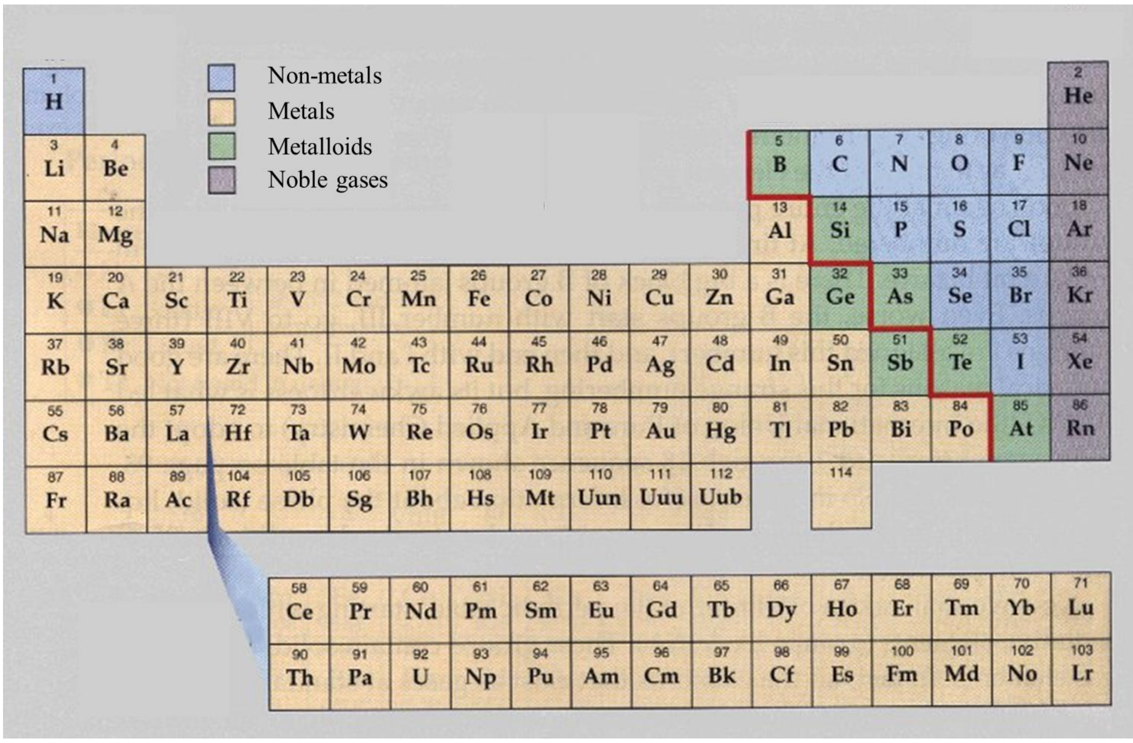

Figure 1. Periodic table of elements.

Most elements in nature are metals. Seven of the main elements found on the earth's surface are metals: aluminium, iron, calcium, sodium, potassium, magnesium and titanium (Giddings, 1973). There are also seven metalloids: boron, silicon, germanium, arsenic, antimony, tellurium and astatine. Metalloids are distinguished by having both metal and non-metal characteristics. They are semiconductors, very unstable in form and can be either shiny or opaque (Prasad, 2001). Heavy metals can also be distinguished between the metals. Most of them are highly toxic and can harm the environment considerably. Heavy metals' main distinguishing characteristic is their high density, above 6 g/cm^{-3}. The metal Ti and the metalloid As are the only exceptions, being classified as heavy metals even though their densities are 4.5 g/cm^{-3} and 5.7 g/cm^{-3} respectively (Manahan, 2007). All transition metals can be classified as heavy metals.

Metallic elements, in both biological and chemical terms, are no degradable and, consequently, are highly durable in the environment. This, along with their toxicity, can cause serious problems. Metallic compounds, on the other hand, can be degraded, but the new metal-containing substances which are obtained through degradation are often even more toxic. Metals' toxicity varies depending on its concentration in each environment and its speciation. Each metal's own characteristics and physical-chemical conditions of the surroundings establish each metal's speciation. As an example, mercury speciation in a water system is shown in Figure 2 (Xavier Domènech, 2006).

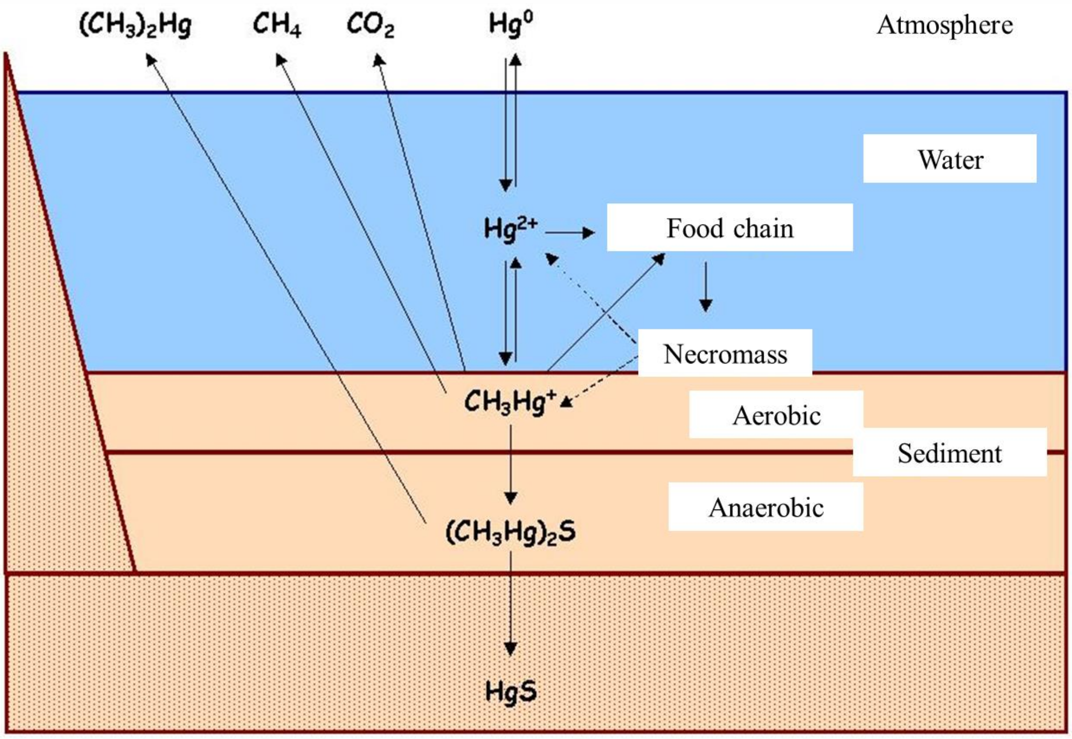

Figure 2. Hg speciation and biogeochemical cycle in a water system.

2. Toxicity of heavy metals

Several metals are necessary for animals' and plants' survival. These are called essential metals and are needed in precise quantities and must be present between their lowest and highest concentration levels (Sarkar, 2005; Yang et al., 2015). They include Mn, Mo, Cu, Co, Zn and V; Ni, Fe, Sn and Cr are essential metals just for animals. Fe, for example, is indispensable for humans for oxygen transport, any shortage in this metal leads to anaemia. Zn is present in more than 100 enzymatic reactions in the human body. Co is an essential metal which is present in vitamin molecule B12, which is why B12 is also called cobalamin and it is needed for the brain and nerve systems to operate properly. However, these elements too become toxic when they exceed a maximum level of concentration (Sarkar, 2005).

Human bodies do not find it easy to metabolise heavy metals and sometimes are unable to. When heavy metals cannot be eliminated from the body, they concentrate in cells and tissues, preventing the cells' activity and harming DNA. Hg, Ni, Pb, As and Cd are the most toxic heavy metals, they play no role in the human body. Many research projects have proven that toxic metals have a harmful effect on all of the body's cells. As well as causing neurological, immunological and endocrine damage, they also harm the digestive and breathing apparatus (Lippmann, 2008).

In water systems metallic element toxicity for plants and microorganisms depends on the concentration, speciation and availability of each metal. Metal availability is limited when connected with sediments of small particles with many adsorption points. These sediments usually have large amounts of clay and organic matter. At the same time, in neutral or neutral alkaline pH waters, metal elements' precipitation is favoured, limiting its availability. The presence of anions created by insoluble salts in water also contributes to metal elements' immobility and, consequently, their toxicity is reduced. The formation of metal oxide in oxygenated places is another factor which limits metal availability (Merian, 1991).

3. Ways in which metals reach the environment

Metals' and metalloids' origin in the environment can be natural or anthropogenic. The substrate lithology is the most important natural source. The contribution of metals and metalloids as a result of rock erosion depends on each area's lithology composition. Al, Ba, Fe, Mn and V are the metals which reach surface and underground waters in the greatest quantities coming from the lithology (Garrett, 2000). Volcanic eruptions, too, are one of the most important natural sources of metallic elements, mostly affecting the atmosphere. Eruptions have considerable impact on the environment's metal and metalloid chemistry balance. As, Sb, Hg, Cu, Pb, Se and Zn are the metals emitted by volcanoes in the greatest quantities. Aerosols created by the sea are also natural sources of metals. Metals in aerosols can travel long distances due to the wind. Finally, particles of biological origin are another source of metals: for instance, pollen, bacteria and viruses from plants (Henley and Berger, 2013).

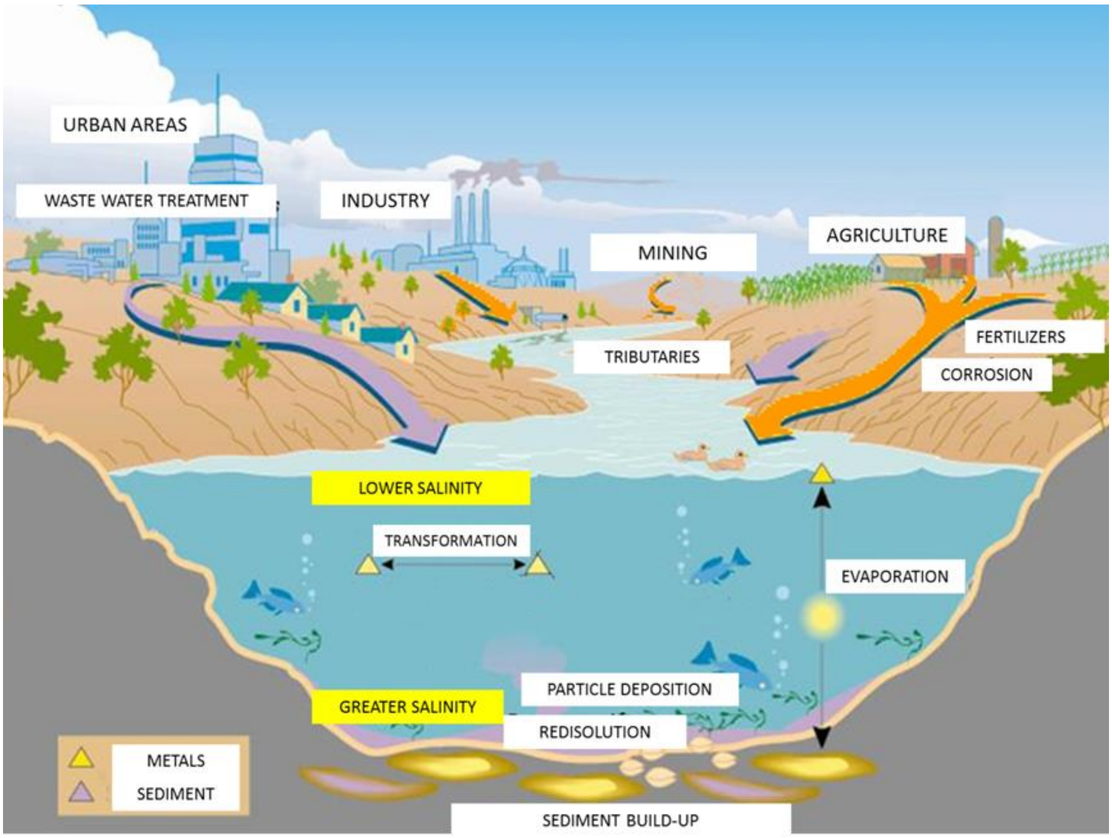

Figure 3. The main anthropogenic origins of metals affecting the environment.

The amount of metal and metalloids of anthropogenic origin in the environment has grown constantly since the industrial revolution. The ways of entry for metals of anthropogenic origin into the environment can be seen in Figure 3. Nowadays, the main anthropogenic sources are the followings (Xavier Domènech, 2006):

- **Mining:** One of the main sources of metals, above all As, Cd, Cu, Ni, Pb and Zn. These metals can be found in mines themselves or in mining-related activities.

- **Mineral smelting:** Many metals are emitted to the atmosphere during the process of extracting metal from minerals. During these processes metals such as As, Hg, Cd, Pb and Tl are emitted.

- **Metallurgy:** Metal particles are emitted to the atmosphere during the thermal processes. In addition to this, the solid waste material which is produced includes many metals, mostly Cr, Cu, Mn, Pb, Sb and Zn.

- **Other industrial activity:** Metal coating, chemical painting, plastic materials, catalysing, electronics and electrode industries also emit large quantities of metal, amongst others: Cu, Sn, Cr, Cd, Ni, Hg, Pt, As, Sb, Se, Mo and Zn.

- **Waste management:** Metals such as Cr, Cu, Mn, Ni, Pb and Zn are emitted to the environment during solid waste combustion, waste disposal area leaching processes and waste water treatment at water treatment plants.

- **Corrosion of metal structures:** Metals are also given off into the environment as a result of instability which unstable environmental conditions produce in materials. Fe, for instance, can be given off when construction materials corrode; Cu and Pb are given off by chimneys; Cr, Ni and Co by steel; Cd and Zn by steel materials coatings; Cr and Pb when paint degrades.

- **Farming and livestock farming:** As, Cu and Zn are used extensively as animal feed additives and, consequently, are to be found in livestock farming waste. The fertilisers and pesticides used in agriculture also contain As, Cu, Mn, Pb, Zn and other such metals.

- **Forestry management, logging and timber industry:** As, Cr and Cu are the main metals and metalloids emitted. They are used to protect wooden materials.

- **Fossil fuel combustion:** Large quantities of metal are given off into the atmosphere during fuel combustion, mostly Cd, Zn, As, Sb, Se, Cu, Mn, Ni, Cr and V.

Nowadays, large amounts of metal and metalloid of anthropogenic origin reach the environment due to contamination. Consequently, the metal concentrations which can be measured in environmental samples are considerably higher than those natural values (contamination). And sometimes the presence of metal in the environment can cause serious problems of toxicity for living organisms (pollution).

4. Metals and metalloids in the environment

Water, atmospheric and earth systems are amongst the most important in the environment. The quality of these three systems has a direct effect on human survival and quality of life. On the one hand, we need uncontaminated land to be able to grow food for human consumption. On the other hand, we have to keep rivers, lakes and underground aquifers clean in order to satisfy our water needs. Finally, we need air of at least minimum quality in order to breathe. It is important to keep the water, land and atmosphere as uncontaminated as possible. Any one of these systems being harmed can have different impact on different ecosystems. Metal contamination is one of these problems. After reaching the environment, metals and metalloids can undergo different physical-chemical processes depending on their geographical distribution and speciation. In order to be able to quantify metal pollution in the water, in soil and in the atmosphere, measurements of different types of metal and metalloid are indispensable. Being aware of metal's natural cycles is also very important in order to foresee the damage they can cause and, as far as possible, try to prevent it from happening (Merian, 1991). In the following section we will summarise the main characteristics of metal and metalloid behaviour in aquatic systems and the atmosphere.

4.1. Water systems

Obtaining quality water is very important for human beings. Polluted drinking waters can produce many diseases and this has had a clear influence on the population of many parts of the world. At present, this problem has still not been solved in many countries. In advanced countries, however, the distribution of quality drinking water has been ensured. So maintaining the quality of the natural water supply, avoiding chemical contamination, is an important subject in today's society (Manahan, 2007). There are organic and inorganic chemical contaminating agents, and heavy metals are the most important in the latter group. As, Cd, Cu, Cr, Fe, Hg, Mn, Mo, Ni, Pb, Se and Zn are the most important metal and metalloids which appear as a result of contaminating natural water (Merian, 1991).

The ways in which metal elements usually reach an aquatic system are the following:

- **Atmospheric deposition:** Dry or wet deposition of metallic elements of natural or anthropogenic origin in the atmosphere.
- **Leeching in rocks, the earth and waste materials:** Leeching of natural (depending on each place's lithology) or anthropogenic (mining, use of fertilisers, industrial activity, etc.) materials in the earth or on the earth's surface (Bradl, 2005).
- **Direct waste material spillage:** Waste materials from urban areas, livestock farming, farming and industry which are spilled straight into different water systems.

Metals and metalloids behave as ions dissolved in water, most of them bonding with other ions or molecules. Metals' stable state depends on the surrounding physical-chemical conditions. In order to reach that situation, metals go through numerous reactions and processes. These reactions and processes include redox reactions, adsorption into other water particles and desorption from particles, precipitation and dissolving processes (Benes and Steinnes, 1995). In each case, the series of processes and reactions which can take place depend on the metal's own characteristics and the physical-chemical properties of the surrounding mediums (Manahan, 2007; Xavier Domènech, 2006). In some water systems, particularly in estuaries, physical-chemical characteristics change considerably for short periods of time. Factors with the greatest influence include the total dissolved solids, salinity, temperature, pH, redox potential, the amount of suspended particles and dissolved oxygen (van Ryssen et al., 1999).

Water pH is a very important characteristic in defining metal elements' speciation. In acid neutral water, metals can be present as hydrated metallic cations or complexed by other complexing agents in solution. Complexing agents may be organic or inorganic. Complexed metals can be more easily adsorbed onto suspended solid particles as these can be of different sizes and types (Censi et al., 2006). The adsorption of complexes, however, happens more easily with clay particles or humic acids (Menicagli et al., 1988). Physical interaction strength between metals and humic acids decreases in the following order (Xavier Domènech, 2006):

$$Fe^{2+} > Pb^{2+} > Cr^{3+} > Hg^{2+} > Cd^{2+} > Cu^{2+} > Mn^{2+}$$

Depending on the size and density of the complexes formed by adsorption, metals can be dissolved or precipitate into the sediment due to flocculation process. In highly acidic water, metals can be replaced by H^+ in adsorption sites limiting the metallic flocculation. If the system's pH is acidified, the metal may be redissolved, sediment becoming a secondary source of metals (Xavier Domènech, 2006).

In water with higher pH, metallic cations tend to have higher precipitation as insoluble hydroxides. Many anions, carbonates, phosphates and sulphates, amongst others, encourage metal precipitation. In water with slightly higher pH than neutrality, the concentration of metal tends to be lower. Thus, the lowest Al concentration is around pH 6 and for Fe, Pb, Ni and Cd are, respectively, around pH 8, 10, 10.5 and 11. In alkaline water, on the other hand, negatively charged hydroxy complexes are considerable and then metal concentration increases (Al-Wabel et al., 2002).

Metal redox reactions are also significant in water systems. Several metals' mobility increases in anaerobic conditions. Fe and Mn, for instance, are present in the lowest oxidation states in reducing environments, forming dissolved metallic cations (Fe^{2+} and Mn^{2+}). At the same time, in water with more oxygen and environments which favour metal oxidation, metals are present in the highest oxidation states, forming insoluble oxides (Fe_2O_3 and MnO_2). Zn, Cd, Pb and Ag, for instance, form insoluble sulfides in anaerobic conditions by reducing of sulphates. In these last conditions metals are easily trapped and remain in sediment or in soil (Menzies, 2007; Schlieker et al., 2001).

Therefore, metals can be dissolved in the water system, they can be attached to particles in suspension or deep sediment depending on their own characteristics and environmental physical-chemical conditions. Deep sediments are important in aquatic systems (Ng et al., 1996). Transition metals such as Al, Mn, Fe, Cu, Zn and Ti, for instance, use to be present in sediments as oxides and aluminosilicates (Xavier Domènech, 2006). One of the main features of sediment is the distribution of the size of particles, in other words, texture. There is usually a greater concentration of metallic elements in the fine fraction of the sediment (<63 µm) (Forstner, 1989). There are usually more places for interaction between sediment and cations in fine particles than in large particles. Water systems surface sediments, which are mostly made up of small particles, have often been used to measure the contamination level (Forstner, 2004). As explained above, metals which are accumulated in sediments can be redissolved in desorption processes if physical-chemical conditions change (He et al., 2006). Both biological and chemical mechanisms can be behind this process (Pagnanelli et al., 2004). Salinity is a parameter which has considerable influence on the desorption process. On the one hand, in water with high salinity Na^+ and K^+ cations can replace metal adsorption sites in sediments. On the other hand, Cl^- anions can create soluble complexes with several metallic elements (Comans and Van Dijk, 1988; Paalman et al., 1994). Amongst other factors which favour the redissolution of metal from sediments are pH decreases, organic matter oxidation and the presence of complexing agents (Schlieker et al., 2001). Due to these factors, the mobility of the sediments metals could be affected by biological activity, the metals own properties, interaction between liquids and solids and water's physical-chemical properties.

4.2. The Atmosphere

The atmosphere is another important part of the environment for human beings. It is the layer which protects the earth and makes it possible to live on the earth. It is the source of CO_2, which plants need for photosynthesis, and of O_2, which humans need for breathing. It is another important factor in water's hydrological cycle, which transports water from the oceans to the land. Unfortunately, the atmosphere is also harmed by several chemical contaminating agents (Manahan, 2007). The World Health Organization (WHO) has blamed indoor and outdoor air pollution for the death of more than 2 million people (Gordon et al., 2004). The main contaminating agents which can be found in the atmosphere are particulate matter (PM), carbon monoxide, nitrogen and sulphur oxide, ozone and organic volatile compounds. Most deaths are caused by particulate matter which is smaller than 10 µm in diameter (Donaldson et al., 2000).

When talking about atmospheric contamination, it is worth differentiating between emission and inmission. The concentration of chemical contaminating agents emitted into the atmosphere by a source of contamination is known as emission and is measured at the contaminating agent's point of outlet. On the other hand, inmission is the chemical concentration in a particular atmosphere. The concentration values given to these two terms are usually completely different. Inmission values are not only affected by emission values. They are also affected by all the physical-chemical processes which suffer the contaminating agents during atmospheric transport and by the climatologic conditions of the area (Orozco et al., 2003). Climatic factors are variable and, depending on them, the damage caused by pollution is less or more serious. Atmospheric chemical contaminating agents can be transformed by many processes and different species of higher or lower toxicity can be created (Junior et al., 2013). Furthermore, differences in inmision values can be identified even in two places located at the same distance from a source of contamination due to orographic differences (Bacardit and Camarero, 2010). With regards to the chemical reactions which a contaminating agent may undergo in the atmosphere, it must be taken into account that the atmosphere is an oxidant environment. Radiation from the sun is another important factor. There are many different types of chemical reaction mechanisms which take place in the atmosphere and, consequently, many new species of contaminating agents can be created too (Meraz et al., 2015).

Metals and metalloids mainly occur as particles or attached to particles in the atmosphere. Only a very few, mercury, for instance, are found in gaseous form. Particulate matter, mixed with small drops of liquid, make up atmospheric aerosol, which moves throughout the atmosphere. The symbol PM is used when talking about particulate matter. The numbers next to the PM symbol show particles' diameter (in µm). In general, fine particles ($<PM_{2.5}$) and coarse particles ($>PM_{2.5}$) are differentiated. PMT is used for the total amount of particles. First level particles are those which are emitted directly into the atmosphere while second level particles are those created as the result of reactions and processes during transport in the atmosphere (Orozco et al., 2003).

The damage which metallic particles of the atmosphere can cause depends on the particles' size and composition (Pirani et al., 2015). Metals in particles can be found in atoms, ions or molecules (salts or oxide). There are many metallic oxide particles (Elom et al., 2014; Liang et al., 2015). Most of them are the result of fossil fuel combustion. As an example, when pyrite (FeS_2) containing coal is burned Fe_3O_4 is emitted to the atmosphere. Organic vanadium, which is used as a fuel supplement, becomes vanadium oxide (V_2O_5) during combustion processes. During the combustion of carbon and other fossil fuels, many Al, Si, Ca, Fe, Na, Mg and K elements oxide particles are created. Even smaller As, Sb, Se, Cd, Pb, Zn (in large quantities) and Ba, Be, Cr, Sn, Ni, Sr, U, V (in small quantities) element-rich particles (Coles et al., 1979). Hg is also emitted when carbon is burned, mostly as a gas. Pb, which is used in painting and as an antiknock agent in petrol, is also emitted into the atmosphere in particulate matter (Berg et al., 1995). As a last example, particles rich in Na are formed in sea aerosols (Schill et al., 2015).

The size of particles, as mentioned above, is another important factor in addition to the particle chemical composition regarding the damages which particles can produce. Size also affects the time which particles remain in the atmosphere. Particles can have different geometrical shapes, but when we refer to size in this context, all particles are spherical. The size of particles varies from 10^{-3} to 10^{3} µm (Orozco et al., 2003) and can be classified in three groups:

- **Aitken particles or fine particles:** 0.1 µm or smaller in diameter.
- **Medium particles:** These exist in suspension in the atmosphere and their size is between 0.1 and 10 µm.
- **Large particles:** Their diameter is greater than 10 µm.

Fine particles move through the atmosphere randomly. They often collide and, consequently, clots are formed. Large particles, on the other hand, last for short periods in the atmosphere and often form clots (Ervens, 2015; Wallace et al., 2013). The particles which spend longest in the atmosphere are medium particles, between 0.1 and 10 µm. They are classified as $PM_{2.5}$ and PM_{10}. $PM_{2.5}$ particles are more harmful due to their smaller size (Heo et al., 2015).

Suspended particulate matter in the atmosphere, after longer or shorter periods, end up falling onto the earth or into the water system. Fine particles clot before falling and large particles fall directly. There are two types of particle deposition:

- **Dry particle deposition:** particles fall as a result of the earth's gravity, either directly or after hitting something in their path. 20% of particles in the atmosphere are eliminated due to this type of deposition (Sabin et al., 2006).

- **Wet deposition:** helped by rain, hail or snow. This type of deposition is the most common (Injuk and Van Grieken, 1995).

5. Analysis of metals and metalloids in environmental samples

As stated above, it is important to monitor metallic contamination in different environmental compartments. In order to do so, and after designing an appropriate monitoring programme, samples must be collected and analysed. Collecting samples, too, must be done properly, making sure that representative samples are taken. Collecting samples, in fact, is one of the most important stages of the analytical process. Mistakes made in the sampling are reflected in the final results and, consequently, the diagnosis of the metal contamination under studied area may be wrong. After sampling all the samples must be carried to the laboratory and then analysed. Samples transport and storage must be correct, in order to avoid contamination, transformation or losing. As far as the treatment of samples in concerned, acidification, filtration, water elimination, the fractioning of different sizes and/or leaching with appropriate liquids must be carried out (Jones, 1995). Finally, analytical detection and quantification are usually carried out using an instrumental technique, the most frequently used techniques being voltammetry, atomic absorption/emission and mass spectrometry (Van Loon, 1985). The main analytical techniques used for metal analysis in aquatic systems and the atmosphere are explained in the following sections.

5.1. Sampling and sample treatment

5.1.1. Water systems: water and sediments

Natural water forms highly dynamic systems. In addition to surface water, other types of water include rain, fresh water (in streams, lakes and rivers), salt water (in the sea) and transitional water (in estuaries). Both microorganisms and organic and inorganic materials can be found in solution and in suspension in water. All of these must be taken into account when designing an appropriate sampling process (EPA, 2007a). Throughout the process of sampling, and afterwards, there may be changes in the samples' composition and/or characteristics, thus losing their original representability. There may be losses of analytes due to precipitation, complexation and adsorption processes and they may also get adsorbed onto containers walls. Either single or composite samples can be collected. Passive sampling is another option (Jones, 1995).

As mentioned above, samples may cease to be representative if they are contaminated. Positive contamination (when samples' concentration is increased), negative contamination (when the concentration decreases) and pseudo contamination (when other compound not being the analyte is added) can all take place (Gasparon, 1998; Van Loon, 1985). Positive and negative contaminations are the most common risks. Positive contamination is the most frequent type. The organic matter present in water can also cause problems complexing or adsorbing the analyte.

The characteristics of the containers used for water samples are also very important. For analysing metal elements in water the most appropriate containers are plastic, and specially polytetrafluoroethylene (Teflon). Borosilicate containers can release Si and Na into samples. Metal can be adsorbed into these container's walls producing negative contamination. Polypropylene containers are cheaper than glass or Teflon ones, and glass containers are fragile (Gasparon, 1998). Teflon caps are also preferred.

Cleaning of materials to be used during analysis should also be taken into account. Metal impurities can cause positive contamination in Teflon containers during their production and supplements used as stabilizers can also cause negative contamination or pseudo contamination. Because of this, plastic container, and, in general, any type of material or container, should be cleaned in acid baths (HNO_3 1 M) before use (Martínez, 2005).

In order to maintain the speciation of the analytes in the sample and minimise negative contamination, acidifying samples using sub-boiled HNO_3 (pH 1-2) is highly recommended. Precipitation of analytes and their adsorption to container walls is avoided in this way. Furthermore, in acidic conditions micro-organisms' biological activity is stopped too (Liess and Schulz, 2000). Before acidifying, the water sample is filtered through a 0.45 µm diameter filter, which separates dissolved metal fraction and metal fractions in particulate matter (Gasparon, 1998). After collecting the sample and pretreating it, analysis of the metallic elements should be carried out as quickly as possible. The water sample is usually kept at 4ºC and in the dark until analysis (Martínez, 2005; Van Loon, 1985).

Sediments have also often been used in order to study metallic contamination in aquatic systems (Gredilla et al., 2013; Venkatramanan et al., 2015). Surface sediments (up to 4 cm) provide information about recent contamination. By analysing surface sediments in a particular geographical area, the spatial distribution of contamination in that area can be examined. At the same time, if the past register for the metallic element is to be studied, deep cores of sediments have to be taken. The concentrations from deeper samples of sediment cores provide reliable background values (Alemayehu et al., 2014; Camarero et al., 1998). When it is possible (when the sediment is exposed to the air and visible), samples can be collected by hand using appropriate gloves and plastic spatulas. When there is a layer of water over the sediment, dredges or similar equipment must be used, normally using a boat. There are many types of dredges, the most commonly used ones being Birge-Ekman, Ponar and Shipek (these are appropriate when there is not much current, above all in ports and lakes), and Van Veen (when there is a strong current) (Figure 4). To collect sediment columns, on the other hand, empty plastic tubes of different diameters and lengths are used. On its way down into the water it allows the water to go through the tube and they have a lid at the top, which is open. Once the tube is submerged in the sediment, the lid is closed and the tube, now full of sediment, is pulled up using a piece of string or rope. Amongst the tools used for collect sediment cores as samples, the most commonly used are manual tools, gravity tools, box-like tools, piston and vibration tools (Figure 5). Sediments in direct contact with sampling tools must not be taken in order to avoid contamination and sediment must be manipulated as little as possible. When more than one sample has to be taken, the tools to be used should be cleaned using the water in the surrounding area (Mudroch and Azcue, 1995).

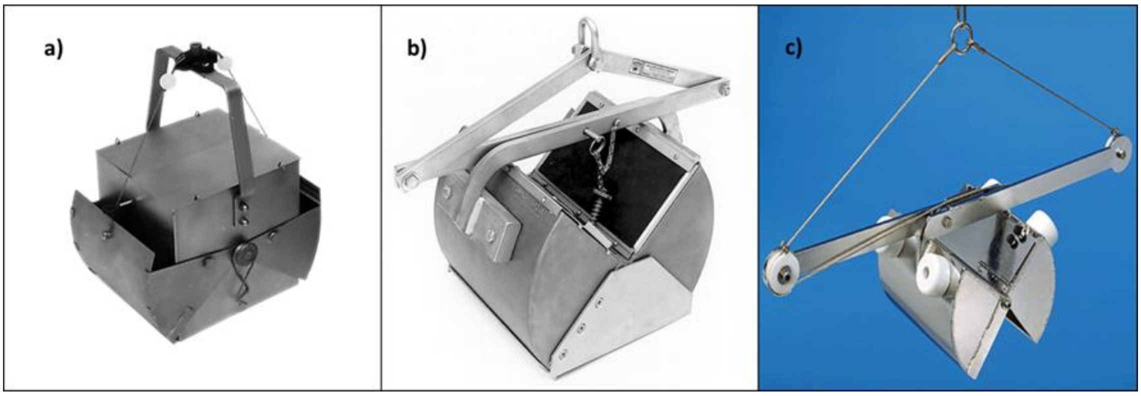

Figure 4. a) Birge-Ekman dredge, b) Ponar dredge and c) Van Veen dredge.

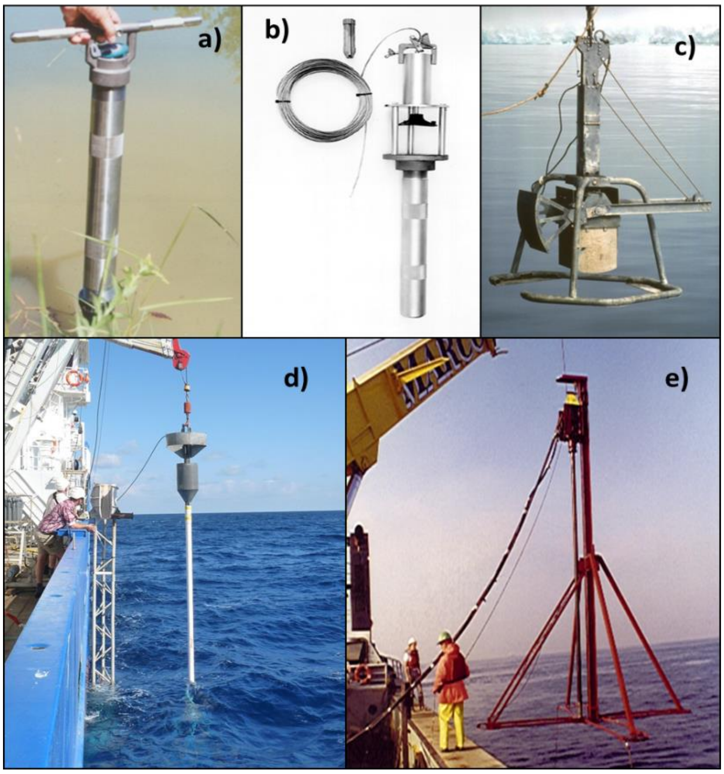

Figure 5. Tools for collecting sediment cores: a) hand tool, b) Kajak-Brinkhurst gravity tool, c) box-like tool, d) piston-type tools and e) vibration tool.

Positive or negative contamination can take place while samples of sediment are being transported to the laboratory. The use of metal containers must be avoided. Teflon is recommended, but polypropylene, polyethylene and borosilicate containers can also be used. With sediments, cleaning the containers is not as important as it is when analysing water because the concentration of metal is far higher. Teflon material which is going to be in contact with sediments, for instance, must first be cleaned using water and no phosphates soap. After removing the remains of the soap with water, it is kept in an acid bath (HNO_3 6M) for 72 hours. Then it is cleaned using distilled water and, finally, dried in a particle-free atmosphere (Van Loon, 1985).

95% of the surface sediments is water. So the first thing to do in the laboratory is to dry the sediment's humidity off in a clean atmosphere, in an oven or by freeze-drying. The drying process in air is longer and there is greater risk of the sample being contaminated. But it is the simplest and cheapest method. Oven dried sediments are put in the oven at 100-110ºC for the required time. If there are volatile metallic elements amongst the analytes, lower temperatures must be used to avoid losses (Tuncel et al., 2007). When freeze-drying is used, the sediments must first be frozen and then they are kept at between -40 and -50ºC and 0.01 and 0.05 Torr for the time needed for the water to be eliminated by sublimation. Depending on the characteristics and quantity of the sample, freeze-drying may take between 24 hours and 14 days. With freeze-drying, volatile metallic element loss is minimised as the process is carried out at a very low temperature. This also prevents aggregation between particles. The cost of freeze-drying, both in energy consumption and in the tools required, is greater than that of other methods used for drying samples (Mudroch and Azcue, 1995).

Once the sediment has been dried, the sample can be divided into different fractions by sieving. Usually metals and metalloids are measured in fractions of less than 63 µm. This fraction, with greater surface density, is made up mostly of mud and clay (Luoma et al., 1990). After homogenising the dry fraction to be analysed, it must be stored correctly way until analysis, trying to prevent transformations, oxidations and volatile elements losing due to micro-organisms activity. Ideally, samples should be kept in completely closed containers to avoid contact with oxygen. The most important parameters for storing sediments are temperature and time. At low temperatures biodegradation and the loss of volatile elements are minimised. On the other hand, the sooner the analysis is carried out after the sampling, the more reliable the results obtained will be (McManus et al., 1985).

With regards to sediments treatment, if the analytes' total concentration is to be measured, then the sample must be totally dissolved using hydrofluoric acid or fusion techniques. For samples fusion with lithium metaborate or alkaline metal hydroxides mixed with sodium peroxide are used (Mudroch and Azcue, 1995). This technique, however, is seldom used because solutions with a lot of mineral salt are obtained, which increases the possibility of interference in the detection process. The basic component of the sediment, silicate, can also be decomposed using hydrofluoric acid, making it possible to measure the total concentration of metal in the sample (Scancar et al., 2000). Sometimes mixtures of acids, which make it possible to oxidise the sulphides and organic matter present in the sample, are used to extract metals from sediments. These mixtures include nitric acid and perchloric acid (with hydrofluoric acid or without) and nitric acid/hydrofluoric acid. At the same time, when adsorbed metals, metals bonded to carbonates or sulphides and metals precipitated as oxides or hydroxides are to be analysed (acid fraction or pseudototal) mixtures of acids which allow sediment oxidation are used (Ongeri et al., 2014), mostly *aqua regia*, a mixture of hydrochloric acid and nitric acid (1:3). Nowadays the method most used to extract metals from sediments, EPA 3051 A, involve samples being heated in *aqua regia* in a microwave oven (EPA, 2007b). In this way the amount of metals and metalloids recovered is a higher fraction than that available for plants or microorganisms. Sequential extraction of sediments is also carried out. In order to perform sequential extractions, mixtures of weak acids, acetic acid and EDTA are used in subsequent extractions, which allow metal speciation analysis. The best known and most widely used methods are those proposed by the BCR and Tessier (Rauret et al., 1999; Tessier et al., 1979).

5.1.2. Biomonitoring metallic contamination in air

Contamination in the atmosphere can be monitored in different ways, using passive, active, automatic or sensor samplers. In passive sampling, analytes are directly absorbed or adsorbed on solid supports. Sampling can last from few hours to several years (Tuduri et al., 2012). In active sampling, on the other hand, the atmospheric air to be analysed goes through a filter (to separate the particles in suspension) using electric energy and, then, through a solid support on a column (in order to attach the metals which may be present in the gaseous phase). With this method daily averages can be measured. Active sampling is appropriate for gases and particles (Mathe et al., 1998). Automatic sampling is the most expensive method, but it also provides the best results. Automatic sampling allows reliable analysis of many chemical contaminating agents in a short period of time. Sensor-based sampling is based on spectrometer technique (Roeva et al., 2015).

The most used method to study atmospheric contamination is the active sampling. Recently, passive sampling has also become more widely used, using artificial tools (dust fall filters (DFF) (Beamer et al., 2014), for instance) or sentinel organisms. Using sentinel organisms is cheap and reliable. Many types of vegetation have been used to investigate metallic element deposition, accumulation and distribution, amongst others lichens (Marques et al., 2004), moss (Fernandez et al., 2002) and bark and leaves of trees (Amores and Santamaria, 2003). Vegetation is affected by changes in environmental physical-chemical characteristics, including atmospheric characteristics and chemical composition.

Biomonitoring is the name given to study biosphere contamination and quality using living organisms. There are several advantages of this technique: in most environments it is possible to find a biological organism which is in contact with its surroundings; in most cases it is easy to take these kinds of samples; and, compared with other techniques, biomonitoring is cheap (Wolterbeek, 2003). Biomonitoring can be direct or indirect. In the former, the concentration of contaminating agents accumulated on the organisms (bioaccumulator) is measured. In the latter, the changes in living organisms' state brought about by the presence of chemical contaminating agents are quantified (Markert et al., 1997). In direct biomonitoring, work can be carried out using bioaccumulators which grow in the study area or control organisms which have grown in a clean environment and are transplanted to the study area. For instance, uncontaminated moss or lichens can be transplanted to a contaminated area, controlling the time during which bioaccumulators are exposed (Ceburnis and Valiulis, 1999).

The organisms most commonly used to study atmospheric contamination are plants (Balasooriya et al., 2009). Plants spend their whole vegetation cycle in a single place and, compared with some other organisms, and in general, they are more sensitive to contamination and reflect specific places' characteristics better (Nali and Lorenzini, 2007). Thus, vascular and non-vascular vegetation is most commonly used world-wide for biomonitoring atmospheric metallic contamination. Several factors can affect the metallic accumulation ability of the plants such as climate, availability of metals, the vegetation's own characteristics, state of health and type of reproduction, and the characteristics of the land on which the vegetation in question grows (Conti and Cecchetti, 2001).

5.1.2.1. Moss

Moss has been used as sentinel organism to analyse air quality since 1960 (Carlberg et al., 1983; Rühling and Tyler, 1968). Moss is a non-vascular plat classified in the land plant division *Bryophyta*. One of its main characteristics is the lack of a developed cuticle, in other words, there is no external wax tissue for protecting the plant's surface. In addition, it does not have a developed root system and takes the nutrition and minerals it needs from the atmosphere (Holoubek et al., 2000). Chemical atmospheric contaminants as metals which are in water solution, in gaseous phase or attached to particles can be accumulated in moss. The three most common accumulation mechanisms are: i) particles which accumulate on the surface of moss, ii) particles which go through moss wall cells due to ion exchange, and iii) contaminating agents which accumulate due to metabolically controlled mechanisms (Poikolainen, 2004). The ability of moss to accumulate metallic elements dropes in this order: Cu > Pb > Ni > Co > Cd > Zn, Mn (Rosman et al., 1998; Szczepaniak and Biziuk, 2003). The species of moss most used for biomonitoring in Europe are: *Hypnum cupressiforme*, *Hylocomium splendens*, and *Pleurozium schreberi* (Figure 6), and they are plentiful all over Europe (Onianwa, 2001).

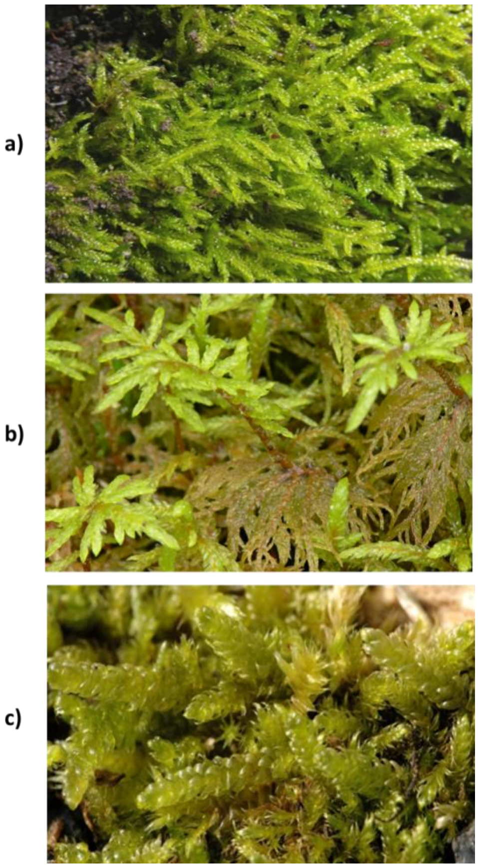

Figure 6. a) *Hypnum cupressiforme*, b) *Hylocomium splendens* and c) *Pleurozium schreberi* moss.

In order to carry out properly the moss sampling, several general norms must be followed (UNECE, 2010). First of all, the moss must be 3 metres away from the leaves of surrounding foliage. In woods where this is not possible, moss must be collected as far as possible from the foliage’s' leaves. Moss must be far from urban areas and at least 300 metres away from motorways, towns and industries and at least 100 metres away from any roads with less traffic. For analysing metallic elements, collecting 5 sub-samples from 50 x 50 m sample areas is recommended. Moss must be taken from dead tree stumps in order to avoid possible contamination from the particles of soil beneath it. Talcum-free latex gloves must be used. Samples must be collected in the shortest possible time in order to avoid changes in the amount of metallic elements which may be caused by climate changes. Moss must be transported to the laboratory in paper bags (UNECE, 2010). Once in the laboratory, samples must be cleaned as quickly as possible in order to eliminate any material and other dirt which may be on them. Nitrogen enriched air can be used for cleaning because distilled water or weak acid may influence the extraction of metallic elements accumulated in the moss (Gonzalez-Miqueo et al., 2010). Green parts of the moss must be taken to analyse the metallic elements of the last 2-3 years. After separating the moss's green parts and cleaning them, the samples must be dried in a constant temperature of 40ºC (Gonzalez-Miqueo et al., 2009). Freeze-drying, too, is often used in general with vegetation. After drying, moss samples must be homogenised and kept in polypropylene containers until analysis. Moss can be sieved in order to obtain homogeneous samples and the contamination must be avoided. Because of that, moss samples are often ground without being sieved, aluminium oxide material being used for grinding (Van Loon, 1985). When the moss has been dried and homogenised, dry incineration, wet incineration or acid extraction can be used to extract the metals and metalloids in liquid samples. Dry combustion must be done at the lowest possible temperature. Usually this is between 450 and 500 Cº. But this method can lead to losses of As, Se and Hg (Lalor et al., 2003). The technique most used in wet combustion is acid digestion in a microwave oven, using mixtures of hydrochloric and nitric acid, sometimes with hydrogen peroxide (Fernandez et al., 2002; Gerdol et al., 2001; Gonzalez-Miqueo et al., 2009; Gonzalez-Miqueo et al., 2010).

5.1.2.2. Lichens

Lichen is a symbiotic association between an alga (photobiont) and a fungus (mycobiont) (Augusto et al., 2010). Lichen does not have leaves, flowers or roots. Like moss, lichen takes its nutrients straight from the atmosphere. Lichen can be found in most ecosystems around the world and is able to withstand extreme climatic conditions, for instance long droughts. Due to its resistance and strength it can be found in many areas. Lichen is a long life organism and, being sensitive to chemical contaminants, has often been used as biomonitor (Conti and Cecchetti, 2001). The main ways in which chemical contaminating agents go into lichen are the same as those mentioned above for moss (Szczepaniak and Biziuk, 2003). The species most used in biomonitoring is leaf-appearance lichen (*Parmelia sulcata*, for example) and bush-form lichen (*Usnea filipendula*, for example) (Figure 7).

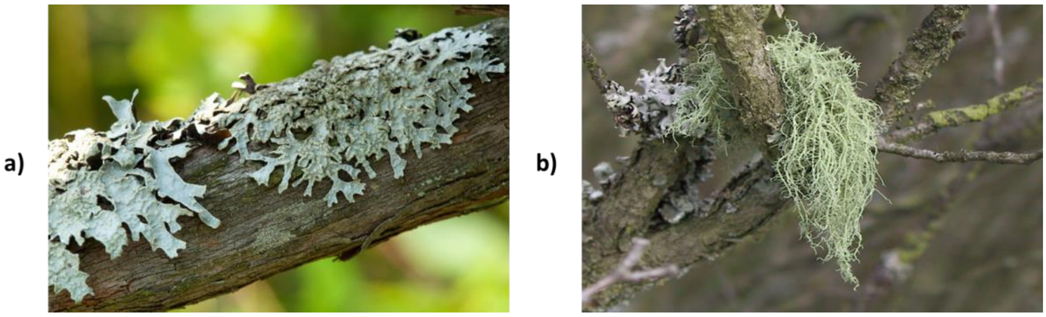

Figure 7. a) *Parmelia sulcata* and b) *Usnea filipendula* lichen.

Lichen sampling as well, has some norms to be followed (Comisión Económica para Europa de Naciones Unidas, 2008). Lichens must be collected in areas which are at great distance away from urban areas and must be collected from tree trunks which are at least 100 metres away from any type of road. One hectare sampling area must be chosen as maximum and lichen samples must be taken from at least 5 different trees. The trees which the lichen is on also have to fulfil with certain criteria. Firstly, they have to be alive and in good condition. The tree trunks must have between 20 and 40 centimetres in diameter and between 60 and 120 centimetres in perimeter, in this way it is possible to check that the sampled lichens are of similar age. The inclination of the trees from which the lichen is taken must be 20º as maximum. Finally, lichen must be taken from a height of between 1 and 2 metres. To collect lichen, a stainless steel scalpel must be used. Throughout the collection of samples talcum powder free latex gloves must be worn, in order to avoid contamination. The lichen must be transported to the laboratory in hermetically sealed polypropylene bags at a recommended temperature of -18ºC. Once in the laboratory, the lichen must be hand-cleaned as soon as possible in order to remove dead material and any other dirt. Freeze-drying is the technique most commonly used for drying lichen. Once dried, lichen has to be homogenized and kept in polypropylene containers. The dry sample can be ground in order to obtain homogeneous lichen, using aluminium oxide material for grinding. When the lichen is ready for analysis, it should be kept in polypropylene containers and at -18ºC until analysis. As with moss, dry or humid combustion can be used to extract metallic elements (Van Loon, 1985). But the most used extraction method is the digestion with a microwave oven, using mixtures of nitric acid and hydrochloric acid and, sometimes, hydrogen peroxide (Adamo et al., 2002; Bajpai et al., 2010; Conti and Cecchetti, 2001; Klos et al., 2011; Poikolainen, 2004).

5.1.2.3. Tree leaves

Since 1950 vascular plant leaves have been used as bioaccumulators. Leaves' most important characteristic in terms of biomonitoring metallic elements in the atmosphere is their morphology and size (Manes et al., 2002). In addition to leaves' characteristics, the solubility, size and linkage of the particles which have fallen onto the leaves are also important (Mankovska et al., 2004). Climatic characteristics can also have a direct effect on metallic elements' ability to accumulate on leaves (Harrision and Chirgawi, 1989). Pine leaves are suitable bioindicators of Fe, Al and Pb (Ataabadi et al., 2010; Oliva and Mingorance, 2006). Beech leaves, on the other hand, have also been used for monitoring heavy metals in the atmosphere (Amores and Santamaria, 2003).

Various factors must be taken into account with regards to tree leaf sampling if they are to be representative and if metallic element concentration of different sample points is to be comparable (Martin and Coughtrey, 1982), including:

- **Species of trees and types of trees:** It must be the same tree species at all sample points. There may be considerable differences between the amount of particles stuck to the leaves of different species of trees. Likewise, the trees to be sampled must have fully developed leaves. Trees which have illnesses or rotten branches must be avoided. Trees which are protected by other trees' foliage must also be avoided.

- **Sampling season:** Samples should be taken between mid-summer and February or March, those being the periods of the year in which trees are subject to greatest stress and metallic element nourishment is at its lowest concentration on leaves.

- **Types of leaves:** The leaves to be sampled must be between 1.5 and 2.5 metres above from the ground and taken from leaves as far as possible from the tree trunks. If it is possible, leaves which are in contact with the air and solar radiation must be sampled. Leaves must be taken from at least two branches in difference positions. The leaves must be taken from their stems and must be healthy. Mature, fully-developed leaves must be taken. Taking leaves of different ages should be avoided. Each sample should have between 50 and 100 leaves.

To collect the leaves, talcum powder-free latex gloves should be worn and the leaves must be kept in paper bags. Leaves must be stored in a dry place as possible and transported in an uncontaminated refrigerator (Tyler and Olsson, 2006). Once the leaves are in the laboratory, external dirt must be cleaned off them using nitrogen enriched air or distilled water. The clean leaves must be dried in an oven at 40ºC for the time required or using freeze-drying (Machava and Barna, 2005). Dry samples may be ground using aluminium oxide materials in order to obtain homogeneous leaves (Van Loon, 1985). The samples should be kept in polypropylene containers until analysis. The most commonly used techniques for extracting metallic elements from leaves are the same for moss and lichen: dry incineration and wet combustion or acid digestion using a microwave oven (Amores and Santamaria, 2003; Mankovska, 1998).

5.2. Detection and quantification of metals and metalloids in water samples and extracts of solid samples

Water samples or extracts of solid samples are liquid mediums to be analysed. The concentrations of the metals in water samples or in environmental solid samples are usually be very low. The elements which show these low concentrations are called trace elements, concretely and following to the definition to those elements which show concentrations below 100 $mg \cdot Kg^{-1}$ (McNaught and Wilkinson, 1997). Therefore, those low concentrations have to be measured with highly sensitive techniques with the aim to detect and quantify them with a confidence level.

The first optical technique used to measure metallic concentrations was the molecular spectrophotometry (Lobinski and Marczenko, 1992). However, due to the high detection limits of this technique, it cannot be used for trace elements analysis.

Electrochemical techniques can be used as well for metal analysis. Potentiometric methods based on selective electrodes show high detection limits and therefore it is not an appropriate detection and quantification technique for trace elements analysis. Stripping voltammetry allows the analysis of the total and labile metallic concentration. One of the advantages of stripping voltammetry techniques is that it allows the preconcentration of the analytes. The metals accumulate in the electrode by means of a faraday or non-faraday process. Afterwards, the stripping process produce the analytical signal (Brainina et al., 2000). A mercury electrode is used in the anodic stripping voltammetry (ASV) which shows high sensitivity for some metallic elements (Ag, Cd, Cu, Pb, Sb and Zn) (Kumar et al., 2006). Cathodic stripping voltammetry (CSV) allows the analysis of more metals (Al, Co, Cr, Fe, Ni and Se) at ppt (ng·Kg^{-1}) levels (Shahbazi et al., 2015).

On the other hand, the detection limits of atomic spectrometry techniques allow trace elements analysis. In these atomic techniques the sample is ionized or atomized at very high temperatures. In Atomic Absorption Spectroscopy (AAS), the ionized elements absorb the radiation at characteristic wavelengths for the elements and at proportional intensities to the concentration of the element. For the samples vaporization AAS techniques can use a flame (Flame Atomic Absorption Spectrometry (FAAS) (Ju et al., 2015)), a quartz furnace (quartz furnace Atomic Absorption Spectrometry (QFAAS) (Hranicek et al., 2010)), or a graphite furnace (graphite furnace Atomic Absorption Spectrometry (GFAAS) (Anal, 2014)).

Following with spectrometric techniques, Atomic Emission Spectrometry (AES) can be used for the analysis of metals. In AES high temperatures are used for the metallic atomization or ionization. When metal ions return to its origin energetic state, they emit photons at characteristic wavelengths for the elements and at proportional intensities to the concentration of the element (Lobinski and Marczenko, 1996). Atomic Emission Spectrometry techniques are classified depending the source used for the metallic ionization as flame (flame Atomic Emission Spectrometry (FAES) (Raposo et al., 2015)), or plasma. There are three ways to produce the plasma: Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Zhao et al., 2015), Direct Current Plasma Atomic Emission Spectrometry (DCP-AES) (Arnold et al., 2006) and Microwave Induced Plasma Atomic Emission Spectrometry (MIP-AES) (Matsumoto and Nakahara, 2004). Atomic Emission techniques allow simultaneous multielemental analysis.

Fluorescence techniques are also used for the detection and quantification of metals which are in liquid mediums. The elements molecules or atoms absorb energy and after absorbing it, they return to its origin energy state emitting energy at the same wavelength (resonance fluorescence) or larger (Stokes fluorescence). The intensity of the emitted energy (fluorescence) is measured. The fluorescence intensity is proportional to the element concentration in the sample and to the radiation intensity used for the sample excitement (Lobinski and Marczenko, 1996). Fluorescence techniques are classified in three different techniques depending on if atoms or molecules are determined and depending on the spectral range used: molecular fluorescence spectrometry (Spectrofluorometry) (Luster et al., 1996), Atomic Fluorescence Spectrometry (AFS) (Frentiu et al., 2015) and X-ray Fluorescence spectrometry (XRF), used for solid samples analysis (McComb et al., 2014).

Mass spectrometry techniques can be used as well in the analysis of metals. In these techniques ions produced at very high temperatures are measured or counted. Ions characteristic of each isotopes are identified by means of its mass-charge ratio. The ions quantity is proportional to the concentration of each isotope. Typical mass spectrometers have an ions source, a mass-charge ratio depending ion spreading mass analyser and a signal reader (Lobinski and Marczenko, 1996). Mass spectrometer techniques are classified according to the ions source: Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (Ni et al., 2015), Thermal Ionization Mass Spectrometer (TIMS) (Li et al., 2015) and Microwave Induced Plasma Mass Spectrometer (MIP-MS) (Chatterjee et al., 2001). ICP-MS is the most used technique for environmental samples metallic analysis due to its very low detection limits and simultaneous multielemental analysis (Brown and Milton, 2005).

Activation Spectrometry can be used as well for the detection and quantification of metallic elements in solid samples. In this technique the radiation emitted by a stable analyte nuclide which receives a neutron or charged particle is measured. The energy of the emitted radiation is characteristic of the nuclide and the intensity is proportional to its concentration in the sample (Parry, 1993). The Activation Spectrometry most used is the Neutron Activation Analysis (NAA) (Aksoy et al., 1998).

6. Monitoring metallic contamination in aquatic systems: European legislation

The awareness of water protection, defend and management comes from the last XXth century decade. In 1998 in a Ministerial Seminar held in Frankfurt, the European Council asked the European Commission to draw up guidelines on improving the ecological quality of water bodies. There already existed 30 different pieces of legislation regarding water quality, but leading member states, aware of gaps and limited effectiveness of existing legislation, began to consider drawing up an unified directive that would integrate all these different bodies of legislation (Henocque and Andral, 2003). In 1995 the European Environmental Agency issued a report emphasising the need for the protection of Community waters in terms of quality and quantity. In 1999 all previous requests were set forth in the European Parliament and, finally, in 2000 the European Water Framework Directive (EWFD) was drawn up and adopted (WFD, 2000).

In compliance with this legislation, all Member States of the European Union must implement the Directive and ensure the Good Ecological Status, GES, of their waters (including territorial waters, transitional waters, coastal and groundwaters) by the year 2015. A body of water achieves Good Ecological Status when the relevant parameters (physical and chemical, hydromorphological and biological) that ensure good biological quality of water show low levels of user induced alterations. That is, good ecological status is achieved when those values differ very little from those of water which has not suffered any kind of alteration. The Directive sets out another important objective which is the complete eradication or progressive reduction of hazardous substances, achieving concentrations near background values for naturally occurring substances but near zero values for man-made synthetic substances. Therefore, each mass body must be deeply analysed, researching its characteristics, assessing anthropogenic influences and making economic analysis. An important step to carry out this kind of analysis is the monitoring of water bodies quality.

EFWD says that first of all the water body which is analysing has to be characterised, defining its physic-chemical, hydromorphologic and biological parameters (ICES, 2006). Afterwards a chemical analysis has to be carried out following an appropriate monitoring program. In order to specify the water body ecologic state, Ecological Quality Ratios (EQR) must be calculated. EQRs are achieved by dividing the observed parameter values for the body of water in question by the reference values (Environmental Quality Standards, EQS) for the zone (ECOTAST, 2003).

The Directive says that a suitable monitoring programme would include the following three levels: i) surveillance monitoring, ii) operational monitoring, and iii) investigative monitoring (Ferreira et al., 2007). Each monitoring programme has its characteristics. Surveillance monitoring is to be carried out in order to assess long-term changes resulting from human activity. Operational monitoring shall be carried out when a body of water is identified as being at risk of failing to meet the objectives of the Directive.

Investigative monitoring is to be carried out only in special cases (to ascertain the impact of point source pollution and/or where the reasons for exceedances of environmental quality standards for a particular area are not known). The main aim of water bodies monitoring is to define the ecological state in the time and in an area.

Priority substances are identified in the Directive regarding the chemical parameters monitoring. Apart of some organic compounds, some metals and its compounds are mentioned (Pb, Ni, Cd, Hg and Sn (tributyltin)). Furthermore, Cd, Hg and Sn are classified as hazardous substances in the Directive (UE, 2013).

On the other hand, the Directive just refers very few times to sediments and biota compartments and they are so important in water bodies (Borja et al., 2004; Crane, 2003). EQS values just were developed for waters. The water ecological state identification needs the analysis of above mentioned sediments and biota compartments as well (Heiskanen et al., 2004; Kimbrough et al., 2008). In fact, many times sediments and biota have been deeply analysed because they give relevant information about some specific contaminants (Balkis and Aksu, 2012; Bastami et al., 2015; Marengoni et al., 2013). Sediments are indicators of chemical pollution as they are able to accumulate organic and inorganic contaminants in long time. Sediments are more stable than waters and for this reason they give more reliable information in a monitoring program (Lasheen and Ammar, 2014; Namiesnik and Rabajczyk, 2010; Witters, 1998). Nevertheless, the EWFD does not define EQS values for sediments. As reference natural, background values are used. Based on those background values, a contamination level of an area can be estimated using the geoaccumulation indexes (Mueller, 1981). Sediment Quality Guidelines (SQG) reference values obtained from toxicological tests are also used to determine an area contamination level (Wenning and Batley, 2002).

With the intention to fulfil the EWFD aims, in the Basque Country has been carried out some improving actions. Basque Country transitional waters can be affected by several factors and against the damaging effects of those factors, some effective actions have been carried out from the 1990s (Bald et al., 2005). It has to be highlighted the treatment of waste waters before being emitted to transitional and coastal water bodies. On the other hand, the closing of some industrial activities due to economic crisis contribute to the water bodies' quality improvement.

The rivers flowing from the Basque Country into the Atlantic Ocean have suffered alterations caused primarily by industrial activity, whereas those flowing into the Mediterranean have suffered pressures from agricultural activities. In 2008 , in order to manage the application of the WFD to all these altered water bodies, the Basque Water Agency was set up, which asked different groups to participate in the decision making process. In this manner, apart from the Basque Government and district councils, other kinds of groups, such as town councils and environmental organisations, would have a say in the measures to be taken. Although most research conducted to date concludes that the status of water bodies in the Basque Country has improved considerably, it also makes it clear that restoring water to its original condition is an impossible task (UNESCO, 2009).

7. Monitoring metallic contamination in air: European legislation

Several norms about protecting air quality have been established in Europe over recent years. These norms have been corrected and updated over time. The latest norms are

Directives 2004/107/CE (UE, 2004) and 2008/50/CE (UE, 2008). Air quality and the conditions which air has to fulfil are established by these directives, in order to guarantee a minimum air quality. Amongst chemical contaminating agents, particles, ozone, nitrogen dioxide, sulphur dioxide, carbon monoxide, heavy metals, benzene and benzopyrene are mentioned, particles and ozone being the ones which cause living beings the greatest problems. Heavy metals are often stuck to particles. As, Cd, Pb, Hg and Ni are amongst the most common heavy metals emitted into the atmosphere as a result of human activity (EEA, 2013).

European Directives 2004/107/CE and 2008/50/CE distinguish between two values with regards to heavy metals. On the one hand, the European Union's objective values with regards to air quality, which, as far as possible, may not be exceeded. On the other hand, limit values, which are concentration values which must not be exceeded. As (6 ng/m^3), Cd (5 ng/m^3) and Ni (20 ng/m^3) objective values (yearly averages) are set by the European directives. In the case of Pb the limit value is fixed (500 ng/m^3). The directive for Hg does not set limits, only recommending that emissions be limited.

As can cause skin and lung cancer. Cd, on the other hand, as well as harming kidneys and bones, can cause lung cancer. Pb increases artery pressure and can cause neurological problems. Hg is volatile and highly toxic. Ni is carcinogenic and can damage the endocrine system. These metals are durable and can travel over long distances in the atmosphere. Consequently, they can appear in remote and unexpected areas (WHO, 2007).

Finally, we should mention that the European contamination and emissions sources inventory is being prepared at the moment. According to the European Pollutant Release and Transfer Register *(E-PRTR)*, companies and public institutions which are involved in contaminating activities voluntarily can declare the quantity of contamination that they emit into the environment, including emissions into the atmosphere, natural water bodies and water treatment plants. This information is public and is stored in a database (E-PRTR, 1995).

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Chapter 2

EXPERIMENTAL

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Some of the procedures, materials and reagents used in this work will be described in this section to avoid unnecessary repetitions along the text.

1. Materials and reagents

Dry solid samples were digested using mixtures of different reagents such as hydrochloric acid (Merck, HCl %37, Tracepur), nitric acid (Merck, HNO_3 %69, Tracepur) and hydrogen peroxide (Fluka, H_2O_2 for trace analysis). Acidification of water samples and preparation of standard solutions for calibration was carried out with sub-boiled portions of the previously mentioned HNO_3. Sample extracts were filtered through 0.45 µm Millipore filters (Milles-HV, Hydrophobic PVDF 0.45 µm) and Omnifix syringes (20 mL, Braun). Elix (Element A10, Millipore) quality water was used for cleaning purposes and Milli-Q (Element A10, Millipore) quality water for calibrant preparation and dilution of samples.

Solid samples were freeze-dried in a Cryodos (Telstar) lyophilizer (Figure 1). Oysters were previously grinded and homogenized using a commercial blender. Dry sediments were sieved at different particle sizes by means of an Octagon sieve shaker (Endecotts) (Figure 1). Dry leaf, moss, lichen and below 2 mm soil samples were ground and homogenized in a planetary ball mill Pulverisette 6 (Fritsch, Germany) (Figure 1). Calibrants for ICP/MS analysis were prepared inside a 100 Class clean room using a XS205 analytical balance (±0.00001 g, Mettler Toledo). The rest of samples and reagents were accurately weighed in an AJ150L analytical balance (±0.0001 g, Metler Toledo). All the solutions and sample dilutions were prepared by weigh.

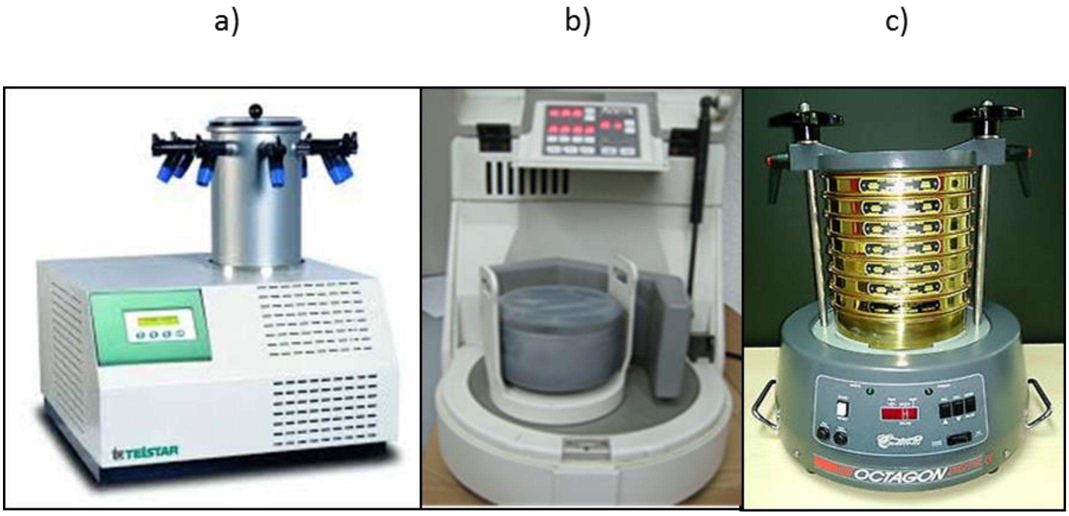

Figure 1. a) Cryodos lyophilizer, b) Pulverisette planetary ball mill and c) Octagon sieve shaker.

The digestion of solid samples was carried out either in a 3000S microwave-assisted oven (Anton Paar, Graz, Austria) with 100 ml PTFE extraction vessels or by a HD2070 Sonopuls Ultrasonic Homogenizer (Bandelin) equipped with a 6 mm glass probe (Figure 2).

Some samples (lichens), however, were digested in a high pressure asher (HPA-S, Anton Paar). The concentration of different elements was simultaneously measured in liquid samples by ICP/MS (NexION 300, Perkin Elmer, Ontario, Canada) inside a 100 Class clean room.

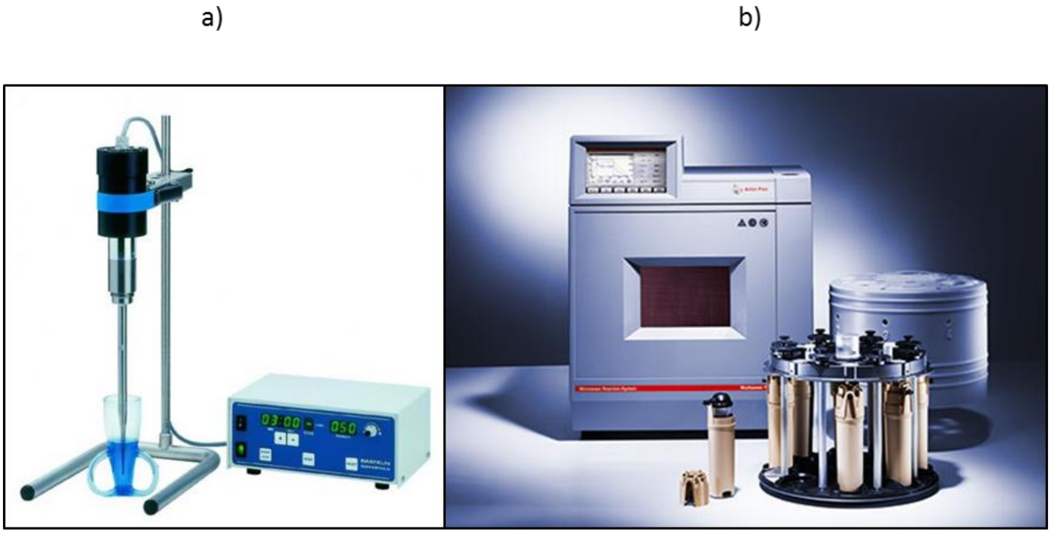

Figure 2. a) HD2070 Sonopuls Ultrasonic Homogenizer and b) 3000S microwave-assisted oven.

2. Experimental procedure

2.1. Cleaning procedure

Glass and plastic material was first thoroughly cleaned with detergent and tap water, then left for 24 hours in a nitric acid bath (10%) and finally rinsed first with Ellix water and then with Milli-Q water. The clean plastic and glass (also PTFE) material was dried in an oven at, respectively, 50ºC and 120ºC.

2.2. Sample pretreatment

2.2.1. Sediments

Estuarine sediments were frozen in the laboratory at −20ºC and further lyophilised in round-bottom glass flasks at 150 mTorr and -52ºC for 48 h. The dry samples were sieved to assure a maximum particle size of 75 µm and kept in the refrigerator at 4ºC until analysis. Lake sediments were dried in an oven at 50 ºC until constant weight (at least for 72 h) and stored in clean glass vials at 4ºC in dark until analysis.

2.2.2. Oysters

Oysters were dissected with a clean scalpel blade to separate the soft tissues from the shells and cleaned using Milli-Q water to remove the dirt. About twenty individuals per sampling site were frozen (-20ºC), homogenised with a blender and freeze-dried in round-bottom glass flasks at 150 mTorr and -52ºC for 48 h. Afterwards, the dried sample was ground in a blender and kept in the refrigerator at 4ºC in dark until analysis.

2.2.3. Beech leaves

Leaves were cleaned with water and then freeze-dried in round-bottom glass flasks at 150 mTorr and -52ºC at least for 48 h. Dried samples were ground in a planetary ball mill and stored in clean glass vials at 4ºC in dark until analysis.

2.2.4. Moss

Moss samples were oven-dried in the laboratory at 40ºC for 72 h until constant weight and the extreme apices of the shoots were milled after removing extraneous plant and dead material. The samples were not washed. Finally, moss samples were stored in clean glass vials at 4ºC in dark until analysis.

2.2.5. Lichen

Lichens were separated from the substrate by hand in the laboratory and freeze-dried under a pressure of 150 mTorr and -52ºC at least for 48 h. Samples were crushed and homogenized in a planetary ball mill and then stored at 4ºC in clean glass vials and dark until analysis.

2.2.6. Soil

Soils were frozen in the laboratory at −20ºC and further lyophilised in round-bottom glass flasks at 150 mTorr and -52ºC for 48 h. The dry samples were sieved to remove the fraction higher than 2 mm and different objects such as bones or stones. The <2 mm fraction was ground in a planetary ball mill and stored in clean glass vials at 4 °C in dark until analysis.

2.2.7. Water

Water samples were filtered through 0.45 μm filters and acidified to 1% HNO_3. The acidified samples were stored in 10 mL polyethylene vials at 4 °C and protected from light until analysis.

2.3. Sample treatment

2.3.1. Lake sediments and soil

About 0.5 g of sample were digested in a microwave oven using $HCl:HNO_3$, 1:3 and following the US Environmental Pollution Agency EPA 3051A method (EPA, 2007). For quality assurance purposes, the BCR 701 (lake sediment) or SRM 2711 (Montana soil) certified reference materials were analyzed in each sample batch. Procedural blanks were also run every sample batch and eight blanks were prepared for estimation of the procedural detection limit. After cooling, the digests were filtered and quantitatively transferred to a polyethylene bottle. The extracts were conveniently diluted with water, their acidity adjusted to 1% HNO_3 and finally stored at 4 °C in dark until ICP/MS analysis.

2.3.2. Estuary sediments

About 0.5 g of dry sediment was transferred to an extraction vessel with 4 mL HNO_3, 6 mL HCl and 10 mL H_2O. Ultrasound energy was applied to the suspension for 6 min, with 6 cycles and 35% of power. Experimental details can be found elsewhere (Fdez-Ortiz de Vallejuelo et al., 2009). The accuracy and repeatability of the method was checked by repetitive analysis (n = 5) of the NIST 1646a certified reference material (estuary sediment, National Institute of Standards and Technology). Eight blanks were at least prepared and analysed in each sample batch for estimation of the procedural detection limit. The extracts were filtered, conveniently diluted with water, their acidity adjusted to 1% HNO_3 and finally stored at 4 °C in dark until ICP/MS analysis.

2.3.3. Oysters

About 0.5 g of the dry oyster was accurately weighed and transferred to an extraction vessel together with 2 ml of HNO_3, 2 mL of H_20_2 and 2 mL of H_2O. The extraction was carried out in a microwave oven for 18 min and 450 W. The NIST SRM 2976 (mussel tissue, National Institute of Standards and Technology) certified reference material was used to validate the analytical method, and at least eight blank samples were analysed per batch of samples to estimate the procedural detection limits. The digests were filtered, conveniently diluted with water, their acidity adjusted to 1% HNO_3 and finally stored at 4 °C in dark until ICP/MS analysis.

2.3.4. Beech leaves

About 0.5 g of beech leaves were digested with 7 mL HNO_3, 3 mL H_2O_2 and 6 mL H_2O in a microwave oven. A ramp of 400 W was applied during 15 min. to reach 200ºC and then this temperature was maintained for 15 min. The SRM 1570a certified reference material (spinach leaf, National Institute of Standards and Technology) was used for quality assurance purposes. Eight blanks were at least prepared and analysed in each sample batch for estimation of the procedural detection limit. After cooling, the digests were filtered, conveniently diluted with water, their acidity adjusted to 1% HNO_3 and finally stored at 4 °C in dark until ICP/MS analysis

2.3.5. Moss

About 0.25 g of sample was digested in a microwave oven using 9 mL of HNO_3 at 180°C for 18 min and following the US Environmental Pollution Agency EPA 3052 method (EPA, 1996). The quality control of the analytical procedure in the case of mosses was carried out by repetitive analysis of the certified reference material SRM 1575a (pine needles, National Institute of Standards and Technology). One blank was included every ten samples for the estimation of procedural detection limits. After cooling to room temperature, all samples were filtered, conveniently diluted with water, diluted to 1%HNO_3 and stored at 4°C in dark until ICP/MS analysis.

2.3.6. Lichen

About 1g of lichen was pre-digested in 5 mL of sub-boiled HNO_3 for 18 hours in a clean glass vial. Then, the vials were sealed with a quartz caps and heated in a high pressure asher (HPA-S, Anton Paar) for 3 hours at 130 bars and 300°C. After cooling, each sample was filtered and quantitatively transferred to a glass vial. The samples were conveniently diluted with water, diluted to 1%HNO_3 and stored at 4°C in dark until ICP/MS analysis. The quality control of the analytical procedure was carried out by repetitive analysis of the certified reference material BCR 482 (lichen). For the detection limit calculations, one procedural blank was included every ten samples.

2.3.7. Water

Filtered and acidified water samples were directly analyzed by ICP/MS analysis. The quality control of the analytical procedure was carried out by repetitive analysis of the certified reference material NIST SRM 1640 (natural fresh water, National Institute of Standards and Technology) and eight independent water blanks were run for the estimation of procedural detection limits.

2.4. ICP/MS analysis of water samples and extracts

The NexION 300 ICP-MS was used for the analysis of water samples and extracts. The analysis was carried out inside a 100 Class clean room (Figure 3). The experimental conditions of the measurements are summarised in Table 1. The external calibration method with internal correction was used. Standard solutions of the analytes and the internal standards (^{45}Sc, ^{115}In, ^{209}Bi, ^{9}Be and ^{74}Ge) were obtained from Alfa Aesar (SpecpureR, Plasma standard solution, Karlsruhe, Germany). The calibrants were prepared in 1% sub-boiled HNO_3 inside the clean room in a weight basis.

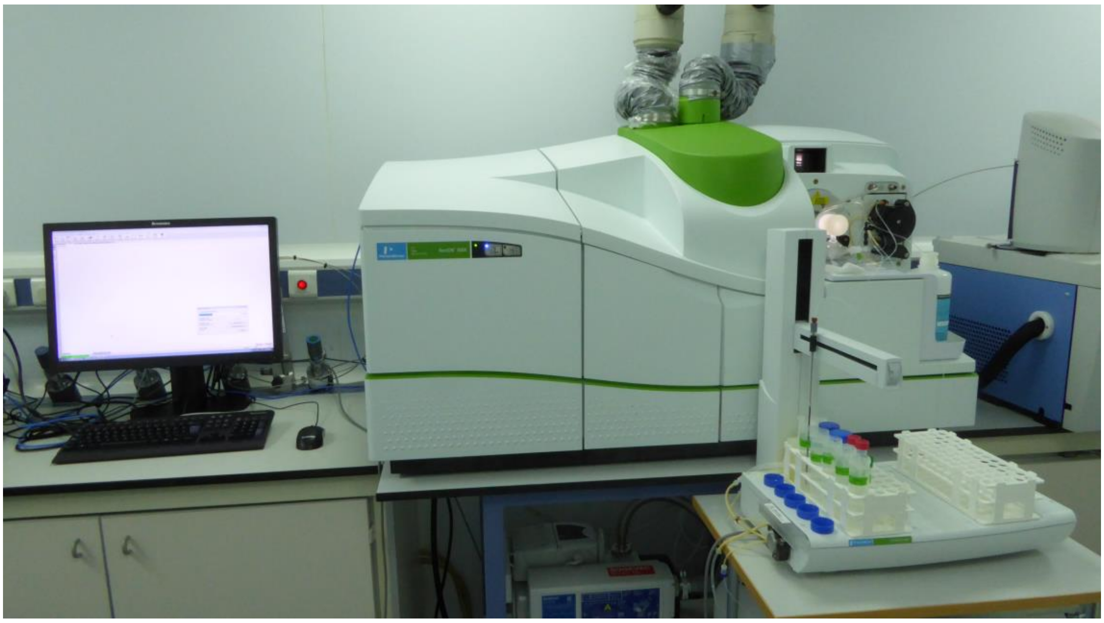

Figure 3. NexION 300 ICP/MS inside the 100 Class clean room.

Table 1. Experimental conditions for the ICP/MS analysis.

Sample flow	0.4 mL/min
Nebulizer gas flow	0.90-1.00 L/min
Plasma gas flow	16 L/min
Auxiliar gas flow	1.2 L/min
RF power	1600 W
Cell gas flow (He)	4.0 mL/min
Dwell time	50 ms
Integration time	1000 ms
Sweeps per reading	20
Readings per replicate	1
Replicates per sample	3

2.5. Direct analysis of solid samples

Some sediment samples were mineralogically characterised by X-ray diffraction spectrometry (XRD) and Raman Spectroscopy.

In the case of XRD characterization, a portion of sediment sample was analysed by a powder diffractometer PANalytical Xpert PRO that incorporates a copper tube ($\lambda Cu_{K\alpha media}$= 1.5418 Å, $\lambda Cu_{K\alpha 1}$= 1.54060 Å, $\lambda Cu_{K\alpha 2}$= 1.54439 Å), vertical goniometer (Bragg-Brentano geometry), programmable divergence aperture, automatic interchange of samples, secondary monochromator from graphite and PixCel detector.

The measurement conditions were 40 kV of voltage and a current of 40 mA, with an angular range (2θ) scanned between 5 and 70°. The treatment of the diffractograms and the identification of the mineral phases was performed with the specific software X`pert HighScore (PANalytical) in combination with the specific powder diffraction file (PDF2) database (International Centre for Diffraction Data - ICDD, Pennsylvania, USA).

The Raman analysis of the sediments was carried out by means of a Renishaw RA 100 Raman Spectrometer (Renishaw, UK) coupled to a fibre optic micro-probe (Oxford, UK) equipped with a 785 nm excitation diodo laser (Figure 4) and a CCD detector (Peltier cooled). The micro-probe mounted on a tripod was joined to different lenses (4×, 20× and 50×) which allow focusing the laser beam between 5 μm and 100 μm at the sample. The nominal power of the excitation source is 150 mW. In order to avoid thermodecomposition of the samples, the laser power was modulated at 1 and 10%. The spectrometer was calibrated daily the Raman signal of Si at 520.5 cm^{-1} of a crystalline silicon chip. The time of integration was between 10 s and 15 s and the number of accumulations was varied in order to achieve the best signal to noise ratio. Spectra were obtained in a spectral range of 200–2000 cm^{-1} approximately with a spectral resolution of 2 cm^{-1}. The Wire 3.2 software package (Renishaw, UK) was used for data acquisition and the analysis and treatment of spectra were carried out by means of the Omnic Nicolet software (Madison, Wisconsin, USA). Interpretation of the results was accomplished by comparison of experimentally obtained spectra with standard Raman spectra contained in the e-Visart and e-Visnich databases (Castro et al., 2005) and other spectra obtained from the on-line database RRUFF (RRuff, 2015).

Figure 4. Renishaw RA100 spectrometer with the 785 nm laser analyzing a sample.

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Section 1

METALLIC CONTAMINATION IN THE ATMOSPHERE

Chapter 3

HIGH-ALTITUDE MOUNTAIN RANGES

CHAPTER 3

HIGH ALTITUDE MOUNTAIN RANGES

Climate change is the result of human activity and its effects can be noted on a global scale (North, 2013). The capacity of an ecosystem to adapt or adjust to new circumstances is often less than the speed at which changes take place. As a result, many ecosystems are altered and, sometimes, harmed (van het Bolcher et al., 2006). The effects of climate change are spread through the atmosphere very quickly on a global scale (Santer et al., 2013). Indeed, the most important reactions occur in the atmosphere, amongst others, the modification of the natural biogeochemical cycle of chemical elements. As examples, the build-up of carbon dioxide in the atmosphere, the thinning of the ozone layer and the increase of nitrogen and phosphorus in the biosphere (Barrett, 1975; Huang et al., 2015; Stolarski et al., 2015). Moreover, the quantities of toxic substances (metals, pharmaceuticals, chemical compounds that produce acid rain) in general have increased (Camarero, 2013).

High mountain ecosystems are highly suitable to study the effects of climate change, due to they are highly sensitive to changes in conditions in their surroundings. On the one hand, climate features change with altitude in these areas. At lower altitudes the climate is more stable and milder. There is also year round snow in some high altitude mountain ranges. On the other hand, the impact of pollution sources on high mountains ecosystems is very limited. Moreover, the deposition rates of air borne particles are usually very high in mountains (Bacardit and Camarero, 2010b) and the drops in temperature that occur with changes in altitude, favour the condensation of volatile chemical pollutants (Bacardit and Camarero, 2010a). As a result of the above factors, ecosystems of high altitude mountain ranges enable the consequences of climate change to be studied to great effect (Camarero, 2013).

1. The Pyrenees

The Pyrenees are a range of mountains that extend from Cape Matxitxako (Basque Country) to Cape Creus (Catalonia) and reach their maximum height of 3404 metres on Aneto Peak. The range is 415 kms long and 150 kms wide at its mid-section. As a result of administrative and political boundaries, the Pyrenees straddle 2 countries. The regions of *Aquitaine*, *Midi-Pyrénées* and *Languedoc-Rosellon* are found on the north side and the Basque Country, Aragon and Catalonia are on the south side. The important cities in the area that may impact the quality of Pyrenean ecosystems are Bilbao, Bordeaux, Zaragoza, Toulouse and Barcelona (Figure 1).

Figure 1. Geographical location of the Pyrenees.

The northern faces of the Pyrenees are much more rugged than the southern faces. This dissymmetry lessens towards the Cantabrian Sea, whereas it becomes more and more pronounced towards the Mediterranean Sea. Many different rock types can be seen throughout the Pyrenees including, amongst others, limestone, arsenopyrite and granite (Barnolas and Pujalte, 2004). The range of temperatures in the Pyrenees is from -10ºC in the winter to 20ºC in the summer and there is an average yearly rainfall of 2,000 mm (Barnolas and Pujalte, 2004).

Pyrenean fauna comprises more than 200 different species. Notable, amongst others, is the presence of the brown bear. The Alpine chamois, deer and roe deer are the main mammals (Barnolas and Pujalte, 2004).

25,900 km^2 of the Pyrenees, of a total of 34,000 km^2, are covered in forest. Scots pine (*Pinus sylvestris*) and beech (*Fagus sylvatica*) are very common. Altitude has a direct impact on the distribution of the species that make up high mountain forests. Above 2,200 – 2,500 metres, the forests give way to grassy fields, as the conditions at these altitudes do not favour forest growth. At subalpine altitudes, between 1,200 and 2,400 metres, conifers predominate, especially black pine (*Pinus uncinata*). Fir trees (*Abies alba*) also grow in damp areas at these high altitudes. Below 1,200 metres, wild pine (*Pinus sylvestris*) and fir trees (*Abies alba*) again are the most numerous. At these altitudes, forests of deciduous species begin to appear, especially beech groves. Beech trees need moisture and so tend to grow in the cooler, cloudy areas. Deciduous forests have great biodiversity, and beech trees, oak (*Quercus*), ash (*Fraxinus*), service trees (*Sorbus domestica*), Norway maple (*Acer platanoides*), white willow (*Salix alba*) and lime trees (*Tilia*) can often be found growing together. Apart from these kinds of trees, numerous types of shrubs can also be found in the forest: boxwood (*Buxus sempervives*), common hazel (*Corylus Avellana*) and holly (*Ilex aquifolium*) for example. As well as trees and shrubs, the deep damp of the forest provides the conditions necessary for organisms such as lichens and bryophytes to live on the smooth bark of the beech trees. There are more than 4,500 species of flora, of which 160 are endemic.

In high altitude mountain ranges, it is common to find forests comprising vascular and non-vascular vegetation that has adapted to high altitudes. In fact, forests are one of the ecosystems that characterise high mountain ranges (Figure 2). Many studies on atmospheric pollution using the leaves of vascular vegetation from the forest can be found in the literature (Dogan et al., 2014; Madejon et al., 2006; Mankovska, 1998; Yildiz et al., 2010). Some other organs of vascular vegetation have also been used to measure the presence of metals in the air, amongst others, the bark of trees, for example (Lippo et al., 1995; Poikolainen, 1997). As well as vascular vegetation, non-vascular vegetation, such as lichens and mosses (Bajpai et al., 2010; Klos et al., 2011), has also been used to monitor atmospheric pollution. In the Pyrenees too, beech leaves (Amores and Santamaria, 2003), lichens (Achotegui-Castells et al., 2013; Barre et al., 2015; Veschambre et al., 2003) and mosses (Fernandez et al., 2002; Gonzalez-Miqueo et al., 2009; Gonzalez-Miqueo et al., 2010) have been used to study atmospheric pollution.

Figure 2. Irati Forest and Pica Palomera Lake (2.250 m), both in the Pyrenees.

High altitude lakes are a special and important kind of ecosystem in mountain ranges (Figure 2). High altitude lakes can be found in many areas, enabling a worldwide comparison of atmospheric pollution (Camarero, 2013). Lakes offer the possibility of studying the effects of long-range air pollution, with the impact of local activities. Consequently, the water and sediment of lakes have often been used to examine metal pollution (Ebrahimpour and Mushrifah, 2008; Magyari et al., 2009; Singare et al., 2013).

In the Pyrenees too, lakes abound. About 1,250 lakes have been counted throughout the range, with 650 on the northern side and 600 on the southern side approximately. Lakes may be either endogenous or exogenous. Endogenous lakes are the result of internal forces and processes in the Earth itself, that is, the result of earthquakes or volcanic eruptions. In contrast, exogenous lakes are formed by forces or processes affecting the Earth’s crust. The lakes in the Pyrenees are exogenous and may be further classified as glacial lakes. These lakes are the result of erosion of the Earth’s crust during glacial periods. Glacial lakes are usually circular and very deep. Lake Estany, for example, in the Pyrenean valley of Valle de Arán, is 880 m in diameter and 80 m deep. There are numerous works that study the presence of metals in Pyrenean lakes (Bacardit and Camarero, 2009; Bacardit and Camarero, 2010c; Bacardit et al., 2012; Camarero et al., 1998; Zaharescu et al., 2009).

This chapter presents the results of research projects carried out on several Pyrenean lakes and in the Irati Forest. The purpose of these works is to study the presence of metals in the Pyrenees, their geographical distribution and evolution over time.

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Chapter 4

SEDIMENTS OF HIGH-ALTITUDE PYRENEAN LAKES: INDICATORS OF POLLUTION IN THE AREA

CHAPTER 4

SEDIMENTS OF HIGH-ALTITUDE PYRENEAN LAKES: INDICATORS OF POLLUTION IN THE AREA

ABSTRACT

High altitude lakes in the Pyrenees are very sensitive ecosystems to detect and quantify metal pollution. With this aim, sediment cores were collected at 18 high altitude Pyrenean lakes in summer 2013. After slicing the cores, the concentration of 24 elements was measured in all the samples. Statistic and chemometric analysis of the results were carried out. Five different lake clusters were identified according to their metal content in surface sediments. Significant correlations (at 95% confidence level) were detected for Zn-Cd, Pb-Sb and Ni-Co pairs of elements. The deep sediment cores obtained in some lakes were used to identify historical records of metal pollution.

Keywords: Pyrenees; Sediments; Metal pollution; High altitude lakes; Natural; Anthropogenic.

1. INTRODUCTION

The ecosystems that exist in high mountain ranges are highly sensitive to changes in environmental conditions. In the Pyrenees, we can find excellent examples of these types of ecosystems (Bacardit and Camarero, 2010a), such as the lakes that are at a height of over 2000 m. The Pyrenees is a mountain range that extends from Matxitxako Cape (Basque Country) to Creus Cape (Catalonia), and its highest point reaches 3400 metres. Cities such as Bilbao, Bordeaux, Zaragoza, Toulouse and Barcelona can affect the quality of its ecosystems. In fact, human activity has had an irrefutable effect on the environment on a global scale. As a consequence of administrative and political borders, the Pyrenean area is divided into two countries, and that has limited significantly the amount of research that has been done about the area as a whole.

In this work, lakes are the subject of investigation to study the contamination in the Pyrenean area. Due to their privileged location, high-altitud lakes are the best observatories to identify the effects of contamination on the environment (Camarero, 2003; Williamson Craig et al., 2009). High mountain lakes are very sensitive to changes in the environment, due to the fact that the presence of pollutants is usually insignificant. Among the pollutants that can be found, we could mention metals and metalloids. From the toxicological point of view, durability and accumulation capacity are the main characteristics of metals and metalloids. Besides, some metals and metalloids are toxic, being able to damage living organisms of different ecosystems. Cd, Hg, Ni, Pb and tributyltin oxide are part of the priority toxic substances list of *The European Water Framework Directive, (EWFD)* (WFD, 2000). Many other metals, for example: As, Cr, Cu and Zn are regarded as toxic in some studies (Naimo, 1995).

A recently published study shows clearly the increase in the concentrations of metals in waters and sediments of high mountain lakes in Europe over the few last decades (Camarero et al., 2009). The origins of these metals and metalloids in the waters and sediments of the Pyrenean lakes can have natural origins (geochemical compositions of the rocks in the area and/or volcanism) or anthropogenic origins (mining, industrial and urban waste that is transported through the atmosphere, combustion of fossil fuels, etc.) (Bacardit and Camarero, 2009). It should be taken into account that chemical pollutants can be tranported long distances in the atmosphere (Bacardit, 2011; Camarero, 2013). Take for example Redó Lake, where high concentrations of lead have been found in its core sediments: those in lower layers were caused by mining acitivities that were carried out long time ago, and those in upper layers, were mainly caused by combustion of fossil fuels (Camarero et al., 1998). Substances such as As, Ag, Cd, Cr, Cu, Hg, Ni, Pb and Zn can be found in complex mixtures of metallic pollutants produced by mining and industrial activities (Nriagu, 1996). Wind direction and wind intensity have a direct effect on the transportation through the atmosphere (Gioda et al., 2011; Groeneveld et al., 1993; Marques et al., 2004). Depending on the time of the year, the magnitude of atmospheric deposition can change: during hotter periods (June-November) it is higher than in cold periods (December -May) (Bacardit, 2011). In hot periods, the southern wind prevails and the quantity of metals and metalloids coming from the south of the Pyrenees is larger (Bacardit and Camarero, 2010b). Metals and metalloids can bond with soil and plants, which given proper conditions could become a significant source of contamination (Bacardit et al., 2012). Furthermore, the altitude at which the lake is situated can affect the metal and metalloids concentration: the higher the altitude of the lake, the fewer metals and metalloids can be found in its waters. The cause of this phenomenon is in part due to orography, contaminants that reach these mountains through the atmosphere can settle in contact with the steep land. As a result, the air rises up the mountains free of pollution. Moreover, at higher altitudes the level of organic matter drops, as a result of decreased vegetation. Organic matter has an affinity for metals. Therefore, as the levels of organic matter that are dissolved in the waters at higher altitudes are lower, the levels of metals and metalloids present are also lower (Bacardit and Camarero, 2010b).

The aim of this study is to analyse the concentrations of metals and metalloids in the sediments of different lakes of the Pyrenees, regarding both the geographical distribution and the historical record, to quantify the effect of human activity on those ecosystems.

2. MATERIAL AND METHODS

2.1. SAMPLING

Sediment cores were taken in August and September 2013 from 18 lakes in the Pyrenees at altitudes higher than 2.000 m: Airoto (AIr), Aixeus (AIx), Anglas (AN), Aube (AU), Baiau Superior (BA), Bersau (BE), Compte (CO), Eriste (ER), Estelat (ES), Gran del Pesso (GR), Llosas (LL), Mariola (MA), Monges (MO), Montoliu (MT), Pica Palomera (PP), Plan (PL), Romero de Dalt (RO) and Siscar (SI) (Figure 1). The geographical location of the lakes can be seen in Table 1. The following are the depths in cm of the sediment cores taken from each lake: AIr: 5, AIx: 22.5, AN: 12, AU: 19, BA: 26, BE: 12, CO: 22.5, ER: 26.5, ES: 36.5, GR: 5, LL: 5, MA: 26, MO: 5, MT: 5, PP: 22.5, PL: 30, RO: 36.5 and SI: 33.

A plastic inflatable boat was used to collect the sediment cores. They were taken from the deepest areas of the lakes with the help of a gravity core sampler. One sediment core was taken from each lake. The cores were divided in-situ into sub-samples depending on the depth. First of all, a 0-1.5 cm layer was separated (the surface layer). From that point on, they were separated into layers of 3.5 cm each. Samples were kept in a double ziploc bag. The surface layers were used to study the distribution of metals and metalloids throughout the Pyrenees in recent years, and the samples from the deeper layers, to examine the historical record of pollution. All the samples were transported downhill in refrigerated backpacks to the laboratory, where they were kept in the dark at a temperature of 4ºC until they were analysed.

Figure 1. Localization of the studied lakes in the Pyrenees: Airoto (AIr), Aixeus (AIx), Anglas (AN), Aube (AU), Baiau Superior (BA), Bersau (BE), Compte (CO), Eriste (ER), Estelat (ES), Gran del Pesso (GR), Llosas (LL), Mariola (MA), Monges (MO), Montoliu (MT), Pica Palomera (PP), Plan (PL), Romero de Dalt (RO) and Siscar (SI).

2.2. ANALYTICAL PROCEDURE

The pretreatment and analysis of the samples was carried out according to the procedures described in Chapter 2, experimental. The concentration of Ag, Al, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Mg, Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, Ti, Tl, V, W and Zn was finally measured by ICP/MS in all the samples.

Table 1. The geographical location of each lake included in the study (UTM 31T).

Lake	X	Y
AIr	339405	4729463
AIx	366449	4718687
AN	722574	4753940
AU	363971	4733677
BA	371347	4716960
BE	704135	4748827
CO	401039	4720626
ER	292437	4724450
ES	435526	4721626
GR	328762	4708588
LL	307658	4720810
MA	354601	4730744
MO	325896	4720922
MT	330366	4738774
PP	325700	4739902
PL	330298	4720754
RO	362790	4729315
SI	397224	4717099

3. RESULTS AND DISCUSSION

3.1. SURFACE SEDIMENTS: GEOGRAPHICAL DISTRIBUTION OF METALS ACCUMULATED IN SEDIMENTS OVER RECENT YEARS

3.1.1. METAL CONCENTRATIONS AND COMPARISON WITH OTHER LAKES

The element concentrations found in the surface sediments can be seen in Table 2. Extreme concentrations have been marked with an asterisk ($c > Q_3+(3*IQR)$, where Q_3 is the %75th percentile and IQR is the interquartile range). After removing the extreme values, the within-lake concentrations of the elements have been summarised in Figure 2 in the form of Box-Whisker plots.

Some elements showed extreme concentrations in some lakes. The appearance of an extreme value in a variable means that the metal concentration in that sample is significantly higher than in the rest of the samples. The lake that shows extreme concentrations is somehow out from the “normality” defined by the rest of the lakes. In the Lake Airoto (Air), extreme concentrations of As (1470 $mg{\cdot}kg^{-1}$) and W (4.96 $mg{\cdot}kg^{-1}$) were found. That area of the Pyrenees is very rich in arsenopyrites (Bacardit and Camarero, 2010b; Camarero, 2003; Camarero et al., 2009; Zaharescu et al., 2009), therefore, As and W have probably a lithogenic origin. It is known that mining activities, mostly to produce Pb and Zn, have been carried out since Roman times around lakes Anglas (AN), Montoliú (MT) and Pica Palomera

(PP) (Birch et al., 1996; Subias et al., 1999). Thus, it is not surprising to have found extreme concentrations of Cd, Mo, Pb, Sb and Zn in the sediments of these lakes. However, the origin of the extreme concentrations of Tl, Ag and Se in the lakes Monges (MO), Plan (PL) and Bersau (BE), respectively, is not clear.

Table 3 shows the metal concentrations found in the surface sediments of the 18 lakes that were examined, compared to the concentrations measured in other lakes. In the case of Lake Respumoso which is situated in Huesca at an altitude of 2130 m, the concentrations in Table 3 correspond to the average concentrations measured in the surface sediments (0-5 cm) obtained from 30 different parts of the lake (Zaharescu et al., 2009). Lake Respumoso was formed after the last glacial period and its waters have been used for drinking and irrigating in the valleys below. The basin of the lake in mainly formed of limestone and detritus. Lake Legunabes is situated in the Ariège department (France). The lithology around the lake is granite, and it is part of what is called Bassiés batholith (Bacardit et al., 2012). Finally, Lake Vidals d'Amunt is situated in the Catalonian Pyrenees, in the Lleida province, at an altitude of 2684 m and it is part of the Maladeta batholith (Bacardit et al., 2012). In lakes Legunabes and Vidals the influence of human activity is low. The 5-7 sediment cores obtained from each of those two lakes were separated into layers, and the averages of the results obtained from the analysis of all the samples have been summarised in Table 3 (Bacardit et al., 2012). For most of the metals, no significant difference has been observed among lakes. Nevertheless, the Cd concentration in Lake Vidals d'Amunt is lower than in the rest of the lakes, and the Zn concentration is lower than that in Lake Lagunabes. The Pb concentration in Lake Lagunabes is, however, slightly higher than that in the sediments of Lake Vidals d'Amunt.

Table 2. The concentration (in mg·kg^{-1}) of Ag, Al, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Mg, Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, Ti, Tl, V, W and Zn measured in the surface sediments (0-1.5 cm) of 18 lakes, together with some related statistics. The table shows the detection limit (DM, µg·kg^{-1}), recovery and reproduciviilty of the analytical method using BCR 701 reference material (n=8). Extreme high concentrations, defined as those concentrations over the 75th percentile of the data (Q3) plus three times the interquartile range (IQR), are marked with an asterisk (*).

	Ag	**Al**	**As**	**Ba**	**Cd**	**Co**	**Cr**	**Cu**	**Fe**	**Hg**	**Mg**	**Mn**	**Mo**	**Ni**	**Pb**	**Sb**	**Se**	**Sn**	**Sr**	**Ti**	**Tl**	**V**	**W**	**Zn**
DL (µg·kg^{-1})	20	175	11	83	34	4	5	1	36	101	19	599	34	162	76	7	950	21	275	8	12	5	2	1
RECOVERY (%)					97		76	71						70	89									86
REPRODUCIBILITY (RSD)	6%	4%	6%	4%	3%	6%	2%	5%	5%	6%	4%	6%	2%	3%	3%	4%	6%	6%	2%	2%	4%	3%	6%	6%
AIr	0.48	8260	1470*	17.6	1.50	3.30	12.1	42.5	7660	0.28	1720	112	0.50	5.45	74.7	0.87	1.51	4.35	5.13	118	0.22	13.0	4.96*	149
AIx	0.35	29600	31.5	46.6	0.46	7.38	20.4	73.3	92700	<LOD	6230	196	0.82	27.0	40.4	0.55	<LOD	1.29	9.11	405	0.052	21.2	0.048	110
AU	0.27	30100	14.4	81.3	1.66	5.54	25.5	16.2	17800	0.19	5810	152	0.89	11.0	135	1.22	1.65	9.21	14.6	830	0.38	35.1	0.27	272
BA	1.13	41700	71.4	61.8	0.38	18.7	22.7	157	66400	0.12	4320	118	0.86	44.0	61.7	1.21	1.21	2.14	5.13	351	0.15	29.0	0.25	183
CO	0.061	10500	7.06	41.4	1.01	2.39	12.2	6.19	7940	<LOD	2010	107	0.24	5.90	25.1	0.12	0.99	2.28	7.02	365	0.23	11.6	0.30	77.3
ER	0.18	25300	181	80.9	0.64	9.38	33.6	19.7	34500	0.12	6300	258	2.42	15.0	100	0.85	0.99	2.86	9.42	644	0.40	43.2	2.60	105
ES	0.23	13100	8.70	125	0.22	3.67	12.4	11.7	11300	<LOD	3600	144	0.081	5.95	26.3	0.20	<LOD	3.53	23.3	1070	0.13	32.1	0.45	63.5
GR	0.082	20300	85.6	60.2	1.06	7.57	20.0	14.4	17400	<LOD	5130	198	1.94	9.49	55.5	0.42	<LOD	1.74	12.2	582	0.26	40.0	0.23	90.0
LL	0.12	22500	59.9	98.0	1.78	7.74	24.2	15.1	22900	<LOD	6340	331	3.76	11.0	106	0.73	<LOD	6.30	11.0	1060	0.45	35.3	1.99	165
MA	2.49	28700	8.78	99.9	1.01	12.0	33.0	46.1	25700	0.12	6750	232	0.57	27.9	199	1.58	1.68	5.92	11.5	274	0.27	38.8	0.34	188
MO	0.51	34100	6.22	98.1	1.03	8.12	33.2	24.6	19600	0.57	6230	161	0.87	14.5	64.0	1.55	2.03	3.02	16.4	645	1.23*	44.6	1.15	138
MT	0.47	18700	79.1	30.9	22.3*	7.12	24.8	88.9	80200	0.27	3100	142	17.7*	22.7	764*	9.86*	2.31	1.70	14.7	86.3	0.24	56.4	0.12	4320*
PL	25.5*	20800	60.8	68.9	1.21	6.69	23.6	29.8	28600	0.38	4220	157	2.45	16.5	111	1.13	<LOD	3.44	15.8	413	0.28	37.5	0.51	199
PP	1.50	22000	52.7	31.5	10.4*	2.42	25.8	253	75800	0.60	2450	71.3	26.3*	10.2	395	8.07*	4.80	1.85	39.2	65.4	0.37	72.7	0.10	2820*
RO	0.32	31600	41.3	104	1.21	8.28	28.6	26.8	29600	0.29	4520	144	1.74	14.6	189	1.96	1.91	8.16	17.1	512	0.37	41.4	0.86	127
SI	0.12	12000	5.90	38.7	0.53	2.86	12.9	8.21	9050	<LOD	2320	79.9	0.31	8.84	38.9	0.18	<LOD	2.79	7.51	333	0.13	14.0	0.05	48.1
AN	0.15	23100	382	61.0	23.5*	15.3	36.9	21.9	39800	1.52	7240	463	0.95	34.4	209	1.21	5.18	3.13	30.5	726	0.15	41.7	0.36	4690*
BE	0.63	36000	242	36.9	0.10	16.0	20.6	188	65500	0.22	4330	126	3.94	32.1	27.8	0.32	24.2*	0.53	18.4	260	0.14	24.5	0.54	150
25th PERCENTIL (Q1)	0.14	17300	8.76	38.3	0.51	3.57	18.2	14.9	15900	0.14	2930	116	0.56	9.33	40.1	0.39	1.28	1.82	8.71	270	0.15	23.6	0.20	102
75th PERCENTIL (Q3)	0.75	30500	109	98.0	1.69	10.0	29.7	77.2	65700	0.52	6250	206	2.78	27.2	191	1.56	4.18	4.74	17.4	666	0.37	42.1	0.93	217
IQR (Q3-Q1)	0.61	13200	100	59.7	1.18	6.43	11.5	62.3	49800	0.38	3320	90.1	2.22	17.9	151	1.17	2.90	2.92	8.69	396	0.22	18.5	0.73	115
Q3+(3*IQR)	2.58	70100	410	277	5.23	29.3	64.2	264	215000	1.66	16200	476	9.44	80.8	644	5.07	12.9	13.5	43.5	1850	1.03	97.6	3.12	562
MEDIAN	0.33	22800	56.3	61.4	1.05	7.47	23.9	25.7	27100	0.28	4430	148	0.92	14.5	87.5	1.00	1.79	2.94	13.4	409	0.25	36.4	0.35	150
AVERAGE	1.92	23800	156	65.7	3.89	8.03	23.5	57.9	36200	0.39	4590	177	3.68	17.6	146	1.78	4.04	3.57	14.9	485	0.30	35.1	0.84	772
STANDARD DEVIATION	5.92	9270	342	30.7	7.28	4.78	7.78	70.5	27400	0.39	1770	95.9	6.92	11.2	180	2.68	6.49	2.38	8.86	301	0.26	15.3	1.24	1500
MIN	0.06	8260	5.90	17.6	0.10	2.39	12.1	6.19	7660	<LOD	1720	71.3	0.081	5.45	25.1	0.12	<LOD	0.53	5.13	65.4	0.052	11.6	0.048	48.1
MAX	25.5	41700	1470	125	23.5	18.7	36.9	253	92700	1.52	7237	463	26.3	44.0	764	9.86	24.2	9.21	39.2	1070	1.23	72.7	4.96	4690

Table 3. Concentrations measured in surface sediments obtained from several Pyrenean lakes (average, standard deviation, mg·kg^{-1}). *Extreme cocnentrations are not included.[1]n=30; [2]n=60;[3]n=48.

Lake	As	Cd	Co	Cr	Cu	Mn	Ni	Pb	Zn	Ref.
18 Pyrenean lakes *	79±101	0.90±0.52	8.1±4.8	23.5±7.8	58±70	177±96	18±11	109±95	133±59	This study
RESPUMOSO[1]	49±42	1.93±0.62	14.5±7.4	31.5±9.6	13±11	1200±1300	23±11	36±29	82±35	(Zaharescu et al., 2009)
LEGUNABES[2]		1.9±1.3			20.6±6.2		15.1±3.6	162±50	230±120	(Bacardit et al., 2012)
VIDALS D´AMUNT[3]		0.37±0.28			13.0±2.8		15.9±2.0	81±46	89±18	(Bacardit et al., 2012)

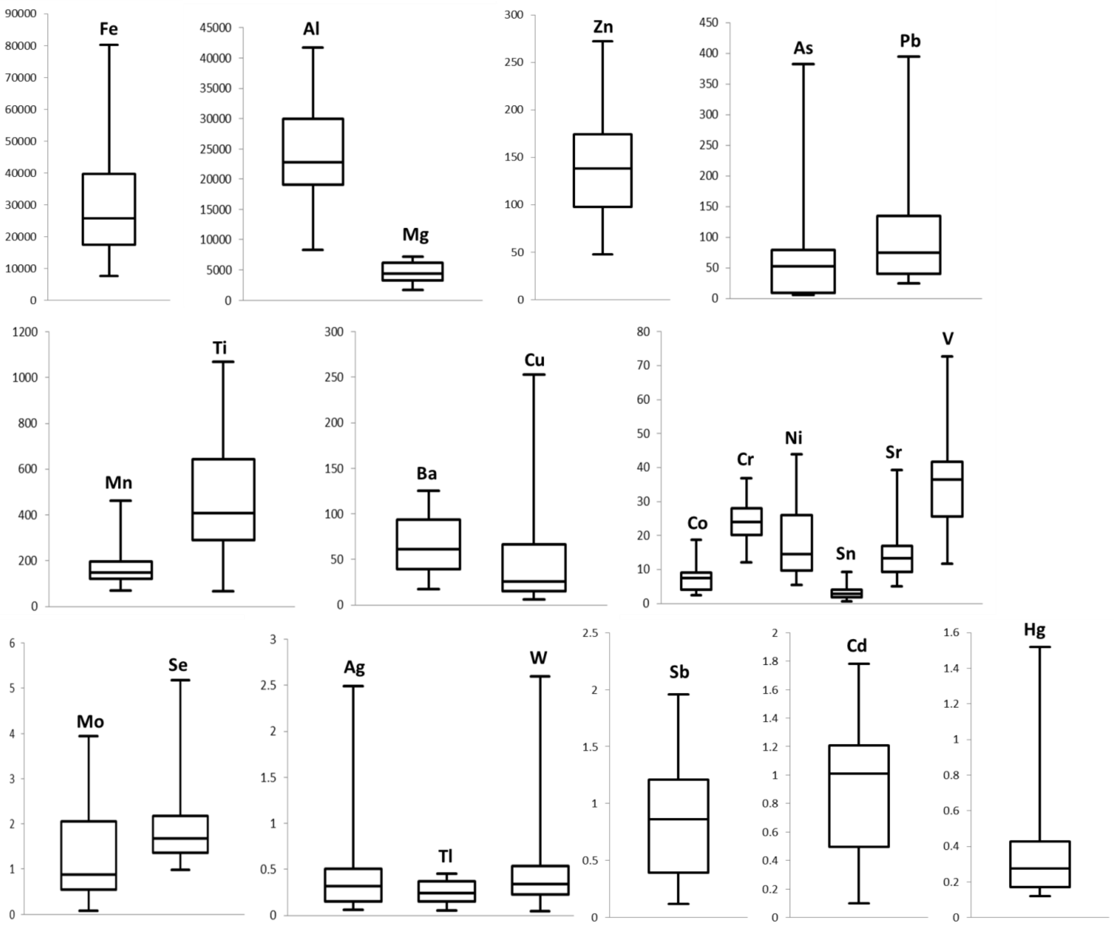


Figure 2. Box-Whixker plots of the concentrations of elements (in mg·kg^{-1}) measured in the surface sediments (0-1.5 cm) of 18 Pyrenean lakes. Extreme values not included in the calculation.

Correlation analysis of the surface sediment concentration data matrix was also carried out. With this aim, Pearson's coefficients were calculated to find out if the element concentrations are correlated each other. The highest correlation coefficients found were those between Zn and Cd (0.99), Pb and Sb (0.94) and Ni and Co (0.90), all of them significant at a 95% confidence level. Cd and Sb usually appear in nature as, respectively, Zn and Pb impurities, also in the Pyrenees. Therefore, the high and significant correlation found between those two elements is not surprising (Bacardit, 2011). Ni and Co also appears together frequently in nature (Hernandez et al., 2003).

3.1.2. PRINCIPAL COMPONENT ANALYSIS (PCA)

The Principal Component Analysis (PCA) of the surface sediment concentration data matrix was carried out by means of The Unscrambler program (v. 9.2 Camo, Oslo, Norway), in order to identify lakes with similar characteristics (Jollifee, 2002). The data matrix consisted on 18 rows (one per lake examinated) and the 24 columns (one per element analysed) (Table 1). Concentrations below the detection limit were substituted by one half of the detection limit. The data matrix was centered and scaled before analysis. To explain variability in the data, a model with three principal components (PC) was chosen. The model managed to explain 63 % of the total variance (PC1: 31%; PC2: 19%; PC3: 13%).

The scores and loadings plots over the first three Principal Components are shown in Figure 3. It can be seen that the studied lakes were separated into 5 different groups depending on the metallic concentrations in their sediments: i) Pica Palomera and Montoliu; ii) Baiau Superior, Aixeus and Bersau; iii) Siscar, Compte and Airoto; iv) Estelat, Gran del Pesso, Eriste, Llosás, Aube, Romero de Dalt, Mariola, Monges and Plan; and v) Anglas.

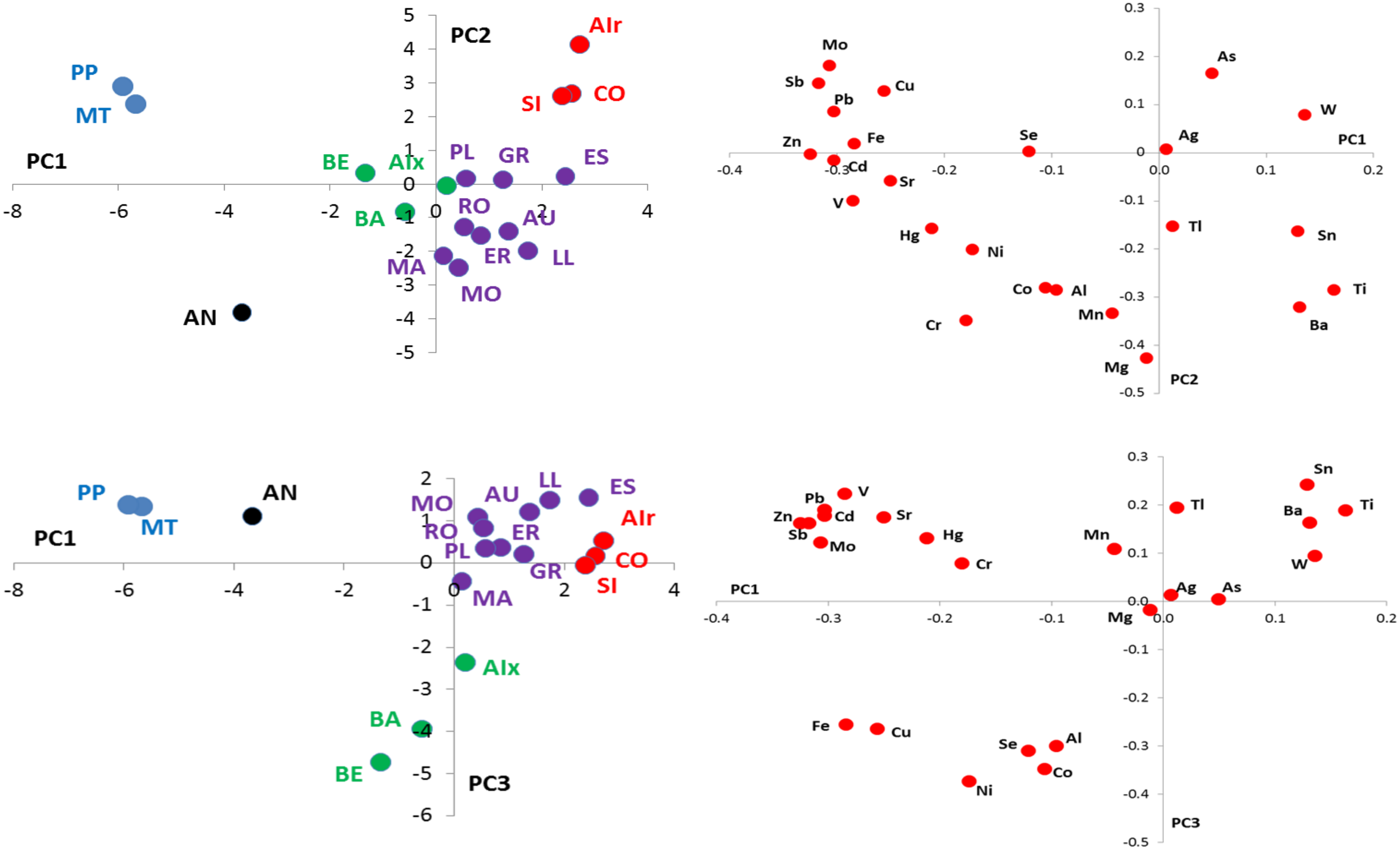
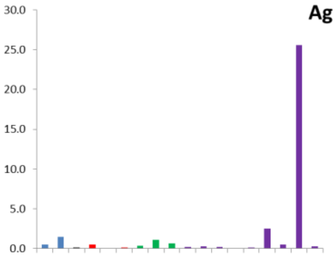


Figure 3. Scores and loadings plots (PC1-PC2 and PC1-PC3) obtained after PCA of the studied dataset.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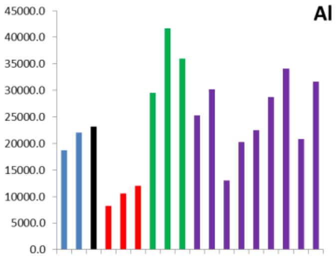
25.0
20.0
15.0
10.0
5.0
0.0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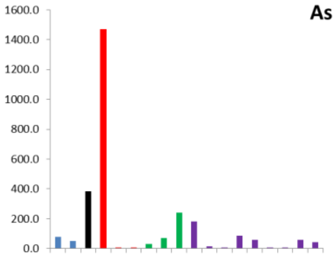
40000.0
35000.0
30000.0
25000.0
20000.0
15000.0
10000.0
5000.0
0.0


As
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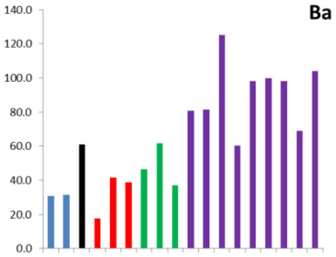
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0


Ba
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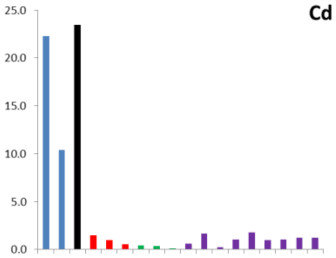
100.0
80.0
60.0
40.0
20.0
0.0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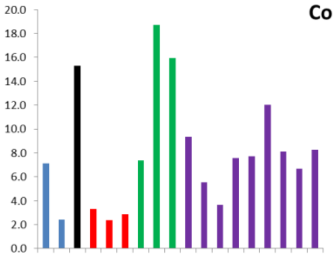
0.0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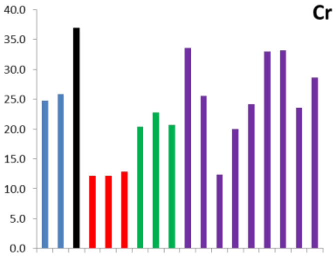
2.0
0.0


Cr
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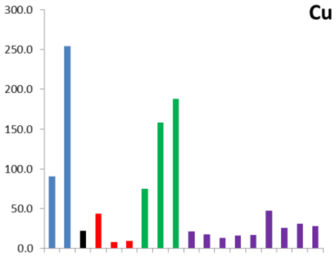
30.0
25.0
20.0
15.0
10.0
5.0
0.0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250.0
200.0
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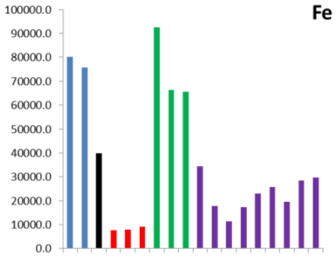
100.0
50.0
0.0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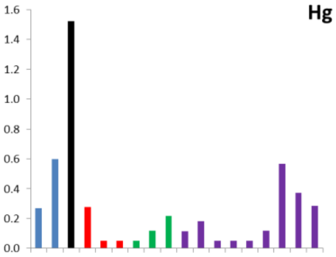
0.0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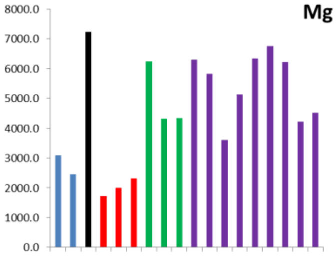
1.2
1.0
0.8
0.6
0.4
0.2
0.0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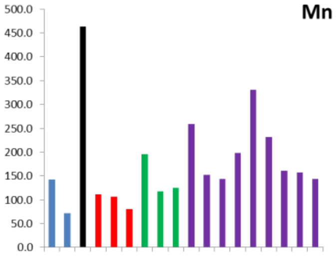
2000.0
1000.0
0.0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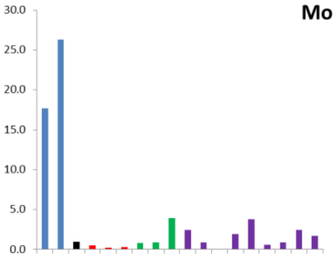
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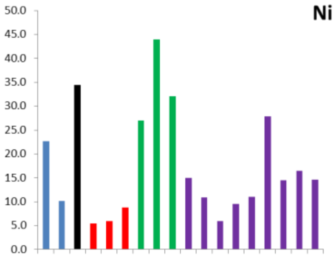
0.0


Ni
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20.0
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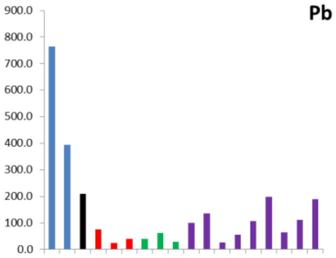
5.0
0.0


Pb
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600.0
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400.0
300.0
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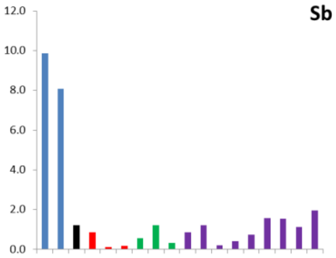
100.0
0.0


Sb
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6.0
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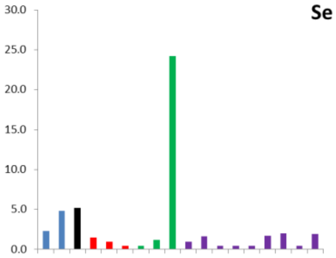
2.0
0.0


Se
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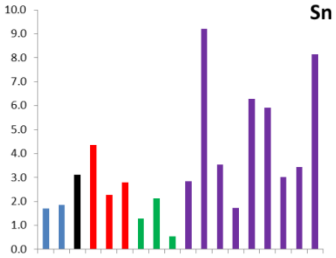
0.0


Sn
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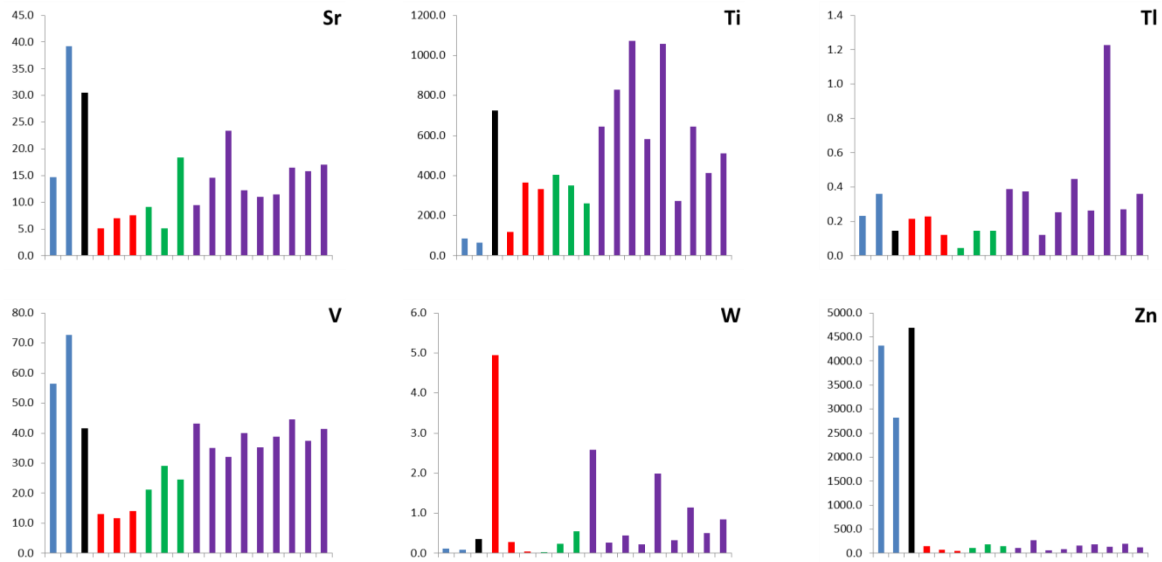

Figure 4. Concentrations (in mg·Kg^{-1}) of metals and metalloids measured in the surface sediments. Different colours have been used to differentiate the lake groups identified as a result of PCA. In axis X, from left to right: MT, PP, AN, AIr, CO, SI, AIx, BA, BE, ER, AU, ES, GR, LL, MA, MO, PL and RO.

Looking at similarities between lakes of the same group, sediments of PP and MT have some of the highest concentration levels of Cd, Cu, Fe, Mo, Pb, Sb, V and Zn, as shown in Figure 4. In lakes BA, Aix and BE, however, A1, Co, Cu, Fe, Ni and Se concentrations are significantly higher than in the rest of lakes (Figure 4). In general, the lowest element concentrations were found in SI, CO and Air. The only exceptions are the levels of As and W found in Lake Airoto. As already stated, this lake is located in an area very rich in arsenopyrites. The group that is formed by ES, GR, ER, LL, AU, RO, MA, MO and PL shows high concentrations of Ti, Mg, Ba, Mn, Sn and Tl, and, finally, the sediments in lake AN are rich in Cd, Cr, Hg, Mn, Sr and Zn (Figure 4).

3.2. SEDIMENT CORES: HISTORICAL RECORD OF METAL CONCENTRATIONS

Sediment cores were used to study the historical record of pollution. The analysis was limited to 12 elements (Cu, Cr, Pb, Zn, As, Cd, Ni, Hg, Co, Mn, Sn and Sb) since, for the rest of the elements, the concentrations found below the detection limits were numerous. As it has already been said in the experimental Section, firstly surface sediments were separated from each core (0-1.5 cm) and from that point on, the sediment cores were divided into 3.5 cm layers to obtain sediment samples. Information of a rather long period of time is stored in a 3.5 cm layer due to the slow sedimentation ratio in lakes. As a consequence, the resulting depth profiles could be so smoothed that historical information remains hidden in them (Camarero et al., 1998). This should be taken into account for a correct interpretation of the results. There are examples in literature, however, where 2.4 cm layers have been used to investigate historical records of pollution (Bacardit et al., 2012). It must also be taken into account that Ti concentration has been used to normalised data, since its origins has been reported to be natural in the Pyrenees (Camarero, 2003). The sediment cores used in this study were the ones which at least had 17 cm long, that is to say, those obtained from ER, PP, PL, MA, Aix, AU, RO, BA, SI, CO and ES. The vertical profile with depth of the normalised concentration of each metal can be seen in Figure 5.

As it can be seen in Figure 5, Cd, Pb, Sb and Sn have a similar tendency in all the lakes. After the first 5 cm from the surface, concentration levels diminish significantly. This tendency exists in 8 lakes out of the 11 that were studied, e.g., AIx, AU, BA, ER, ES, MA, PL and RO, with the following exceptions: Pb in AIx, and Pb and Sn in BA. In all these lakes, the concentrations of elements such as Cd, Pb, Sb and Sn have risen significantly in recent years. In fact, another study has estimated that the settling ratio in the first 30 mm of sediments in the Pyrenean Lake Redó is of 0.23 mm per year (Camarero et al., 1998). In other words, that means that the upper 5 cm layer in the lake has accumulated in the last 200-300 years. According to this estimation, the rises in concentration levels of Cd, Pb, Sb and Sn in the lakes considered in this study started, approximately, with the industrial revolution (Bacardit et al., 2012; Camarero et al., 1998).

The case of PL lake is worth mentioning, as it shows the same tendency in all the metals and metalloids that have been studied, except for Hg and Zn. The first 5 cm layer contains the highest concentrations of most of the elements and, as it could be expected, the concentration levels drop as we go deeper. However, the highest relative levels of Hg and Zn are found at a depth of 5-10 cm and 25-30 cm. In the sediment cores of PP lake, the highest concentrations of most elements were measured at a depth between 5 and 10 cm. Mining activities in this area could come from the Middle Ages, and this could explain the presence of these maximums of concentration. Lakes MA, AIx and CO show similar profiles. Apart from the general tendency that was observed for Cd, Pb, Sb and Sn, the highest concentration levels of the rest of the elements were found at a depth of around 15 cm; this may have been caused by a faster sedimentation ratio in these lakes.

Most of the results obtained after the analysis (taking into account the limitations that having divided the columns into 3.5 cm causes) coincide with those in other studies where Pyrenean lakes have been examined. For example, Camarero et al. in 1998 registered high metal concentrations originating from anthropogenic inputs in Lake Redó, in the layers that correspond to the period between 500 AD, and 1000 AD (Camarero et al., 1998). Camarero's study suggests that the appearances of higher concentrations are the consequence of mining activities which were active at that time. Sedimentation ratio is, however, an important variable that must be taken into account when studying historical records. Depending on the location of the lake and the topography around it, the sedimentation ratio varies, and this may induce profound differences in the datation of the cores of each emplacement.

A correlation analysis of the whole concentration data matrix (concentrations from all the lakes and all the depths all together) was carried out. The highest correlation coefficients were found between the same pairs of elements than in the analysis of surface sediments, that is, between Zn and Cd (0.94), Pb and Sb (0.85) and Ni and Co (0.94). They were all significant at a 95% confidence level.

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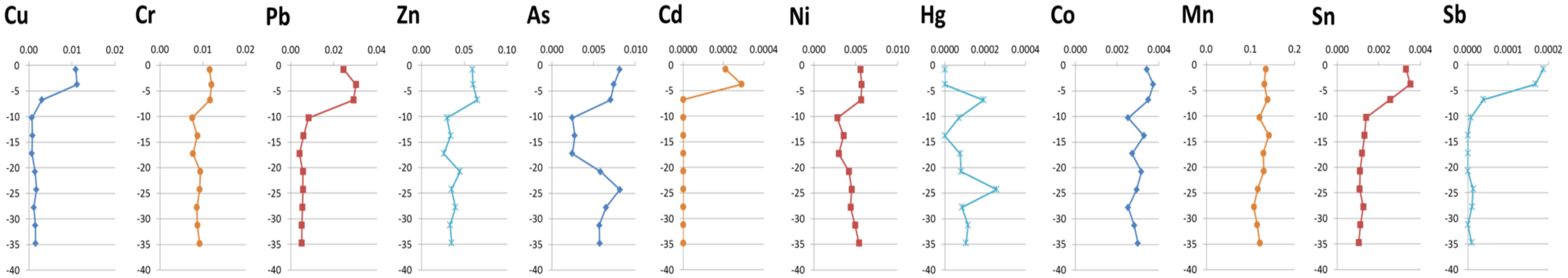

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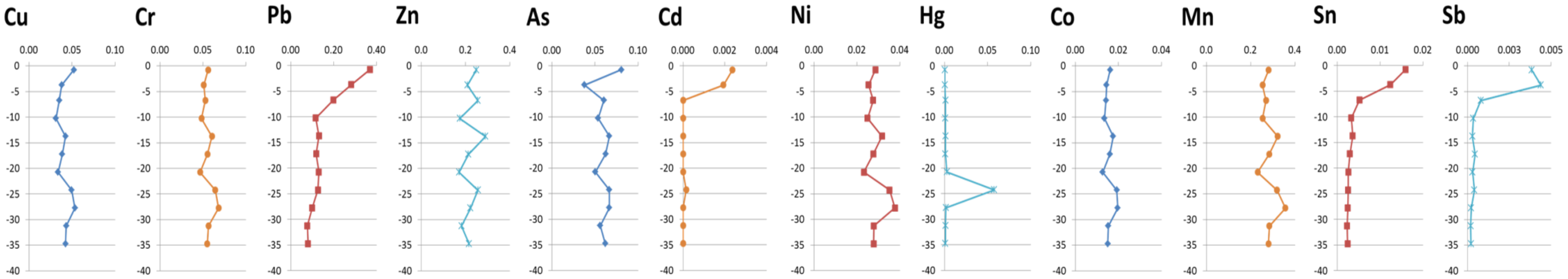

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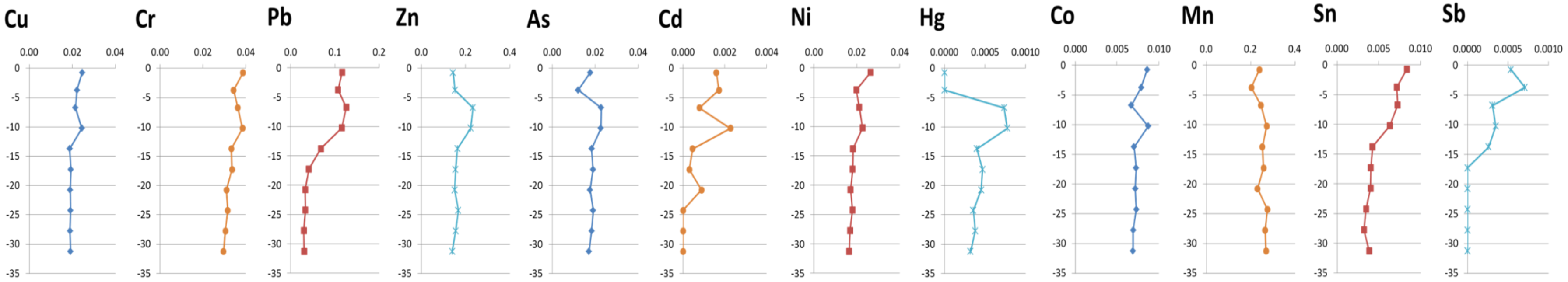

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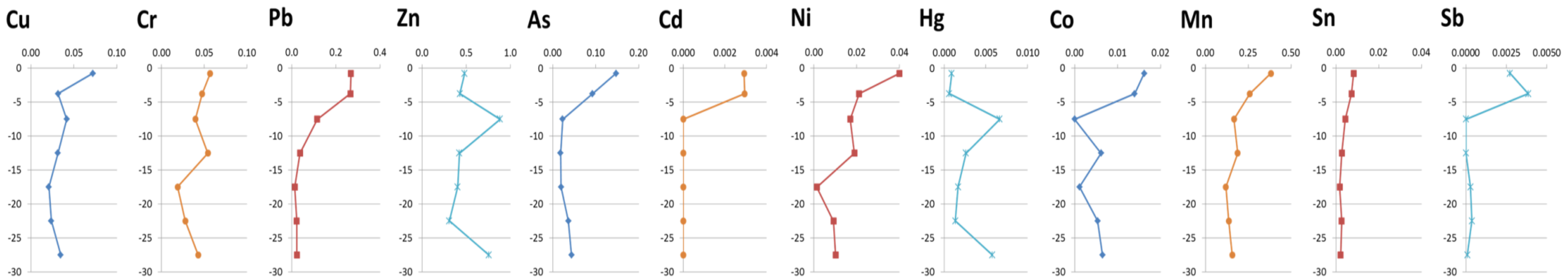

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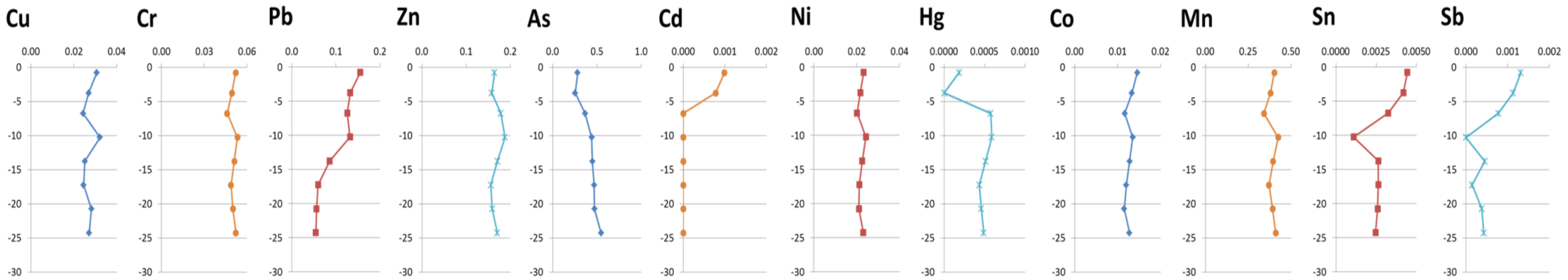

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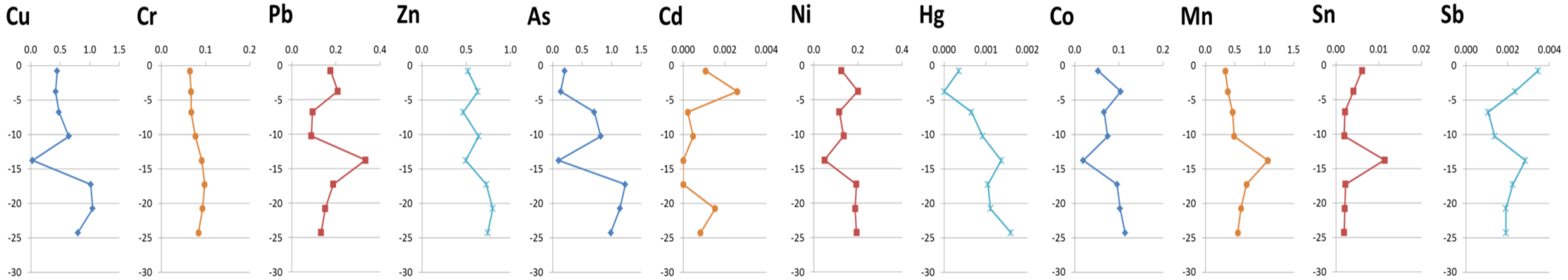

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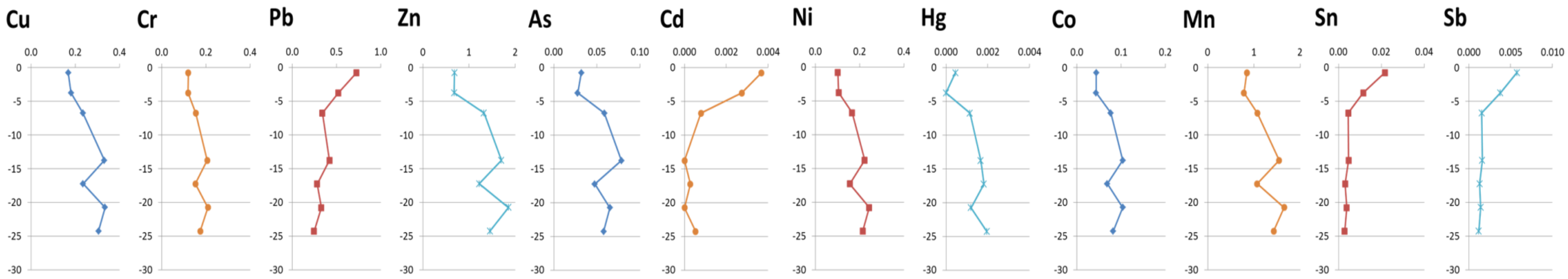

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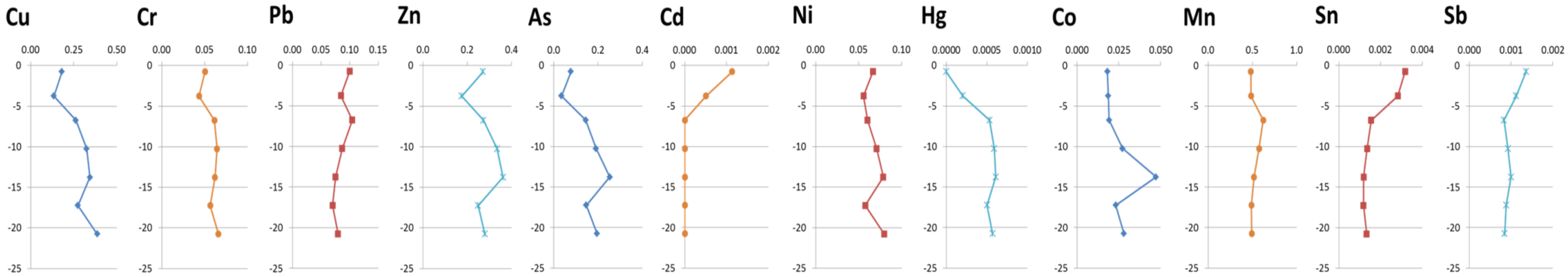

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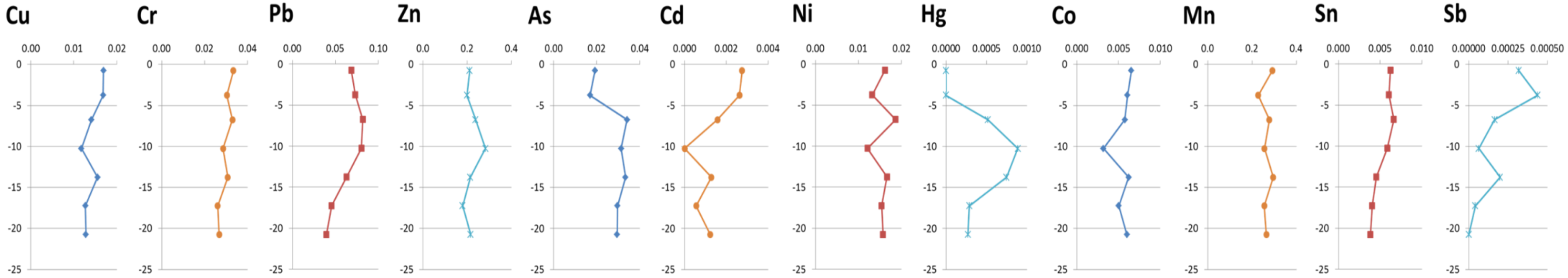

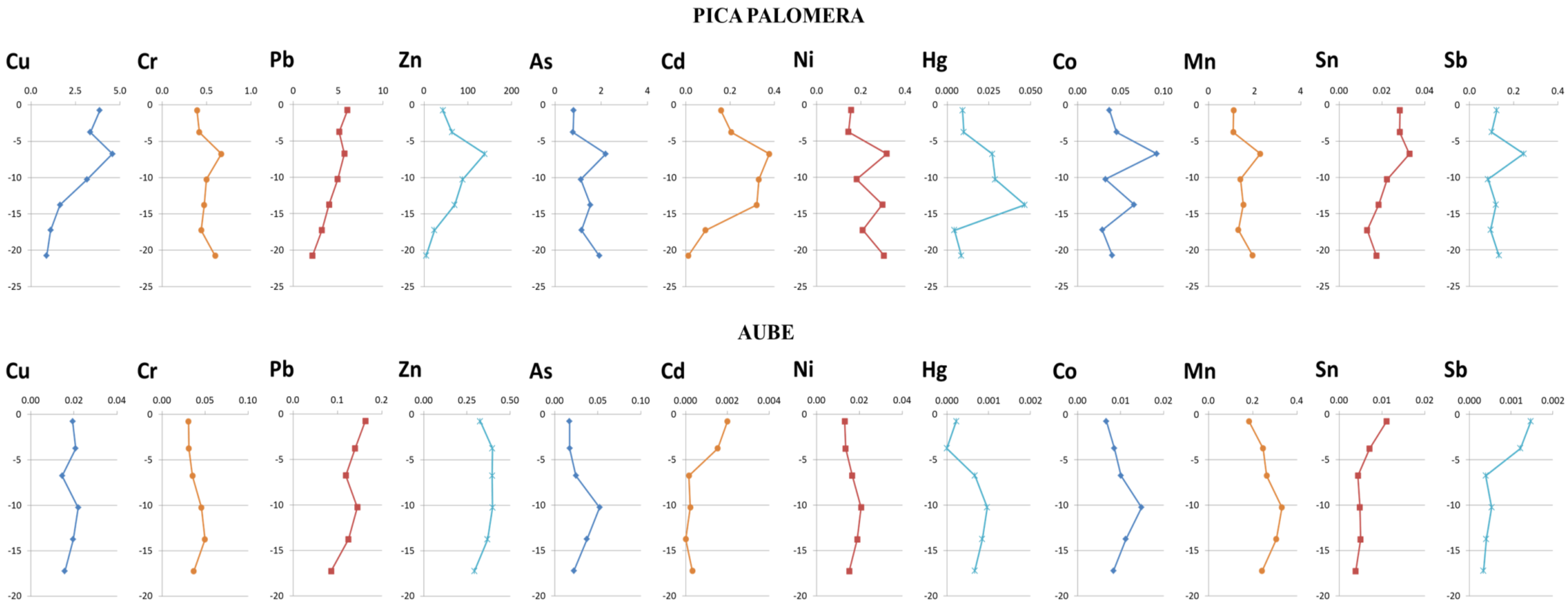

Figure 5. Vertical concentration profiles of the elements measured in the sediment cores obtained from Pyrenean lakes. Depth in cm is plotted in axes Y. Concentrations normalised with Ti are plotted in axes X.

3.3. ENRICHMENT FACTORS (EF): ANTHROPOGENIC INPUTS

The enrichment factor (EF_M) is an index used to determine if a pollutant has natural or anthropogenic origin. In this study concentrations have been normalised with Ti to calculate the EF_M (Equation 1).

$$EF_M = \frac{C_M / C_{Ti}}{C_M^0 / C_{Ti}^0} \qquad \text{(Equation 1)}$$

M stands for the studied metal, C represents the metal concentration in the sediment sample, and C^0 is the estimated background value for that metal in the area (Camarero et al., 1998). According to other studies, the sediments that were at a depth of 17 cm could be 700-900 years old. In this study, it has been assumed that the anthropogenic origin of metals in sediment layers deeper than 17 cm is negligible. Consequently, the average of concentrations deeper than 17 cm has been used to estimate the background values. EFs higher than 2, show non natural metallic input and indicate the presence of contamination (Camarero, 2003).

Table 4. EF values calculated from upper layers (0-1.5, 1.5-5.0, 5.0-8.5, 8.5-12.0, 12.0-15.5 cm) of the sediment cores in some Pyrenean lakes. The average, minimum and maximum values of the EFs calculated are provided.

Lake	**As**	**Ba**	**Cd**	**Co**	**Cu**	**Fe**
Alx	0.7 (0.2-1.3)	1.0 (0.9-1.0)	-	0.9 (0.6-1.7)	0.7 (0.4-0.8)	0.7 (0.4-0.9)
ER	0.7 (0.5-0.9)	1.1 (1.0-1.3)	-	1.0 (0.9-1.1)	1.0 (0.9-1.1)	0.9 (0.8-1.1)
AU	1.3 (0.8-2.4)	1.1 (1.0-1.2)	2.4 (<DM-6.2)	1.2 (0.8-1.8)	1.2 (0.9-1.3)	1.1 (0.8-1.4)
BA	0.6 (0.1-1.2)	1.2 (0.9-2.2)	1.4 (0.3-3.1)	0.6 (0.2-0.9)	0.7 (0.1-1.3)	0.8 (0.2-1.7)
CO	0.9 (0.6-1.2)	1.5 (1.0-2.2)	1.2 (<DM-2.2)	0.9 (0.5-1.1)	1.1 (0.9-1.3)	0.9 (0.8-1.1)
ES	1.0 (0.4-1.4)	1.3 (0.9-1.8)	-	1.0 (0.8-1.2)	2.2 (0.5-7.2)	1.0 (0.8-1.2)
MA	0.9 (0.5-1.4)	1.2 (1.0-1.4)	2.4 (<DM-7.0)	0.9 (0.5-1.3)	0.8 (0.6-1.0)	0.8 (0.6-1.1)
PL	1.3 (0.4-3.3)	1.1 (0.7-1.6)	-	1.1 (<DM-2.5)	1.1 (0.6-2.0)	1.4 (0.4-2.8)
PP	0.7 (0.4-1.1)	1.0 (0.8-1.2)	22.6 (8.2-34)	1.4 (0.7-2.3)	3.4 (1.3-5.2)	0.6 (0.4-1.1)
RO	1.0 (0.6-1.3)	1.1 (0.9-1.5)	-	1.1 (0.8-1.3)	1.0 (0.7-1.2)	1.1 (0.8-1.5)
SI	1.1 (0.7-1.3)	1.3 (0.9-2.0)	-	1.1 (1.0-1.3)	1.1 (0.9-1.2)	1.6 (0.3-1.7)

Lake	**Mn**	**Ni**	**Pb**	**Sb**	**Sn**	**Zn**
Alx	1.0 (0.9-1.2)	0.8 (0.6-0.9)	1.0 (0.8-1.3)	1.2 (0.9-1.6)	0.9 (0.8-1.1)	0.9 (0.6-1.2)
ER	0.9 (0.8-1.0)	0.9 (0.8-1.0)	1.9 (1.0-2.8)	1.4 (<DM-3.0)	1.2 (0.4-1.8)	0.9 (0.9-1.1)
AU	1.0 (0.7-1.3)	1.0 (0.8-1.3)	1.6 (1.3-1.8)	2.3 (1.1-4.3)	1.6 (1.1-2.8)	1.2 (1.1-1.3)
BA	1.0 (0.6-1.9)	0.7 (0.2-1.0)	1.3 (0.6-2.5)	1.1 (0.5-1.8)	2.2 (1.0-5.8)	0.8 (0.6-1.0)
CO	1.0 (0.8-1.1)	0.9 (0.7-1.1)	1.7 (1.1-2.0)	-	1.4 (1.0-1.7)	1.0 (0.8-1.3)
ES	1.0 (0.8-1.1)	0.8 (0.5-1.0)	2.4 (0.7-5.8)	4.3 (<DM-19)	1.7 (1.0-3.3)	1.2 (0.7-1.8)
MA	0.8 (0.5-1.1)	0.7 (0.4-1.1)	1.7 (1.1-2.9)	2.1 (1.0-4.8)	2.8 (1.0-7.6)	0.8 (0.4-1.2)
PL	1.3 (0.7-2.4)	1.7 (0.1-3.9)	5.0 (0.6-11.)	13.8 (<DM-44)	2.0 (0.8-3.7)	0.6 (0.4-1.1)
PP	0.7 (0.5-1.1)	0.7 (0.4-1.0)	2.2 (1.4-2.8)	0.9 (0.6-1.8)	1.3 (0.7-1.8)	14 (4.7-27)
RO	1.0 (0.8-1.2)	1.0 (0.8-1.3)	2.0 (0.9-4.6)	5.2 (0.8-20)	2.1 (0.9-6.5)	1.0 (0.8-1.3)
SI	0.9 (0.7-1.0)	1.2 (1.0-1.6)	2.4 (0.9-4.0)	-	1.3 (0.8-2.1)	1.2 (1.0-1.6)

The EF_M values corresponding to the upper 5 layers (0-1.5, 1.5-5.0, 5.0-8.5, 8.5-12.0, 12.0-15.5 cm) of each core were calculated, and the average, maximum and minimum values obtained in each case are shown in Table 4. If some elements do not appear in the table, it is due to the fact that all their EF_M values were lower than 2. On the one hand, the case of PP lake is the most significant, as its average EF_M values for Cd, Cu, Pb and Zn were far above 2. Moreover, regarding Cd and Zn, the lowest EF_M was above 2. The anthropogenic origin of these metals is, therefore, evident, and it is probably a consequence of the historic mining activities. Also in RO, three elements (Sb, Sn and Pb) show average EF_M values above 2. The situation is very similar in lake MA: the EF_M for Cd in this lake is also higher than 2. The average EF_M values found in PL for Pb (5.0) was quite high and very high for Sb (13.8). On the other hand, in lakes AIx, ER and CO all the elements showed EF_M values below 2. On the other hand, Cd, Pb, Sb and Sn are the elements which presented more average EFs above 2, (3, 5, 4 and 4 lakes respectively, out of 11 lakes), which showed that the anthropogenic input of those metals is important all over the Pyrenees.

The EF_M of the surface sediments (0-1.5 cm) can be used to study the anthropogenic input over recent years (Figure 6). It can be estimated that this layer has been formed over the last 60-70 years (Camarero et al., 1998). Lake AIx did not show EF_M above 2 in any of the studied elements, showing that the recent anthropogenic input of metals and metalloids in this area is negligible. However, in PL lake, an EF_M above 2 has been obtained for elements such as As, Co, Cu, Fe, Mn, Ni, Pb, Sb and Sn. Furthermore, in the case of Sb, this lake has shown the highest EF_M out of all the lakes (31.4). Cd, Cu, Pb, Sb and Sn are the elements that showed the highest EF_M in surface sediments, and also the ones that have the widest geographical distribution (Figure 6).

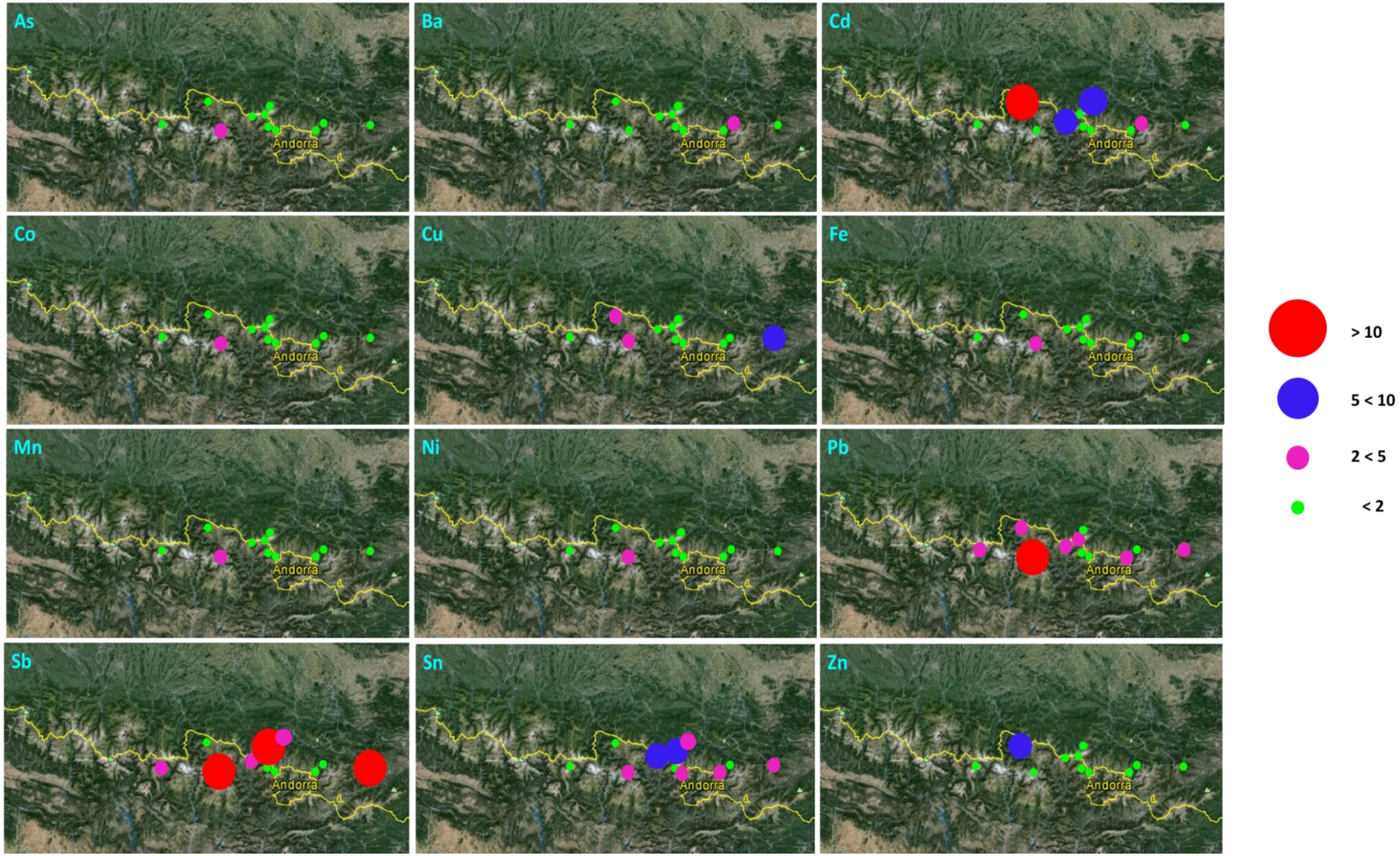

Figure 6. EF$_M$ values calculated for surface sediments in different Pyrenean lakes.

4. CONCLUSIONS

Regarding metallic pollution in the Pyrenees, the results obtained after the analysis of lake sediments confirm that the influence of human activity is significant. The mining activities which started long time ago (probably in the Middle Age) and the combustion of fossil fuels over recent years have had a direct effect on the lake ecosystems. The metal emissions released into the atmosphere via industrial activities can reach the lakes, airborned long distances by the winds.

Lithogenic composition should be taken into account to understand the accumulation of some elements (i.e. As and Ti) in the Pyrenees, as the presence of rocks rich in Ti and arsenopyrites is high.

The lakes under study can be divided into 5 groups depending on the metallic composition of their sediments: i) Pica Palomera and Montoliu showed high concentrations of Cd, Cu, Fe, Mo, Pb, Sb, V and Zn; ii) Baiau Superior, Aixeus and Bersau showed high concentrations of Al, Co, Cu, Fe, Ni and Se; iii) Siscar, Compte and Airoto showed the lowest concentrations of metals in general; iv) Estelat, Gran del Pesso, Eriste, Llosás, Aube, Romero de Dalt, Mariola, Monges and Plan showed high concentrations of Ti, Mg, Ba, Mn, Sn and Tl, and finally, v) Anglas showed high concentrations of Cd, Cr, Hg, Mn, Sr and An.

According to the EF_M values calculated for the surface sediments, Cd, Cu, Sb, Sn, and particularly Pb, seem to be the elements with the highest anthropogenic impact in the Pyrenees. Tradicional mining activities and the massive use of fossil fuels over recent years could be important contributing factors.

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Chapter 5

MOSSES, LICHENS AND BEECH LEAVES AS BIOINDICATORS OF ATMOSPHERIC METALLIC POLLUTION: A CASE STUDY, THE IRATI FOREST (WESTERN PYRENEES)

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ABSTRACT

The Irati Forest, an area of about 17.000 ha in the Western Pyrenees, is one of the most important forests in Europe. Monitoring of air contamination with living organisms provides low-cost information on the nature and quantity of contaminants. In this study beech leaves, lichens and mosses collected at 26 sampling sites of the Irati Forest in November 2011 were used to i) compare the potential of these three organisms as biondicators of atmospheric metallic contamination, ii) provide a preliminary assessment of the distribution of metal contamination in the area and iii) study the possible origin of metals in the forest. The calculated enrichment factors (EFs) suggested that most of the metals considered in this study were of anthropogenic origin. Metal emissions from steelworks and road traffic, transported by the predominant winds from the West, could be partially responsible of the presence of metals in the Irati Forest. The geomorphology of the Irati River water catchment seems to affect deeply the geographical distribution of metals. All the organisms investigated showed a potential to be used as bioindicator of atmospheric metal contamination, but it must be taken into account that, due to their different longevity, they provide us with information characteristic of different periods of time. The results obtained suggested that the accumulation of certain metals in beech leaves highly depends on the mountainside orientation of the sampling site.

Keywords: Beech leaf; Moss; Lichen; Bioindicators; Atmospheric metal contamination; Irati Forest.

1. INTRODUCTION

There are many pristine forests around the world which allow the survival of different organisms. At the same time, these organisms contribute to maintain forest ecosystems close to their original conditions. Forest ecosystems are also important because they provide us with food, oxygen, different materials and leisure spaces. The number of Ha covered with forests, however, is decreasing constantly around the world due to deforestation, global climate change, contamination, diseases and pathogens. All of these threats are increasing in the last decades due to human activity (Amores and Santamaria, 2003).

Regarding contamination in forests, one of the most common and damaging pollution is that caused by heavy metals. Metal and metalloids can be transported in the atmosphere long distances (Gioda et al., 2011; Marques et al., 2004). They can reach, consequently, even unexpected areas like pristine mountain ecosystems such as forests. Biomonitoring is regarded as a means to assess metallic elements concentrations in aerosols and deposition. This implies that the monitor should concentrate the element of interest and quantitatively reflect its ambient conditions (Wolterbeek and Verburg, 2002). Furthermore, monitoring of air contamination with living organisms provides low-cost information on the nature and quantity of contaminants (Loppi and Bonini, 2000; Markert et al., 1997).

Lichens have been recognized as sensitive indicators of atmospheric metallic contamination. Lichens have no roots or well-developed cuticle. In addition, the lichen longevity, stability, growth form, roughness and the direct dependence on atmosphere for nutrients facilitate the interception and retention of particles which emphasize the applicability of lichen for atmospheric monitoring purposes (Nayaka et al., 2003). Lichens have been used as active (transplantation) and passive (lichen living in situ) atmospheric metal biomonitors from a long time. The metallic accumulation of metals in lichens can take place in three different ways: i) bounding metal ions to the cell wall by an ion exchange mechanism, ii) by intercellular active accumulation and iii) by entrapment of particles which contain metals (Szczepaniak and Biziuk, 2003).

Bryophytes and especially the carpet-forming species like mosses are considered suitable atmospheric biomonitors as well. Mosses show long-term airborne metals accumulation because they obtain most of their elemental supply from the atmosphere due to a lack of a root system. As lichens, they do not have a well-developed cuticle. Moreover, mosses high cationic exchange capacity and high surface-area-to-volume ratio favour the accumulation of atmospheric contaminants (Fernandez et al., 2002; Gerdol et al., 2001). Mosses widespread occurrence enables them to reflect the spatial and temporal variations of atmospheric metals through their accumulation (Faus-Kessler et al., 2000; Gerdol et al., 2001). Furthermore, moss biomonitoring seems to be the most popular, because it causes fewer technical and analytical problems than lichens and trees (Szczepaniak and Biziuk, 2003).

The leaves of the trees are also very efficient for atmospheric particles biomonitoring (Tomasevic et al., 2011). The morphology of leaves is important for the atmospheric particles deposition ratios and absorption. Beech leaves have been used to biomonitor many atmospheric contaminants (Baker and Jickells, 2006; Fromm et al., 1987; Olszowski et al., 2012; Tomasevic et al., 2011; Tyler and Olsson, 2006). Besides, the contribution of metallic elements to leaves by deposition is higher than by roots uptake and the following transportation to leaves (Tyler and Olsson, 2006). The annual autumn leaves shedding is an important event in which concentrated metals can be excluded at regular intervals as detoxification mechanism (Fromm et al., 1987).

These three indicators of atmospheric contamination retain atmospheric metallic elements of different contamination sources. One of those sources can be the lithology of the area itself, which produces mineral particles that can be windblown an incorporated to the atmosphere (Demirbas, 2005; Hubbard, 1995; Szczepaniak and Biziuk, 2003). Al and Fe are considered to be constituents of resuspended soil particles (Baker and Jickells, 2006). Anthropogenic emissions due to industrial activities (Olszowski et al., 2012), road traffic (Berg and Steinnes, 1998), fires (Demirbas, 2005; Hubbard, 1995; Sippula et al., 2009) and wood treatment industry (Stilwell and Gorny, 1997) are also important. In order to assess the anthropogenic or natural sources of metals and metalloids in biomonitoring works, the enrichment factor (EF) is usually calculated (Chiarenzelli et al., 2000). The EFs compares the relative concentration of an analyte in the organisms used for the biomonitoring to that in soil (Bergamaschi et al., 2005; Klos et al., 2011). While unit or near-unit EFs are taken as an indication of soil origin, substantial enrichment over the natural (crustal) background levels points to anthropogenic sources. The widely accepted threshold for such a distinction is EF > 10 (do Carmo Freitas et al., 2007).

The aims of this work are i) to compare the efficiency of mosses, lichens and beech leaves as bioindicators of atmospheric metallic contamination, ii) to investigate the geographical distribution of metals in the Irati Forest (Western Pyrenees) and iii) to study their origin and possible sources in the area.

2. EXPERIMENTAL

2.1. STUDY AREA AND SAMPLING

The Irati Forest, one of the widest beech forests in Europe (17000 ha), is located in the Western Pyrenees (Figure 1). It is a vast territory between the Ortzanzurieta Hill in the West and the Orhi and Pikatua Mountains in the East. The Abodi mountain range constitutes the southern limit of the forest, while the ridgeline from Harsudurra to Errozate defines the northern limit. The upper part of the Irati River water catchment takes an important part of the forest. The river rises near the Orhi Mountain and flows first to the West and then turns quite sharply to the South in the surroundings of Orbaitzeta, forming a characteristic funnel-like corridor which for sure influences the dominant wind currents in the area (Figure 1).

Two local roads, one from Orbaitzeta (NA-2032) and another one from Otsagabia (NA-2012), drive a considerable amount of visitors, especially from late spring to early autumn, to the surroundings of the reservoir of Irabia, in the heart of the forest, where most of the leisure activities are concentrated. One main road (N-135) with heavy traffic all the year around crosses the western part of the forest from the South to the North. One secondary road, the NA-140/NA-2011 runs in parallel with the Southeast boundary of the forest. In the northern boundary, a 4X4 track is sporadically used by local cattle ranchers. In addition, a relatively complex system of 4X4 tracks with traffic limitations is present in the forest. The nearest cities are Iruñea (55 km to the Southwest), Donostia-Biarritz (80 km to the Northwest) and Orthez-Pau (80 km to the Northeast), with a remarkable urban and industrial activity (Figure 1). Livestock and agricultural activities are still important in the surroundings of the forest, and even in a few punctual exploitations inside it. Controlled grass burning has also been reported in the area, especially in the north sector (Barre, 2013).

The Irati Forest presents mountain climate characteristics, with subalpine rank. The average annual temperature is about 8ºC, with an annual precipitation of about 2.000 mm and predominant winds from the northwest (Acedo and Ganuza, 2007; Ezcurra et al., 2013; Gonzalez-Miqueo et al., 2009). It is considered to be a young forest (less than 12000 years old). Initially oak (*Quercus petraea*) was the most abundant tree but nowadays beech (*Fagus sylvatica*) is the most widespread species (Martínez et al., 1991). Other species can also be found, such as rowan (*Sorbus domestica*), maple (*Acer platanoides*), willow (*Salix alba*), linden (*Tilia*), mountain elm (*Ulmus glabra*), boxwood (*Buxus sempervirens*), hazel (*Corylus avellana*), holly (*Ilex aquifolium*), hawthorn (*Crataegus monogynav*), juniper (*Juniperus communis*) and blackthorn (*Prunus spinosa*). A large variety of mosses and lichens is also present.

The area to be sampled was first divided in 26 quadrants. Once in the field, an area of about 50×50 m was selected as sampling site inside each quadrant, taking care that there was no main road closer than 300 m nor track or building closer than 100 m. Composite samples were collected within the 50×50 m area as representative of the quadrant. The sampling sites are shown in Figure 1, and their UTM coordinates, altitude and mountain side orientation are summarised in Table 1.

Beech (*Fagus sylvatica*) leaves, lichens, mosses (Figure 2) and soil were collected in November 2011. Not all kind of samples were collected in all the sampling sites (Table 1). Healthy and mature leaves exposed to sun light were collected from different living trees at a height between 1.5 and 2.5 m and as far as possible from the trunk. Composite samples of at least 50 leaves were collected according to the procedure described elsewhere (Martin and Coughtrey, 1982). Regarding mosses (*Hypnum cupressiforme*), composite samples of five subsamples were collected from stumps using a scalpel and following the guidelines of the UNECE ICP-Vegetation (Harmens, 2010). Lichens (*Parmelia sulcata*) were collected from the trunks of different living beech trees at an approximate height of 1.5 m with a scalpel. Finally, surface soils (top 10 cm) were collected in the same area than leaves, mosses and lichens. The sampling was carried out using latex globs. Moss samples were put inside paper bags, while soil, lichen and leaves were placed in Ziploc plastic bags. The paper and plastic bags were labelled and transported to the laboratory inside mobile fridges at 4ºC.

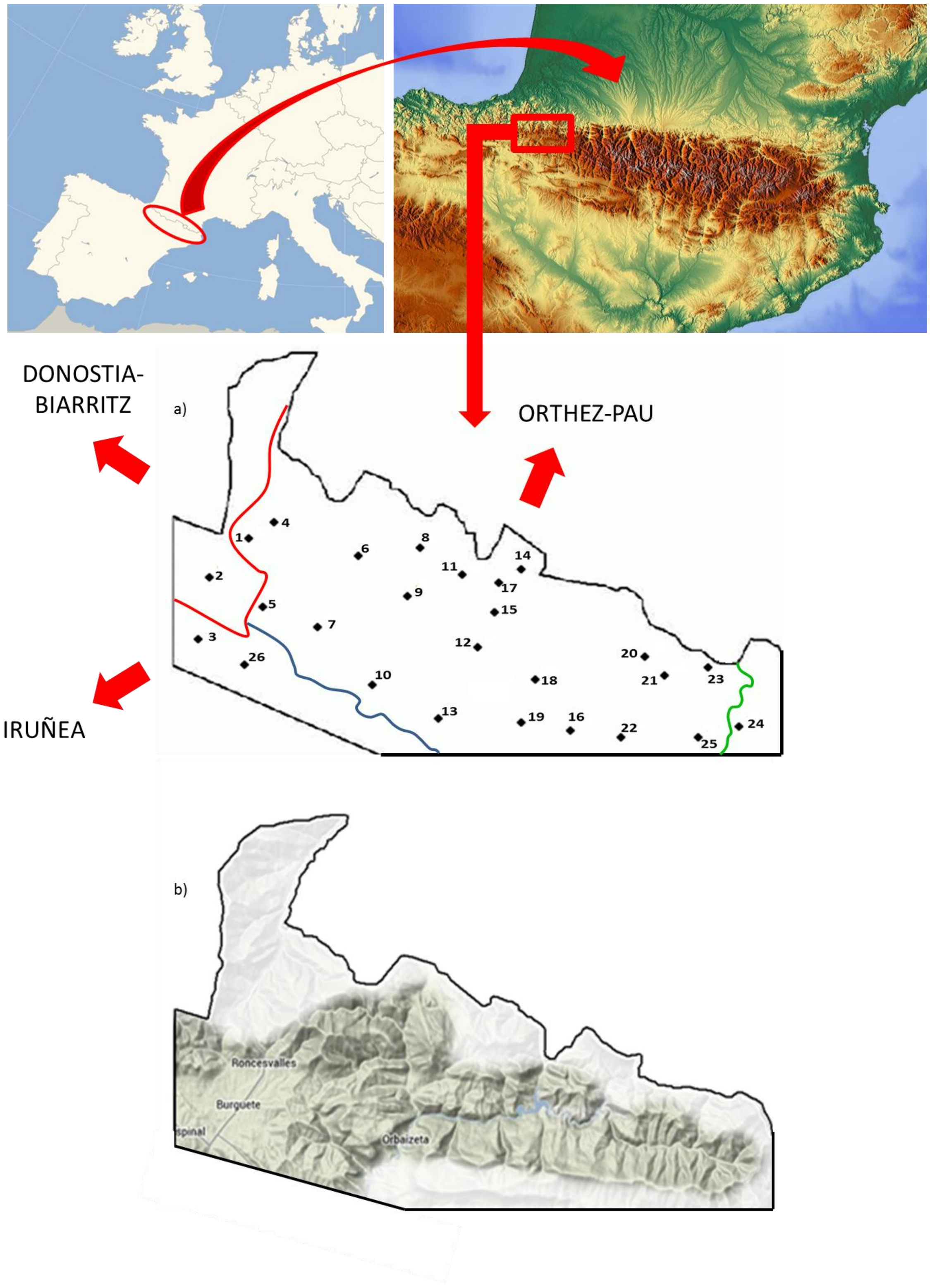

Figure 1. a) Situation of the Irati forest in the Western Pyrenees. The sampling points, the most important roads (NA-135 in red, NA-140 in blues and NA-2011 in green) and the main towns are also shown in the map; b) the water catchment of the Irati River with its characteristic funnel-like form constitutes the heart of the Irati Forest.

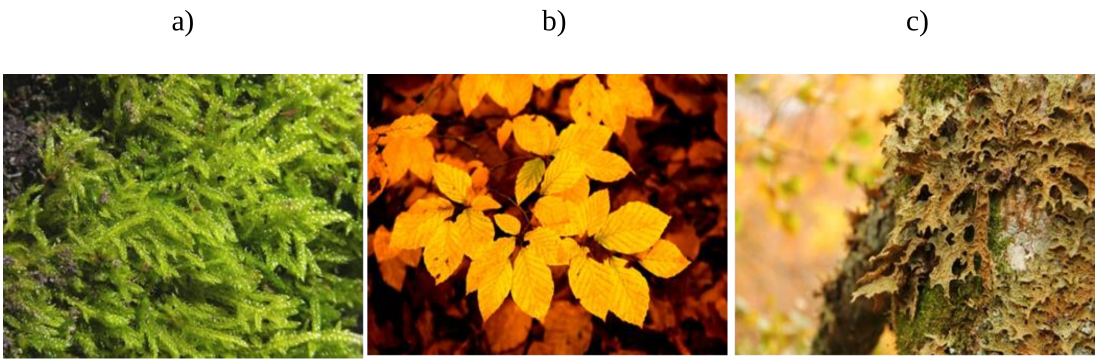

Figure 2. Samples collected in the Irati Forest: a) moss (*Hypnum cupressiforme*), b) beech leaves (*Fagus sylvatica*) and c) lichen (*Parmelia sulcata*).

Table 1. Sampling date, geographical coordinates (UTM 30T), altitude, mountainside orientation and the type of sample collected (+ collected, - not collected) for each sampling site.

SAMPLING SITE	SAMPLING DATE	X COORDINATE	Y COORDENATE	ALTITUDE (m)	ORIENTATION	LEAVES	LICHEN	MOSS	SOIL
1	02/11/2011	636966	4765064	1030	W	+	+	+	+
2	02/11/2011	634717	4762013	901	SE	+	+	+	-
3	04/11/2011	634044	4758807	927	E	+	+	+	+
4	02/11/2011	638736	4765086	1174	W	-	+	+	+
5	02/11/2011	637427	4760143	965	SW	+	+	+	+
6	02/11/2011	643481	4763701	903	S	+	+	+	+
7	04/11/2011	640643	4758562	918	N	+	+	+	+
8	02/11/2011	647461	4763827	962	SE	+	+	+	-
9	03/11/2011	645956	4760126	1026	N	+	+	+	-
10	03/11/2011	643357	4756580	851	N	+	+	+	+
11	02/11/2011	649290	4762189	920	E	+	+	+	+
12	03/11/2011	649917	4758109	1340	N	+	+	+	+
13	03/11/2011	647916	4753916	1047	E	-	+	+	+
14	03/11/2011	652768	4761366	994	W	+	+	+	+
15	03/11/2011	652507	4759363	1130	NW	+	+	+	+
16	04/11/2011	656078	4755008	925	W	+	+	+	+
17	03/11/2011	654771	4761889	910	SW	+	+	+	+
18	03/11/2011	655032	4756924	1288	SW	+	+	+	+
19	04/11/2011	654858	4755095	927	NW	+	+	+	-
20	03/11/2011	658975	4759752	1192	SE	+	+	-	+
21	03/11/2011	659302	4758371	1248	NE	+	+	+	+
22	03/11/2011	656628	4754332	893	W	+	+	+	+
23	03/11/2011	662225	4759366	1340	N	+	+	+	+
24	03/11/2011	663897	4754837	976	SW	+	+	+	+
25	03/11/2011	661888	4754408	991	W	+	+	+	+
26	04/11/2011	636293	4757459	993	NE	+	+	+	+

2.2. ANALYTICAL PROCEDURE

The pretreatment and analysis of the samples was carried out according to the procedures described in Chapter 2, experimental. The concentration of 21 elements was finally measured by ICP/MS, but not all the elements were measured in all the samples. Statistical and chemometric analysis of data was performed by means of The Unscrambler (v. 9.2 Camo, Oslo, Norway). Contour maps were produced with the aid of the 3D Field programme (v. 3.8.8.0, by Vladimir Galouchko).

3. RESULTS AND DISSCUSION

3.1. SOIL

The concentrations found in soil samples and some related statistics are given in Table 2. Extreme values, defined as those concentrations higher than the 75th percentile plus three times the interquartile range (Otto, 2007), are marked with an asterisk in Table 2. The existence of extreme values in a variable indicates unexpected high concentrations of a given metal at specific sites in comparison with the rest of values which could be considered as "normal". Most of the extreme values (for Cd, Cr, Fe, Mg, Mn, Sb, Sn, Ti, V and Zn) were found in sampling site 26, which also presented the highest concentrations of Ni, Pb and Tl within the studied soils. This fact may be due to a punctual contamination or to the presence of a livestock farm in the surroundings of this sampling site. Extreme concentrations of Ti and Sb were also found in sampling site 4, and of Mg in sampling sites 13 and 20. After removing extreme values, statistics of the variables were recalculated and plotted in Figure 3. The concentrations measured were in most of the cases below the estimated abundance of elements in Earth's crust (Barbalace, 1995). The only exceptions were As, Cd, Pb, Sb and Tl (estimated abundance in $mg\cdot Kg^{-1}$, respectively, 1.5, 0.11, 14, 0.2 and 0.6). There are many studies which report high concentrations of As in soils and lake sediments of the Pyrenees due to a natural presence of arsenopirites (Bacardit and Camarero, 2010; Camarero, 2003; Camarero et al., 2009). Mining exploitation of Pb and Zn (with As, Cd, Sb and Tl as associated elements) has also been reported in different parts of the Pyrenees including their western end (Ansorena and Marino, 1990). It can be concluded consequently that the metals and metalloids contained in soils of the Irati Forest have basically a lithogenic natural origin. This is confirmed by comparison of the concentrations measured with the threshold values (EBA-A, EBA -B and EBA -C) defined in the Basque legislation on contaminated soils currently in force (Gobierno Vasco, 2005). Briefly, non-altered soils are characterised by concentrations below the EBA -B value, while soils with the concentration of any contaminant over the EBA-C value should be considered as contaminated. The As concentration exceeded the EBA -C value in all the sampling sites, while the EBA -B values was only exceeded for Zn in sampling site 26. The rest of elements presented concentrations below the EBA-B threshold in all the sampling sites.

Table 2. Element concentrations (mg·kg^{-1}) found in soils from the Irati Forest, together with some related statistics, the detection limits (LOD), accuracy (as recovery from the replicate analysis of the SRM 2711 reference material) and reproducibility (as relative standard deviation from the replicate analysis of the SRM 2711 reference material) of the analytical method. The threshold values EBA-A, EBA-B and EBA-C defined in the Basque legislation on contaminated soils (Gobierno Vasco, 2005) are also provided. L: clay content estimated for soils of the Irati Forest, 3.3% (Kapur S. et al., 2008); H: organic matter content estimated for soils of the Irati Forest, 10.2% (Kapur S. et al., 2008). Extreme concentrations (c>Q3+3IQR) are marked with an asterisk (*).

	Al	**As**	**Ba**	**Cd**	**Co**	**Cr**	**Cu**	**Fe**	**Mg**	**Mn**	**Ni**	**Pb**	**Sb**	**Sn**	**Sr**	**Ti**	**Tl**	**V**	**Zn**
L.O.D ($\mu g \cdot kg^{-1}$)	195	21	102	41	141	182	86	182	194	891	241	980	81	24	611	911	112	504	286
RECOVERY (%)	80%	103%	86%	98%	114%	78%	99%	70%	82%	70%	94%	80%	82%		79%	91%	68%	70%	100%
REPROD. (RSD)	8%	8%	9%	4%	5%	10%	9%	2%	14%	12%	4%	5%	14%	16%	10%	8%	4%	2%	11%
1	11600	123	75.1	0.22	2.63	10.9	8.15	15000	790	385	4.81	38.0	0.62	0.94	12.1	40.0*	1.47	12.3	43.5
3	6920	106	17.5	0.051	2.29	10.2	5.83	10800	681	167	4.20	7.37	0.35	0.30	3.05	13.7	1.42	11.4	17.0
4	8550	42.7	32.1	0.092	1.98	8.90	10.1	13000	926	207	5.10	37.7	1.27*	0.81	7.75	33.3*	1.40	11.1	36.4
5	15900	79.1	64.3	0.12	4.33	17.6	6.78	13900	1910	371	9.28	15.7	0.36	0.89	9.15	12.1	1.62	22.9	52.9
6	18800	94.7	22.8	0.098	1.26	19.2	7.86	21500	1090	134	7.58	37.1	0.38	1.14	12.0	10.3	1.53	19.4	40.7
7	14800	105	45.7	0.12	5.38	15.8	6.39	15700	2360	388	11.04	13.7	0.38	0.68	4.71	9.68	1.41	19.5	44.3
10	13800	154	37.3	0.11	3.69	15.0	5.56	13500	1770	130	8.92	13.6	0.27	0.89	5.85	8.73	1.54	17.1	43.8
11	14500	106	43.0	0.081	5.31	16.6	6.40	15700	2290	256	10.17	14.6	0.33	0.88	4.63	11.2	1.60	20.3	45.5
12	16700	19.0	48.2	0.13	4.04	18.5	6.98	18900	1930	333	8.47	30.2	0.47	1.93	5.02	8.13	1.58	25.5	64.8
13	11300	112	40.8	0.099	4.67	13.1	7.10	12700	6690*	322	8.14	11.8	0.34	0.60	27.2	11.5	1.51	16.5	34.5
14	13100	47.6	41.5	0.083	2.87	15.8	6.41	11900	1610	121	7.23	14.1	0.25	0.92	4.95	8.23	1.52	18.8	41.2
15	12500	105	30.7	0.42	3.86	21.9	5.10	10300	2940	198	9.74	14.7	0.30	1.12	4.94	22.0	1.48	16.8	40.7
16	10500	52.0	43.1	0.23	4.99	13.2	6.28	11900	1820	386	10.09	11.8	0.33	0.52	6.86	13.6	1.46	16.7	39.4
17	11500	116	36.9	0.28	7.42	11.9	5.84	16900	1810	513	15.82	20.0	0.33	0.75	7.14	6.17	1.47	16.2	47.8
18	12700	118	44.0	0.13	6.20	14.9	7.88	17200	2500	470	11.82	14.9	0.42	0.72	4.60	6.96	1.52	17.3	48.9
20	11000	113	29.7	0.11	3.70	14.6	8.33	12700	8250*	254	8.50	13.1	0.24	0.68	32.9	5.07	1.44	15.5	39.8
21	15500	102	59.7	0.14	6.09	18.6	8.00	19600	3330	425	14.38	18.8	0.42	0.70	6.84	6.65	1.61	21.2	63.5
22	13200	112	57.0	0.097	4.77	14.6	5.39	12400	2040	346	9.32	15.4	0.33	0.76	5.02	10.7	1.50	19.1	39.7
23	10600	101	45.5	0.25	5.32	16.2	7.59	14400	2260	478	12.49	28.3	0.26	1.63	6.57	4.71	1.38	16.2	75.5
24	14800	116	61.0	0.17	4.78	19.4	6.37	15200	2630	315	13.36	14.7	0.24	0.98	12.1	7.57	1.52	21.6	48.7
25	18600	111	22.8	0.098	1.22	18.9	7.66	21500	1080	131	7.29	37.4	0.38	1.12	12.2	9.94	1.52	19.1	41.7
26	15200	35.4	58.9	0.66*	7.00	36.3*	6.50	41800*	6800*	1360*	18.78	50.0	2.59*	4.91*	9.38	76.2*	1.71	61.1*	153*
25 PERCENTILE	11200	72.3	31.7	0.092	2.81	13.2	6.17	12600	1480	190	7.51	13.6	0.29	0.70	4.95	7.42	1.45	16.2	39.7
75 PERCENTILE	15300	114	57.5	0.22	5.34	18.7	7.87	17600	2700	397	12.0	31.9	0.42	1.12	12.0	13.6	1.55	20.5	49.9
IQR (Q3–Q1)	4100	41.7	25.8	0.13	2.53	5.50	1.70	5000	1220	207	4.49	18.3	0.13	0.42	7.05	6.22	0.10	4.30	10.2
Q3+(3*IQR)	27500	239	135	0.62	12.9	35.2	13.0	32700	6370	1020	25.4	86.8	0.80	2.39	33.3	32.3	1.84	33.4	80.4
MEDIAN	13200	105	43.0	0.12	4.50	15.8	6.64	14700	1990	327	9.30	15.2	0.35	0.88	6.85	10.1	1.52	18.0	43.7
MEAN	13300	94.1	43.5	0.17	4.26	16.5	6.93	16200	2610	350	9.84	21.5	0.49	1.09	9.31	15.3	1.51	19.8	50.2
DESVEST	2980	33.6	14.7	0.14	1.72	5.52	1.17	6550	2020	257	3.57	11.7	0.52	0.92	7.32	16.2	0.08	9.86	25.9
MIN	6920	19.0	17.5	0.051	1.22	8.90	5.10	10300	681	121	4.20	7.37	0.24	0.30	3.05	4.71	1.38	11.1	17.0
MAX	18800	154	75.1	0.66	7.42	36.3	10.1	41800	8250	1360	18.78	50.0	2.59	4.91	32.9	76.2	1.71	61.1	153
EBA-A		23	80+2.5L	0.8	6+0.5L	25+L	10+0.5L				12+L	16+0.7L+2H							50+2L
EBA-B		30		5	20	53	24				40	120							106
EBA-C		35		18	30	75	250				280	330							840

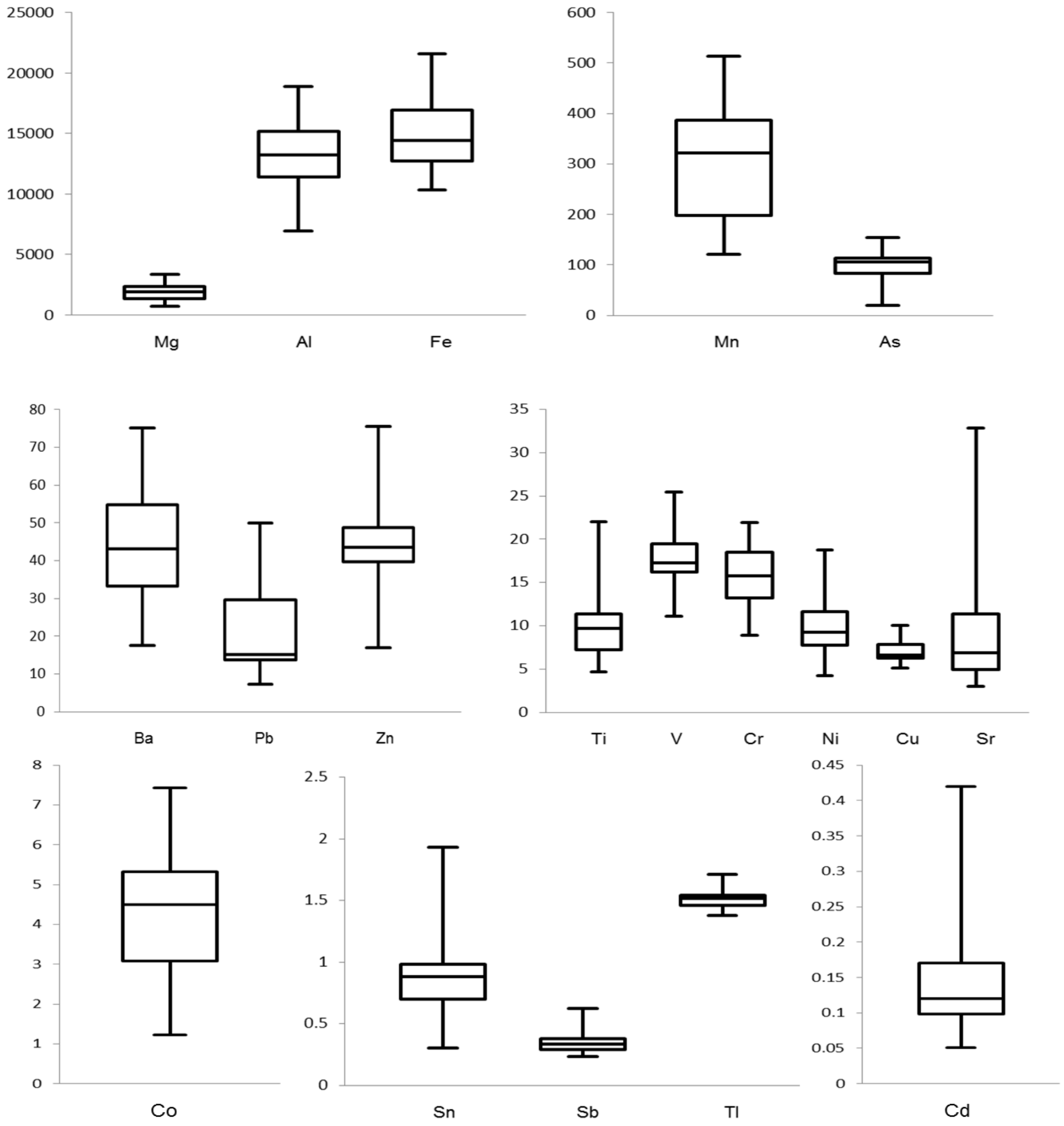

Figure 3. Within sampling sites concentrations (mg kg^{-1}) found in soils of the Irati Forest after removing extreme values. The box shows the 25th percentile and the 75th percentile, and the whiskers represent the smallest and largest concentrations, while the line inside the box is the median of the population.

3.2. BEECH LEAVES (*Fagus sylvatica*)

The element concentrations measured in beech leaves are shown in Table 3 together with some related statistics. Samples from sampling points 4 and 13 were accidentally lost. Extreme values, which are marked with an asterisk in Table 3, were identified for Al in sampling sites 3 and 10, for Ba in sampling site 22 and for Mg in sampling site 26. Figure 4 summarises the results after removing the extreme values.

Information in the Literature about the elemental composition of beech leaves in European forests is scarce. Amores and Santamaría reported, however, concentrations of several macro and micro elements in beech leaves collected in Pyrenean forests from the

North of Navarre, including the Irati Forest, in 1995 and 1997 (Amores and Santamaria, 2003). The concentrations measured in our study are comparable to those of Amores and Santamaria in the case of Fe, Mg, Mn and Zn, and slightly lower in the case of Cu (3.5±1.3 mg·Kg^{-1} *vs*. 6.1±1.4 mg·Kg^{-1}). Data have also been published for some German (Zech et al., 1990), Swedish (Tyler and Olsson, 2006) and Slovakian (Mankovska, 1998) forests. A comparison with the concentrations reported in those works and those measured in our study is provided in Table 3.

Table 3. Element concentrations (mg·kg^{-1}) found in beach leaves from the Irati Forest, together with the detection limits (LOD), accuracy (as recovery from the replicate analysis of the SRM 2711 reference material) and reproducibility (as relative standard deviation from the replicate analysis of the SRM 2711 reference material) of the analytical method. Extreme concentrations (c>Q3+3IQR) are marked with an asterisk (*). Concentrations found in beech leaves from other locations are also provided for comparison.

	Ag	Al	As	Ba	Cd	Co	Cr	Cu	Fe	Hg	Mg	Mn	Ni	Pb	Sb	Sn	Sr	Tl	V	Zn
L.O.D. (mg·kg^{-1})	0.02	1.02	1.23	0.42	0.36	0.06	0.44	1.11	1.08	0.05	1.24	0.98	0.03	0.27	0.07	0.17	0.85	0.59	0.43	0.58
RECOVERY (%)		92%	82%		96%	85%		98%		128%		97%	88%	84%			84%		82%	96%
REPRODUCIBILITY (RSD)	8%	4%	12%	10%	3%	4%	8%	2%	9%	21%	4%	8%	2%	8%	14%	16%	9%	17%	8%	2%
1	0.57	264	1.41	48.4	0.45	0.35	24.0	3.35	444	1.89	2000	931	11.1	2.75	0.10	0.23	51.0	<LOD	1.08	38.3
2	<LOD	152	<LOD	48.7	<LOD	0.68	6.47	4.23	172	0.22	896	956	5.04	0.76	<LOD	<LOD	22.9	0.62	0.44	25.7
3	<LOD	485*	<LOD	61.0	<LOD	0.85	19.5	5.05	534	0.24	1030	1440	10.1	1.20	<LOD	<LOD	20.9	0.61	0.93	26.3
5	<LOD	136	<LOD	50.4	<LOD	0.62	5.48	3.74	156	0.23	1100	693	5.01	0.66	<LOD	<LOD	27.3	<LOD	<LOD	27.9
6	<LOD	153	<LOD	17.7	<LOD	0.10	3.95	5.75	172	0.10	1200	306	2.98	0.39	<LOD	<LOD	25.0	<LOD	<LOD	30.1
7	0.041	221	<LOD	72.7	<LOD	0.72	7.75	3.50	216	0.24	1350	765	6.44	0.74	<LOD	<LOD	27.0	0.63	0.58	26.3
8	<LOD	156	<LOD	42.1	<LOD	0.78	16.0	5.37	232	0.29	1460	570	11.2	1.11	<LOD	<LOD	23.9	0.70	<LOD	25.7
9	<LOD	170	<LOD	25.6	<LOD	0.12	3.11	4.37	178	0.11	968	393	3.49	0.68	<LOD	<LOD	49.2	<LOD	<LOD	26.1
10	<LOD	393*	<LOD	32.7	<LOD	0.91	25.3	3.85	433	0.28	1120	410	13.3	0.93	<LOD	<LOD	31.8	0.62	0.47	32.3
11	0.61	120	1.35	29.8	0.43	0.29	11.8	2.31	176	2.01	1500	1220	5.97	1.31	0.09	0.23	29.5	0.61	0.99	26.5
12	<LOD	218	<LOD	50.1	<LOD	0.73	7.98	5.51	246	0.28	1310	745	4.88	1.17	<LOD	<LOD	26.1	0.65	0.57	34.9
14	0.53	105	<LOD	64.3	0.46	0.21	6.40	2.13	172	1.81	1320	1190	3.82	0.90	0.08	<LOD	34.9	<LOD	1.09	27.6
15	<LOD	96.6	<LOD	52.6	<LOD	0.08	1.55	4.29	134	0.10	1640	345	4.09	0.34	<LOD	<LOD	31.9	<LOD	<LOD	44.2
16	0.56	166	<LOD	46.2	0.44	0.41	38.4	2.04	364	1.93	1230	894	18.3	0.73	0.09	0.18	26.2	0.59	0.58	29.7
17	0.53	101	<LOD	26.7	0.51	0.27	21.8	2.20	267	1.81	1430	754	14.3	0.51	0.08	<LOD	41.2	<LOD	0.80	20.6
18	0.58	120	<LOD	42.2	0.43	0.22	15.7	1.80	238	1.98	1390	650	8.69	0.54	0.08	<LOD	23.9	0.60	0.90	28.7
19	0.52	138	<LOD	32.8	0.40	0.39	36.3	2.89	351	1.74	2080	162	16.8	0.67	0.08	0.20	54.8	<LOD	1.41	30.1
20	<LOD	151	<LOD	47.8	<LOD	0.10	4.52	4.60	178	0.09	1270	442	6.01	0.54	<LOD	<LOD	34.8	<LOD	<LOD	38.5
21	0.61	163	<LOD	41.8	0.55	0.29	19.7	3.22	293	2.07	948	296	13.5	1.22	0.10	0.20	40.3	0.63	1.15	32.5
22	0.54	104	<LOD	141*	0.44	0.22	12.7	2.03	211	1.82	1790	1720	9.94	0.76	0.08	<LOD	46.7	<LOD	0.96	26.8
23	<LOD	172	<LOD	64.6	<LOD	0.72	16.4	5.02	224	0.30	1430	194	17.6	1.70	<LOD	<LOD	33.5	0.64	<LOD	30.4
24	<LOD	111	<LOD	59.4	<LOD	0.09	2.80	4.12	152	0.051	1410	314	6.25	0.50	<LOD	<LOD	52.8	<LOD	<LOD	23.4
25	0.57	144	<LOD	35.2	0.39	0.32	27.7	2.11	314	1.91	1430	601	14.6	1.20	0.09	0.19	37.1	<LOD	0.97	26.3
26	0.56	114	<LOD	9.33	<LOD	0.18	13.2	1.63	241	1.81	2620*	374	4.97	0.89	0.09	0.18	7.60	<LOD	0.89	35.6
25 PERCENTILE	0.53	115		32.7	0.42	0.19	5.71	2.15	173	0.23	1140	352	4.98	0.57	0.08	0.18	25.3	0.61	0.58	26.3
75 PERCENTILE	0.58	172		57.7	0.47	0.71	21.2	4.54	309	1.87	1490	922	13.4	1.19	0.09	0.23	41.0	0.64	1.06	32.5
IQR (Q3-Q1)	0.05	57		25	0.05	0.52	15.5	2.39	136	1.64	350	570	8.42	0.62	0.01	0.05	15.7	0.03	0.48	6.20
Q3+(3*IQR)	0.72	341		133	0.63	2.27	67.8	11.7	716	6.81	2560	2630	38.8	3.05	0.10	0.37	88.2	0.73	2.50	51.2
MEDIAN	0.56	151		47.0	0.44	0.30	12.9	3.62	228	0.30	1370	625	7.57	0.76	0.09	0.20	31.8	0.62	0.92	28.3
MEAN	0.52	173		47.6	0.45	0.40	14.5	3.55	254	0.97	1410	683	9.11	0.93	0.09	0.20	33.3	0.63	0.86	29.8
DESVEST	0.15	92.6		25.1	0.05	0.27	10.4	1.30	104	0.87	395	407	4.88	0.51	0.01	0.02	11.6	0.03	0.27	5.39
MIN	0.04	96.6		9.33	0.39	0.08	1.55	1.63	134	0.051	896	162	2.98	0.34	0.08	0.18	7.60	0.59	0.44	20.6
MAX	0.61	485		141	0.55	0.91	38.4	5.75	534	2.07	2620	1720	18.3	2.75	0.10	0.23	54.8	0.70	1.41	44.2
NORTH OF NAVARRE (Amores and Santamaria, 2003)								6.1±1.4	113±41		1.700±500	556±420								30±11
SLOVAKIA (Mankovska, 1998)		50-150	<0.2	<100	<0.5	<0.1	<0.1	6-14	200-2000		1000-1500	<1000	1-2	2-6			<10		<1	20-80
GERMANY (Zech et al., 1990)		115±14						7.8±2.4	130±10		530±150	530±40								25.3±9.4
SWEDEN (Tyler and Olsson, 2006)	0.01	57	0.055	55	0.14	0.04	0.37	7.7	74	0.03	2100	2800	0.83	0.71	0.05		40	0.005	0.20	24

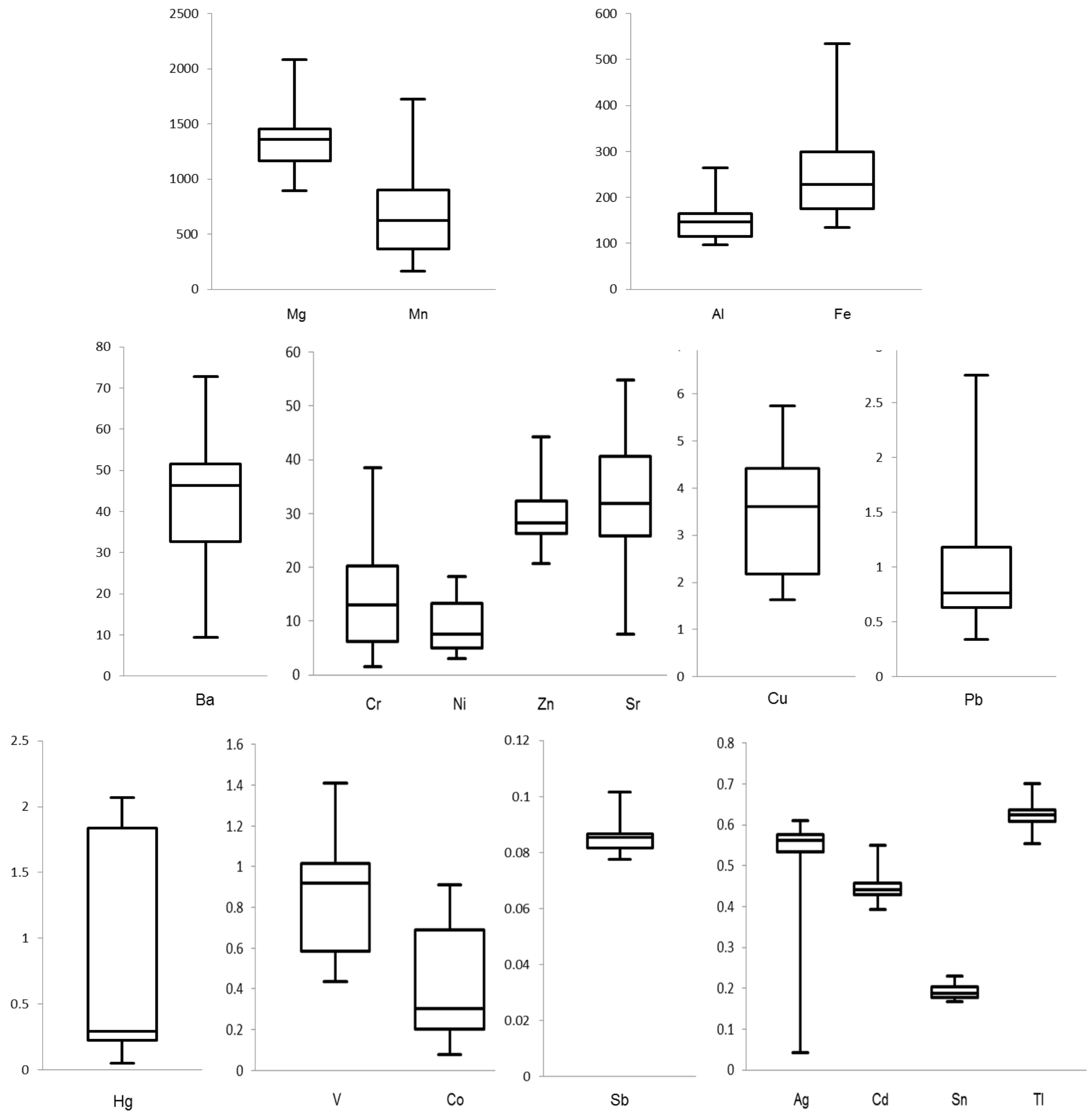

Figure 4. Within sampling sites concentrations (mg kg^{-1}) found in beech leaves of the Irati Forest after removing extreme values. The box shows the 25th percentile and the 75th percentile, and the whiskers represent the smallest and largest concentrations, while the line inside the box is the median of the population.

The enrichment factors (EF) in beech leaves were calculated according to Eq. 1 (Chiarenzelli et al., 2000) in order to assess the antropoghenic or natural origin of metals and metalloids.

$$EF_M = \frac{C_M / C_{Al}}{C_M^{soil} / C_{Al}^{soil}} \qquad \text{Eq. 1}$$

In Eq. 1, M is the element considered, and c and c^{soil} are the concentrations of the element in, respectively, the sample and the soil. As it can be observed, concentrations normalized with aluminium have been used in the calculation. While unit or near-unit EFs are taken as an indication of natural origin, substantial enrichment over the concentrations found in soil points to anthropogenic sources. The widely accepted threshold for such a distinction is EF >

10 (do Carmo Freitas et al., 2007). Within-sites average EFs for each element are shown in Figure 5. All the elements analysed exceeded the EF threshold of 10 except As, Fe, V, Pb and Co. An important input of metals of anthropogenic origin should be consequently concluded from the results of beech leaves for most of the elements investigated.

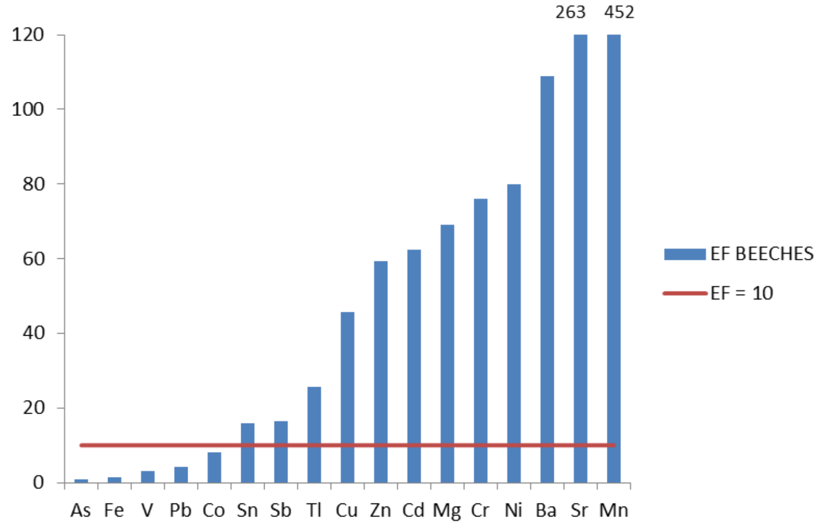

Figure 5. Within-sites average enrichment factors (EF) calculated for the elements in beech leaves. The red line indicates the threshold value (EF = 10) above which anthropogenic origin should be concluded.

Correlation analysis of data was carried out. Pearson correlation coefficients were calculated for all pairs of variables. No element showed a significant correlation between its concentrations in soil and in leaves. The concentrations of Cr and Ni in beech leaves, however, were highly correlated (0.89), indicating that this two elements may have a common origin in beech leaves of the Irati Forest. An study of the total diffuse emissions carried out in Sweden, showed that road traffic contributes in more than 99% to the total Cr and Ni of the atmosphere (Hjortenkrans et al., 2006). Moreover, it has also been reported that combustion of fossil fuels, especially diesel and biodiesel, releases an important amount of Cr and Ni to the atmosphere (Agarwal et al., 2011; de Miguel et al., 1997; Wang et al., 2003). The presence of diesel motor fuel vehicles in the complex 4x4 track network of the Irati Forest is rather abundant and could be partially responsible of the Cr/Ni contamination (Figure 5) in the area. The most striking point in the case of leaves, however, is the marked negative correlation between the concentration of Cu and those of Ag, Cd, Sb, Hg and Sn. These five elements present, in addition, a high positive correlation among each other.

Principal Component Analysis of the data set of beech leaves was also carried out. Principal Component Analysis (PCA) is a multivariate statistical technique capable of discerning patterns in large environmental datasets (Jolliffe, 2002). The dataset consisted on a matrix with the concentrations of the elements in columns (19, As was not considered because the concentrations measured in most of the sampling sites were below the detection limit) and the sampling sites in rows (26). Concentrations below the detection limit were substituted by the half of the detection limit. The dataset was centred and scaled before PCA analysis. A model with 3 PCs was finally selected to explain the variability of data. The first, second and third PCs explained, respectively, 38%, 19% and 11% of the total variance of the dataset. The corresponding scores and loadings plots are shown in Figure 6. As it can be observed, sampling sites were separated in two different groups, the first one with negative scores on PC1 and characterized by relatively high concentrations of Cu, Co, Al, Tl, Ba and Zn (2, 3, 5, 6, 7, 8, 9, 10, 12, 15, 20, 23 and 24), and the second one with positive scores on PC1 and characterised by relatively high concentrations of Ag, Hg, Sb, Cd, Sn, V, Cr, Ni, Mg, Fe, Pb, Mn and Sr (1, 11, 14, 16, 17, 18, 19, 21, 22, 25 and 26). Interestingly, most of the sampling sites in the second group have a west (W, NW or SW) component in their mountainside orientation, while most of the sites with any other orientation (N, NE, E, SE or S) form the second group. In general terms, PC1 discerned between sites with high (positive scores) and low (negative scores) metal concentrations. West component winds are prevalent in the western Pyrenees and thus metallic contamination can be transported from highly industrialized areas and steelworks located in the Basque Coast from Bilbao to Baiona and in the surroundings of Iruñea (Gonzalez-Miqueo et al., 2009). Steelworks have a considerable impact on the atmospheric particle load and are characterized by fine metallic particles input containing Cr, Fe, Mn, Ni and Zn (Choel et al., 2010; Damek-Poprawa and Sawicka-Kapusta, 2004; Vestergaard et al., 1986). After emission, these metals can be mixed with marine and continental compounds and windblown large distances from the source (Choel et al., 2010; Damek-Poprawa and Sawicka-Kapusta, 2004; Vestergaard et al., 1986). The N-135 main road from Iruña to Donibane-Garazi, with considerable truck traffic, might be another important source of metals to the atmosphere that finally impacts in the Irati Forest. Another interesting observation that arises from the combination of the results from the PCA and the correlation analysis is that it seems that beech orientated to the West (W, NW or SW) are more likely to accumulate less Cu and proportionally more Ag, Cd, Sb, Hg and Sn than those orientated to any other cardinal point.

In order to visualize the distribution of metals in beech leaves of the Irati Forest, the Normalised-and-Weighted Average Concentrations (NWACs) were finally calculated. The NWAC is a cumulative index, to sort samples according to their contaminant content. Samples are characterised by a single score in a scale from 0 to 10, the NWAC, which is calculated taking into account exclusively the concentration of contaminants freely selected by the user and present in the sample (Gredilla et al., 2014). The kriging interpolation of the obtained NWACs in a contour map allows us identifying, in a very simple and intuitive way, the points of higher concern in the area investigated, in this case, the Irati Forest. The results obtained using the concentrations of the 20 elements measured in the beech samples are shown in Figure 7. Two main hotspots of metallic contamination were identified. The first one in the North-West corner of the studied area, in the surroundings of sampling site 1, clearly influenced by the N-135 main road. The second one is at the closed end of the funnel-like corridor that forms the water catchment of the Irati River, around the sampling site 21.

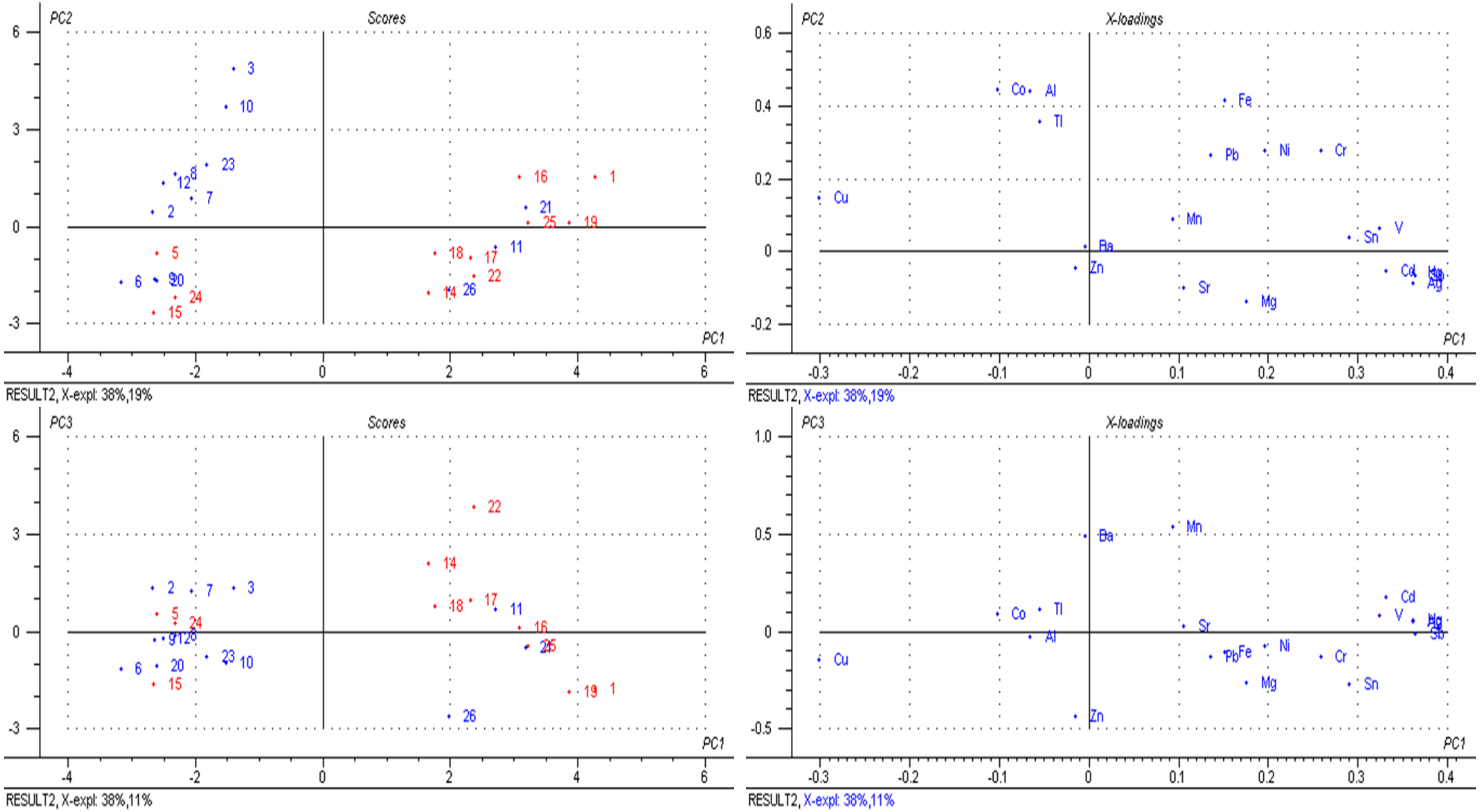

Figure 6. Scores and loadings plots (PC1-PC2 and PC1-PC3) obtained after PCA of the beech leaves dataset. In red: sampling sites with NW, W or SW orientation; in blue: sampling sites with N, NE, E, SE and S orientation.

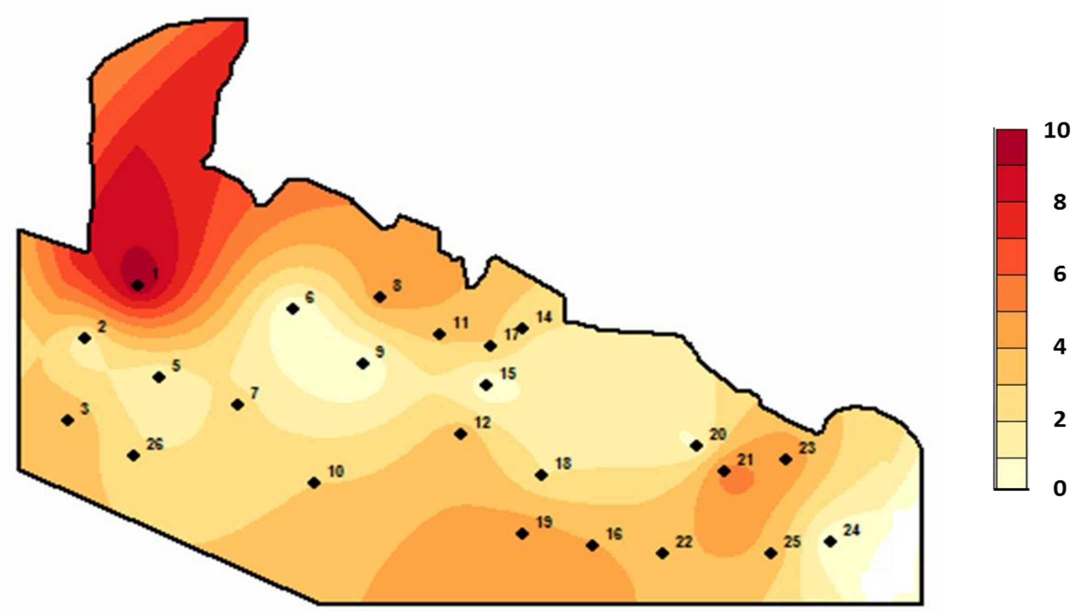

Figure 7. Hotspots of metal contamination in the Irati Forest according to the NWAC values calculated using the concentration of the 20 elements measured in beech leaves.

3.3. LICHEN (*Parmelia sulcata*)

The element concentrations measured in lichens are shown in Table 4 together with some related statistics. Extreme values, which are marked with an asterisk in Table 4, were identified for Cd (in sampling sites 14, 19 and 20), for Cr (in 4, 17 and 22), and for Ag (in 16), Pb (in 22), Sb (in 22) and Sn (in 19). Figure 8 summarises the results after removing the extreme values.

Lichens have been widely used to monitor metal pollution in mountain regions. As an example, the concentrations measured in this work have been compared with those obtained from the analysis of *Parmelia sulcata* lichens collected in the Aspe valley (Central Pyrenees) (Veschambre et al., 2003) and *Xanthoria parietina* lichens from the Prades Mountains (Tarragona, Catalonia)(Achotegui-Castells et al., 2013) (Table 4). The Aspe valley is a basically pristine area with the main road E-7 connecting Pau and Sabiñanigo as the most important potential source of pollution. Concerning the Prades Mountains, a remarkable industrial activity (petrochemical, paper and glass plants) takes part in their surroundings. Similar concentrations of Cu, Pb, Sb, V and Zn were observed in all the areas, while the concentrations of Cd, Mn and Rb were higher in the Irati Forest than in the Axpe Valley, and those of Cr and Ni lower in the Irati Forest than in the Prades Mountains.

Since the Al concentration was not measured in lichens, the calculation of the corresponding EFs was not possible.

Table 4. Element concentrations (mg·kg^{-1}) found in lichens from the Irati Forest, together with the detection limits (LOD), accuracy (as recovery from the replicate analysis of the BCR 482 reference material) and reproducibility (as relative standard deviation from the replicate analysis of the BCR 482 reference material) of the analytical method. Extreme concentrations (c>Q3+3IQR) are marked with an asterisk (*).Concentrations found in lichens from other locations are also provided for comparison.

	Ag	**Cd**	**Cr**	**Cu**	**Hg**	**Mn**	**Ni**	**Pb**	**Rb**	**Sb**	**Sn**	**Sr**	**V**	**Zn**
L.O.D. (µg·kg-^{1})	12.8	7.47	9.27	19.6	0.08	1610	6.62	9.78	6.83	9.90	10.0	137	10.0	8.10
RECOVERY (%)		86%	61%	100%	78%	69%	115%	80%		70%	63%	78%	80%	93%
REPRODUCIBILITY (RSD)	23%	1%	7%	7%	8%	6%	4%	2%		12%	2%	6%	6%	8%
1	0.043	0.20	2.50	19.9	0.14	302	6.15	9.91	17.6	0.041	0.26	19.9	2.33	55.9
2	0.072	0.20	2.89	22.2	0.35	181	2.77	12.9	21.3	0.032	0.19	25.2	3.91	21.9
3	0.058	0.20	2.18	16.7	0.69	454	2.85	18.2	13.8	0.048	0.19	15.5	4.46	35.8
4	0.074	0.32	16.8*	17.1	0.23	278	3.38	22.6	7.52	0.068	0.25	10.1	5.99	41.7
5	0.032	0.12	1.41	5.39	0.18	219	1.64	7.86	21.0	0.030	0.12	30.8	0.95	25.2
6	0.045	0.20	6.58	10.9	0.17	232	2.68	38.3	17.8	0.031	0.20	26.2	3.91	27.3
7	0.042	0.18	2.23	10.5	0.19	252	2.94	14.9	11.1	0.032	0.21	14.2	4.03	25.5
8	0.057	0.18	0.90	12.6	0.16	129	1.34	8.17	27.6	0.048	0.20	31.7	0.82	30.7
9	0.063	0.25	3.99	16.5	0.14	71.9	2.08	5.62	12.3	0.032	0.23	13.2	2.63	26.5
10	0.077	0.24	2.72	12.6	0.18	176	2.25	6.93	12.0	0.033	0.16	13.5	3.43	33.3
11	0.072	0.45	1.71	8.98	0.36	116	1.75	4.95	22.6	0.032	0.21	27.0	2.12	19.6
12	0.072	0.28	3.40	13.9	0.22	313	3.10	22.3	14.7	0.055	0.21	19.4	6.26	37.9
13	0.054	0.26	3.57	19.9	0.21	192	3.89	20.1	11.8	0.041	0.23	16.3	6.13	35.8
14	0.048	1.28*	1.97	8.99	0.22	498	1.91	11.5	11.2	0.030	0.33	16.1	2.03	42.6
15	0.048	0.26	3.83	9.71	0.17	333	4.00	14.8	12.4	0.032	0.25	17.9	5.47	36.6
16	0.17*	0.21	2.95	8.76	0.15	124	2.61	15.5	6.07	0.036	0.19	10.7	4.68	23.6
17	0.042	0.33	9.58*	6.60	0.17	201	3.87	19.1	9.16	0.022	0.18	16.3	5.51	41.2
18	0.041	0.22	2.97	8.61	0.18	217	2.60	10.3	13.5	0.022	0.13	14.9	5.19	28.1
19	0.062	0.72*	6.20	6.69	0.12	89.2	1.83	5.33	4.90	0.028	6.81*	8.7	2.20	24.6
20	0.042	0.88*	3.96	15.4	0.36	249	2.51	9.38	13.7	0.036	0.25	16.0	4.57	45.6
21	0.056	0.22	3.56	9.25	0.30	130	4.88	16.5	7.58	0.036	0.15	11.5	5.43	52.5
22	0.049	0.30	12.9*	10.2	0.35	433	3.82	87.5*	9.79	0.21*	0.18	15.1	6.28	32.4
23	0.031	0.16	3.44	7.13	0.26	103	2.92	9.84	5.72	0.025	0.15	9.08	3.61	13.2
24	0.032	0.18	2.71	6.18	0.17	72.0	1.22	5.75	12.9	0.029	0.20	12.8	2.46	15.6
25	0.031	0.09	1.83	5.53	0.20	74.8	1.12	2.21	8.68	0.013	0.12	8.05	1.74	11.6
26	0.030	0.20	0.49	7.45	0.41	207	1.46	8.93	9.91	0.045	0.29	10.5	2.54	33.6
25 PERCENTILE	0.038	0.19	2.12	7.37	0.17	122	1.81	7.62	9.04	0.029	0.17	11.3	2.29	24.3
75 PERCENTILE	0.062	0.30	3.96	15.7	0.31	284	3.49	18.4	15.4	0.041	0.25	19.5	5.44	38.7
IQR(Q3-Q1)	0.024	0.11	1.84	8.33	0.14	161	1.68	10.8	6.37	0.012	0.08	8.20	3.15	14.4
Q3+(3*IQR)	0.134	0.62	9.48	40.7	0.73	769	8.53	50.8	34.5	0.077	0.47	44.1	14.9	81.9
MEDIAN	0.047	0.22	2.96	9.93	0.19	204	2.64	10.9	12.1	0.032	0.20	15.3	3.91	31.5
MEAN	0.053	0.31	4.13	11.5	0.24	217	2.75	15.7	13.0	0.041	0.46	16.6	3.80	31.5
DESVEST	0.026	0.26	3.72	4.85	0.12	118	1.19	16.5	5.57	0.035	1.30	6.64	1.69	11.2
MIN	0.031	0.09	0.49	5.39	0.12	71.9	1.12	2.21	4.90	0.013	0.12	8.05	0.82	11.6
MAX	0.17	1.28	16.8	22.2	0.69	498	6.15	87.5	27.6	0.21	6.81	31.7	6.28	55.9
ASPE VALLEY (Veschambre et al., 2003)		0.05	2.93	10.5		34.2		11.1	3.47				3.88	52.4
PRADES MOUNTAINS (Achotegui-Castells et al., 2013)		0.113-0.121	14.1-24.8	5.39-7.36			7.74-13.2	1.52-3.52		0.035- 0.057			1.91-3.08	26.8-38.1

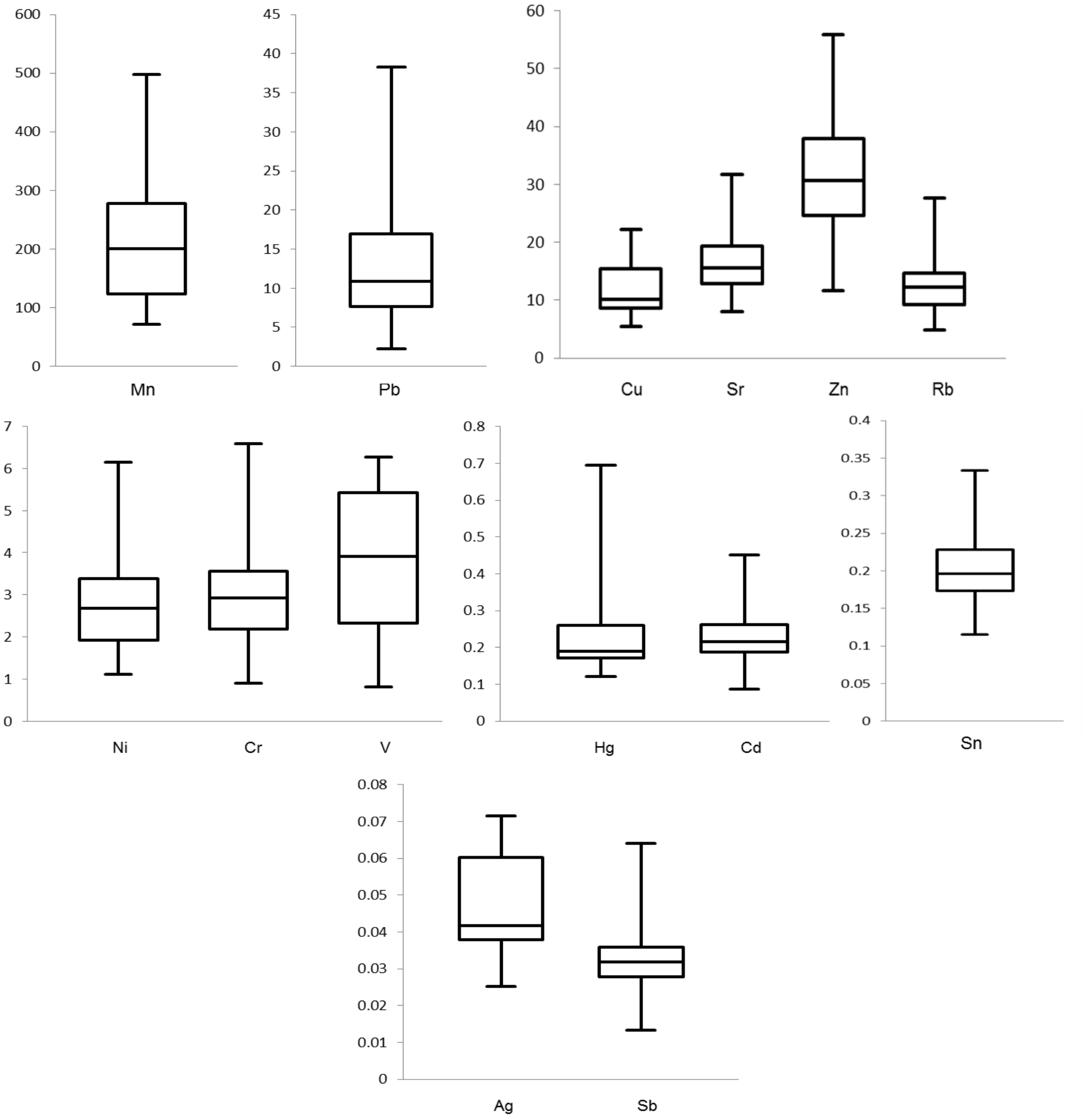

Figure 8. Within sampling sites concentrations (mg kg^{-1}) found in lichens of the Irati Forest after removing extreme values. The box shows the 25th percentile and the 75th percentile, and the whiskers represent the smallest and largest concentrations, while the line inside the box is the median of the population.

Correlation analysis of data revealed a significant correlation between the concentrations of Rb and Sr (0.93) in lichen. These pairs of metals probably have a common origin in the Irati lichens. No element showed a significant correlation between its concentrations in soil and in lichen.

The dataset of lichens, consisting on a matrix with 14 columns (concentrations of the elements measured in lichens) and 26 rows (the sampling sites), was also subjected to Principal Component Analysis. Concentrations below the detection limit were substituted by the half of the detection limit. The dataset was centred and scaled before PCA analysis. A model with 3 PCs was finally selected to explain the variability of data. The first, second and third PCs explained, respectively, 28%, 18% and 12% of the total variance of the dataset. The corresponding scores and loadings plots are shown in Figure 9. Clustering is not evident, but sampling sites 4 and 22 clearly differ from the rest, with high positive scores on PC1 and high concentrations of elements like Cr, V, Ni, Pb, Sb, Mn and Zn. Sampling sites 1, 3, 12, 13, 15, 17 and 21 seem to form another cluster characterised by high concentrations of Cd, Cu, Hg and Ag. Sampling sites 2, 5, 8 and 11 presented high concentrations of Rb and Sr as common characteristic. No evident relationship between clustering and altitude or mountainside orientation could be identified.

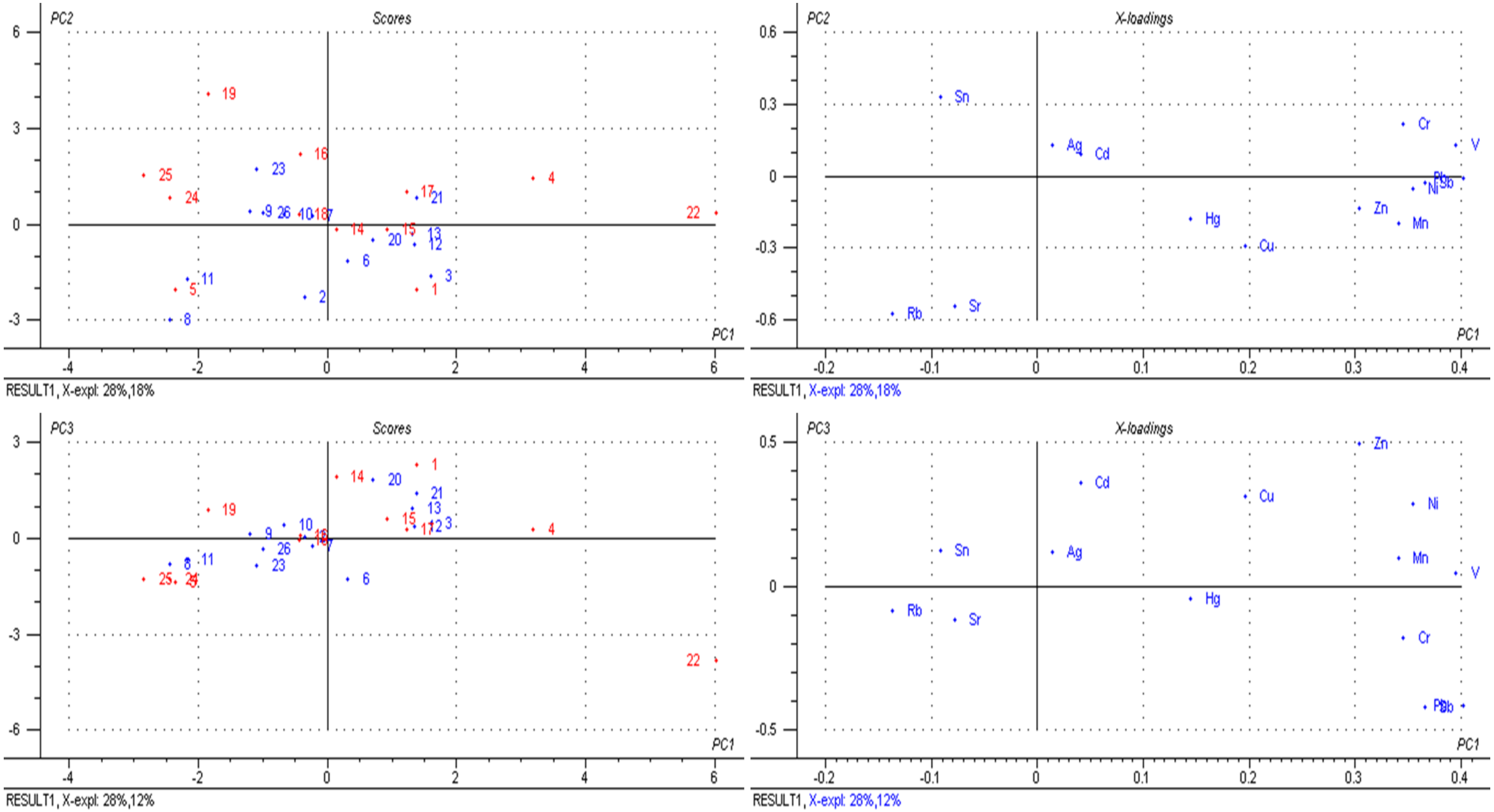

Figure 9. Scores and loadings plots (PC1-PC2 and PC1-PC3) obtained after PCA of the lichens dataset. In red: sampling sites with NW, W or SW orientation; in blue: sampling sites with N, NE, E, SE and S orientation.

The concentrations of the 14 elements measured in lichens were used to calculate the NWACs and plot them in a contour map following the same methodology than for leaves (Section 3.2) (Figure 10). A very marked hotspot of metal contamination can be identified in sampling site 22, not far from that identified in the case of leaves (sampling point 21). Other points of concern are around sampling points 4, on the one hand, and 14, on the other, both of them with West orientation. Again, the predominant winds from the West might be partially responsible of this situation.

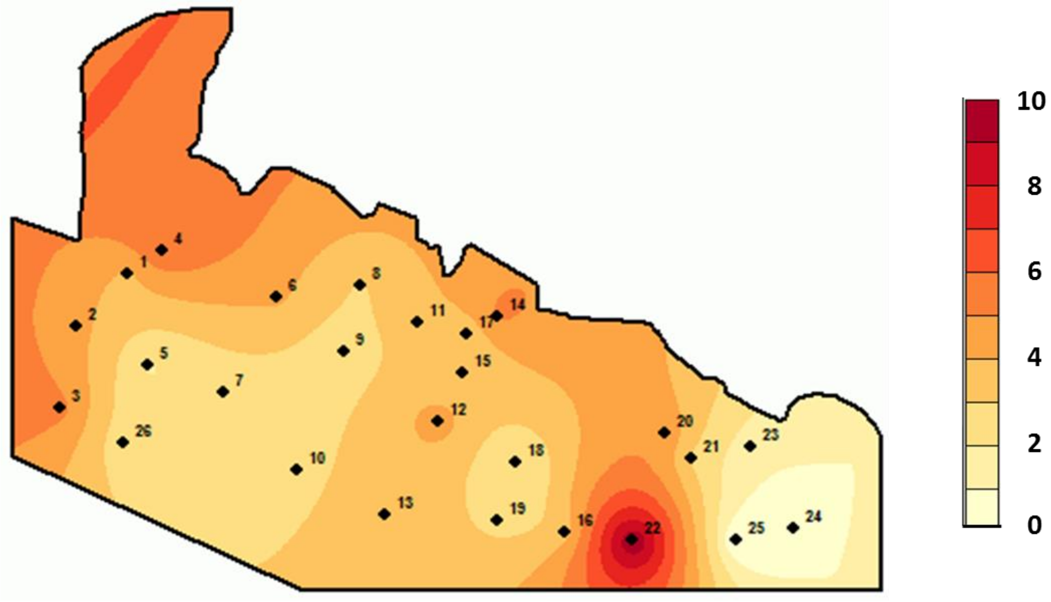

Figure 10. Hotspots of metal contamination in the Irati Forest according to the NWAC values calculated using the concentration of the 14 elements measured in lichens.

3.4. MOSS (*Hypnum cupressiforme*)

The element concentrations measured in mosses are shown in Table 5 together with some related statistics. Samples from the sampling point 20 were accidentally lost. Extreme values, which are marked with an asterisk in Table 5, were identified in sampling sites 21 (for Al, Ba, Ni and Zn), 24 (for Al and V), 25 (for Cr), 18 (for Al), 16 (for V) and 8 (for Zn). The sampling point 21 presented, in addition, the maximum concentrations of Fe and Ni. Figure 11 summarises the results after removing the extreme values.

Mosses have also been largely used to monitor atmospheric pollution. The concentrations measured in mosses from the Irati Forest have been compared with those measured in mosses collected in other mountain regions, such as the Prades Mountains (Achotegui-Castells et al., 2013) and several forested areas of the north of Navarra (Gonzalez-Miqueo et al., 2009) (Table 5). In all the cases, *Hypnum cupressiforme* has been the moss species considered. Similar concentrations of Cd, Cr, Mn, Pb, V and Zn were observed in all the cases. While the concentrations of Al, and As were slightly lower in the Irati Forest than in the other two areas, considerably higher concentrations of Ni, and especially Cu and Hg, were found in mosses from the Irati Forest.

Table 5. Element concentrations (mg·kg^{-1}) found in mosses from the Irati Forest, together with the detection limits (LOD), accuracy (as recovery from the replicate analysis of the SRM 1575a reference material) and reproducibility (as relative standard deviation from the replicate analysis of the SRM 1575a reference material) of the analytical method. Extreme concentrations (c>Q3+3IQR) are marked with an asterisk (*).Concentrations found in mosses from other locations are also provided for comparison.

	Al	**As**	**Ba**	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Sr**	**V**	**Zn**
L.O.D. (mg·kg^{-1})	5.05	0.01	0.02	0.002	0.01	0.05	4.5	0.4	3.4	0.05	0.02	0.004	0.001	0.2
RECOVERY (%)	88%	72%	98%	91%		94%	96%	68%	78%	91%	83%			98%
REPRODUCIBILITY (RSD)	9%	8%	6%	9%		3%	12%	12%	6%	4%	8%			4%
1	311	0.15	60.2	0.14	0.15	4.98	125	54.2	338	1.83	5.59	27.1	1.48	22.6
2	336	0.12	21.8	0.1	0.54	3.58	402	24.4	254	0.96	1.59	15.7	1.02	13.6
3	272	0.10	31.7	0.16	17.7	151	391	39.3	328	16	2.03	16.3	0.75	14.6
4	645	0.34	25.7	0.18	0.20	5.51	128	48.2	357	1.48	6.18	19.7	1.57	25.8
5	489	0.16	35.8	0.33	1.40	6.65	355	47.3	167	1.83	2.88	33.5	1.24	26.3
6	522	0.21	17.4	0.15	9.87	364	700	46.9	211	5.36	3.37	19.4	1.14	21.9
7	547	0.31	46.8	0.34	8.38	731	1694	47.5	231	6.84	2.98	28.3	1.76	37.4
8	445	0.10	44.7	0.19	4.01	22.6	300	24.2	126	29.1	3.29	29.4	1.17	81.0*
9	655	0.17	16.1	0.28	1.36	125	730	29.6	106	5.01	2.97	27.7	0.66	17.4
10	517	0.12	32.5	0.26	1.10	7.80	328	45.9	133	1.2	2.11	26.0	0.50	20.4
11	204	0.34	34.7	0.13	0.33	1430	2610	39.2	195	13.3	1.82	20.8	0.041	22.3
12	584	1.01	60.4	0.43	0.75	1360	6840	34.4	200	51.6	4.05	27.3	2.44	27.8
13	619	0.21	71.0	0.26	1.57	6.65	558	64.0	200	2.53	2.94	26.8	1.77	29.7
14	358	0.70	16.6	0.12	0.62	1810	5440	23.3	331	14.6	2.16	13.7	0.31	16.6
15	474	0.11	22.1	0.22	1.24	27.39	66.1	30.6	154	21.4	2.77	20.8	5.69	36.5
16	264	0.53	39.8	0.17	0.86	1360	4930	37.4	85.6	16.5	1.75	30.9	17.5*	23.7
17	1800	0.48	29.0	0.30	4.31	6.05	1560	50.8	190	4.11	3.87	22.9	0.99	21.9
18	3370*	0.77	47.5	0.12	6.11	47.7	3280	37.4	242	40.9	4.64	16.4	2.83	34.8
19	780	0.13	32.3	0.25	1.59	5.48	653	44.5	140	1.33	1.96	25.5	0.93	18.0
21	3830*	0.93	137*	0.24	3.86	699	7120	42.2	201	170*	6.58	37.0	3.65	281*
22	408	0.48	40.6	0.55	1.22	516	1490	32.4	261	16.0	3.10	25.7	5.51	21.1
23	2210	0.42	58.0	0.40	4.16	128	1980	47.4	112	5.94	1.90	28.5	4.84	21.3
24	5550*	0.84	54.7	0.19	9.95	8.60	4240	45.8	127	7.07	4.25	41.3	13.1*	24.8
25	895	0.46	31.7	0.20	66.9*	949	3590	28.5	125	39.8	2.25	28.8	1.16	21.3
26	332	0.26	12.5	0.14	26.0	299	890	47.6	149	17.8	2.89	9.91	1.02	18.6
25 PERCENTILE	347	0.14	23.9	0.15	0.81	6.65	373	31.5	130	2.18	2.07	19.6	0.96	19.5
75 PERCENTILE	838	0.51	51.1	0.29	7.25	715	3440	47.5	248	19.6	3.96	28.7	3.24	28.8
IQR(Q3-Q1)	491	0.37	27.2	0.15	6.44	708	3060	16.0	118	17.4	1.89	9.10	2.28	9.25
Q3+(3*IQR)	2310	1.60	133	0.73	26.6	2840	12600	95.3	602	71.9	9.63	56.0	10.1	56.5
MEDIAN	522	0.31	34.7	0.20	1.57	125.0	890	42.2	195	7.07	2.94	26.0	1.24	22.3
MEAN	1060	0.38	40.8	0.23	6.97	403	2020	40.5	199	19.7	3.20	24.8	2.92	36.0
DESVEST	1330	0.28	25.4	0.11	13.9	556	2170	10.3	78.2	34.2	1.37	7.32	4.08	52.7
MIN	204	0.10	12.5	0.10	0.15	3.58	66.1	23.3	85.6	0.96	1.59	9.91	0.041	13.6
MAX	5550	1.01	137	0.55	66.9	1810	7120	64.0	357	170	6.58	41.3	17.5	281
NORTH OF NAVARRA (Gonzalez-Miqueo et al., 2009)		1.005		0.214	5.84	7.55		0.121	155	3.67	9.19		4.01	31.7
PRADES MOUNTAINS (Achotegui-Castells et al., 2013)	3100-6594	0.42-0.74		0.09-0.25	9.07-24.3	6.03-7.80				6.66-13.4	1.89-3.93		2.41-3.97	28.1-29.9

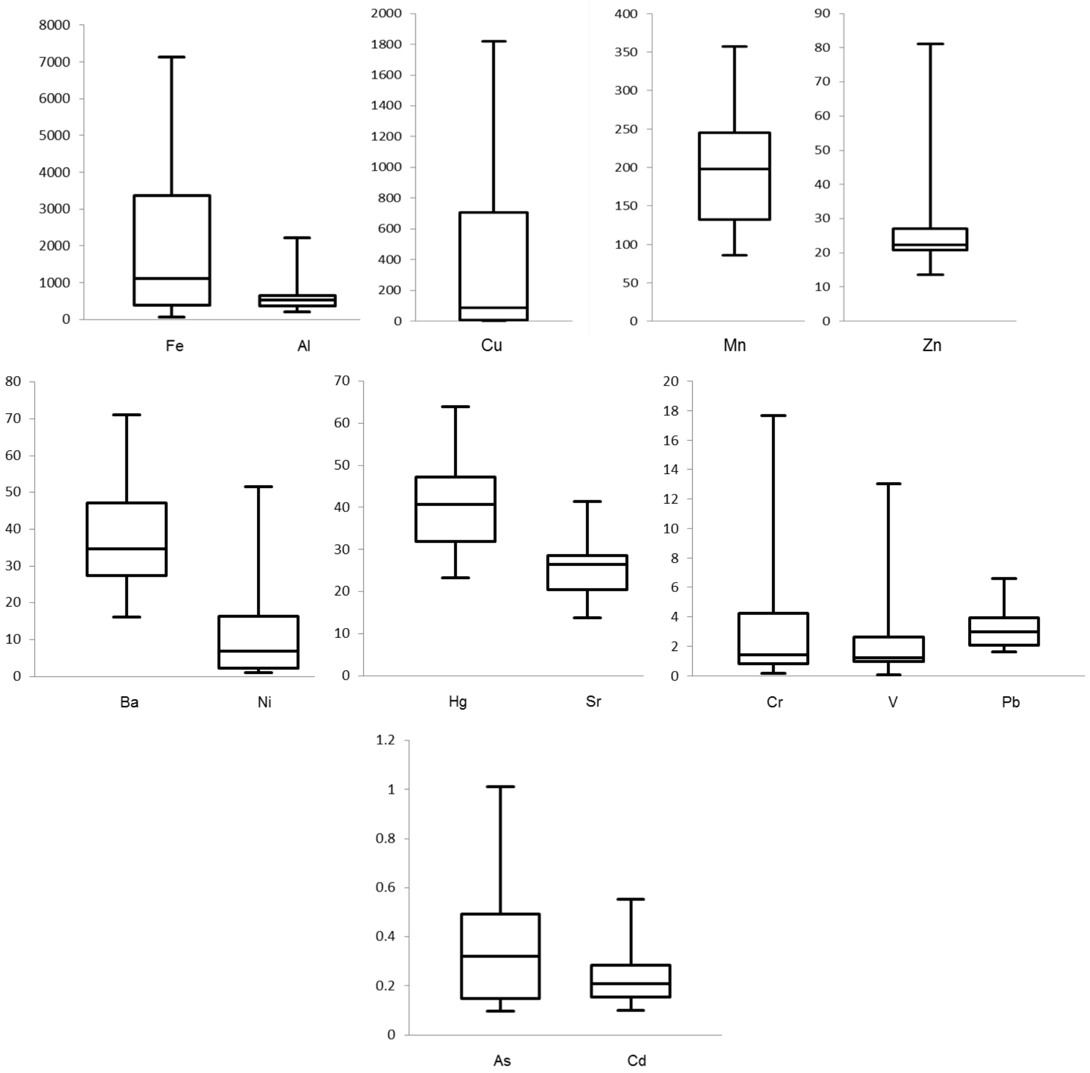

Figure 11. Within sampling sites concentrations (mg kg^{-1}) found in mosses of the Irati Forest after removing extreme values. The box shows the 25th percentile and the 75th percentile, and the whiskers represent the smallest and largest concentrations, while the line inside the box is the median of the population.

The EFs in mosses were also calculated like in Section 3.2. Within-sites average EFs for each element are shown in Figure 12. All the metals considered exceeded the EF threshold of 10 except As, Fe, Pb and V. The concentrations of As and Fe were, in addition, highly correlated in mosses (0.92), suggesting their common and natural lithogenic origin. These results confirm, however, the existence of an important input of other metals of anthropogenic origin in the Irati Forest.

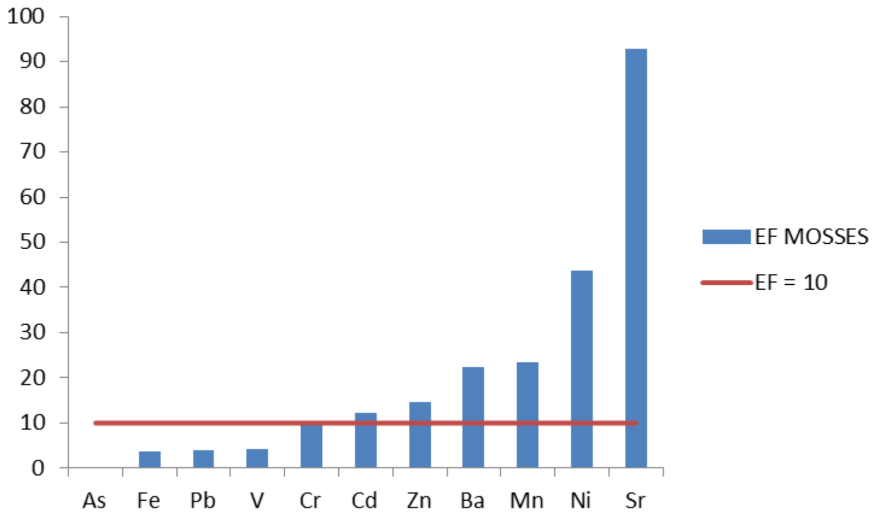

Figure 12. Within-sites average enrichment factors (EF) calculated for the elements in mosses. The red line indicates the threshold value (EF = 10) above which anthropogenic origin should be concluded.

The dataset of mosses was a matrix with 14 columns (concentrations of the elements measured in mosses) and 25 rows (the sampling sites). Concentrations below the detection limit were substituted by the half of the detection limit and the resulting dataset was centred and scaled before PCA analysis. A model with 3 PCs was finally selected to explain the variability of data. The first, second and third PCs explained, respectively, 35%, 16% and 14% of the total variance of the dataset. The corresponding scores and loadings plots are shown in Figure 13. Like in the case of lichens, the clustering model is not evident, but sampling sites 12, 16, 18, 21 and 24 form a separated group in the positive part of PC1. These sites are characterised by relatively high concentrations of Fe, As, Ni, Pb, Sr, Al, Zn and Ba. The rest of sampling sites present lower scores on PC1 and, in general, lower metal concentrations. No evident relationship between clustering and altitude or mountainside orientation could be identified.

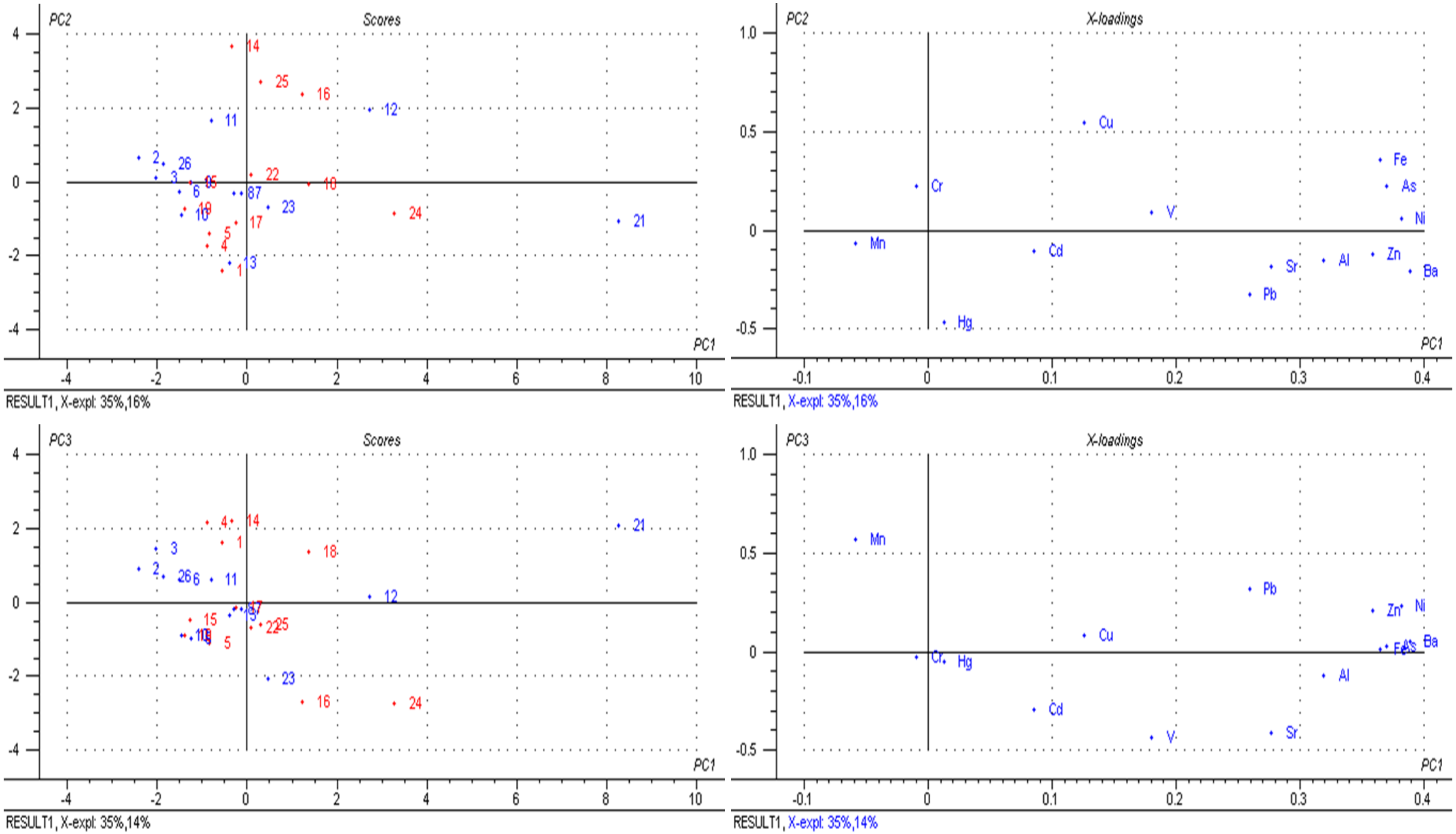

Figure 13. Scores and loadings plots (PC1-PC2 and PC1-PC3) obtained after PCA of the mosses dataset. In red: sampling sites with NW, W or SW orientation; in blue: sampling sites with N, NE, E, SE and S orientation.

The concentrations of the 14 elements measured in mosses were used to calculate the NWACs and produce the corresponding contour map (Figure 14). In good agreement with the PCA results, the hotspot of metal contamination identified in the sampling site 21 prolongs throughout the water catchment of the Irati River and takes all the final part of the characteristic funnel-like corridor (sampling sites 12, 18, 16 and 24). Winds from the SW could be consequently partially responsible of the metal content measured in moss of the Irati Forest.

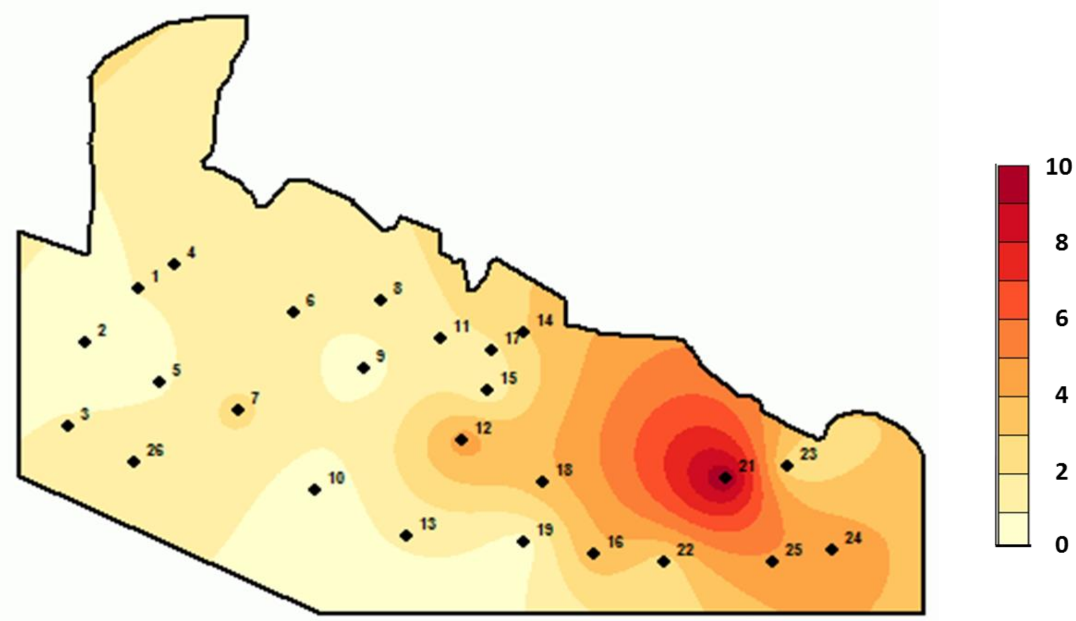

Figure 14. Hotspots of metal contamination in the Irati Forest according to the NWAC values calculated using the concentration of the 14 elements measured in mosses.

4. DISCUSSION: COMPARISON AMONG BEECH LEAVES, LICHENS AND MOSSES AS BIOINDICATORS OF ATMOSPHERIC METAL CONTAMINATION

A normally distributed variable does not include outlier or extreme values, so that it can be conveniently described by the mean and standard deviation of randomly distributed values. Extreme values clearly lay out the normality described by the rest of the values and may appear in a variable due to a wide variety of reasons. In environmental monitoring data, like those we are managing in this work, extreme concentrations indicate the existence of sampling points with a significant higher concentration of a given metal in comparison with the rest of sampling sites, that is, the existence of hotspots of that metal within sampling sites. 10 extreme values were identified in the datasets of lichens and mosses and only four in that of beech leaves. It seems that lichens and mosses are more sensitive than leaves to the existence of metallic local contaminations. After removing extreme values a close-to-normal distributions were obtained for most of the elements in the three bioindicators considered (Figures 4, 8 and 11). Looking at the corresponding means and standard deviations (Figure 15) the elements can be divided in four different groups according to their accumulation rates in leaves, lichen and moss: i) the accumulation of Cr, Mn, and probably Ag, Sb and As, is significantly higher in leaves than in lichen and moss, ii) mosses accumulate Cu, Hg, and probably Al and Fe, in a higher extent than leaves and lichens do, iii) Pb and V accumulate more efficiently in lichen than in moss, and in moss more efficiently than in leaves, iv) finally, leaves, lichen and moss present similar accumulation rates for Cd, Ni, Zn, Sr, and probably Sn and Ba. It is not possible to arrive to any definitive conclusion in the case of Co, Mg, Tl and Rb because their concentration was measured in a single matrix, leaf, lichen or moss.

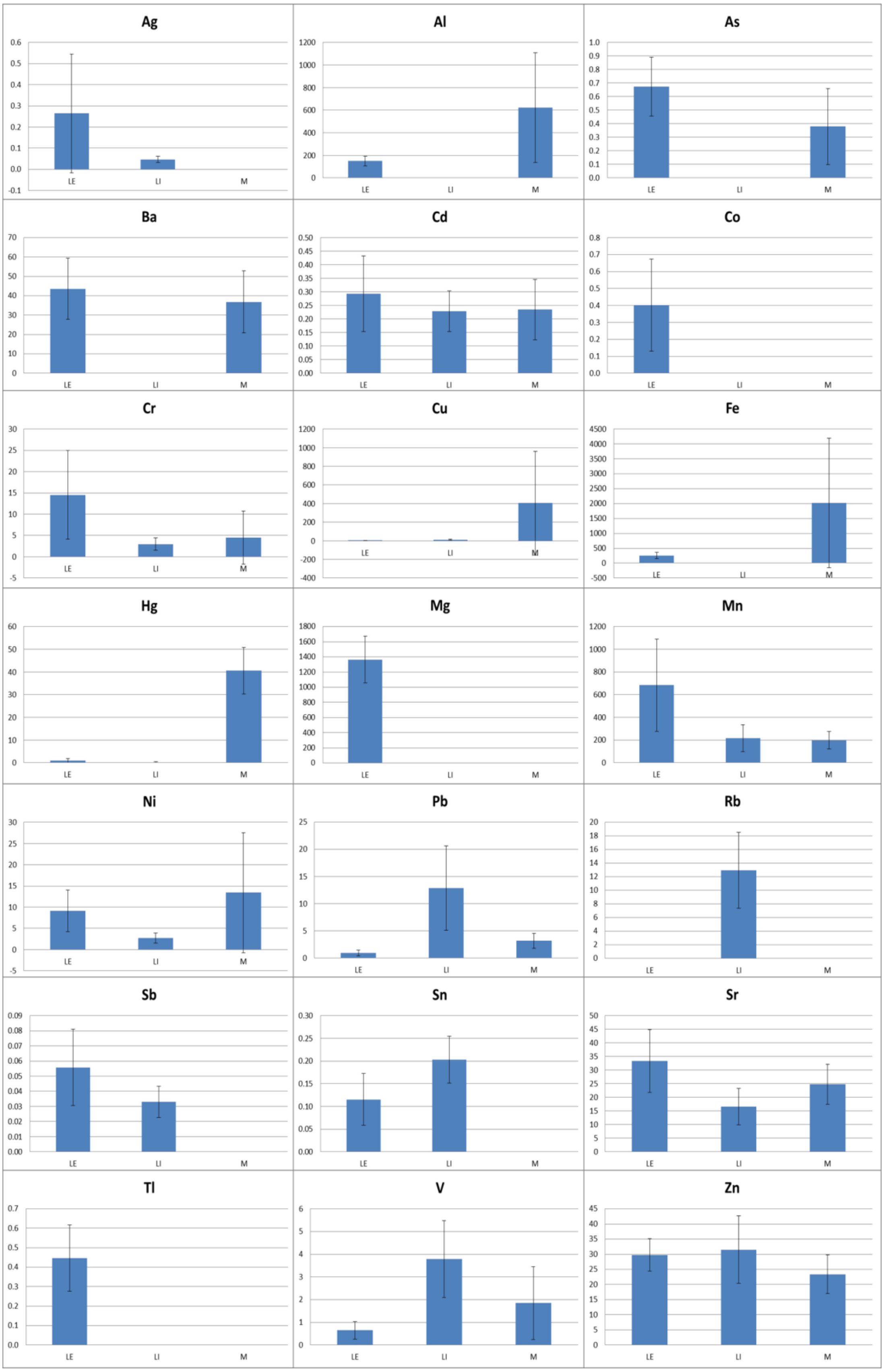

Figure 15. Means and standard deviations of the element concentrations (mg·kg^{-1}) found in beech leaves (LE), lichens(LI) and mosses (M) from the Irati Forest.

The enrichment factors calculated for both beech leaves and mosses suggest an anthropogenic origin of certain toxic metals such as Cu, Sr, Ni, Mn, Ba, Zn, Cd and Cr, most of them closely related to steelwork emissions and diesel exhausts, in the Irati Forest. It is also important to highlight that while the concentrations of metals in lichen and moss are not influenced by variables like altitude or mountainside orientation (Figures 9, 13), those in leaves are strongly affected by orientation (Figure 6).

The NWAC, on the other hand, is a cumulative index that integrates the concentrations of all the elements measured in each matrix. The contour maps produced using the NWAC values obtained for leaf, lichen and moss (Figures 7, 10 and 14) can be used to investigate the distribution of atmospheric metal contamination. In general terms, the distributions suggested by each bioindicator are rather similar, and identify a hotspot of contamination at the end of the funnel-like corridor formed by the water catchment of the Irati River (around sampling points 21 and 22), and another one in the W-NW sector of the studied area (in the surroundings of the N-135 main road). This last hotspot, however, is not evident in the distribution obtained using moss as bioindicator. Looking at the contour maps produced specifically for each element in each bioindicator (Figure 16), however, it becomes evident that the distribution is element-and bioindicator-dependent, although, with the help of the results from the correlation analysis, several similarities can be listed. The distributions of Cr and Ni, on the one hand, and Ag, Cd, Sb, Hg and Sn, on the other, in beech leaves are rather similar, while that of Cu is inversely proportional to those of the last five elements. Concerning lichens, Rb and Sr follow a similar distribution pattern in the studied area. The same can be concluded for As and Fe in mosses. In all these cases, the corresponding correlation coefficients are over 0.7 (or below -0.7 in the case of Cu with Ag/Cd/Sb/Hg/Sn). In addition, and as an exception, the concentrations of Mn in leaves, lichen and moss are highly correlated (Beech Leaves-Lichens: 0.64; Beech Leaves-Mosses: 0.65; Lichens-Mosses: 0.75). It is the only element whose distributions are rather similar in the three matrices investigated (Figure 16). The fact that no other element presents a similar behaviour can be explained by the different longevity of the species considered as bioindicators. The longevity of beech leaves is about 6 months, from early spring to late summer, while mosses build carpets during a period of 3-5 years, and their metal content is generally considered to reflect the atmospheric deposition during that period (Wolterbeek, 2003). Finally, even if lifespan is difficult to measure in a lichen because the definition of what constitutes the same individual is not precise, lichens are considered to be among the oldest living organisms (Conti and Cecchetti, 2001). These differences in longevity certainly affects the amount of contaminant accumulated in the bioindicator and might partially explain the differences observed in metal distribution when estimated using leaves, lichen or moss.

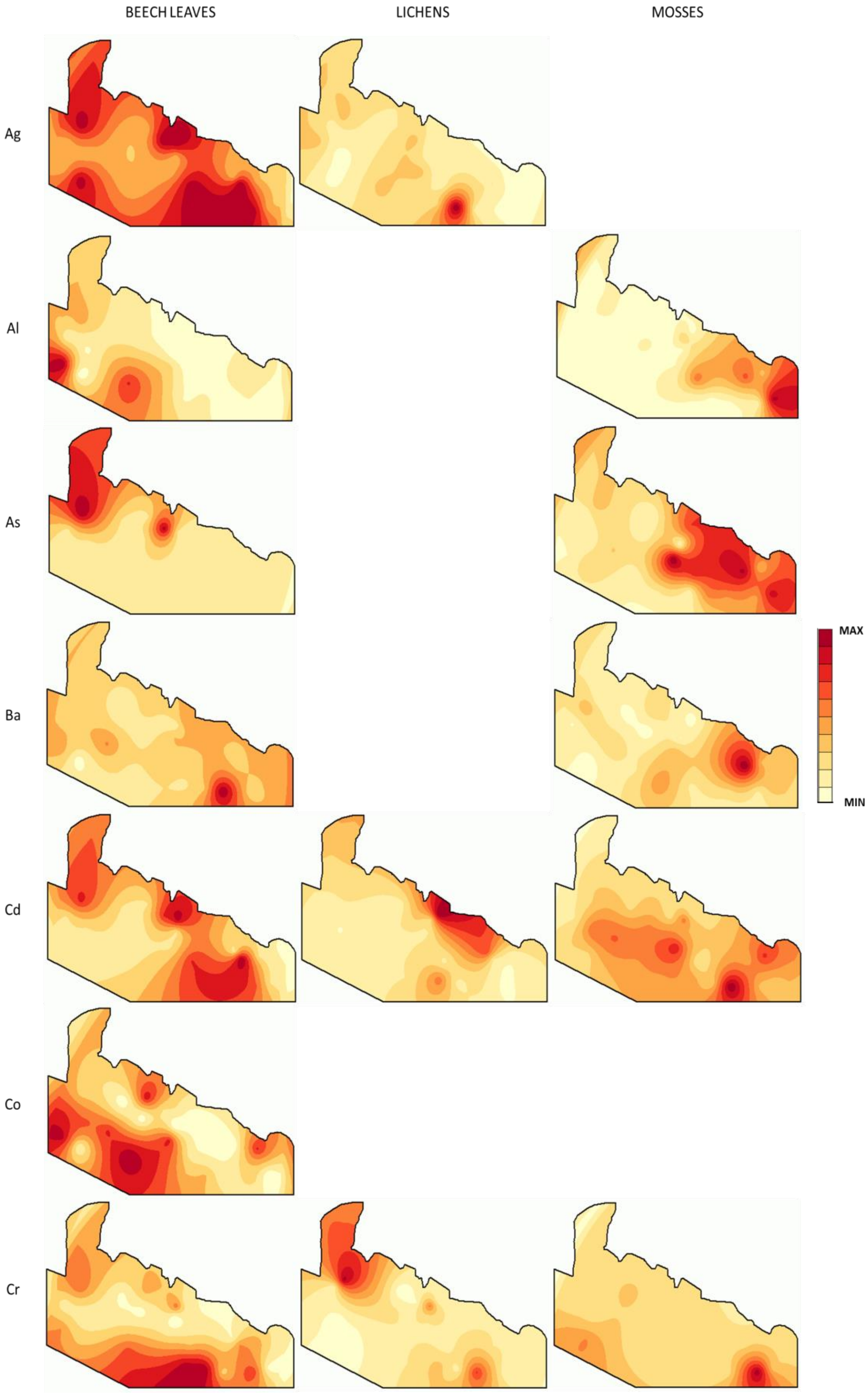

Figure 16. Geographical distribution of several elements in beech leaves, lichens and mosses from the Irati Forest.

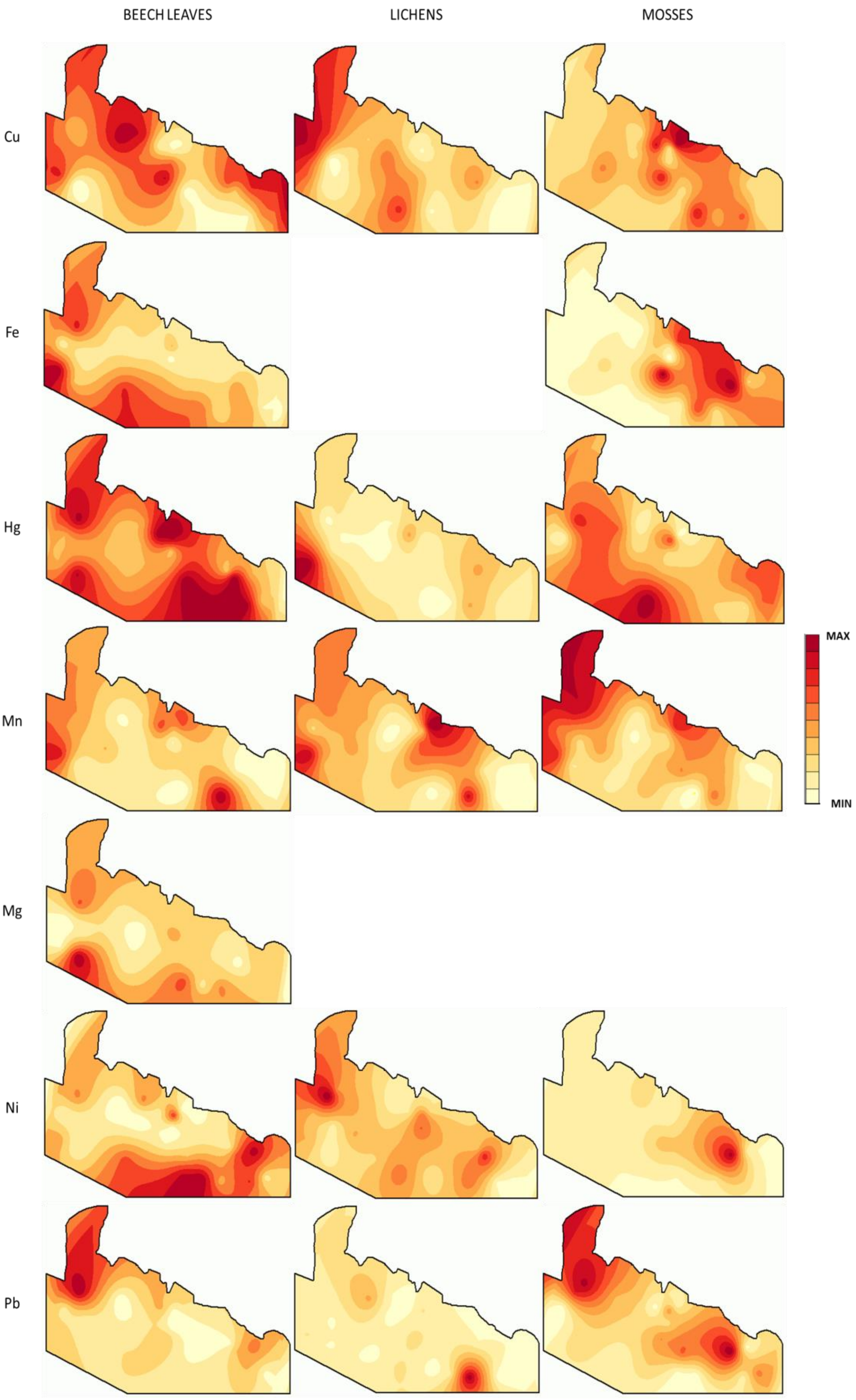

Figure 16 (Cont.). Geographical distribution of several elements in beech leaves, lichens and mosses from the Irati Forest.

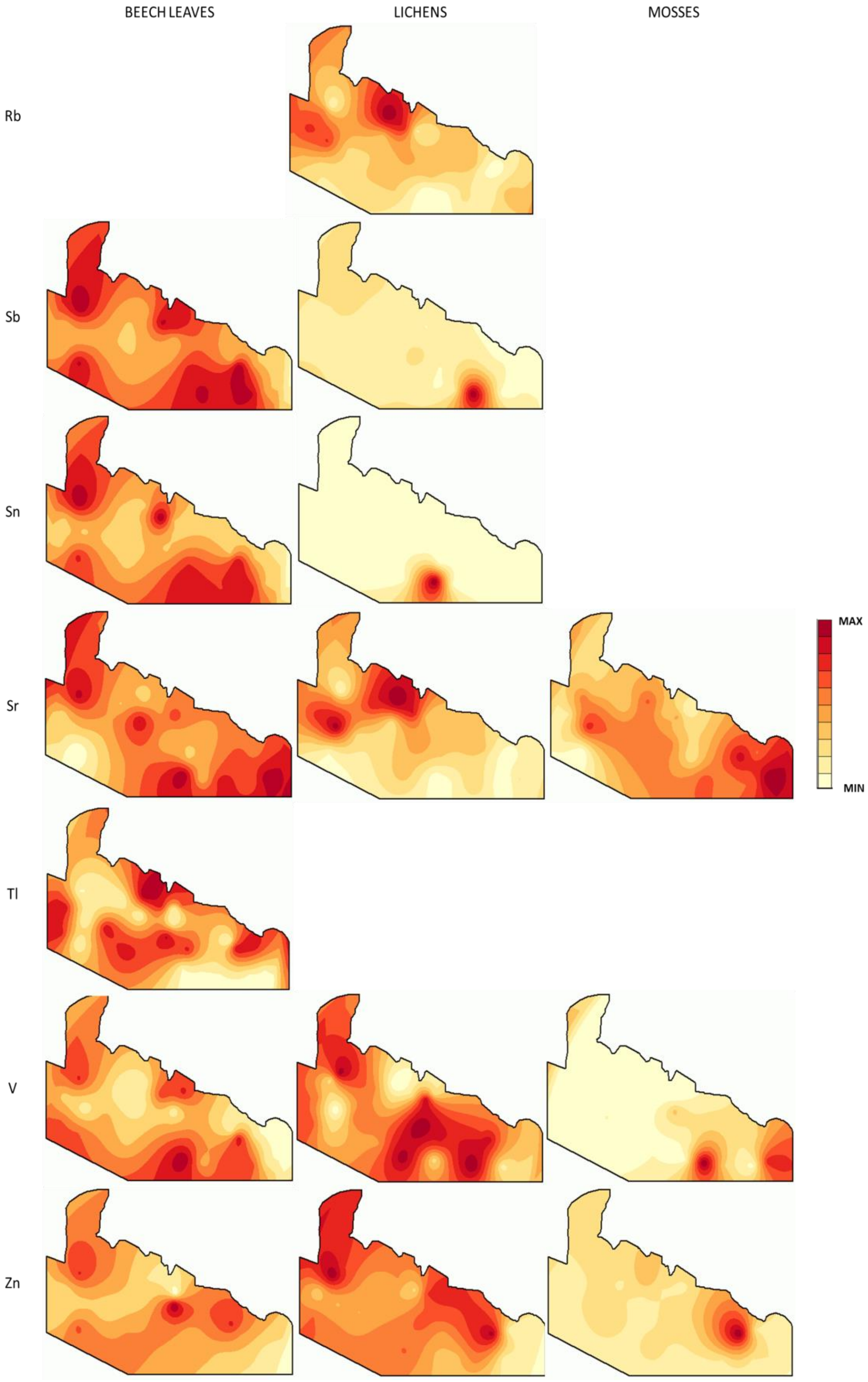

Figure 16 (Cont.). Geographical distribution of several elements in beech leaves, lichens and mosses from the Irati Forest..

5. CONCLUSIONS

Elements like Cu, Sr, Ni, Mn, Ba, Zn, Cd and Cr probably have an anthopogenic origin in the atmosphere of the Irati Forest. Hotspots of metal atmospheric contamination concentrate in the upper part of the water catchment of the Irati River and in the NW sector of the studied area. The three bioindicators investigated, beech leaves, lichens and mosses, lead, in general terms, to this conclusion. Diesel exhaust and emissions from steelworks located in the industrialized areas of the Basque Coast and Iruñea (transported by the dominant NW-W-SW winds towards the Irati Forest) might be responsible of this geographical distribution of pollution.

The accumulation rate of Cr and Mn is higher in beech leaves than in the other two matrices, while those of Cu and Hg are higher in moss than in leaves and lichen. Lichens are more efficient than moss and leaves to accumulate elements like Pb and V. The accumulation of Cd, Ni, Zn and Sr is rather similar in the three bioindicators. It is to be highlighted that the accumulation of metals in beech leaves is highly influenced by mountainside orientation.

Although the three organisms investigated have demonstrated ability to accumulate metals in their tissues and, consequently, their potential to be used as indicators of atmospheric metal contamination, the combined use of beech leaves, lichens and mosses in monitoring exercises must be handled with care because, due to their different longevity, they provide us with information that is not representative of the same period of time.

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Section 2

METALLIC CONTAMINATION IN ESTUARIES

Chapter 6

ESTUARY SISTEMS

CHAPTER 6

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1. Estuaries: description, classification and main features

Estuary is the name given to the widest part of a river that flows into the sea and is affected by the tides. Consequently, an estuary is the meeting point between a river and the sea, where salt water and fresh water mix. Estuaries may be divided into three parts: a sea section, closest to and directly linked to the sea; a mid-section, where salt water from the sea mixes with fresh water from the river; and lastly, a river section, consisting mainly of fresh water but, depending on the tides, it may also be affected by salt water.

A wide variety of shapes and sizes of estuary can be found all over the world. Most often, the form of an estuary is determined by natural barriers in the form of corals, land, sand or mud, that offer protection from the harsh conditions created by the sea (waves, strong winds, storms). The different shapes and sizes of estuaries may be determined by the sea conditions, different features of the river and climate factors. Estuaries constitute a diverse range of ecosystems rich in biodiversity.

Estuaries are classified according to geological origin and the mix of salt water and fresh water. Taking into account its geological features, an estuary can be classified into four categories (Pritchard, 1955; Pritchard, 1967):

- **Coastal plain:** Estuaries formed at the end of the Ice Age, when ice that had piled up over the years melted and the sea swallowed up the lowest part of the river valley. This kind of estuary is mostly found in mild latitudes: the Thames, in the United Kingdom (Figure 1); Chesapeake Bay, in the United States; Si-King, in Hong Kong, amongst others.

- **Tectonic:** Estuaries formed as a result of tectonic plate movements, where land closest to the sea sinks and the resulting basin is filled by the sea. San Francisco Bay, for example (Figure 1)

- **Delta:** Estuaries formed by the sea depositing large quantities of sand and silt close to the sea. They are usually shallow and can change depending on the transportation and movement of sand and silt, which can result in the position of estuary mouth changing. These estuaries form deltas. Most estuaries in Colombia are delta estuaries (Figure 1).

- **Fjords:** Estuaries formed by glaciation and seawater filling very steep sided valleys. In this kind of estuary there is little mixing of deep water and seawater. The best examples of this kind of estuary can be found in Canada, Chile, Greenland, Alaska, Norway (Figure 1) and Siberia.

Some estuaries have mixed features and, consequently, cannot be classified into one of the preceding four categories.

Figure 1. a) Thames Estuary (UK); b) San Francisco Bay (USA); c) Buenaventura Estuary (Colombia); d) A Norwegian fjord.

Depending on tidal action, an estuary may be **microtidal** (tidal range is smaller than 2 m), **mesotidal** (tidal range is between 2 and 4 m) or **macrotidal** (tidal range is greater than 4 m).

Regarding hydrological features, a clear classification of estuaries is not easy (Bowden, 1967). The salinity of estuary water ranges from 0-1‰ to 35‰ and with many factors coming into play, salinity in an estuary may change over time and in space. However, the following general classification can be made:

- **Salt-wedge:** in these estuaries the flow of fresh water from the river is greater than the flow of salt water from the sea. The less dense fresh water floats above the salt water, creating two sharply defined masses of water. The two strata create a pronounced vertical salinity gradient known as a halocline. In these estuaries the quantity of salt water that enters the estuary depends on the flow of the river. Salt-wedges are common in microtidal estuaries

- **Highly stratified:** This kind of estuary is commonly found in fjords, where there is a strong flow of river water. The boundary between salt water and fresh water is sharply defined in this kind of estuary, which is usually very deep and has salt water right at the bottom.

- **Slightly mixed:** As a result of strong tides, salt water and fresh water mix to a large extent. Water reflux generates near-bed currents, which can have a marked impact on sediment transport processes. Slight mixing is commonly found in mesotidal estuaries.

- **Vertically mixed:** In this kind of estuary salt water and fresh water mix completely and, as a result, salinity does not vary with depth (isohaline), as it does in highly stratified and salt-wedge estuaries. These estuaries are usually shallow and have a large tidal range.

- **Reverse flow:** In this rare kind of estuary, the evaporation of surface water is greater than the influx of water. In consequence, surface waters have greater salinity than deeper salt water (hiperhaline). Density increases, causing the water on the surface to sink. Seawater, as a result, flows to the edges to a large extent.

As well as the geomorphological and hydrological features mentioned above, sedimentation processes also play an important role in estuaries and, moreover, are determined by geomorphological and hydrological features. Sediment can reach an estuary via the sea or the river. Sediment coming from the sea includes sediment previously transported into the sea by the river and other floating particles suspended in the sea itself (Guilcher, 1967). Suspended particles brought by the river and the sea and suspended matter from resettlement of sediments in the estuary itself, deposit in different quantities and in different places along the estuary. Deposition depends on the geomorphological and hydrological characteristics of the estuary. Where the estuary current is weaker and the river current becomes one with the sea current, most depositing particles are clay particles (< 1 µm) and dirt particles (<20 µm). In contrast, in areas affected by sea currents, the depositing particles are sandier. The settling rate of clay particles is 10^{-5} cm/s, whilst that of dirt particles is 0.04 cm/s.

Solid particles in the estuary may be made up of both agglomerate and aggregate particles and flocculation processes can occur (Taghon et al., 1984; Turner, 2002). Agglomerate particles are formed in reversible processes, whereas the processes by which aggregates are formed are irreversible (Aleman et al., 2007). Flocculation is the term for the process whereby aggregate particles are created from isolated suspended particles (Postma, 1967). Flocculation has a direct effect on particle settling rate. Flocculation arises from Van der Waals forces between molecules. Flocculation does not occur in fresh water as clay particles usually have negative electrostatic charges and the forces of repulsion between them are considerable. By contrast, in salt water, concentrations of cations are high, neutralising the clay particles' negative charges. In this manner, if close enough to each other, clay particles can form aggregates through the action of Van der Waals forces. Salinity, therefore, has a direct effect on flocculation processes.

Estuaries constitute ecosystems that are necessary for the survival of many species and are usually areas abundant in nutrients, as much for birds as for mammals, fish and many other life forms. Many migrating birds use estuaries on their migrations to feed and rest. Fish and shellfish that fetch high prices use estuaries for biological processes that occur at some point in their life. In addition, the vegetation that grows on the flats of estuaries is also very important. The vegetation limits flooding resulting from tidal action, protecting species that live there and diminishing the movement of land and sediment.

Estuaries and coastal waters are very productive water bodies. As such they have always been intensively exploited. They have been, and still are, the most important socio-economic resources, because of the industrial and urban centres that have developed around them. Furthermore, activities such as mining, forestry, agriculture and fish farming are often carried out close to estuaries, often resulting in serious ecological damage to their water quality, such as increases in organic matter and drops in oxygen levels, for example (Uriarte and Borja, 2009). Estuaries have often been used for transporting both goods and people. Waste matter generated by activities in the area has often been discharged into them and, as a result, a large number of estuaries have become a storehouse of pollutants. Both organic and inorganic pollutants from the sea, from streams, from the atmosphere or from water treatment plants on the very banks of the estuary, easily accumulate in the estuary, especially in the sediment. At the same time, estuaries are extremely valuable from an ecological, biological, social and economic point of view (Bierman et al., 2011).

2. Metals and metalloids in estuaries

The metals and metalloids that reach an estuary have either a natural or an anthropogenic source (Figure 2). Rock leaching and volcanic eruptions are the most important natural sources (*11*). Amongst others, metals reach estuaries from urban areas and as a result of activities such as agriculture, industry, animal farming and mining (*12*-*15*). Metallic waste discharge can also occur directly. Another path metals have into estuaries is atmospheric deposition, be it wet or dry deposition.

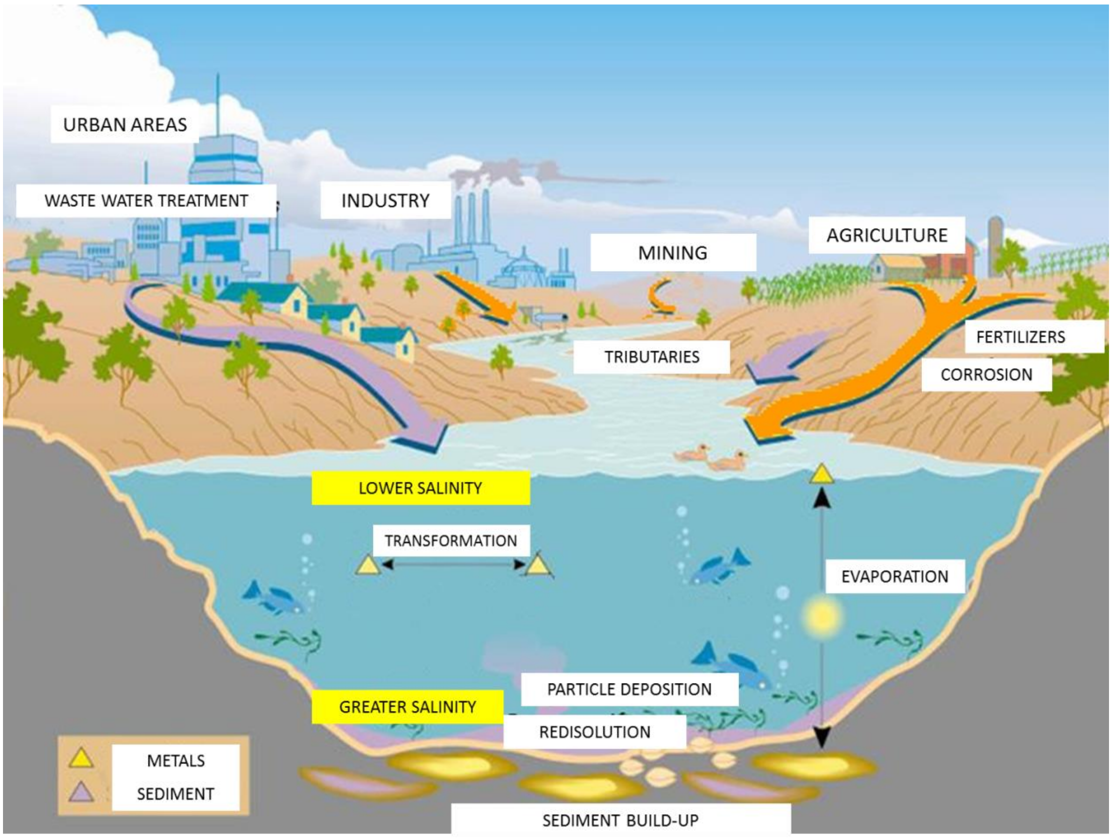

Figure 2. metals in estuaries: sources and natural processes

Metals dissolved in water tend to react with different particles in the water, through ion exchange, adsorption, chelation or other chemical processes (Dekov et al., 1998). These particles may be later deposited in sediment (Reboreda et al., 2008).

Metal elements can also react directly with particles in sediment, with sediment texture and composition having a direct impact on metal reactions (Abrahim et al., 2007; Szava-Kovats, 2008). Humic substances in the estuary, for example, form stable complexes with metals (Takahashi et al., 2002).

Metals present in sediment can be dissolved into the water (He et al., 2006). The factors that most cause the mobility of metals are the characteristics of the sediment (pH, redox potential, quantity and nature of organic matter), the characteristics of the metal itself and the physical and chemical properties of the water in the area (pH, redox potential, salinity...) (van Ryssen et al., 1999). Consequently, it may be said that the water itself may become a source of pollution, if the right conditions occur.

The mass of chemical pollutants dissolved in water is very small compared to that found in sediment (Izquierdo et al., 1997; Salomons, 1998). The concentration of metals in surface sediments can give important information as to water quality in the estuary and can often be easily obtained (Spencer et al., 2003), which has given rise to their frequent use in determining metal pollution in estuaries. Both in Europe and around the world works on monitoring metal elements in estuarine sediments abound. Table. 1 shows an example of results obtained in numerous such studies.

So, bearing in mind the importance of estuarine ecosystems for a large number of life forms, the harm caused by the industrial and urban activities that have developed along them and the fact that these are areas of great value from an ecological, biological, social and economic point of view, in the following chapters of this study the results of monitoring work carried out in the Nerbioi-Ibaizabal and Urdaibai estuary, both in the Basque Country, the Tubarão estuary in Brazil, the Cávado estuary in Portugal and the estuary of the Hugli River in India will be presented, showing the geographical distribution of metal pollution and examining its evolution over time.

Table 1: Concentrations of various metals measured in sediments from various estuaries around the world (in mg·kg^{-1}).

Estuary	Ag	As	Ba	Cd	Co	Cr	Cu	Fe	Mn	Mo	Ni	Pb	Sb	Sn	V	Zn	Ref
Tamar (England)	-	-	-	0.4-0-5	-	-	129-161	30500-36500	348-390	-	25.0-28.0	139-165	-	-	-	246-282	(Singh and Turner, 2008)
Restronguet (England)	1.2	-	-	-	-	-	2890	-	-	-	-	168	-	-	-	2200	(Rainbow et al., 2011)
Odiel (Spain)	-	330	-	14.4	-	-	2109	-	-	-	-	590	-	-	-	1154	(Vicente-Martorell et al., 2009)
Tinto (Spain)	-	339	-	8.4	-	-	1897	-	-	-	-	496	-	-	-	1115	(Vicente-Martorell et al., 2009)
Canal de Santo Padre (Spain)	-	384	-	7.1	-	-	2215	-	-	-	-	630	-	-	-	1431	(Vicente-Martorell et al., 2009)
Sado (Portugal)	-	-	-	0.3-0.4	-	-	59.0-136	34000-45000	-	-	-	55.7-77.0	-	-	-	370-391	(Almeida et al., 2008)
Minho (Portugal)	-	-	-	0.02-0.3	-	-	2.8-22.4	14000-39000	-	-	-	4.8-15.9	-	-	-	37.7-91.6	(Almeida et al., 2008)
Douro (Portugal)	-	-	-	0.1-0.3	-	-	1.0-229	2700-30000	-	-	-	0.25-192	-	-	-	6.2-457	(Almeida et al., 2008)
Tagus (Portugal)	-	-	-	0.9-11.0	-	-	9.7-214	31000-50000	-	-	-	11.8-350	-	-	-	88.2-1086	(Almeida et al., 2008)
Cavado (Portugal)		6.1-11.7	-	0.13-0.37	3.9-5.8	20.2-34.7	54.9-113	8600-10400	75-449	-	9.4-16.4	30.3-40.9	-	2.9-4.7	10.1-12.8	94-170	(Gredilla et al., 2015)
Patras estuary (Greece)	3.3	8.8	385	0.5	17.0	202	82.5	35000	1015	1.7	110	49.5	1.6	-	-	120	(Papaefthymiou et al., 2010)
Girondi (France)	-	18.7	-	0.50	-	78.4	24.5	-	-	-	32.0	46.8	-	-	-	168	(Larrose et al., 2010)
Drin (Albania)	-	-	-	0.10-0.13	-	-	12.0-13.0	725-749	289-299	-	-	0.40-0.50	-	-	-	6.9-7.1	(Ianni et al., 2010)
Macaé (Brazil)	-	-	89-234	-	-	44-94	31-52	35000-62000	147-950	-	17-32	38-52	-	-	-	105-150	(Molisani et al., 2015)
Tirumalairajan (India)	-	-	-	-	-	-	20	2053	19.7	-	-	5.2	-	-	-	29	(Venkatramanan et al., 2014)

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Chapter 7

EFFECTS OF A TROPICAL STORM IN A POLLUTED ESTUARY: TUBARÃO RIVER AS A CASE OF STUDY

CHAPTER 7

EFFECTS OF A TROPICAL STORM IN A POLLUTED ESTUARY: TUBARÃO RIVER AS A CASE OF STUDY

ABSTRACT

Tropical storm can produced unprecedented changes in the metal distribution of water and sediments in a polluted estuarine area. Tubarão River (Santa Catarina, Brazil) estuary is affected by metal and metalloid pollution from exploitation and processing of coal mining, agricultural activities, urban discharges, industrial and leisure zones, etc. In order to study the distribution, sources and risk assessment of metal contamination in a polluted estuary after a tropical storm, waters and surface sediments were collected from 15 selected sampling sites along Tubarão River four days after the strong storm (January 2013). The concentration of 24 elements (Ag, Al, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Mg, Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, Ti, Tl, V, W and Zn) were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and the mineralogical composition of the sediments by Raman spectroscopy and X-ray diffraction (XRD). The metal concentrations in waters and in bed sediment showed wide spatial variation due to the variability in water discharges after a storm and variations of anthropogenic inputs along the estuary. In general, high metal concentration in water samples and low in the sediments were found in upstream sites of the estuary (near of coal mining areas). The sampling sites next to the sea and in Oratorio River (one of the seven tributaries of the estuary) showed the highest values in the sediment samples. Normalized and Weighed Average Concentrations (NWAC) were calculated for the identification of hotspots. The results of this study were compared with a previous study in the same area during dry season at 2012 by Principal Component Analysis (PCA), showing changes in the sediments metallic pollution after a strong storm event.

Keywords: Water; Sediment; Metal and metalloids; Physic-chemical parameters; Tropical estuary, Tubarão River; Storm.

1. INTRODUCTION

Estuarine waters receive contaminants such as metals and metalloids via anthropogenic activities and/or through riverine inputs. The distribution of soluble and particulate trace metals are controlled by physical-chemical processes (Windom et al., 1988), daily tidal currents and regimes of alluvial loading. This last factor can be environmentally relevant in the case of tropical estuaries (Balachandran et al., 2005). Strong tropical storm events can resuspend superficial sediments with different particle size, dispersed and accumulated across different sedimentary environments (Guillen et al., 2006). Furthermore, contributions of soil water to the stream from the adjacent soils increase during storm events (Zimmer and Lautz, 2014). Fine-grained sediments tend to accumulate metal and metalloids due to their sorptive nature, and thus can act as an important reservoir of contaminants with risk of mobilization (Eggleton and Thomas, 2004; Towler and Smith, 2000). The increase on the solubility of the mentioned metals from sediment could carry out an increasing of the total concentration of metals in the estuarine water and can affect the bioavailability and toxicity of metal ions in the aquatic ecosystem (Sparks, 2005).

Tubarão River (Santa Catarina, Brazil) has been affected by coal mining activities for over 50 years (Kagey and Wixson, 1983; Sekine et al., 2008; SIECESC, 2008; Zheng et al., 2007). Nowadays, most of the mines in the Santa Catarina region are disabled, but as this region is highly drained by streams, trenches and water channels among others, the waters and sediments of the river have the undesirable effects of these activities, e.g., piles of waste, acid drainages, abandoned mines, and the acidification of water bodies, remain in full view (Prefeitura de Lauro Muller, 2014). The estuary has other sources of contamination such as coal-combustion power plant, traffic road, agricultural and industrial zones, and industry and wastewater effluents (Prefeitura de Lauro Muller, 2014). In addition, the rapid population growth of the area together with the lack of efficient urban planning and economic development has brought about serious problems of environmental degradation (Henrique et al., 2013).

Tubarão estuary is affected regularly by strong storms, which could change or affect the biogeochemistry of the area. The environmental effect of these strong rainfall events on a polluted aquatic system is not usually studied (Birch and Taylor, 1999). No known studies have been focused on differences in metal concentration of water and sediment samples after one of these extreme storm events in a polluted tropical estuarine. Therefore, the aims of the present work were: i) determine the spatial variability and the tendencies of metal pollution along the estuary, ii) identify the potential contamination sources, and iii) estimate the effect of a storm event in the mobility of the sediments metallic content in a polluted tropical estuary.

2. MATERIALS AND METHODS

2.1. STUDY AREA AND SAMPLING PROCEDURES

Tubarão estuary (Santa Catarina, Brazil) is a 120 km long, with a water flow that increases toward the estuary and a drainage area of 4,728 km^2. It has seven principal freshwater inputs: Bonito, Rocinha, Orotoria, Capivaras, Braco do Norte, Palmeiras and Capivari River (Silva et al., 2012; Prefeitura de Lauro Muller, 2014).

Waters and sediments samples were collected at 15 sampling sites (BO, RO, TBLM, OR, ORTB, LA, TBLA, PL, PLTB, BN, PD, NCTB, CA, FITB, and ES) along Tubarão River in January of 2013 (Figure 1). The sampling was carried out after four days of rainy season with strong storms; the storms caused the increases of the water fluxes in the estuary and floods in many places.

Superficial sediments (0–2 cm) were collected at each sampling sites in the centre of the riverbed by hand using latex gloves or by means of Ponar dredge depending on the depth and the flow). Superficial water (top 10 cm) was also collected with a 50 mL HNO_3 pre-cleaned polypropylene bottle. The first water sample was used to homogenise the bottle and then to fill it to the top. At the same time pH, electrical conductivity and redox potential were measured *in situ* in the water samples. The sediment samples were stored in 500 mL polyethylene bottles and water bottles protected against light using aluminium foil were transported to the laboratory at 4ºC in a cool box. Blank samples (pre-cleaned bottles filled with Milli-Q quality water at the beginning of the sampling day) were handled in a similar way.

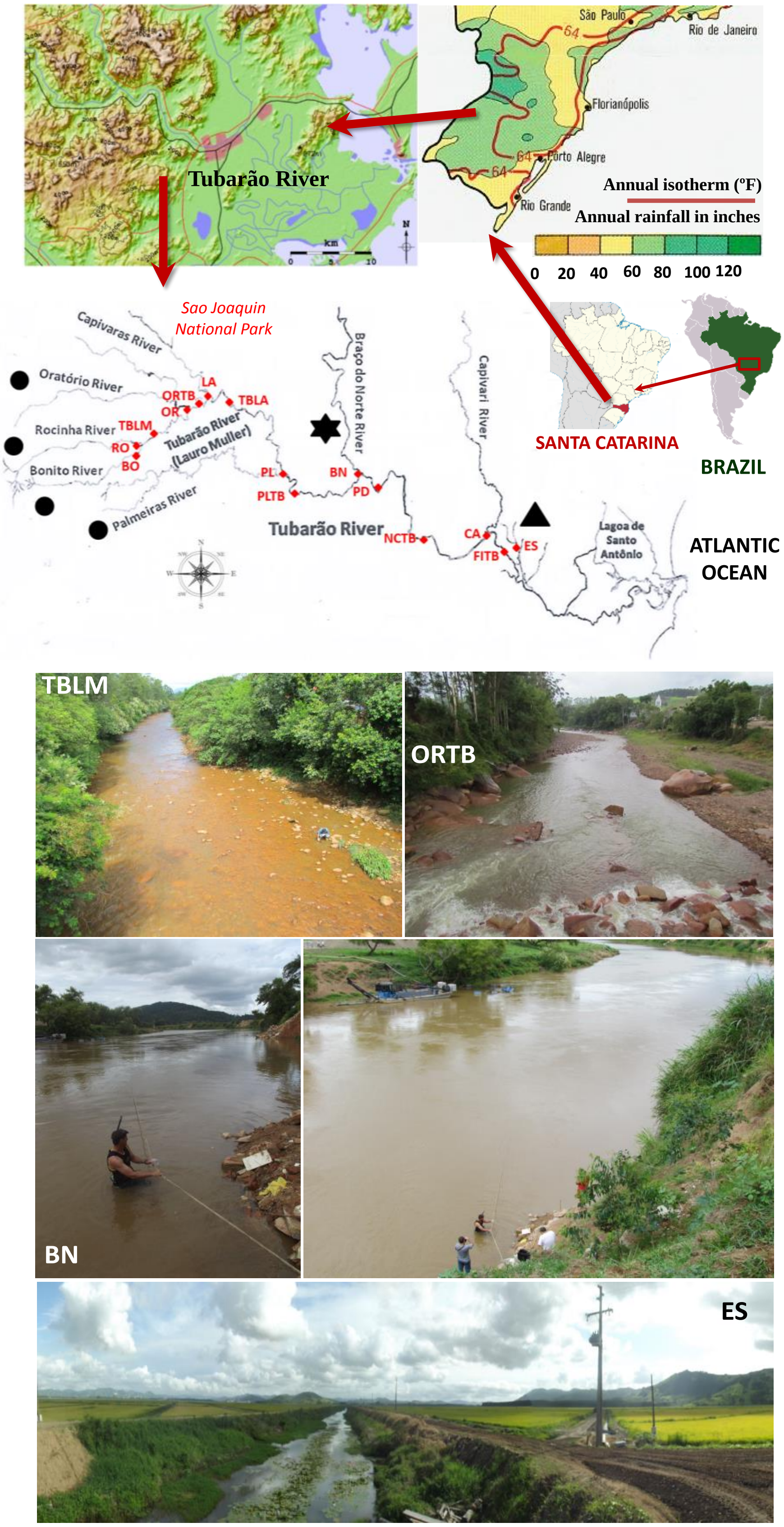

Figure 1. Geographical location of the Santa Catarina State (Southern Brazil), annual precipitations, temperature and orography of the studied area, and images and location of the sampling sites in Tubarão estuary. Black circle: coal mines; black star: pig farm and black triangle: thermoelectric plant.

2.2. ANALYTICAL PROCEDURE

The pretreatment and analysis of the samples was carried out according to the procedures described in Chapter 2, experimental. The concentration of 24 elements was finally measured by ICP/MS. All the sediments samples were mineralogically characterised as well by powder X-ray diffraction (XRD) of dust and Raman Spectroscopy (RS) as described in Chapter 2, experimental. Statistical and chemometric analysis of data was performed by means of The Unscrambler (v. 9.2 Camo, Oslo, Norway). Contour maps were produced with the aid of the 3D Field programme (v. 3.8.8.0, by Vladimir Galouchko). The detection limits, accuracy and reproducibility of the analytical methods are summarised in Table 1.

Table 1. Detection limits (LOD, in μg·kg^{-1} for water samples and mg·kg^{-1} for sediment samples), accuracy (as percent of recovery) and reproducibility (as relative standard deviation, RSD) of the waters analytical method.

	WATER			SEDIMENT		
	LOD (μg·kg^{-1})	Recovery (%)	RSD (%)	LOD (mg·kg^{-1})	Recovery (%)	RSD (%)
Ag	0.16	95	7	0.06	82	9
Al	1.29	103	12	1.8	78	8
As	0.59	90	3	0.34	89	4
Ba	16.3	93	7	0.88	81	8
Cd	0.39	93	10	0.14	69	12
Co	0.3	94	6	0.28	78	6
Cr	0.64	95	3	0.81	89	2
Cu	4.16	106	2	0.69	92	2
Fe	5.99	85	4	1.4	78	5
Hg	53.5	52	16	0.244	61	18
Mg	2.49	78	11	0.82	81	6
Mn	1.97	101	4	0.74	88	7
Mo	0.31	80	8	0.203	81	11
Ni	2.62	98	1	0.38	91	6
Pb	0.026	92	8	0.40	92	5
Sb	0.33	94	13	0.008	78	14
Se	0.51	92	2	0.344	79	11
Sn	9.99	72	6	0.08	88	9
Sr	4.99	94	5	0.202	92	4
Ti	0.37	89	8	0.069	88	6
Tl	2.03	97	11	0.005	89	8
V	0.43	94	4	0.23	81	6
W	13.6	72	16	0.31	84	7
Zn	0.81	95	8	1.2	98	4

3. RESULTS AND DISSCUSION

3.1. ELEMENT CONCENTRATIONS AND SPATIAL DISTRIBUTION IN THE WATER SAMPLES

The representation of the concentrations found for each metal and metalloids at each sampling site can be seen in Figure 2 and Table 2. Extreme Fe concentrations were identified in RO and TBLM sampling sites, while Pb showed an extreme concentration in PL.

Al, Fe, Mg, Zn and Mn were major elements, with concentration values between 8390 and 36.5 $\mu g \cdot kg^{-1}$. Ba, Ni and Sr would be in a second position, and their concentration varied between 48.7 and 4.91 μg·kg-1. As, Cd, Co, Cr, Cu, Pb, Se, Ti and V were the trace elements with the lowest concentrations. Ag, Hg, Mo, Sb, Sn and W concentrations were in all the cases below the detection limit.

Although the spatial distribution is specific for each analysed element, some general trends can be listed. For Al, As, Cr, Se, V and Zn the concentrations found were higher in the upstream sampling sites, while the behaviour of Mg and Pb (except PL) was the opposite, with higher concentrations in downstream sampling sites. The sampling sites in PL and RO presented high concentration of As, Co, Cr, Mg, Mn, Ni, Pb, Se, Sr, Ti and Zn which could be affected by the coal mining wastes still located Near Palmeiras and Rocinha Rivers (Silva et al., 2011). In the case of rainfall events, the disturbed lands near the mine are susceptive to erosion and can finish at the bank of these rivers, and thus increase the amount of suspended particulate matters and soluble metal load in the river waters.

Higher conductivity and lower redox values were found at the mouth of the estuary. pH varied from 3.5 to 7.0, being minimal in Palmeiras, Oratorios, Bonito and Rocinha Rivers (Table 2). The acid pH values (3.5-4.5) could be due to pyritic containing wastes from the abandoned coal mines near of these effluents rivers. The pHs of the water could influence on the concentrations of metals dissolved in the water and the concentrations in bed sediments of the upper part of Tubarão River.

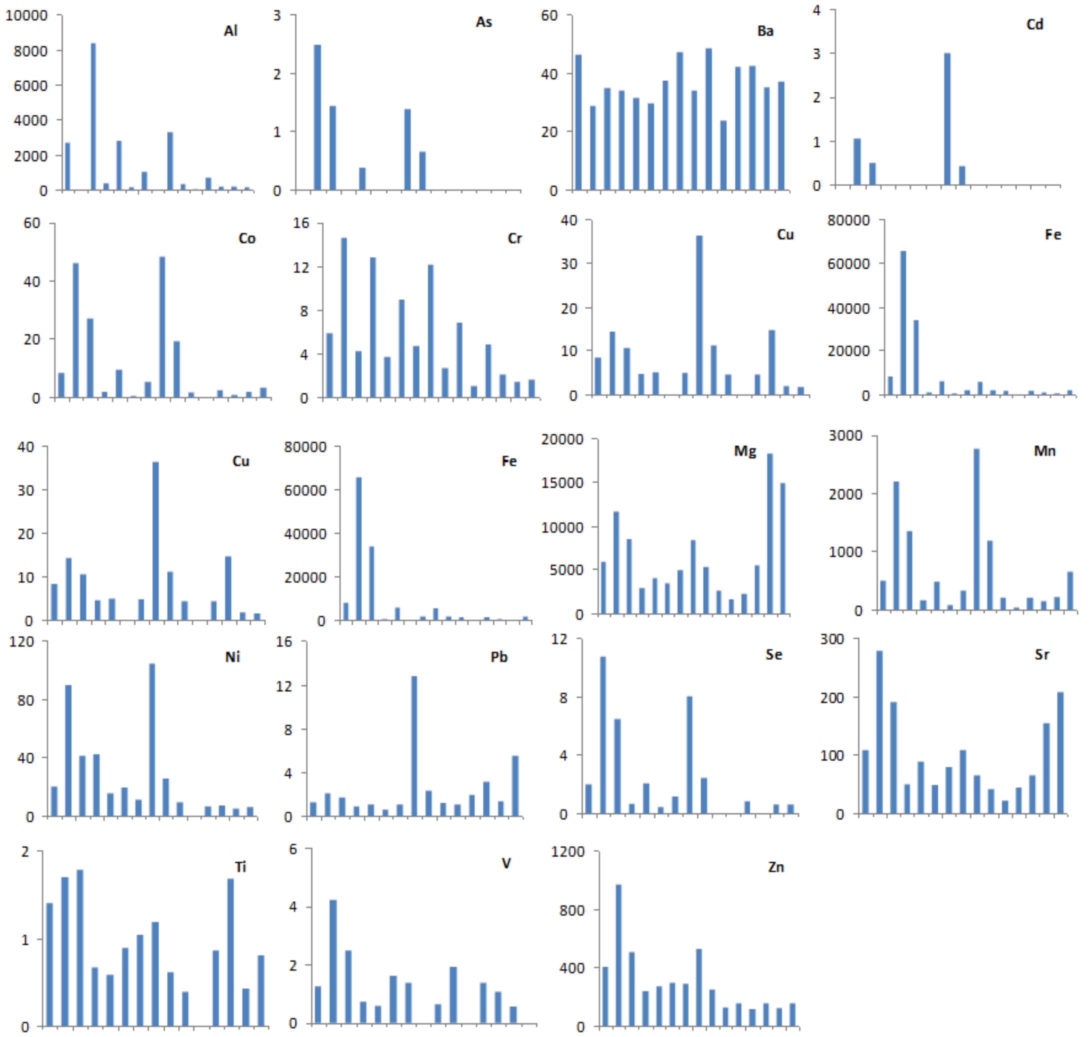

Figure 2. Water metal concentration (μg·kg^{-1}) of the Tubarão estuary in the next order from the left side of the X axis, which is the inner side of the estuary to the right side of the X axis, which is the outer part of the estuary and nearest to the sea: BO, RO, TBLM, OR, ORTB, LA, TBLA, PL, PLTB, BN, PD, NCTB, CA, FITB and ES.

Table 2. Water metal concentration (µg·kg^{-1}), pH, Eh (mV) and conductivity (µS cm^{-1}) of Tubarão estuary together with some related statistics. Extreme high concentrations, defined as those concentrations over the 75th percentile of the data (Q3) plus three times the interquartile range (IQR), are marked with an asterisk (*).

	Al	As	Ba	Cd	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Se	Sr	Ti	V	Zn	pH	Eh	Cond
BO	2700	<LOD	46.4	<LOD	8.44	5.86	8.36	8360	5830	502	20.1	1.24	2.01	108	1.41	1.25	405	4.70	126	115
RO	S	2.49	28.7	1.06	46.0	14.6	14.3	65500*	11700	2210	89.9	2.11	10.8	280	1.70	4.21	973	4.06	163	314
TBLM	8390	1.44	35.0	0.50	27.1	4.21	10.7	33900*	8500	1360	41.6	1.74	6.47	192	1.78	2.47	507	4.46	144	265
OR	379	<LOD	34.2	<LOD	2.03	12.8	4.69	702	2890	167	42.1	0.91	0.69	50.5	0.67	0.73	241	6.31	39	70
ORTB	2780	0.38	31.7	<LOD	9.36	3.75	4.98	5930	4010	495	15.5	1.07	2.07	88.8	0.58	0.59	273	4.15	156	190
LA	125	<LOD	29.6	<LOD	0.69	8.92	<LOD	406	3420	81.9	19.7	0.63	0.46	49.0	0.89	1.62	294	6.55	8	45
TBLA	1020	<LOD	37.3	<LOD	5.17	4.72	4.92	1960	4980	330	11.0	1.05	1.18	79.1	1.05	1.36	291	5.70	25	80
PL	S	1.38	47.1	3.00	48.4	12.1	36.2	5490	8380	2760	104	12.8*	8.01	109	1.19	<LOD	532	3.46	201	355
PLTB	3310	0.65	34.0	0.43	19.2	2.68	11.1	1950	5320	1190	25.8	2.27	2.43	65.8	0.61	0.65	249	3.86	174	162
BN	340	<LOD	48.7	<LOD	1.56	6.83	4.44	1430	2600	207	9.33	1.19	<LOD	42.3	0.39	1.93	128	6.60	22	38
PD	47.7	<LOD	23.8	<LOD	<LOD	1.04	<LOD	179	1600	36.5	<LOD	1.07	<LOD	22.5	<LOD	<LOD	157	6.98	37	58
NCTB	720	<LOD	42.1	<LOD	2.49	4.83	4.39	1320	2240	211	6.55	1.93	0.82	44.6	0.87	1.38	116	6.32	27	55
CA	164	<LOD	42.3	<LOD	0.90	2.09	14.8	755	5490	152	7.10	3.14	<LOD	65.2	1.69	1.06	156	6.27	32	304
FITB	208	<LOD	35.1	<LOD	1.99	1.42	1.84	237	18200	217	4.91	1.36	0.59	155	0.43	0.56	123	6.16	40	1172
ES	135	<LOD	37.0	<LOD	3.29	1.63	1.76	1730	14900	651	6.17	5.54	0.59	208	0.81	<LOD	155	5.60	51	677
25 PERCENTILE	149	0.51	31.7	0.45	1.88	2.09	4.41	702	2890	167	6.97	1.07	0.62	49.0	0.61	0.67	155	4.15	27	58
75 PERCENTILE	2740	1.96	42.3	2.52	21.1	8.92	12.7	5930	8500	1190	41.7	2.27	5.46	155	1.48	1.85	405	6.32	156	314
IQR(Q3-Q1)	2591	1.45	10.6	2.07	19.2	6.83	8.29	5228	5610	1023	34.7	1.20	4.84	106	0.87	1.18	250	-	-	-
Q3+(3*IQR)	10513	6.31	74.1	8.73	78.8	29.4	37.6	21614	25330	4259	146	5.87	19.9	473	4.09	5.39	1155	-	-	-
MEDIAN	379	1.38	35.1	0.78	4.23	4.72	4.98	1730	5320	330	17.6	1.36	1.60	79.1	0.88	1.31	249	5.70	40	162
MEAN	1560	1.27	36.9	1.25	12.6	5.83	9.42	8650	6670	704	28.9	2.54	3.01	104	1.00	1.48	307	5.41	83	260
DESVEST	2350	0.82	7.23	1.20	16.5	4.38	9.15	17900	4870	825	31.4	3.09	3.46	73.5	0.48	1.04	227	1.17	68	305
MIN	47.7	0.38	23.8	0.43	0.69	1.04	1.76	179	1600	36.5	4.91	0.63	0.46	22.5	0.39	0.56	116	3.46	8	38
MAX	8390	2.49	48.7	3.00	48.4	14.6	36.2	65500	18200	2760	104	12.8	10.8	280	1.78	4.21	973	6.98	201	1172

3.2. ELEMENT CONCENTRATIONS AND SPATIAL DISTRIBUTION IN THE SEDIMENT SAMPLES

The representation of the concentrations found for each metal and metalloids at each sampling site in sediment samples from Tubarão estuary can be seen in Figure 3 and Table 3. Mn showed an extreme concentration in PD sampling site and Cu in OR sampling site.

Al, Fe, and Mg were major elements, with concentration values between 112.000 and 334 mg·kg^{-1}. Ba, Mn, Ti, V and Zn had an intermediate concentration, and their concentration varied between 1800 and 8.01 mg·kg^{-1}. As, Co, Cr, Cu, Mo, Ni, Pb, Sb, Se, Sn, Sr and Tl were as trace elements, and Ag was found to be next or below detection limit. Cd, Hg and W were in all the cases below the detection limit.

High elemental concentration (Al, Ba, Co, Cr, Cu, Mg, Mn, Ni, Se, Sn, Sr, Ti, V, and Zn) was found in OR sampling site. With the runoff after rain, disturbed suspended particulate near the mines from upper Bonito, Rocinnha and Oratorio Rivers could get deposited at the confluence of these rivers, where is located OR sampling site, affecting to the metallic concentrations of its sediments.

In the middle part of the estuary (PD, NCTB and CA) were found quite high concentrations of Al (only NCTB), Ba, Co, Cr, Mn (only PD), Ni, Pb (PD and CA), Se, Sn, Tl (only PD) and Zn (only NCTB). PD, NCTB and CA sampling sites are close to agricultural areas (including rice crops and husbandry areas) and close to the biggest cities of the studied area, which are Tubarão and Capivari de Baixo cities with 97.300 and 21.000 inhabitants respectively. The storm events could affect to the runoffs of this areas finishing in the estuarine sediments. This area also suffer the effects of a thermoelectric plant ("Complexo Termoelétrico Jorge Lacerda"), which could emit some of the studied elements and volatile trace compounds, finally reaching the Tubarão River stream by the wind masses (northeast predominantly) or transported through the terrain orography after being deposited in the soil (Possamai et al., 2010).

Finally, three general trends can be listed: i) Cr and Ni with higher concentrations in the upper part of the river, ii) Mg, Se, Ti and Zn with higher concentrations in the mouth of the river, and iii) As and Cu with lower concentration in the middle of the estuary.

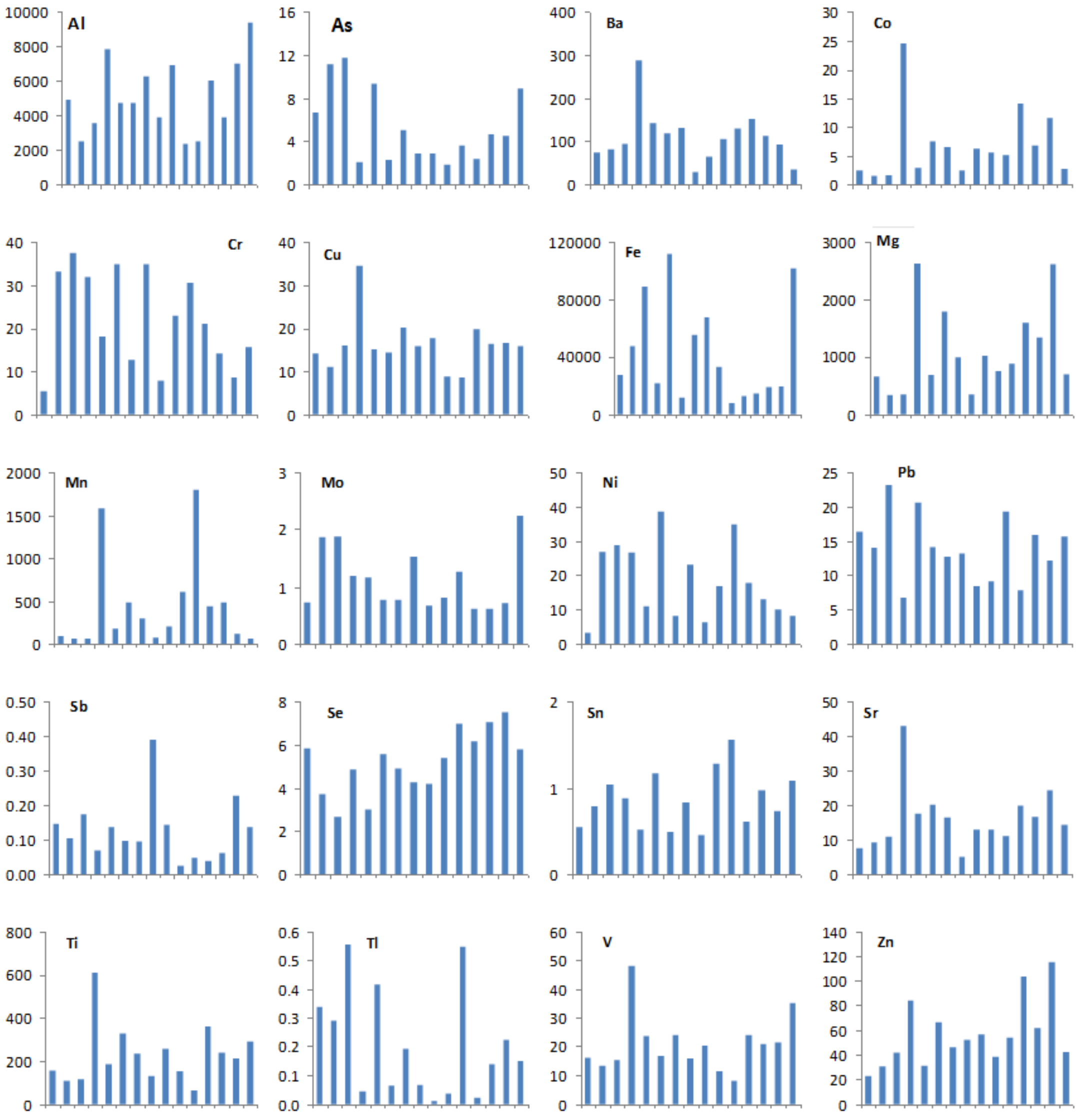

Figure 3. Sediment metal concentration (mg·kg^{-1}) of the estuary in the next order from the left side of the X axis, which is the inner side of the estuary to the right side of the X axis, which is the outer part of the estuary and nearest to the sea: BO, RO, TBLM, OR, ORTB, LA, TBLA, PL, PLTB, BN, PD, NCTB, CA, FITB and ES.

Table 3. Sediment metal concentration (mg·kg^{-1}) of the Tubarão estuary together with some related statistics. Extreme high concentrations, defined as those concentrations over the 75th percentile of the data (Q3) plus three times the interquartile range (IQR), are marked with an asterisk (*).

	Ag	Al	As	Ba	Co	Cr	Cu	Fe	Mg	Mn	Mo	Ni	Pb	Sb	Se	Sn	Sr	Ti	Tl	V	Zn
BO	<LOD	4920	6.67	73.6	2.49	5.43	14.2	27600	661	96.7	0.73	3.26	16.3	0.15	5.84	0.55	7.72	155	0.34	16.1	23.1
RO	<LOD	2500	11.2	82.4	1.60	33.2	11.0	47700	334	61.7	1.87	26.8	14.0	0.10	3.72	0.79	9.21	109	0.29	13.4	30.4
TBLM	<LOD	3570	11.7	94.9	1.74	37.4	15.9	88800	347	61.7	1.88	28.7	23.1	0.17	2.70	1.05	11.0	115	0.56	15.3	42.0
OR	0.06	7870	2.10	289	24.6	31.8	34.3*	21900	2620	1580	1.19	26.7	6.81	0.07	4.87	0.88	43.0	711	0.04	48.1	83.9
ORTB	<LOD	4750	9.34	143	2.95	18.1	15.1	112000	681	173	1.17	11.1	20.5	0.14	3.01	0.52	17.7	185	0.42	23.8	31.0
LA	<LOD	4740	2.32	119	7.48	34.8	14.3	11700	1790	488	0.77	38.5	14.1	0.10	5.59	1.18	20.2	328	0.06	16.8	66.7
TBLA	<LOD	6300	5.03	132	6.59	12.6	20.1	55200	994	301	0.77	8.23	12.7	0.10	4.93	0.49	16.5	233	0.19	24.0	46.0
PL	<LOD	3930	2.90	30.0	2.47	34.8	15.8	67400	344	78.1	1.53	23.2	13.2	0.39	4.28	0.84	5.15	130	0.07	15.8	52.1
PLTB	0.06	6950	2.90	65.1	6.34	7.82	17.6	32700	1020	210	0.68	6.20	8.38	0.14	4.22	0.46	13.0	257	0.01	20.5	56.9
BN	<LOD	2370	1.88	105	5.64	22.8	8.83	7550	757	609	0.81	16.8	9.06	0.02	5.39	1.29	12.9	153	0.04	11.4	38.5
PD	<LOD	2530	3.67	129	5.22	30.6	8.62	12800	878	1800*	1.26	34.9	19.2	0.05	7.01	1.57	11.2	63.9	0.55	8.01	53.9
NCTB	<LOD	6060	2.35	153	14.1	21.0	19.8	14700	1590	437	0.62	17.8	7.84	0.04	6.17	0.61	20.1	360	0.02	24.1	103
CA	<LOD	3910	4.69	114	6.87	14.1	16.4	18900	1340	485	0.62	13.1	15.9	0.06	7.07	0.97	16.7	236	0.14	21.0	62.0
FITB	0.17	7050	4.57	92.3	11.6	8.50	16.6	19400	2610	120	0.71	10.0	12.2	0.23	7.50	0.73	24.4	211	0.22	21.5	115
ES	<LOD	9420	8.91	35.0	2.78	15.6	15.8	102000	701	61.4	2.24	8.21	15.7	0.14	5.80	1.09	14.3	289	0.15	35.2	42.2
25 PERCENTILE	-	3570	2.35	73.6	2.49	12.6	14.2	14700	661	78.1	0.71	8.23	9.06	0.06	4.22	0.55	11.0	130	0.04	15.3	38.5
75 PERCENTILE	-	6950	8.91	132	7.48	33.2	17.6	67400	1590	488	1.53	26.8	16.3	0.15	6.17	1.09	20.1	289	0.34	24.0	66.7
IQR(Q3-Q1)	-	3380	6.56	58.4	4.99	20.6	3.4	52700	929	409	0.82	18.6	7.24	0.09	1.95	0.54	9.10	159	0.30	8.70	28.2
Q3+(3*IQR)	-	17090	28.6	307	22.5	95.0	27.8	225500	4377	1717	3.99	82.5	38.0	0.42	12.0	2.71	47.4	766	1.24	50.1	151
MEDIAN	-	4750	4.57	105	5.64	21.0	15.8	27600	878	210	0.81	16.8	14.0	0.10	5.39	0.84	14.3	211	0.15	20.5	52.1
MEAN	-	5120	5.35	110	6.83	21.9	16.3	42600	1110	437	1.12	18.2	13.9	0.13	5.21	0.87	16.2	236	0.21	21.0	56.5
DESVEST	-	2110	3.40	61.3	6.09	11.1	6.02	34700	747	541	0.53	11.0	4.75	0.09	1.45	0.32	9.01	156	0.19	9.93	26.5
MIN	-	2370	1.88	30.0	1.60	5.43	8.62	7550	334	61.4	0.62	3.26	6.81	0.02	2.70	0.46	5.15	63.9	0.01	8.01	23.1
MAX	-	9420	11.7	289	24.6	37.4	34.3	112000	2620	1800	2.24	38.5	23.1	0.39	7.50	1.57	43.0	711	0.56	48.1	115

3.3. MINERALOGICAL CHARACTERIZATION

Table 4 shows the mineralogical composition of the sediment samples identified by XRD and Raman Spectroscopy. The most common minerals found were quartz, albite and amazonite (Figure 4). Sediment enriched in quartz tends to have lower levels of trace element than aluminosilicates (as amazonite, anorthoclase, orthoclase, and anorthite) for its lower cation exchange capacity (Harland, 1994). Quartz and silicates are natural compounds that by erosion of metamorphic and sedimentary rocks of the zone can be incorporated into the estuarine waters and thus finish in the sediments. Hematite, goethite, birnessite, rutile and anatase were also identified along the estuary. Muscovite and halite has been only identified by XRD in ES and FITB sampling site respectively.

Table 4. Identification of different mineral phases present in sediments of each sampling sites in Tubarão River.

		BO	RO	TBLM	OR	ORTB	LA	PL	PLTB	BN	PD	NCTB	CA	FITB	ES
Quartz	**SiO_2**	X	X	X	X	X	X	X	X	X	X	X	X	X	
Albite	**$NaAlSi_3O_8$**	X	X	X	X		X	X	X		X	X	X	X	
Amazonite	**$KAlSi_3O_8$**							X	X	X	X	X	X	X	
Anorthoclase	**$Na,K)AlSi_3O_8$**	X													
Orthoclase	**$KAlSi_3O_8$**		X												
Diopside	**$MgCaSi_2O_6$**			X	X	X	X								
Anorthite	**$CaAl_2Si_2O_8$**			X		X									
Hematite	**Fe_2O_3**		X	X	X	X	X	X		X			X		
Goethite	**$Fe(OH)_3$**	X		X											
Gypsum	**$CaSO_4{\bullet}2H_2O$**					X	X			X				X	X
Litharge	**PbO**			X											
Birnessite	**$(Na,Ca,K)_{0.6}$ $(Mn^{4+},Mn^{3+})_2O_4 \cdot 1.5\ H_2O$**					X						X	X		X
Muscovite	**$KAl_2(AlSi_3O_{10})(F,OH)_2$**														X
Coal	**C**		X			X	X								
Anatase	**TiO_2**		X				X			X	X			X	X
Rutile	**TiO_2**											X			
Halite	**NaCl**													X	

Gypsum is the unique sulphate phase identified in LA, ORTB, BN, FITB and ES sampling sites. Gypsum can be considered secondary mineral formed after the oxidation of the pyrite (FeS_2), mineral present in acid drainages of coal mines. Its presence in downstream sampling sites near the mouth of the estuary could be explained by the high particulate loads due to the strong storms, which contribute to the transport of minerals from upstream sites close to the coal mining wates to downstream ones. Other sulphate mineral which could be formed after pyrite oxidation is jarosite ($KFe_3(SO_4)_2(OH)_6$). In a previous study (Silva et al., 2013), jarosite was identified in sediments of rivers near of the coal mines. In contrast, it was not identified in the present study, possibility due to terrestrial matter from the surrounding lands that has been deposited on the bed sediments by storm-driven freshwater pulses.

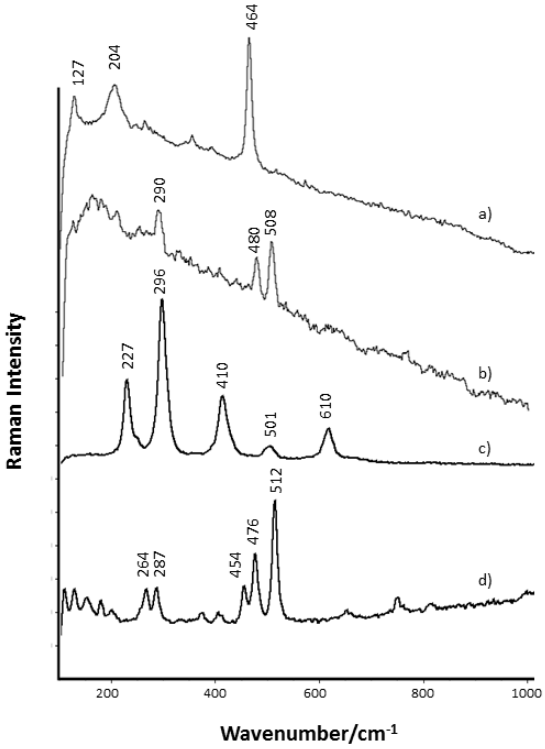

Figure 4. Raman spectra (785 nm laser) of the quartz (a), albite (b), hematite (d) and amazonite (d) found in sediment samples from the Tubarão River.

3.4. SEDIMENT-WATER PARTITIONING COEFFICIENTS

Partitioning coefficients (K_d) were also calculated: i) to describe the distribution of metals and metalloids between analysed sediments and waters samples, and ii) to know the tendencies of the metal and metalloids fate in Tubarão estuary system (Kucuksezgin et al., 2008). K_d values are affected by the water physic-chemical properties and sediments or solids surface properties. Therefore, they can change depending on the studied geographic area and even in the same area in the different locations and changing properties. The equation for partitioning coefficient is expressed as follows (Equation 1):

$$K_d = Cs / Cw \qquad \text{Equation 1}$$

where Cs is the sediments metal concentration ($mg \cdot kg^{-1}$) and Cw is the metal concentration in water ($mg \cdot L^{-1}$). Thus, high K_d values indicate that the metal has been preferentially retained by the sediment, and low values suggest remains in the water (Anderson and Christensen, 1988).

The K_d values varied between $53 \cdot 10^3$ for Al (PD) to 69 for Sr (FITB). Figure 5.a shows the average values of the Log K_d which decrease in the following order: Ti > V > Al > Fe > Pb > As > Cr > Se > Ba > Cu > Co > Ni > Mn > Zn > Sr > Mg. The higher values of K_d found for Ti, V and Al indicate preferential association with sediment and low mobility of these elements to the water. Mg, Sr, Zn, Mn, Ni, Co and Cu showed the lowest partition coefficients of Tubarão River, being the metals that seem to have more tendencies to be dissolved after high precipitation days. Our results is in concordance with a previous study of Silva et al. 2011(Silva et al., 2011), in the mentioned study, Cd, Co, Cu, Mn, Ni, and Zn appear as metals of high mobility and Fe and Pb with practically null mobility in geochemical modelling simulating of the compounds from Santa Catarina coal mining areas.

It was also observed that in general the partition coefficients for Al, Co (except OR and LA), Cu, Fe, Mg (except OR), Ni (except LA), Se, S (except OR) and Zn increased in downstream sampling sites (near of the sea) indicating higher adsorption capacity of the sediment. Finally, the results indicated that K_d for Al, Co, Cu, Fe, Mg, Mn, Ni and Se increased while the water pH increases (Figure 5.b).

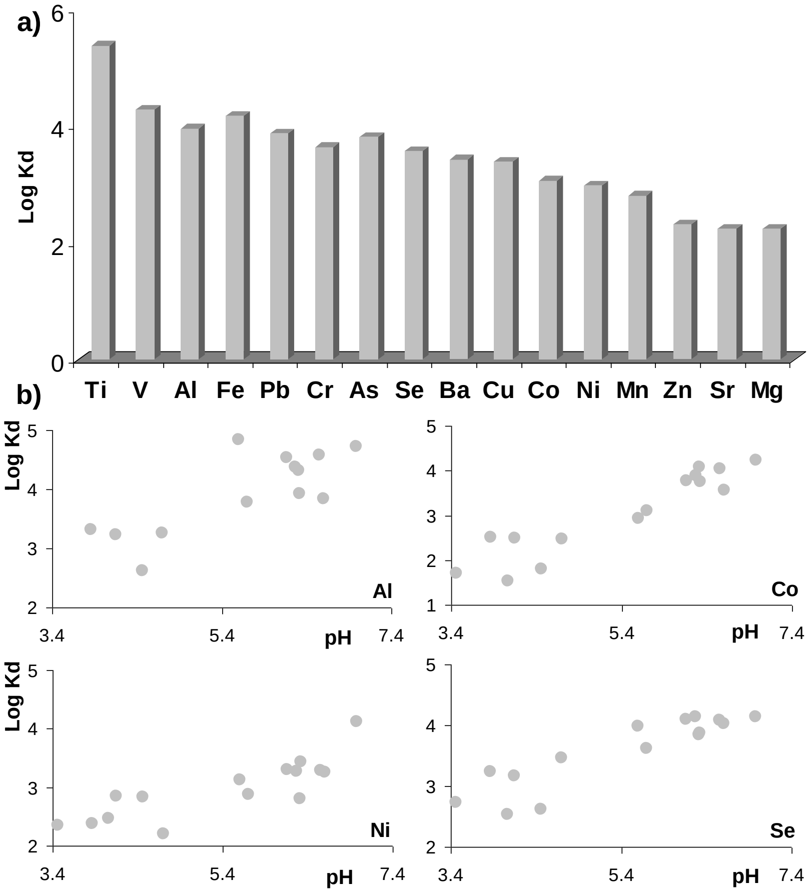

Figure 5. a) Average values of partition coefficients between sediment and water (K_d) calculated for the studied elements, and b) Log Kd versus pH relationships for Al, Co, Ni and Se in Tubarão Estuary.

3.5. NORMALIZED-AND-WEIGHTED AVERAGE CONCENTRATIONS (NWACS)

Normalised-and-Weighted Average Concentration (NWAC) is a cumulative index to sort samples (in our case, water or sediment) according to their content of selected elements. Samples are characterised by a single score in a relative scale from 0 to 10, the NWAC value. More information about the NWACs and the methodology for their calculation can be found elsewhere (Gredilla et al., 2014). The calculation of the NWACs and their representation on a map according to a colour based code allow us identifying, in a very simple and intuitive way, the sampling sites of higher concern in the studied area. The concentrations of the elements measured (with concentration > LOD) in the samples were used to calculate the corresponding NWACs separately for waters and sediments (Figure 6.a).

Regarding the NWAC values calculated using 17 metal concentrations of the waters samples, two effluents, Rocinha and Palmeira Rivers were considered as sites of major concern regarding all studied elements. In contrast, the NWACs calculated with 21 metal concentrations of the sediment samples, showed that the highest values were obtained in Oratorio River and in the mouth of Tubarão estuary. The opposite behavior showed by the water and sediment calculated NWACs could be explained as a consequence of the previous day’s strong rainfalls, affecting to the transport of suspended sediments, surroundings soil particles and mining waste leachings by water to the downstream sampling sites, finally being deposited in its bed sediments, thus increasing the NWAC of the downstream sediment samples. Trying to confirm this hypothesis new NWAC values were calculated taking into account metals typical leached from the Santa Catarina mining wastes, such as Al, Cu, Fe, Mn, Ni, Zn and Pb (Silva et al., 2013). In the case of water samples, with the new calculated NWAC values (Figure 6.b), the area upper the river continue as the site of major concern regarding the elements with mining wastes origin, and in sediment, the NWAC values were higher in a punctual site of confluence of tributaries (Oratorio, Rocinha and Bonito) and in the middle of the Tubarão estuary. These results confirmed that other metal sources such as the same Tubarão River upstream sediments, Tubarão and Capivari City´s direct urban discharges, thermoelectric plant emissions, farming and livestock of the surroundings area could affect as well to Tubarão River metallic contamination.

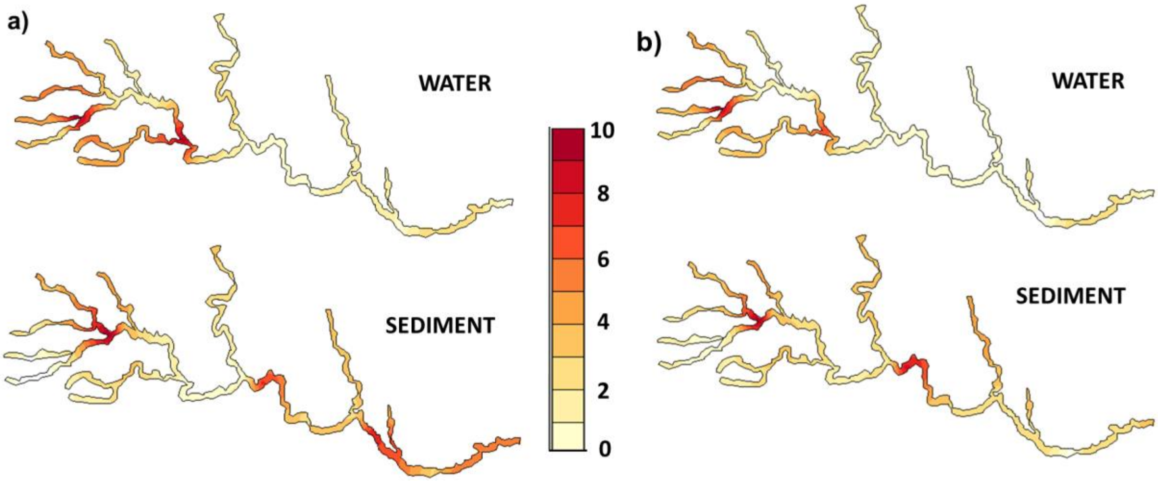

Figure 6. Colour based on the visualization of the Normalized-and-Weighted Average Concentration (NWAC) values: a) calculated with studied elements concentrations of water and sediment samples and b) calculated with Al, Cu, Fe, Mn, Ni, Zn and Pb concentration of water and sediment samples from Tubarão estuary. NWAC values are classified as follows: 0<NWAC<2: very low concern, 2<NWAC<4 low concern, 4<NWAC<6 medium concern, 6<NWAC<8 high concern and 8<NWAC<10 very high concern.

3.6. CHEMOMETRIC ANALYSIS

Principal component analysis (PCA) is a multivariate statistical technique capable of discerning patterns in large environmental datasets. PCA transforms a two dimensions multivariate data array into a new data set, so that some of the new variables (principal components, PCs) are linear combinations of the original variables and can explain most of the variability of the original data (Jolliffe, 2002). The first component represents the maximum variation of the data set and corresponds to the direction explaining the maximum variance; the second PC corresponds to the direction orthogonal to the first PC, explaining the maximum variance not explained by the first PC, and so on.

PCA was carried out in the case of the sediments data matrix. Variables (Ag, Cd, Hg and W) with values below of the detection limits were removed from the dataset. The data set (15 sampling sites x 20 variables) was centred and scaled before carrying out the PCA analysis. The model with three components, explaining more than the 71% of the variance (PC1: 41%; PC2: 18%; PC3: 16%) was chosen to explain the variability of data. OR sampling site showed extreme PC1 values and the scores and loadings (PC1-PC2) without the mentioned sample can be seen in Figure 7.a. Sediments from the estuarine channel and near of the mouth are on the left side of the scores plot and are related to Al, Ba, Co, Cu, Mg, Se, Sr, V and Zn. As it has been mentioned, some sources of these elements are the coal mining, urban, farming activities and the thermoelectric plant (Gobeil et al., 2005; Horowitz et al., 2012). Sediments from sampling sites near of the Braco do Norte River (BN and PD) are located in the positive side of PC1 and PC2, and are characterized by Mn, Sn, Ni and Cr. Finally, with negative values of PC2 were sediments of the sampling sites near the coal mining areas, and basically characterized by Fe, Sb, As, Mo and Pb.

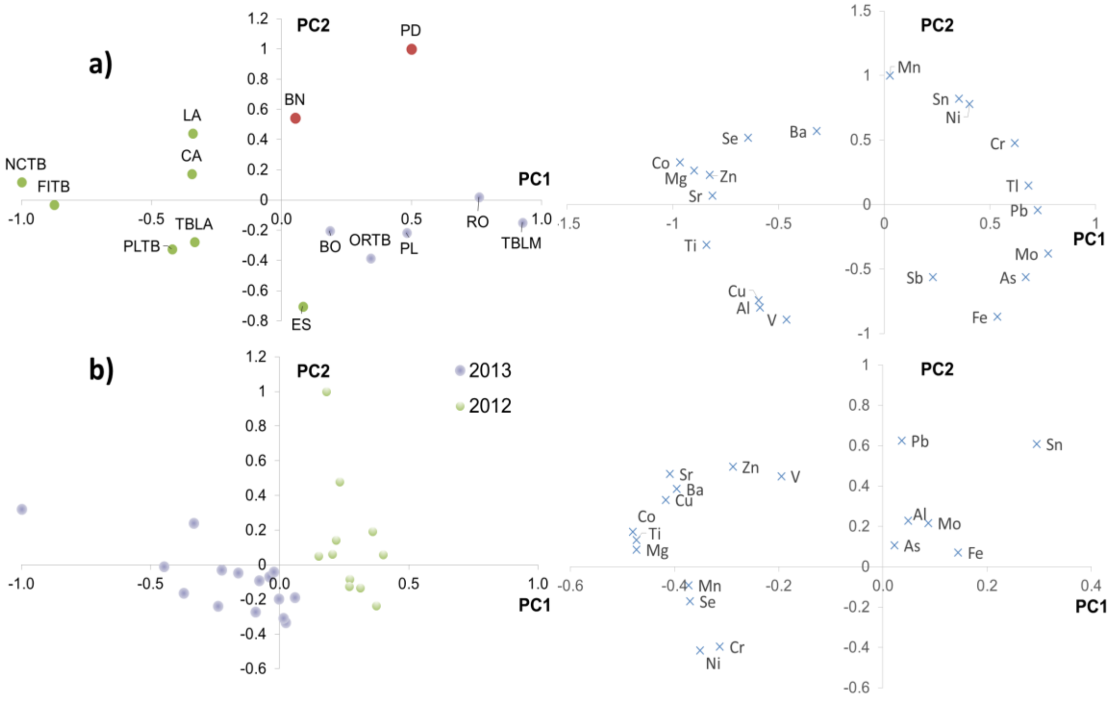

Figure 7. Scores and loadings plots (PC1-PC2) obtained after PCA of: a) sediment dataset of 2013 (the colours correspond to the situation of each sampling sites; green: estuarine channel, red: Braco do Norte River and blue: tributaries near coal mines), and b) sediment samples of two samplings: 2012 (green) vs 2013 (blue) in Tubarão estuary.

To identify possible changes in the sediment concentration of the estuary by strong storms, the results of this study were compared with a previous study in the same area during dry season at 2012 (Silva et al., 2013). PCA was realized with a new sediment dataset including the results of 2012 sampling to our data matrix. The data set (26 sampling sites x 18 variables) was centred and scaled before carrying out the PCA analysis. The model with the three first principal components (37%, 14%, and 12% of explained variance, respectively) was selected. Figure 7.b shows the distribution of the scores and loadings, which reveals the existence of two different groups of sediment samples with specific pollution profiles and date of sampling. The first one includes all the sediments collected at 2012 (positive PC1 values) with higher concentrations of Al, As, Fe, Mo, Pb, and Sn, and the second group of sediments collected in the present study with higher concentrations of Ba, Co, Cr, Cu, Mg, Mn, Ni, Sr, Se, Ti, V and Zn. This result showed significant changes in metal and metalloid concentrations of the sediments at Tubarão estuary after a strong storm event.

4. CONCLUSIONS

In general, the water metal concentrations were higher in upstream sampling sites of Tubarão River, especially at two estuarine tributaries, Rocinha and Palmeira Rivers, both localized in a coal mining area. The mine water after a storm often contains high level of metals and could have negative effects on estuarine water quality. On the other hand, higher metallic concentrations were identified in the sediment samples of the middle or downstream sampling sites.

The metallic geographical distribution variation could be attributed to: i) anthropogenic inputs such as mining, industrial/domestic discharges, etc. ii) a difference in hydrodynamic conditions regulated by freshwater flow after the storms, and iii) a dispersal of sediments into the estuary after the strong storms.

The mobility of the metals and metalloids express as partitioning coefficients (K_d) decrease in the order: Ti > V > Fe > Al > Pb > As > Cr > Se > Ba > Cu > Co > Ni > Mn > Zn > Sr > Mg. These results suggest that the metals with lower K_d could be mobilized during storm flow. K_d was related with the water pH in the case Al, Co, Cu, Fe, Mg, Mn, Ni and Se.

Comparing sediment metal concentrations of the present study with a previous study in the same area after dry season, higher values were found for Ba, Co, Cr, Cu, Mg, Mn, Ni, Sr, Se, Ti, V and Zn and lower for Al, As, Fe, Mo, Pb and Sn.

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Chapter 8

SPATIAL DISTRIBUTION OF SOME TRACE AND MAJOR ELEMENTS IN SEDIMENTS OF THE CÁVADO ESTUARY (ESPOSENDE, PORTUGAL)

CHAPTER 8

SPATIAL DISTRIBUTION OF SOME TRACE AND MAJOR ELEMENTS IN SEDIMENTS OF THE CÁVADO ESTUARY (ESPOSENDE, PORTUGAL)

ABSTRACT

For the first time, the occurrence and spatial distribution of several elements, including some toxic metals, have been investigated in sediments of the estuary of the Cávado River (Esposende, Portugal). The pseudo total concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Sn, V and Zn in sediments collected at 24 sites covering the tidal part of the estuary were determined. According to the concentrations measured, the Cávado estuary can be considered as non-contaminated. For most of the elements, the concentrations tended to be higher upstream. Only a moderate enrichment in Cr and Ni was detected in a few sites. An important decrease in concentration was observed for the most toxic metals compared to historical data reported in the literature. Several hotspots were identified, but even in those cases the amount of metals stored in the sediments does not represent a toxicological threat to living organisms.

Keywords: Trace elements; Major elements; Sediments; Estuaries; Pollution monitoring; Cávado estuary.

1. INTRODUCCION

Coastal areas are fragile environments usually subjected to important anthropogenic pressures. Specifically, estuaries are scenarios of global concern. They are areas of high biological production but, unfortunately, a wide range of potentially polluting activities are usually carried out in their surroundings affecting the ecosystem services (Holder, 2002).

In this paper, the geographical distribution of metal and metalloid elements in surface sediments of the Cávado estuary was studied. The Cávado estuary is located on the Atlantic coast near Esposende, in North-Western Portugal (41.5 N; 8.70 W) (Figure 1). The river has two principal tributaries, the Homem River and the Rabagão River, with drainage areas of 246 km^2 and 257 km^2, respectively (Almeida et al., 2008; Vieira et al., 1998). The estuarine part of the Cávado River covers an area of 2.56 km^2 (Machado et al., 2012). It retains its natural banks, with the exception of the downstream northern bank which is artificial. The average population density around the estuary is 200 inhabitants/km^2, living mainly in the communities of Fão and Esposende.

Currently, Cávado estuary encloses a fishing harbour and two marinas (Ramos et al., 2012). Additionally, there are four other municipalities upstream which have considerable influence on the estuary: Amares, Vila Verde, Braga and Barcelos. Agriculture is the main activity in the area but recreational uses of the estuary, including fishing and yachting, are also important, especially during summer time. Small shipyards are also present. Previously an important mining activity existed upstream, in the headwater region of the Rabagão and Homem rivers. The principal ore minerals mined include wolframite ($FeWO_4$), chalcopyrite ($CuFeS_2$), pyrite (FeS_2) and blende (ZnS) (Gonçalves et al., 1994). This activity ceased several decades ago but drainage waters from inactive mines may still affect the quality of the sediments downstream. Other industrial activities in the entire water catchment area include textile and ceramic manufacture, electroplating, paperboard mills and slaughter-houses.

Few articles have been published regarding metal pollution in the Cávado estuary. Reis et al. (2014) used the macroalga *Fucus spiralis* to investigate Cd, Cr, Cu, Fe, Mn and Zn contamination in water. With regards to sediments, the influence of salt marsh plants on the concentrations and potential mobility of metals (Almeida et al., 2008; Machado et al., 2012; Reis et al., 2014) has been studied. These two investigations used pseudo total metal concentration (Cd, Cr, Cu, Fe, Pb, Mn, Ni, Zn) data obtained from the analysis of bulk surface sediment samples (5-20 cm) collected at salt marshes of the Cávado estuary in the summer and winter of 2006, with emphasis on the influence of salt marsh plants and microbial communities. A single study has been found which specifically investigates pseudo total metal content (Cd, Cr, Cu, Ni, Pb, Zn) in the surface sediments fraction of less than 63 µm (Gonçalves et al., 1994). Gonçalves' group collected samples in 1988 and 1989 at 21 different sampling points covering the whole water catchment of the Cávado River, from the headwater regions to, approximately, the upper limit of the tide, thus excluding the estuarine area of about 9 km in length. These 9 km are in fact the area covered in our study, in such a way that only one of Gonçalves's sampling points (the most downstream) lies in the area investigated in our work, near our most upstream sampling point. Gonçalves et al.

(1994) also estimated background concentrations for several metals in the catchment area. The results of these works will be commented in more detail later.

2. MATERIAL AND METHODS

2.1. STUDY AREA AND SAMPLING

In our study, sediment samples, labelled as *A*-*X*, were collected at 24 sites in the estuary in June 2012 (Figue 1). When accessible, both sides of the river banks were covered, representing the whole estuarine part of the Cávado River. Some sites are situated in urbanised areas of Esposende (*A*, *B*, *C*, *D*, *E*) and Fão *(L*). Other sites are in a salt marsh close to the mouth of the estuary (*F*, *G*, *H*, *J*). The remaining sites correspond to agricultural or forested areas (*I*, *K*, *M*-*X*). The sampling points *K*, *L* and *M* are relatively close (50-100 m) to a road bridge joining the villages of Esposende and Fão. The sampling points *O* and *P* are close to a motorway bridge (A28 from Porto to Viana do Castelo), and separated by an artificial dike: *P* is on the outer side of the dike in the main channel of the river and fully affected by the off-stream of a small municipal waste water treatment plant (WWTP), while *O* is on the inner side of the dike.

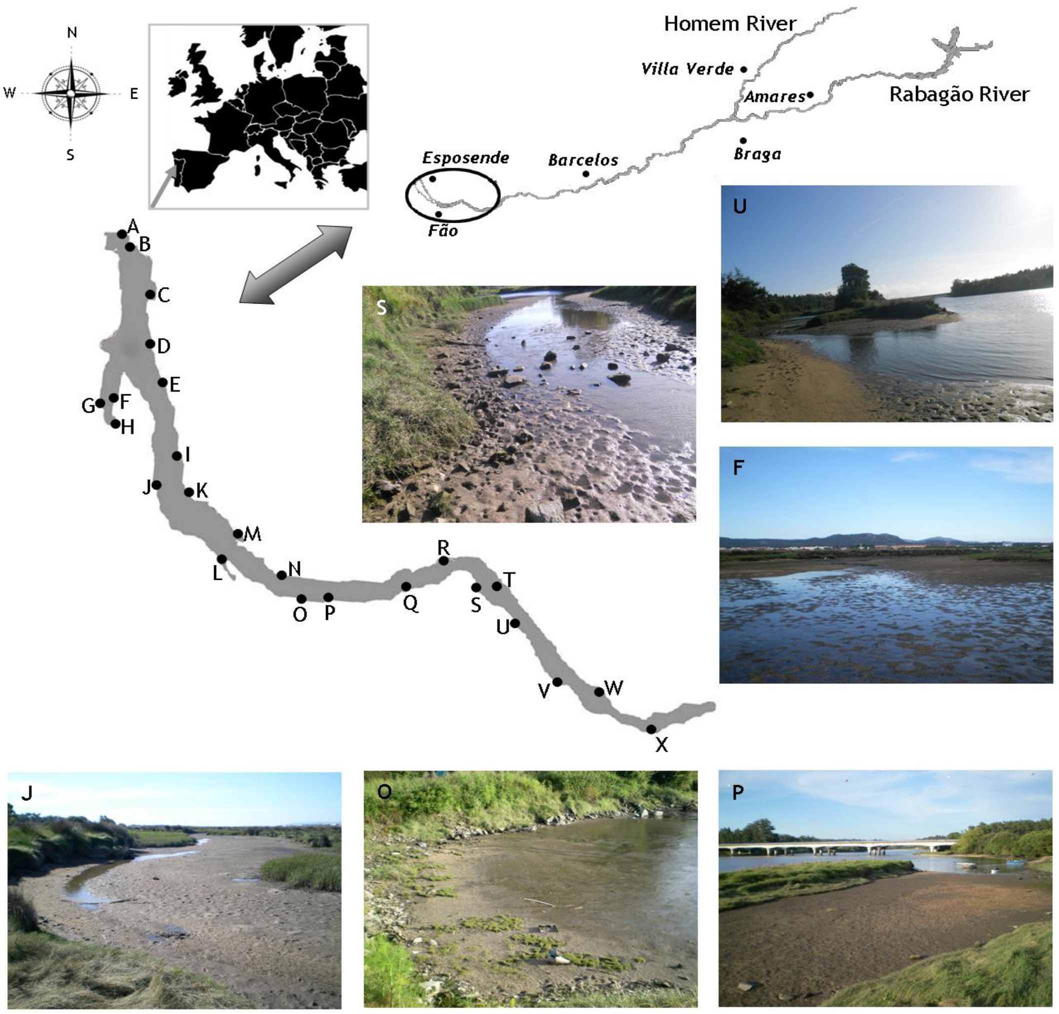

Figure 1. Location of the sampling sites in the Cávado estuary (Esposende, Portugal).

Surface sediments (0-5 cm) were collected by hand (using plastic gloves) at low tide from the river banks in muddy areas. About 500 g of sediment from each sampling point were collected, preserved in double zip-lock bags and transported to the laboratory at 4 ºC in a cool box.

2.2. ANALYTICAL PROCEDURE

The pretreatment and analysis of the sediment samples were carried out according to the procedures described in the Chapter 2, experimental. The pseudo total concentrations of a wide range of elements (Al, As, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Sn, V and Zn) were simultaneously determined by ICP/MS. The reproducibility of the method, as relative standard deviation (RSD), was calculated after triplicate analysis of the marine sediment CRM PACS-2, and the detection limits were estimated from 8 replicates of procedural blanks (Table 1).

Table 1. Detection limits (mg·Kg^{-1}), calculated after replicate analysis of procedural blanks) and reproducibilities (expressed as relative standard deviation, %, and calculated after triplicate analysis of the CRM PACS-2) of the analytical method. Comparison between our data and those obtained by Gonçalves et al. (1994) in 1988-89 and (Machado et al., 2012) in 2006. All concentrations in mg·Kg^{-1}.

	Detection limit (mg·Kg^{-1})	**Reproducibility (RSD, %)**	**Background (mg·Kg^{-1})** [1]	**(Goncalves et al., 1994)** [2]	**Our data** [3]	**(Machado et al., 2012)** [4]	**Our data** [5]
Al	4.4	3	-	-	13300	-	7400
As	2.3	5	-	-	11.7	-	6.1
Cd	0.13	4	0.33	1.67	0.37	0.10	0.13
Co	0.23	2	-	-	5.8	-	3.95
Cr	1.3	2	17	45.6	34.7	34.5	20.2
Cu	1.1	1	94	220	113	63.8	54.9
Fe	2.7	4	-	-	10400	2700	8600
Mg	2.3	0.5	-	-	2000	-	4100
Mn	1.5	2	-	-	449	162	75
Ni	0.44	2	8.5	22.0	16.4	16.8	9.4
Pb	0.52	2	30	65.1	40.9	49.8	30.3
Sn	0.070	5	-	-	4.7	-	2.9
V	1.3	2	-	-	10.1	-	12.8
Zn	2.2	2	102	199	170	118	94

[1] Background concentrations estimated by Gonçalves et al. (1994)

[2] Concentrations in sediments collected in the most downstream sampling point, CV01 (average of two campaigns, 1988-1989)

[3] Concentrations measured in the most upstream sampling point, *X* (June 2012)

[4] Average concentrations in sediments collected in a salt marsh (colonized and non-colonized by *Halimione portulacoides*) in summer and winter of 2006

[5] Average concentrations measured in sediments from *F*, *G*, *H* and *J* (June 2012)

3. RESULTS AND DISCUSSION

3.1 ELEMENT CONCENTRATIONS IN SEDIMENTS: GEOGRAPHICAL DISTRIBUTION AND TRENDS IN TIME.

The concentrations found were compared with those reported in the literature for the same area (Gonçalves et al., 1994; Machado et al., 2012). As above-mentioned, the work of Gonçalves et al. (1994) included data corresponding to two sampling campaigns in 1988 and 1989. Since it did not cover the estuarine area, only the metal concentration found at *X* sampling point (Figure 1) could be used for comparison. Although Gonçalves et al. (1994) concluded that the area close to *X* was unpolluted, even lower concentrations of most of the metals were detected in 2012 (Table 1). This difference was clearer for Cd, for which 4.5 times lower concentrations were found in 2012 (sample *X*) than in 1988-89 (Gonçalves et al., 1994).

The publication of Machado et al. (2012) included data compiled after two sampling campaigns carried out in a salt marsh area close to the river mouth, near *F*, *H*, *G* and *J* sampling points (Figure 1). The sediments collected in Cávado estuary were characterized by a high content of organic matter, and in general, low metal concentration. The concentrations observed in 2012 (*F*, *H*, *G* and *J* sampling points) are compared with those found in 2006 in Table 1. Lower concentrations (1.6-2.2 times) of Cr, Mn, Ni and Pb were measured in 2012. Unexpectedly Fe, concentration was about 3-fold higher in 2012. It is worth to note again that Machado et al. (2012) studied bulk sediment samples which could be one explanation for these discrepancies (De Gregori et al., 1996). Since metals are primarily retained in the fine fraction, the lower concentration of metal measured in the present work compared with those found by Machado et al. (2012) would be even a stronger indication of decreased anthropogenic pressure in the last years. It is to be highlighted that the metal concentrations measured in 2012 (5th and 7th columns of Table 1, respectively) were close or even below the background values estimated for the area (Gonçalves et al., 1994), except in the case of Cr, Ni, Pb and Zn at the most upstream point (*X*).

The concentrations found for each metal at each sampling point are shown in Figure 2. The sampling points are ordered from left to right according to increasing distance from the mouth of the estuary. Although the spatial distribution is element-specific, some general trends can be listed. For most of the elements (Al, Cd, Co, Cu, Mn, Pb, Sn and Zn) the concentrations found in sediments from the upper part of the estuary are higher than those measured in sediments from the lower part. Mg presented an opposite behaviour since higher Mg concentration values were found in sites near the river mouth. The presence of Mg is usually higher in sediments from marine zones than in those from fresh water environments, due to its fixation by clay minerals or calcite (Libes, 2009).

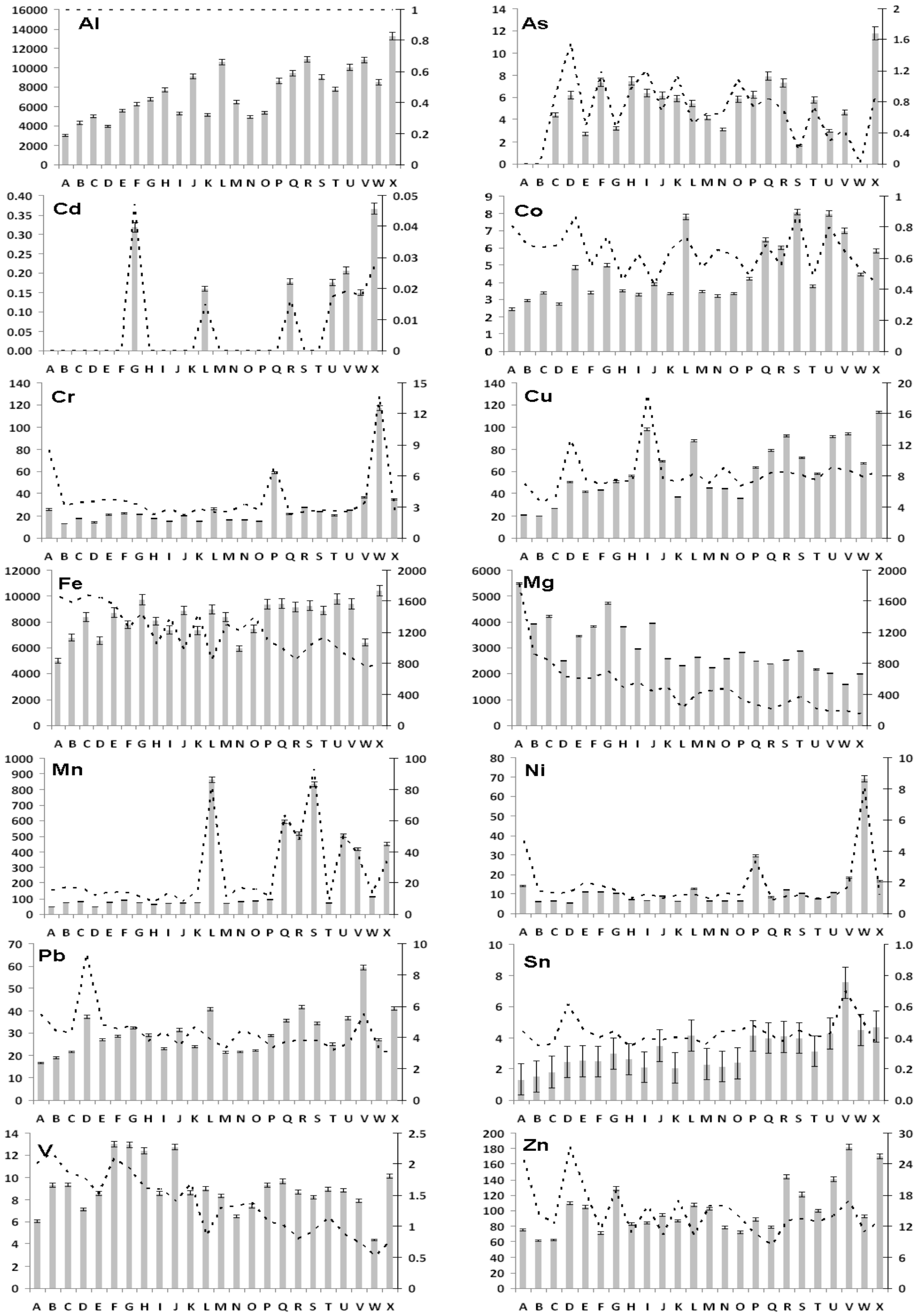

Figure 2. Metal concentrations (mg·kg^{-1}) found in sediment samples collected at each sampling site. Discontinuous lines indicate concentrations normalised to aluminium (multiplied by 1000 for better visualization).

There were some specific sampling sites with different behaviour. This was the case of *G* and *L*, in the middle part of the estuary, with quite high concentrations of Cd, Co, Cu (only *L*), Mg (only *G*), Mn (only *L*), Pb and Sn (only *L*). The sampling site *G* is located in the most inner part of a natural marsh with a comparatively higher water residence time due to its semi-closed nature, which implies an easier accumulation of contaminants at this station. Conversely, *L* is located under a bridge with traffic, a fact that certainly influences the metal content in its sediments (Hjortenkrans et al., 2006). The sampling point affected by the effluent of the WWTP (*P*) presented concentrations only slightly higher than those of the site protected by the dike (*O*), but comparable to those of the remaining sampling locations. Remarkably high concentrations of both Ni and Cr were found at *P* and *W* sampling sites. In the case of *W*, which can considered an agricultural site, the accumulation of Ni and Cr might be the consequence of the use of phosphate fertilizers (Whitby et al., 1978). The high amount of Cr and Ni found at *P* has probably the same origin, but the effect is multiplied due to the presence of a dike close to this site. The concentrations of As, Fe and V did not show any clear geographical distribution throughout the estuary. Arsenic concentration varied from not detectable at *A*, *B* and *W* sampling sites to 12 $mg \cdot kg^{-1}$ at *X*. The concentrations of V were slightly higher at *F*, *G*, *H* and *J*.

The possible existence of correlations between pairs of variables (metal concentrations) was studied by correlation analysis of the dataset (Unscrambler 7.5, Camo Asa, Trondheim, Norway). A Pearson coefficient (in absolute terms) higher than 0.62 indicates a correlation significantly different from zero at a confidence level of 99.9% ($N=24$, $t_{crit}=3.745$) (Miller and Miller, 2005). The correlation analysis is highly influenced by the spatial distribution of the elements, as high correlations were found between the elements with similar spatial partitioning. The element that presented the highest correlations, Al, was positively and highly correlated with Co (0.78), Cu (0.85), Fe (0.78), Mn (0.70), Pb (0.76), Sn (0.84), Zn (0.72) and Cd (0.61). Other elements that were not significantly correlated with Al when all data were considered, such as Cr (0.33) and Ni (0.27), showed significant positive correlations with Al (0.88 for Cr, 0.80 for Ni), when data from samples *A*, *P* and *W* were not considered in the calculation. Therefore, significant correlations of the most of the studied elements with Al suggest their very probable lithogenic origin due to a close association to aluminosilicates (Summer et al., 1996). A negative correlation with Al was found only for Mg (-0.54). In the case of V no significant correlation was observed with the rest of the elements considered in this study. The correlation between Ni and Cr was extremely high (0.99), which corroborates their common origin. Similarly, high correlation was also found between the concentrations of Zn and Cd (0.76), probably due to the existence of Cd impurities in Zn ores (Chrastný et al., 2015).

The estimation of the anthropogenic input of pollutants is most usually attempted by comparison of the actual concentrations measured in the samples with background values. The enrichment factors (EFs) are also based on this approach and, additionally, use concentrations normalised to Al to account for the heterogeneity of the samples due to differences in texture and organic content (Loring and Rantala, 1992). In general, an EF lower than 1-1.5 denotes no significant enrichment, from 1.5 to 3 indicates a moderate enrichment and between 3-5, a moderate to severe anthropogenic pollutant enrichment. Similar qualitative information was obtained by the observation of both normalised concentrations and EFs. The concentrations of elements, normalised to Al, are shown in Figure 2 and were used to calculate the corresponding EFs for Cd, Cr, Cu, Ni, Pb and Zn. The background values (Table 1) used in the calculation (only reported for those six elements) were those estimated by Gonçalves et al. (1994). As the authors did not make an estimation in the case of Al, we used the lowest Al concentration measured in our sediments as Al background concentration (3020 $mg \cdot Kg^{-1}$ in site *A*). All the EFs calculated were lower than 1 for Cd (0.06-0.43), Cu (0.15-0.59), Pb (0.31-0.95) and Zn (0.24-0.80). Compared with the remaining sampling sites, however, relatively higher values were found for Cd at *G*, for Cu at sites *I* and *D*, for Pb at *D* and for Zn at sites *D* and *A*. For both Cr and Ni slightly higher values of EFs were found at *P* (1.2 for both metals), *A* (1.51 and 1.66, respectively) and *W* (2.42 and 2.88, respectively) sampling sites. It should be noted, however, that these EF values might be slightly underestimated due to the use of overestimated background levels. Concerning the normalised concentrations (Figure 2), the distribution of As and Co was found to be homogeneous throughout the estuary. The normalized concentrations of several elements were found to be higher at specific sampling sites, e.g., Mn at *S*, *L*, *Q*, *U*, *R*, *V* and *X*; Cr and Ni at *A*, *P* and *W*; Cu at *D* and *I*; Zn at *A* and *D*; Pb at *D*, and Sn at *V* and *D*. For Mg, Fe and V a clear increase in normalised concentration was observed downstream, highlighting the importance of the estuarine mixing on the enrichment of sediments with these metals.

3.2 NORMALIZED AND WEIGHTED AVERAGE CONCENTRATIONS (NWAC)

The Normalised-and-Weighted Average Concentrations (NWACs) were also calculated to identify areas of major concern regarding metallic contamination in the surface sediments of the Cávado estuary. The NWAC methodology has recently been proposed (Gredilla et al., 2014) providing each sediment with a single score from zero (minimum concern) to ten (maximum concern) according to the concentration of selected contaminants. The NWACs obtained for the sediments from the Cávado estuary, simultaneously taking into account the concentrations of the most toxic elements (As, Cd, Cr, Cu, Ni, Pb and Zn), are shown in Table 2. The NWACs have also been used to produce a contour map (Figure 3) after kriging interpolation by means of the 3DField software (version 3.8.8.0, by Vladimir Galouchko, http://3dfmaps.com). Kriging produces a smoother surface over the area of interest and facilitates an easier visual identification of the areas of higher concern within the estuary, interpolating information from discrete stations to cover the entire study area (Leecaster, 2003). As can be appreciated, one of the areas most affected by the above mentioned elements was the salt marsh close to the mouth of the estuary. Specifically, the *G* sampling point is of concern with a NWAC of 5.2. The longer residence time of water in the marsh compared to the rest of the estuary may explain the high NWAC value found. It is surprising, however, that the other points from the salt marsh (*F*, *H*, *J*) presented NWAC values below 1. The most upstream area investigated in this study was also affected (*W* was the sampling site with the highest value of NWAC). Sediments farther from the ocean (*U*-*X*) are probably more influenced than others by drainage water from the upstream mines. Sampling points *L*, *P* and *R* are of medium concern with NWAC scores between 3 and 4. All these sites are located in agricultural areas, affected by urban and agricultural wastewaters (Rocha et al., 2012).

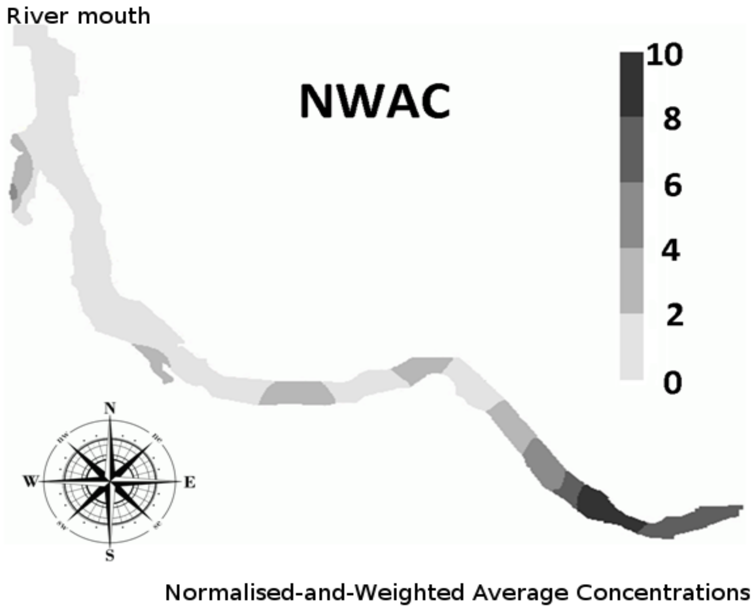

Figure 3. Kriging interpolation obtained from the NWAC values calculated for the 24 sediments collected in the Cávado estuary.

3.3 MEAN EFFECTS RANGE-MEDIAN QUOTIENTS: ESTIMATION OF TOXICITY

Finally, the mean effects range medium quotients (mERMq) were calculated in order to have a rough estimation of the probability of sediments from the Cávado estuary to be toxic. Briefly, mERMqs were calculated by dividing each pollutant concentration measured in the sediment by its respective ERM (effects range median) to obtain the corresponding sediment quality guideline quotient (ERMq) (Long, 2006). Following this, mERMqs for each sample were obtained as the average of ERMqs previously calculated. ERMs indicates the pollutant concentration above which effects are expected to be frequent and have been only defined for very toxic elements such as As (70 mg·Kg^{-1}), Cd (9.6 mg·Kg^{-1}), Cr (370 mg·Kg^{-1}), Cu (270 mg·Kg^{-1}), Ni (51.6 mg·Kg^{-1}), Pb (218 mg·Kg^{-1}) and Zn 410 (mg·Kg^{-1}) (Long, 2006).

Consequently, only these elements were considered in our calculation. Values of mERMq in the ranges of 0-0.1, 0.1-0.5, 0.5-1.5, and >1.5 correspond to the probabilities of toxicity: 9% (non-toxic), 21% (slightly toxic), 49% (moderately toxic) and 76% (highly toxic), respectively. The mERMq values obtained for the sediments collected in the Cávado estuary varied from 0.07 to 0.33 (Table 2), which means that the sediments are unlikely to be more than just slightly toxic. Therefore, the sites previously defined as of higher concern, according to their NWAC scores, do not imply a potential toxicity risk. In any case, as expected, the sites of higher concern coincide with those of higher toxicity risk.

Table 2. NWAC and mERMq values calculated for the sediments collected in the Cávado estuary, using the concentrations of As, Cd, Cr, Cu, Ni, Pb and Zn.

Site	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**	**W**	**X**
NWAC	1.1	0.0	0.24	0.41	0.93	0.89	5.2	0.49	0.31	0.75	0.29	3.0	0.40	0.25	0.22	3.6	0.75	3.4	1.1	0.68	3.14	5.0	10	7.3
mERMq	0.10	0.0	0.08	0.12	0.12	0.12	0.14	0.12	0.13	0.14	0.10	0.17	0.11	0.10	0.10	0.20	0.14	0.19	0.15	0.12	0.17	0.23	0.33	0.23

4. CONCLUSIONS

In summary, data for Al, As, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Sn, V and Zn in surface sediments collected in the Cávado estuary have been reported. The elements´ distribution along the estuary is element-specific but, in general, we have measured higher concentrations of toxic metals, such as Co, Cu, Sn and Zn, in the upper part of the estuary than in the lower part. However, we have identified only two sites with a concentration of Ni and Cr significantly higher than the background values estimated for the area, but even in these two sites, the concentrations measured in sediments do not represent a toxicological threat to the environment. Decreased anthropogenic pressure in the last decades has led to lower concentrations of some metal pollutants like Cd, in the surface sediments. To our knowledge, this is the first time that the occurrence and spatial distribution of several elements, including some toxic heavy metals, in sediments of the estuary of the Cávado River have been systematically investigated. The baseline provided in this study is expected to serve as a useful guide for a correct management of the basin concerning potentially polluting activities such as dredging, recreational activities or fishing/agriculture.

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Chapter 9

OCCURRENCE AND GEOGRAPHICAL DISTRIBUTION OF METALS AND METALLOIDS IN SEDIMENTS OF THE NERBIOI-IBAIZABAL ESTUARY (BILBAO, BASQUE COUNTRY)

CHAPTER 9

OCCURRENCE AND GEOGRAPHICAL DISTRIBUTION OF METALS AND METALLOIDS IN SEDIMENTS OF THE NERBIOI-IBAIZABAL ESTUARY (BILBAO, BASQUE COUNTRY)

ABSTRACT

Pollution in estuaries is an issue of high concern for scientists, local stakeholders and authorities. Sediments have been frequently used as sentinels of chemical pollution, including metals and metalloids. The estuary of the Nerbioi-Ibaizabal River was subjected to an important input of metals since the late XIXth century until about 1975. Afterwards, a significant decrease in chemical pollution has occurred due to a progressive closure of the most polluting activities and the pre-treatment of waste waters. However, an important actuation, including a large movement of highly polluted sediments, has recently started in order to reduce the effects of floods and improve the urban image of the city. It is therefore of interest to have a precise description of the situation in terms of chemical pollution, in order to make feasible a future quantification of the effects derived from the above-mentioned actuation. With this aim, we collected sediments at about 50 sites in the inter-tidal part of the estuary in January 2009, 2010, 2011 and 2014, and the concentration of fourteen elements (Al, As, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Sn, V and Zn) in the acidic extract of the samples was simultaneously measured by ICP/MS. Geoaccumulation indexes to estimate the metal fraction of anthropogenic origin, Normalized and Weighed Average Concentrations (NWAC's) to identify areas of higher concern and mean Effect-Range-Median quotients (mERMq's) to estimate the toxicity associated to the samples were computed. The results show that i) the geographical distribution of metals is rather heterogeneous within the estuary and changes from sampling campaign to sampling campaign, ii) hotspots of metal pollution are concentrated in a few points (the surroundings of the Gobela and Galindo tributary rivers and the closed dock of Deusto in 2009, 2010 and 2011, and the Abra Bay, the dock of Portu and the point where the effluent of the biggest waste water treatment plant of the area is released into the river in 2014), and iii) most of the sediments collected in those hotspots of pollution are highly toxic, mainly due to the presence of Pb, Cd, Zn, Cu, As, and probably Sn, V and Mn, of anthropogenic origin.

Keywords: Sediments; Metals; Estuary; Hotspot.

1. INTRODUCTION

Estuaries are very sensitive areas susceptible to be affected by contamination inputs of different origin, such as industry, domestic sources, agriculture, traffic road run-offs, shipping and atmospheric deposition (Spencer, 2002). Pollutants can enter the estuary from tributary rivers and direct discharges. The impact of anthropogenic perturbation is more important in estuaries which drain densely populated and industrialized areas.

Trace elements are important pollutants that threaten the equilibrium of estuaries due to their toxicity, long persistence and bioaccumulation (Chon et al., 2010; Kumar et al., 2008). In extreme situations, they may represent a serious risk for living organisms and even for human health (Farias et al., 2007). In fact, trace metals are included in the indicative list of the main pollutants of the Water Framework Directive (2000/60/EC, WFD) of the European Union; furthermore, Cd, Hg, Pb and Ni are classified as priority substances.

The fate of metal and metalloids in estuaries depends on the pH, salinity, redox potential, dissolved oxygen and the concentration of organic chelators in the water column (Ahdy and Khaled, 2009; DelValls et al., 2004). Furthermore, estuaries are very dynamic systems in which different biogeochemical processes can affect the fate and bioavailability of metals and metalloids. In water, they are frequently associated to organic matter and/or suspended particles (Alomary and Belhadj, 2007; Filgueiras et al., 2004; Moor et al., 2001). In sediments, they can be stabilized as silicates (lower mobility) or get associated to aluminium silicates, iron or manganese hydroxides and carbonates (higher mobility) (Caccia Valentina et al., 2003). Under certain physico-chemical conditions sediments may act as a secondary source of contamination to the estuary (Kennish, 1998).

Sediment is more conservative than water and most of the metals and metalloids are finally stored in the sediments. Consequently, sediments accumulate historical data on processes within water bodies and the effect of anthropogenic factors on these processes (Birch et al., 2001; Christophoridis et al., 2009). They have been used as environmental indicators and their ability to trace and monitor contamination sources is largely recognised (Lipnick et al., 2002). Sediments are not specifically mentioned in the WFD but, as they also play an important role in the chemical and ecological state of the water due to the interactions between both mediums (Borja and Heinrich, 2005), the directive stablishes an objective for sediments, which is just to avoid an increase in pollutant concentrations with time.

The estuary of the Nerbioi-Ibaizabal River (Bilbao, Basque Country) was strongly impacted by industrial activities, such as iron and steel production and transformation, untreated domestic sewages and mining activities since the late 19th century (Garcia-Barcina Jose et al., 2006; Jesus Belzunce et al., 2001). The gradual implementation of a system to collect and treat most of the sewage waters of industrial and urban origin, together with the closure of the most polluting companies, have resulted in an evident improvement of the environmental quality of the estuary (Garcia-Barcina et al., 2006). In fact the image of the city in the surroundings of the estuary has been completely renewed in a process that continues nowadays. One of the actuations that have recently started (beginning of 2015) consists on the transformation of an isthmus in an island in the area of Zorrozaurre, with an important movement of highly polluted sediments. This actuation represents an evident threat due to a possible mobilisation of pollutants to the water layer that may affect the ecological and chemical status of the estuary.

The aim of this work was to characterise the estuary of the Nerbioi-Ibaizabal River in terms of metal pollution, in order to make feasible a future quantification of the effects derived from the Zorrozaurre island actuation. To this end, four sampling campaigns were conducted in 2009, 2010, 2011 and 2014 to collect surface sediments in about 50 points throughout the tidal part of the estuary. The concentration of fourteen metals and metalloids was measured in all the samples and the results were statistically investigated to i) define the geographical distribution of the analytes in the estuary, ii) identify hotspots of contamination, iii) estimate the toxicity associated to the sediments and iv) check if there is any significant change in metal and metalloid concentration with time.

2. MATERIALS AND METHODS

2.1. STUDY AREA AND SAMPLING PROCEDURES

The estuary of the Nerbioi–Ibaizabal River is located in the continental shelf of the Cantabrian coastline in the northern coast of the Iberian Peninsula (Figure 1). It crosses the city of Bilbao, one of the most important urban areas in the Cantabrian coast. The main fresh water input comes from the Nerbioi and Ibaizabal rivers (68%), while the rest comes from the smaller tributaries Kadagua (27%), Galindo (4%), Asua (0.7%) and Gobela (0.3%) (Landajo et al., 2004).

At the end of the XIXth century, Bilbao city experienced a dramatic increase in population due to the exploitation of the local iron and the beginning of an incipient industrial activity (manufacture of steel, electroplating, ship construction,…). All the wastewaters from urban and industrial origin were directly dumped into the estuary in that period, which led to an environmental collapse of the Nerbioi-Ibaizabal estuary by the middle of the XXth century (Cearreta et al., 2002; Jesus Belzunce et al., 2001; Saiz-Salinas, 1997). However, the situation of the estuary improved significantly at the end of the XXth century due to the closure of the most polluting industries and mine activities, and to the implementation of the so-called “strategy for the integral recovery of the estuary of Bilbao” (Garcia-Barcina et al., 2006). The estuary still suffers nowadays, however, the pressure coming from a million people living around it (Leorri et al., 2008). Several works have investigated the occurrence and distribution of metals and metalloids in the estuary with different objectives and using a variety of approaches (Bartolome et al., 2006; Fdez-Ortiz de Vallejuelo et al., 2010; Fdez-Ortiz de Vallejuelo et al., 2014; Gredilla et al., 2014; Leorri et al., 2008; Sanz Landaluze et al., 2004).

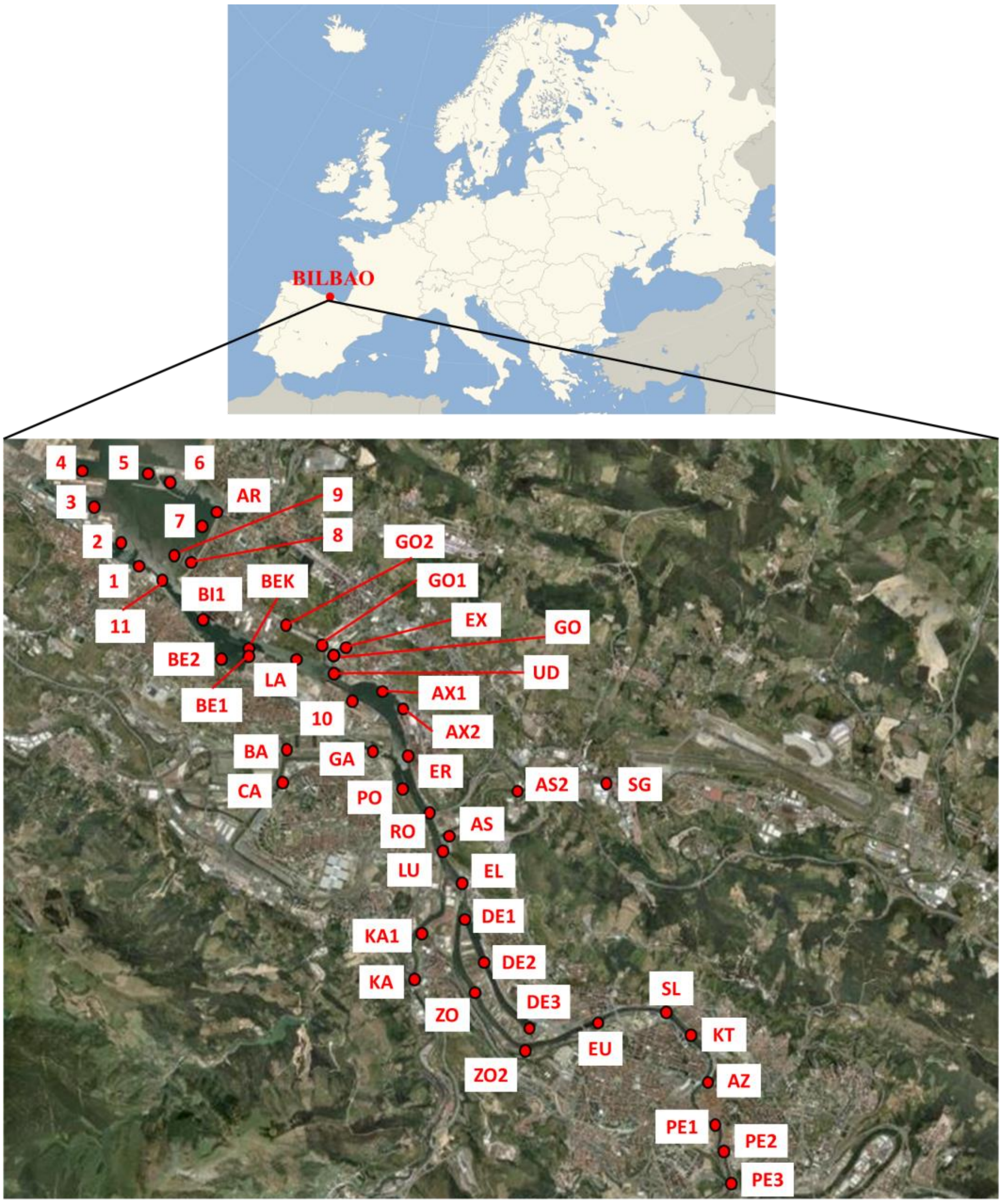


Figure 1. Location of the study area and sampling points in the Nerbioi-Ibaizabal estuary

Forty nine sites were strategically selected in order to carry out a representative sampling in the Nerbioi-Ibaizabal estuary (Figure 1). The samples were collected in January 2009, January 2010, January 2011 and January of 2014. Surface sediments (0–2 cm) were collected by hand using latex gloves from the river bank at low tide. In the sampling points 1 to 11 sediments were collected from a boat using a VanVeen type grab (capacity: 2L; sampling surface: 260 cm^2; weight: 10.42 kg: dimensions: 55 cm × 30 cm × 15 cm). In all the cases, samples were transported to the laboratory in cleaned plastic bags at 4ºC.

2.2. SAMPLE PREPARATION AND ANALYSIS

The pretreatment and analysis of the samples were carried out according to the procedures described in the Chapter 2, experimental. The concentration of 14 elements was finally measured by ICP/MS. The detection limits (LOD) were estimated by replicate analysis of procedural blanks (n=8). The certified reference material NIST 1646a (estuary sediment, National Institute of Standards and Technology) was used to check the accuracy and repeatability of the method (n = 5). The results expressed as LOD (in mg·kg^{-1}), percent of recovery (accuracy) and relative standard deviation (RSD, reproducibility) are summarised in Table 1.

Table 1. Detection limits (LOD, in mg·Kg^{-1}), accuracy (as percent of recovery) and reproducibility (as relative standard deviation, RSD) of the analytical method.

	Al	**As**	**Cd**	**Co**	**Cr**	**Cu**	**Fe**
LOD (mg·kg^{-1})	0.48	0.15	0.043	0.003	0.001	0.004	0.015
Recovery (%)	68	84	74	88	74	98	64
RSD (%)	10	8	8	7	8	4	9
	Mg	**Mn**	**Ni**	**Pb**	**Sn**	**V**	**Zn**
LOD (mg·kg^{-1})	0.54	0.002	0.003	0.086	0.002	0.001	0.34
Recovery (%)	70	80	78	94	81	92	96
RSD (%)	8	12	6	4	5	6	2

3. RESULTS AND DISCUSSION

3.1 ELEMENT CONCENTRATIONS IN SEDIMENTS: GEOGRAPHICAL DISTRIBUTION AND TRENDS IN TIME

The concentrations found in the sediment samples are shown in Tables 3, 4, 5 and 6. Samples from BE2 and BEK (in 2010) and 5 and BE2 (in 2011) were accidentally lost. The extreme concentrations found in each variable (element concentration) and sampling campaign, defined as those concentrations higher than the 75th interquartile plus three times the interquartile range (Otto, 2007), are marked with an asterisk in Tables 3-6, and have been summarised in Table 2. The existence of extreme values in a variable indicates unexpected high concentrations of a given metal at specific sites in comparison with the rest of values which could be considered as “normal”. According to the occurrence and distribution of extreme concentrations, the estuary can be divided in different sectors: i) the mouth of the estuary, downstream the BI1 sampling point, ii) the surroundings of the Gobela River, in the right bank of the estuary, including the docks of Lamiako, Udondo and Axpe, iii) the area of influence of the Galindo River, were the effluent of the WWTP is located, and the dock of Portu, iv) the closed channel of Deusto and v) the main channel of the estuary from the RO sampling point upstream, including the Asua and Kadagua tributaries.

Table 2. Sampling campaigns and sampling sites at which sediments with extreme concentrations of metals were found.

	Al	As	Cd	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Sn	V	Zn
2						2014		2014						2014
9					2014							2014		
BI1		2014			2014						2014		2014	
LA		2009												
		2010												
GO						2009								
GO1			2010											
			2014											
GO2			2009						2009					
			2010						2010					
	2011	2011	2011			2011			2011		2011			
								2014						
UD		2009												
		2014												
AX1				2010	2010	2010	2010			2010	2010	2010		2010
AX2						2009					2009	2009		2009
				2010		2010	2010	2010				2010		2010
							2011					2011		
GA										2009				
										2010				
							2011							2011
		2014									2014			2014
BA									2009					2009
			2010						2010					2010
									2011		2011			
									2014		2014			2014
PO									2009					
		2014	2014	2014			2014				2014			2014
SG														
											2011			
DE3		2009		2009			2009							
							2010		2010					2010
													2011	

For the 2009 and 2010 sampling campaigns, most of the extreme values were found in the sector of the estuary between the docks of Lamiako (LA) and Axpe (AX1-AX2), including most of the sampling points located influence area of the Gobela River (GO/GO1/GO2/UD). Elements like As, Cd, Cu, Mn, Pb, Sn and Zn presented extremely high concentrations at these sites. The most inner part of the channel of Deusto (DE3) also presented a large amount of extreme concentrations (As, Co, Fe, Mg and Zn) in 2009 and 2010. Concerning the 2011 campaign, most of the extreme concentrations (for Al, As, Cd, Cu, Mn and Pb) were identified in the site located most upstream in the Gobela River (GO2), with a few isolated cases in the Galindo/Ballonti system (GA and BA) and, again, the inner part of the channel of Deusto (DE3). The situation substantially changed in 2014. Extreme concentrations appeared in three of the sampling sites located in the mouth of the estuary (points 2, 9 and BI1), in the dock of Portu (PO), and in the BA sampling site. Finally, it is to be highlighted that in three sites extreme concentrations of several metals were found in at least three of the sampling campaigns (Mn and Zn in BA; Cd and Mn in GO2; and Sn in AX2), suggesting a chronic contamination with those elements at those points. BA is directly affected by the effluent of the biggest WWTP of the area, while shipyard activities have been carried out for decades close to the dock of Axpe (AX2). The surroundings of the Gobela tributary were one of the last ones to be connected to the sewage water collector system of the WWTP.

The concentrations of each metal within sampling sites for each sampling campaign have been plotted in the form of Box plots after removing the extreme values (Figure 3). These plots can be used to investigate the change in the overall metal concentration in the estuary with time during the period investigated (2009-2014). For most of the elements (Al, As, Cd, Cu, Pb, Sn and V), the 2011 sampling campaign presented the lowest metal concentrations. The concentrations of Cr, Ni and Zn remained rather constant within the period investigated, while a slight but constant decrease from 2009 to 2014 was observed in the case of Co and Mn. The overall concentration of Fe in the estuary was significantly lower in 2010 and 2011 than in 2009 and 2014. Finally, an increase in Mg concentration was also detected from 2009 to 2014. The precipitations (in L/m^2) accumulated in the 15 days previous to each sampling campaign were 30.7 (2009), 36.2 (2010), 79.5 (2011) and 37.7 (2014) (Meteorología). The heaviest precipitations registered in 2011 may explain the lower overall concentrations found in that sampling campaign, due to a higher input of fresh particulate matter which resulted in an important dilution effect and a lower dispersion of the concentration values (metals more homogeneously distributed within the estuary). It might be assumed that metals not affected by this dilution effect, especially Co, Cr, Ni and Zn have a natural origin in the estuary. In fact, high Pearson correlation coefficients (between 0.53 and 0.73) were found between pairs of these elements when data from the four sampling campaigns were simultaneously considered. Similarly, the observed behaviour would suggest the existence of important anthropogenic point sources of Al, As, Cd, Cu, Pb, Sn and V in the estuary. The Pearson correlation coefficients between As and V (0.88), and Cu and Sn (0.87) were also extremely high. The magnitude of the anthropogenic origin of metals in the estuary of the Nerbioi-Ibaizabal River will be further discussed in following sections.

The concentrations found for each metal at each sampling site are shown in Figure 2 for the sampling campaigns carried out in 2009, 2010, 2011 and 2014, in order to investigate their geographical distribution within the estuary. The general conclusions drawn from the occurrence and distribution of extreme values are confirmed after observation of Figure 2. In general, higher concentrations were registered in the middle part of the estuary and the closed chanel of Deusto in 2009, 2010 and 2011, while in 2014 the situation reversed with higher metal concentrations in the Abra Bay and the dock of Portu. In 2009 and 2010, Sediments from AX1 and AX2 are especially rich in Al, As, Co, Cr, Cu, Fe, Ni, Pb, Sn and Zn. Those from the Gobela River (GO, GO1 and GO2) are rich in Cd and, to a lesser extent, in Cu and Mn. In the surroundings of the effluent of the WWTP, high concentrations of Ni, Cd, Mn, Pb and Zn were found. The sediments from the most inner part of the closed channel Deusto (DE3) were specially rich in Al, As, Cd, Co, Cu, Fe, Pb, Sn, V and Zn. Concerning the situation in 2014, comparatively higher concentrations of Cd, Cr, Cu, Fe, Sn and Zn were found in the Abra Bay (sampling sites 2, 9 and BI1), and As, Cd, Co, Cu and V in the dock of Portu (PO). The high metal concentrations found in 2014 in the Abra Bay in comparison with those encountered in previous sampling campaigns may be due to the construction of a new terminal for long tourist cruises in the area. The works started in November 2012 and finished in February 2014, just one month after our sampling campaign in January 2014 (Izagirre, 2012).

Table 3. Element concentrations found in the sediments collected in January 2009 together with some related statistics. Extreme high concentrations, defined as those concentrations over the 75th percentile of the data (Q3) plus three times the interquartile range (IQR), are marked with an asterisk (*).

	Al	As	Cd	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Sn	V	Zn
1	2150	13.4	1.45	5.81	29.5	52.9	15400	2590	289	17.1	238	13.4	5.86	297
2	1770	16.7	1.45	4.15	27.6	59.5	15400	2960	283	12.2	286	12.0	10.8	321
3	2100	16.4	1.46	4.43	29.4	53.4	16500	3500	266	14.3	286	10.1	10.9	297
4	2160	18.2	1.30	4.32	33.0	54.7	17600	4020	338	15.0	305	10.0	15.1	357
5	1840	29.4	1.59	4.51	28.8	64.8	16000	2770	284	13.1	377	10.6	13.4	332
6	2330	36.0	1.75	4.11	40.0	112	20800	3480	324	11.1	565	23.3	21.6	413
7	2250	29.0	1.78	4.18	37.5	88.7	18300	3510	290	12.5	451	18.1	18.4	377
8	2150	29.1	3.51	3.84	33.6	105	18000	3450	292	12.0	416	13.3	15.5	344
9	1960	17.7	1.48	3.90	30.1	64.7	15600	3180	267	13.3	316	11.4	13.0	279
10	1340	5.18	0.57	6.46	19.2	26.3	12800	624	483	14.1	144	14.6	1.18	161
11	1660	11.8	1.28	5.36	23.6	42.8	13800	1530	376	15.3	244	10.1	3.14	269
AR	3910	14.5	5.92	5.34	20.3	109	11500	3170	404	12.5	251	8.83	8.48	394
BI1	2220	10.4	2.62	7.49	25.9	42.7	15700	1550	434	18.2	219	18.6	2.86	226
BE1	1960	12.1	2.33	5.44	26.8	38.1	15100	1590	455	15.3	259	14.3	4.06	317
BE2	2420	18.3	2.85	4.98	34.8	88.9	14800	2520	261	27.6	327	10.8	12.7	374
BEK	1390	8.68	0.96	5.54	23.6	35.3	13700	638	402	14.9	186	12.1	1.62	199
LA	1630	114*	4.04	2.95	38.9	103	14900	1020	207	11.0	751	18.8	24.9	528
GO	2850	51.0	5.47	6.81	32.1	375*	18800	2650	684	19.9	751	24.5	15.8	935
GO1	3990	14.9	5.47	8.12	24.4	148	19400	2830	630	21.4	327	9.46	21.9	453
GO2	3070	30.3	17.9*	7.37	24.4	235	13400	1190	1630*	17.9	666	11.0	6.38	478
EX	2780	49.9	1.46	5.47	31.2	118	16500	1770	314	23.4	548	12.9	13.5	435
UD	2640	116*	5.62	7.27	52.6	198	19300	1690	200	29.1	890	29.3	21.4	830
AX1	2630	15.0	1.25	8.42	28.0	55.0	16900	1140	484	20.4	324	18.4	3.58	336
AX2	4280	53.1	3.48	12.2	56.7	332*	34200	3100	560	38.7	2040*	64.6*	14.1	1260*
GA	3200	16.8	3.46	9.07	40.5	98.8	21900	1800	519	64.6*	439	15.9	5.80	684
CA	1410	4.36	0.38	4.24	10.0	27.2	10700	431	448	8.50	108	3.07	1.00	122
BA	3660	7.60	8.52	8.56	42.6	103	24700	2680	2980*	28.7	1070	9.69	12.5	1900*
ER	2230	9.45	0.86	7.78	20.6	32.4	18500	713	498	16.3	189	15.4	1.74	193
PO	4090	11.6	0.81	5.56	9.51	49.1	20700	4940	1090*	13.8	250	4.74	13.7	196
RO	1190	9.40	0.78	5.05	17.2	29.2	11800	609	376	13.4	279	4.21	< LOD	141
AS	1850	12.4	1.64	5.41	15.5	40.1	10300	957	235	12.6	314	3.10	2.06	255
AS2	1390	35.7	3.29	5.80	26.3	64.8	9510	183	387	19.5	999	4.65	3.25	324
SG	1080	22.1	1.73	5.48	38.3	33.2	8340	505	412	21.3	731	2.59	3.65	122

Table 3 (Cont.). Element concentrations found in the sediments collected in January 2009 together with some related statistics. Extreme high concentrations, defined as those concentrations over the 75th percentile of the data (Q3) plus three times the interquartile range (IQR), are marked with an asterisk (*).

	Al	**As**	**Cd**	**Co**	**Cr**	**Cu**	**Fe**	**Mg**	**Mn**	**Ni**	**Pb**	**Sn**	**V**	**Zn**
LU	1120	3.61	0.53	5.42	21.8	24.7	11300	513	360	15.7	118	10.6	< LOD	141
KA	1150	2.03	0.23	4.13	6.35	12.6	10600	1140	415	7.39	71.1	2.01	0.66	78.6
KA1	800	0.95	0.15	3.10	3.97	7.78	9630	623	319	4.73	78.7	< LOD	< LOD	39.0
EL	1230	4.06	0.42	5.83	15.6	20.9	14300	698	508	13.0	121	10.3	< LOD	134
DE1	1220	4.03	0.43	5.75	19.9	22.2	11700	516	419	16.0	100	9.85	0.95	131
DE2	1370	19.2	0.39	11.3	11.3	20.3	25300	588	206	18.8	126	3.80	11.17	111
DE3	1860	117*	3.16	15.9*	20.6	206	39900*	657	211	26.3	1270	24.2	16.3	521
ZO	1470	5.05	0.66	4.88	34.8	38.6	13400	1120	256	23.2	165	6.15	0.69	213
ZO2	1130	2.66	0.36	4.99	19.6	19.9	13200	608	458	15.2	106	4.65	0.52	116
EU	727	1.19	0.23	4.30	10.8	10.3	9330	366	336	9.18	68.5	2.46	< LOD	81.6
SL	816	2.58	0.29	4.61	14.1	17.4	11000	266	364	12.1	93.8	3.77	< LOD	102
KT	690	2.01	0.24	3.72	17.8	17.1	10200	413	318	12.8	104	2.98	< LOD	82.7
AZ	1110	2.22	0.31	5.07	30.6	23.0	11100	448	465	23.3	73.5	3.46	< LOD	187
PE1	760	2.10	0.26	4.46	19.2	18.3	10100	390	391	14.1	96.6	3.90	< LOD	90.6
PE2	990	3.63	0.32	5.52	34.9	29.9	13600	976	521	25.5	130	4.72	< LOD	108
PE3	1000	5.27	0.43	7.52	61.4	73.7	22900	750	916	38.8	271	17.4	< LOD	172
25th PERCENTILE (Q1)	1170	4.21	0.43	4.37	19.2	25.5	11400	608	290	12.7	124	4.65	2.66	133
75th PERCENTILE (Q3)	2370	25.6	3.01	7.04	34.2	101	18400	2720	483	21.4	445	15.2	14.4	385
IQR (Q3-Q1)	1200	21.4	2.58	2.67	14.9	75.3	7010	2120	194	8.64	322	10.5	11.7	253
Q3+(3*IQR)	5960	89.7	10.7	15.0	79.0	327	39500	9070	1070	47.3	1410	46.8	49.5	1140
MEDIAN	1840	12.4	1.45	5.42	26.3	49.1	15000	1140	387	15.3	271	10.4	9.62	269
AVERAGE	1940	21.2	2.18	5.96	26.8	74.4	15900	1620	481	18.3	379	12.0	9.32	336
DESVEST	947	27.7	2.94	2.40	12.2	77.7	6080	1230	438	9.78	373	10.2	7.18	327
MIN	690	0.95	0.15	2.95	3.97	7.78	8340	180	200	4.73	68.5	2.01	0.52	39.0
MAX	4280	117	17.9	15.9	61.4	375	39900	4940	2980	64.6	2040	64.6	24.9	1900

Table 4. Element concentrations found in the sediments collected in January 2010 together with some related statistics. Extreme high concentrations, defined as those concentrations over the 75th percentile of the data (Q3) plus three times the interquartile range (IQR), are marked with an asterisk (*).

	Al	As	Cd	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Sn	V	Zn
1	4650	17.5	0.05	5.42	35.1	102	11500	2130	195	14.7	122	19.1	12.0	492
2	4160	16.6	< LOD	5.52	27.5	83.1	8780	1920	200	14.5	105	15.5	10.7	434
3	4500	15.4	1.55	1.83	23.4	50.0	10800	2100	225	9.95	93.8	11.7	15.6	237
4	4280	21.0	< LOD	4.98	25.0	58.1	11200	2280	305	14.2	94.2	11.2	16.8	299
5	4300	15.9	< LOD	5.09	25.5	60.6	8460	1690	207	15.0	89.7	11.8	9.88	314
6	4980	34.2	< LOD	4.89	35.3	111	12400	2290	244	14.9	140	16.7	16.9	380
7	4750	22.1	1.18	5.16	33.3	120	10900	2230	208	14.8	122	16.2	15.3	391
8	4940	30.2	1.16	5.29	34.6	140	11000	2480	229	15.1	155	16.3	17.4	473
9	4450	26.4	0.55	4.70	33.8	104	10500	1990	221	12.4	128	19.1	16.8	387
10	4320	13.0	< LOD	6.40	24.9	67.5	8030	1440	191	18.1	82.4	9.92	4.61	353
11	3810	9.85	1.44	1.67	21.9	33.2	7890	2160	135	13.2	74.7	6.77	4.37	214
AR	7410	27.6	0.33	7.16	29.4	100	9830	2720	484	15.0	126	16.5	14.5	527
BI1	1790	32.4	2.77	4.80	37.3	110	14400	3380	290	22.3	168	18.3	17.3	556
BE1	5070	18.3	0.56	5.02	38.3	90.5	9930	1590	357	17.9	131	17.7	8.38	520
LA	4040	119*	1.54	4.05	46.4	124	10800	662	175	17.1	211	23.4	25.6	600
GO	5340	53.3	2.24	7.27	33.4	769*	13300	1580	432	22.9	250	58.6	13.5	867
GO1	5440	52.7	11.2*	8.43	39.7	382	8430	1030	642	29.6	163	21.5	10.7	845
GO2	5160	44.1	17.3*	8.44	26.7	265	7490	410	1615*	20.0	195	13.2	5.18	590
EX	4930	61.5	< LOD	7.52	31.8	141	10800	1190	410	24.8	191	17.9	15.3	579
UD	4310	97.4	3.86	6.45	52.9	213	10300	1010	153	25.2	237	33.7	20.2	900
AX1	6390	94.6	3.59	28.0*	107*	954*	29500*	2330	856	101*	1380*	150*	13.1	3320*
AX2	7010	110	5.31	16.8*	66.1	461*	29000*	6010*	541	39.6	474	56.0*	39.3	1900*
GA	1790	105	6.07	10.6	60.3	207	18000	1960	577	66.3*	488	29.9	18.3	1390
CA	1820	10.2	2.38	4.62	32.9	131	12400	2330	691	24.2	76.4	15.6	10.9	447
BA	3470	11.2	10.2*	6.67	43.5	134	15300	2680	1660*	31.1	469	17.3	16.8	2180*
ER	1500	18.1	1.86	3.38	27.5	65.3	12200	2480	219	21.6	133	11.9	7.47	420
PO	2320	11.8	0.76	4.24	6.25	34.7	16000	4680	400	15.1	55.1	3.38	9.82	147
RO	1570	17.2	1.60	4.73	24.0	82.2	11800	2180	230	17.3	479	11.1	5.55	372
AS	1150	23.5	2.74	3.76	21.4	74.3	6560	1540	115	15.1	192	6.86	6.28	357
AS2	1170	10.9	2.02	3.39	15.7	38.2	5960	1460	154	14.0	189	4.47	4.48	258
SG	239	10.8	1.06	3.09	25.1	22.0	3990	732	301	14.5	116	1.96	1.47	140
LU	926	12.2	1.85	2.72	28.2	72.2	9550	2360	200	17.7	113	12.5	9.59	333
KA	541	0.55	0.62	3.37	5.46	31.6	11300	2170	401	8.60	40.5	3.15	3.29	146

Table 4 (Cont.). Element concentrations found in the sediments collected in January 2010 together with some related statistics. Extreme high concentrations, defined as those concentrations over the 75th percentile of the data (Q3) plus three times the interquartile range (IQR), are marked with an asterisk (*).

	Al	As	Cd	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Sn	V	Zn
KA1	334	1.88	0.56	1.45	3.04	14.6	7130	1800	293	7.03	30.4	1.79	2.11	91.0
EL	401	4.04	0.64	4.20	14.3	18.0	7040	1060	339	14.7	34.0	4.8	1.97	127
DE1	1250	7.20	1.21	5.91	31.1	40.7	10100	2100	338	24.8	60.5	12.9	5.53	260
DE2	214	15.9	0.53	5.66	6.92	11.4	14200	1260	225	10.6	28.8	1.43	13.0	86.7
DE3	3010	69.9	7.18	10.1	61.3	185	26500*	4160	1150*	35.3	593	41.1	20.9	1740*
ZO	1110	3.56	1.15	3.40	32.1	42.2	9930	1780	197	19.8	57.9	11.9	3.65	209
ZO2	1080	3.76	0.66	4.17	25.2	27.8	9360	1290	361	21.0	36.6	5.37	3.19	164
EU	9.01	1.40	0.66	3.29	15.5	14.6	6400	1020	306	14.0	34.8	3.12	1.22	113.5
SL	916	3.43	0.78	5.49	33.6	30.9	8680	1290	422	24.4	45.0	6.13	3.79	188
KT	362	0.08	0.68	3.90	24.1	24.2	7470	1270	295	16.5	30.6	4.57	2.59	145.8
AZ	725	2.35	0.80	4.39	30.4	36.6	8780	1460	299	20.3	40.0	6.36	1.89	192
PE1	9.10	0.45	0.68	2.73	26.3	29.3	6780	1170	296	17.6	33.8	8.33	2.92	132.0
PE2	9.04	2.42	0.77	2.69	49.7	39.2	7550	1340	348	35.3	48.3	6.69	1.83	163
PE3	475	1.67	0.85	5.39	44.1	40.5	12300	1560	386	29.5	50.1	6.72	3.72	189
25th PERCENTILE (Q1)	916	4.04	0.67	3.40	24.1	34.7	8020	1290	208	14.7	50.0	6.36	3.72	188
75th PERCENTILE (Q3)	4640	32.4	2.56	6.40	37.3	131	12300	2290	410	24.4	191	17.9	15.6	556
IQR (Q3-Q1)	3730	28.4	1.89	3.00	13.1	96.4	4310	1000	202	9.70	140	11.5	11.9	368
Q3+(3*IQR)	15800	117	8.2	15.4	76.7	420	25200	5290	1010	53.5	612	52.3	51.2	1660
MEDIAN	2320	15.9	1.18	4.89	30.4	72.2	10300	1800	299	17.6	116	11.9	9.82	357
AVERAGE	2820	27.3	2.51	5.71	32.3	127	11300	1950	394	21.9	175	17.5	10.3	536
DESVEST	2150	31.8	3.44	4.23	17.6	181	5310	1010	328	15.4	226	23.3	7.70	622
MIN	9	0.08	0.05	1.45	3.04	11.4	3990	410	115	7.03	28.8	1.43	1.22	85.7
MAX	7410	119	17.3	28.0	107	954	29500	6010	1660	101	1380	150	39.3	3320

Table 5. Element concentrations found in the sediments collected in January 2011 together with some related statistics. Extreme high concentrations, defined as those concentrations over the 75th percentile of the data (Q3) plus three times the interquartile range (IQR), are marked with an asterisk (*).

	Al	**As**	**Cd**	**Co**	**Cr**	**Cu**	**Fe**	**Mg**	**Mn**	**Ni**	**Pb**	**Sn**	**V**	**Zn**
1	1320	21.3	0.72	3.73	20.8	38.0	14900	1680	226	7.01	69.1	6.71	0.48	368
2	1140	17.8	0.39	2.96	17.0	26.5	12800	1540	223	9.72	53.4	5.32	0.25	275
3	1140	11.7	0.31	1.82	13.8	19.7	11300	1620	250	12.0	48.2	5.48	1.31	201
4	1110	5.36	0.25	1.73	15.0	15.8	10500	1600	252	11.3	41.1	4.85	3.42	162
6	1310	7.32	0.31	1.72	19.6	33.4	12700	1580	208	9.92	62.3	5.73	4.53	199
7	1160	4.70	0.60	1.61	20.0	32.4	10300	1420	183	9.78	55.9	5.74	5.24	191
8	1140	10.4	0.98	1.52	22.5	39.2	12400	1480	197	9.44	67.9	6.14	5.03	248
9	925	12.6	0.38	1.39	18.8	24.4	10000	1250	188	9.15	59.7	5.22	5.31	171
10	2830	9.10	0.57	6.43	22.1	46.9	10400	1030	337	16.14	113	12.3	2.89	257
11	2730	10.8	1.36	3.52	22.8	38.0	10800	1840	255	14.32	159	8.41	3.75	242
AR	1050	6.81	0.50	2.76	20.0	33.8	9380	1160	149	8.24	57.1	5.24	4.96	262
BI1	1270	6.02	0.48	3.12	21.2	20.8	9780	1140	139	8.53	48.3	4.90	5.68	184
BE1	1580	6.91	0.51	2.25	26.2	24.8	13500	1420	350	8.74	60.2	5.39	6.53	295
BEK	1270	6.83	0.44	3.15	22.5	22.4	10600	1340	218	8.11	51.2	4.41	5.68	264
LA	1040	20.2	0.68	1.16	26.0	34.6	12300	889	179	7.32	92.2	6.20	6.40	297
GO	1510	11.7	1.07	4.53	29.0	89.1	10500	1570	406	8.19	61.1	6.21	5.59	393
GO1	1570	9.21	1.09	4.51	28.8	86.4	10100	1550	398	7.28	61.8	5.98	6.02	392
GO2	4120*	37.2*	17.6*	7.91	25.5	250*	10400	800	1620*	19.0	431*	12.1	5.78	534
EX	1500	17.9	0.51	2.87	24.7	47.1	15800	1540	198	7.28	120	6.62	5.85	322
UD	1800	21.1	0.69	6.77	24.7	55.2	19400	2040	576	6.48	140	7.33	7.46	628
AX1	1050	2.72	0.47	2.77	20.9	34.4	9230	1170	147	7.34	56.4	5.02	5.27	256
AX2	1620	9.64	0.51	9.34	42.0	93.8	29800*	1950	282	5.63	163	21.8*	6.76	767
GA	1440	14.6	1.68	5.82	26.0	50.9	22100*	1220	562	6.22	151	9.32	6.08	901*
CA	1290	0.48	0.71	3.23	30.6	55.4	12600	671	707	5.59	41.1	8.64	6.31	287
BA	2250	7.40	2.57	9.40	29.3	56.2	18300	1800	2580*	28.0	223*	5.92	1.11	382
ER	652	12.5	0.42	4.31	12.5	15.6	8590	907	183	13.0	64.2	4.12	0.27	111
PO	2520	12.7	0.27	5.60	8.46	16.9	13800	1720	894	16.1	45.5	8.38	1.18	110
RO	1020	17.4	0.79	6.59	22.5	36.2	12800	1170	240	26.0	134	16.2	0.43	206
AS	947	15.9	0.77	6.36	12.7	20.9	9190	1070	207	15.1	81.8	9.02	0.34	177
AS2	1590	11.5	1.11	6.05	29.7	48.0	6350	1480	146	18.6	91.2	7.82	3.32	431
SG	1110	29.5	2.39	7.49	33.4	46.8	4730	661	293	21.6	225*	4.93	3.46	285
LU	1570	7.08	0.93	4.56	29.9	42.2	9730	1810	195	16.0	70.1	11.6	4.80	253

Table 5 (Cont.). Element concentrations found in the sediments collected in January 2011 together with some related statistics. Extreme high concentrations, defined as those concentrations over the 75th percentile of the data (Q3) plus three times the interquartile range (IQR), are marked with an asterisk (*).

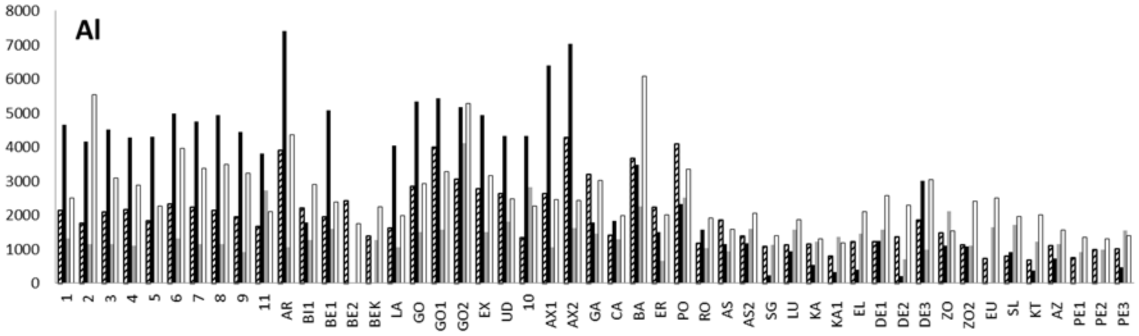
	Al	As	Cd	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Sn	V	Zn
KA	1210	5.10	0.22	4.53	10.1	20.4	6870	1760	317	7.68	49.8	3.84	2.68	114
KA1	1370	3.71	0.21	5.23	8.06	21.3	7690	1730	337	7.85	43.0	2.56	2.57	88.2
EL	1450	12.6	0.81	5.66	31.1	45.8	9760	1840	208	16.7	84.8	15.4	4.17	326
DE1	1570	10.4	0.54	8.09	25.0	32.9	11400	1810	212	18.0	50.6	11.8	3.16	176
DE2	700	11.0	0.05	1.81	7.21	9.26	16700	1050	160	2.79	43.6	1.22	9.71	21.1
DE3	983	8.21	0.46	5.74	5.86	12.2	4960	1750	122	9.72	39.5	2.10	19.6*	163
ZO	2120	16.5	0.63	7.49	33.4	47.1	15300	2800	245	24.9	65.4	8.08	8.57	243
ZO2	1100	3.21	0.51	4.58	22.4	23.8	11300	949	410	18.1	71.1	5.01	1.85	140
EU	1630	7.44	0.49	5.94	28.2	32.5	10300	2550	250	22.5	45.7	6.44	4.64	195
SL	1710	8.48	0.35	8.12	25.5	30.4	10300	2060	228	23.8	37.3	5.02	2.76	165
KT	1210	5.00	0.37	5.91	21.1	21.3	8480	1870	239	17.9	31.3	3.56	2.62	136
AZ	1150	5.53	0.31	6.47	20.5	24.5	8760	1460	385	19.4	35.7	4.45	1.71	140
PE1	912	11.0	0.24	7.20	30.5	45.4	11200	1670	341	28.4	41.8	6.20	1.19	158
PE2	987	6.57	0.25	6.33	30.1	48.4	10400	1600	472	22.8	49.2	6.31	1.50	138
PE3	1550	7.03	0.36	7.25	35.6	61.9	10000	1320	585	23.5	66.1	20.2	3.26	217
25th PERCENTILE	1100	6.76	0.36	2.87	19.6	22.4	9740	1170	197	7.91	48.1	4.96	1.85	163
75th PERCENTILE	1580	12.7	0.79	6.47	29.0	47.1	12800	1760	385	18.6	91.4	8.41	5.8	297
IQR (Q3-Q1)	484	6.0	0.43	3.60	9.4	24.6	3080	597	188	10.8	43.0	3.51	3.9	134
Q3+(3*IQR)	3030	30.7	2.1	17.3	57.1	121	22000	3550	948	50.9	221	18.9	17.6	698
MEDIAN	1290	9.6	0.51	4.58	22.5	33.6	10500	1540	245	11.3	61.1	6.10	4.17	242
AVERAGE	1450	11.0	1.02	4.84	22.8	41.9	11700	1500	372	13.6	84.2	7.36	4.28	265
DESVEST	616	6.9	2.52	2.28	7.9	36.3	4330	439	413	6.89	69.1	4.28	3.25	167
MIN	652	0.48	0.05	1.16	5.86	9.26	4730	661	122	2.79	31.3	1.22	0.25	21.1
MAX	4120	37.2	17.6	9.40	42.0	250	29800	2800	2580	28.4	431	21.8	19.6	901

Table 6. Element concentrations found in the sediments collected in January 2014 together with some related statistics. Extreme high concentrations, defined as those concentrations over the 75th percentile of the data (Q3) plus three times the interquartile range (IQR), are marked with an asterisk (*).

	Al	**As**	**Cd**	**Co**	**Cr**	**Cu**	**Fe**	**Mg**	**Mn**	**Ni**	**Pb**	**Sn**	**V**	**Zn**
1	2500	18.9	1.71	5.78	25.1	59.7	15200	2830	166	13.1	72.0	12.1	14.4	311
2	5530	51.9	4.76	8.82	50.6	373*	27900	13300*	571	17.1	315	40.8	30.1	2010*
3	3090	28.9	1.10	3.79	26.6	78.8	17900	4320	297	11.1	145	11.4	27.1	545
4	2880	22.4	1.40	5.15	21.1	44.3	17600	4140	277	10.9	80.2	9.81	22.8	315
5	2270	47.3	2.00	5.28	22.6	52.9	16100	3840	303	10.0	98.8	13.2	20.0	309
6	3960	29.6	0.49	3.56	32.4	107	17500	3480	219	10.6	144	14.5	25.1	372
7	3370	30.2	1.69	5.63	28.6	85.9	18000	3800	235	11.7	98.9	15.6	20.5	317
8	3480	33.5	2.29	3.33	33.6	124	16800	3230	214	11.2	139	15.3	22.6	444
9	3230	79.0	5.20	5.89	72.9*	206	25000	3730	233	12.6	280	49.5*	37.4	877
10	2250	18.3	0.67	3.88	25.3	51.8	13700	2630	160	14.6	79.1	7.01	11.5	280
11	2090	14.8	0.70	3.42	19.1	49.6	11100	2250	146	12.4	69.1	6.32	8.44	262
AR	4360	27.8	1.40	4.91	29.2	95.1	15000	4160	334	16.5	132	16.3	20.8	480
BI1	2890	418*	6.03	4.71	106*	272	35900	3450	239	19.3	566*	38.1	151*	920
BE1	2380	14.5	0.38	4.49	20.2	47.4	13400	4100	173	15.8	60.2	6.52	8.18	262
BE2	1740	3.91	< LOD	3.38	31.0	20.6	6880	593	456	24.2	16.1	1.22	5.61	63.1
BEK	2250	30.7	1.74	4.20	42.9	76.4	14600	2640	216	13.2	132	24.3	11.1	445
LA	1980	9.11	0.92	3.20	29.4	78.3	28300	2210	734	18.1	62.6	7.91	5.86	351
GO	2930	45.6	3.64	4.98	30.8	257	17500	3220	424	17.9	159	18.4	17.4	685
GO1	3280	30.5	6.47*	5.69	32.2	200	13700	3450	411	22.3	84.4	14.1	14.8	541
GO2	5280	13.2	0.5	2.41	25.7	28.8	9370	12000*	438	10.5	63.7	2.12	27.0	238
EX	3150	74.5	1.05	4.86	32.5	140	14900	2890	240	20.8	194	14.2	20.3	565
UD	2470	89.2*	1.88	4.80	35.6	136	16200	2330	251	17.5	196	15.7	19.7	560
AX1	2440	20.3	2.32	4.93	30.6	75.1	12700	2430	196	17.4	95.9	13.6	10.0	350
AX2	2430	13.5	0.93	5.82	14.7	64.9	12500	3050	97.1	14.9	83.8	5.88	13.8	393
GA	3020	81.7*	3.84	4.81	36.4	180	23600	2150	557	34.5	521*	31.0	12.9	1590*
CA	1980	9.25	0.92	3.20	29.4	77.7	28300	2210	734	18.1	62.3	7.93	5.80	351
BA	6080	14.3	5.69	6.91	65.7	226	22700	3400	3900*	27.7	656*	21.9	22.7	4410*
ER	2010	12.6	0.79	3.29	24.3	47.2	9520	1450	123	15.2	86.2	7.29	7.04	253
PO	3360	172*	13.0*	13.7*	76.0	345	37200*	3140	341	25.2	520*	34.2	46.3	1280*
RO	1920	16.7	1.08	3.21	18.6	50.3	9610	2230	113	11.8	90.0	6.06	8.30	303
AS	1570	9.14	0.31	4.94	9.71	25.3	6090	663	396	7.81	49.1	2.09	4.62	165
AS2	2040	14.0	1.52	5.15	16.0	40.4	7500	1120	104	12.7	91.8	3.24	6.25	253
SG	1400	19.0	0.50	4.67	14.5	26.1	5150	353	261	9.64	136	1.45	4.26	149

Table 6 (Cont). Element concentrations found in the sediments collected in January 2014 together with some related statistics. Extreme high concentrations, defined as those concentrations over the 75th percentile of the data (Q3) plus three times the interquartile range (IQR), are marked with an asterisk (*).

	Al	As	Cd	Co	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Sn	V	Zn
LU	1860	21.5	1.19	3.02	23.0	58.8	12600	2740	150	12.1	83.6	7.61	9.83	327
KA	1300	4.77	< LOD	1.73	4.70	13.8	6580	957	221	3.97	40.3	3.55	3.41	98.7
KA1	1180	3.91	< LOD	1.17	2.95	9.02	5450	1120	164	2.36	24.8	1.58	2.55	51.2
EL	2090	21.0	0.50	3.57	15.0	62.2	14400	2450	219	11.6	83.4	6.14	10.5	264
DE1	2580	18.5	0.69	3.90	22.9	52.1	16000	3300	155	15.2	68.6	8.07	11.7	319
DE2	2290	18.8	0.99	4.62	28.9	67.3	14900	2650	241	18.5	82.1	9.86	8.94	322
DE3	3030	20.2	1.50	3.91	29.4	84.1	17500	4140	156	16.6	120	11.5	14.2	451
ZO	1520	11.7	0.32	5.75	17.8	69.8	12200	1100	335	17.7	140	3.61	4.42	219
ZO2	2400	20.2	1.42	5.82	42.0	81.0	15200	2020	154	27.7	105	19.6	7.93	385
EU	2500	13.9	0.51	4.53	26.8	56.3	15100	2780	131	21.6	65.5	7.64	11.3	272
SL	1950	9.02	< LOD	5.46	18.4	27.2	13600	2630	162	20.0	32.3	2.86	6.04	194
KT	2000	8.25	< LOD	5.52	22.9	28.3	13600	2350	131	23.2	33.2	3.61	5.22	198
AZ	1560	16.9	0.29	6.61	26.9	120	13900	999	365	24.5	223	4.09	4.78	261
PE1	1340	13.3	0.08	5.38	21.9	122	12900	1120	391	18.2	257	4.65	4.54	246
PE2	1300	11.5	0.11	4.80	19.0	111	12400	762	353	17.0	210	5.33	4.12	216
PE3	1380	14.4	0.77	6.55	30.4	145	16200	813	437	22.5	379	8.68	5.11	243
25th PERCENTILE	1930	13.3	0.57	3.57	19.6	48.5	12400	1730	161	11.7	68.4	4.99	5.80	250
75th PERCENTILE	3060	30.3	1.97	5.57	32.3	123	17500	3450	378	19.6	176	15.4	20.4	465
IQR (Q3-Q1)	1120	17.1	1.40	2.01	12.7	74.5	5100	1710	217	7.93	108	10.5	14.6	216
Q3+(3*IQR)	6450	81.5	6.21	11.6	70.4	347	32800	8610	1030	43.4	500	46.8	64.2	1110
MEDIAN	2380	18.8	1.09	4.80	26.8	75.1	14900	2640	239	16.5	95.2	8.14	11.1	317
AVERAGE	2570	35.5	1.94	4.80	29.8	98.9	15700	2910	354	16.3	153	12.2	16.3	505
DESVEST	1070	62.9	2.37	1.87	18.1	82.2	6960	2310	539	6.15	146	10.8	21.8	676
MIN	1180	3.91	0.08	1.17	2.95	9.02	5150	353	97.1	2.36	16.1	1.22	2.55	51.2
MAX	6080	418	13.0	13.7	106	373	37200	13300	3900	34.5	656	49.5	151	4410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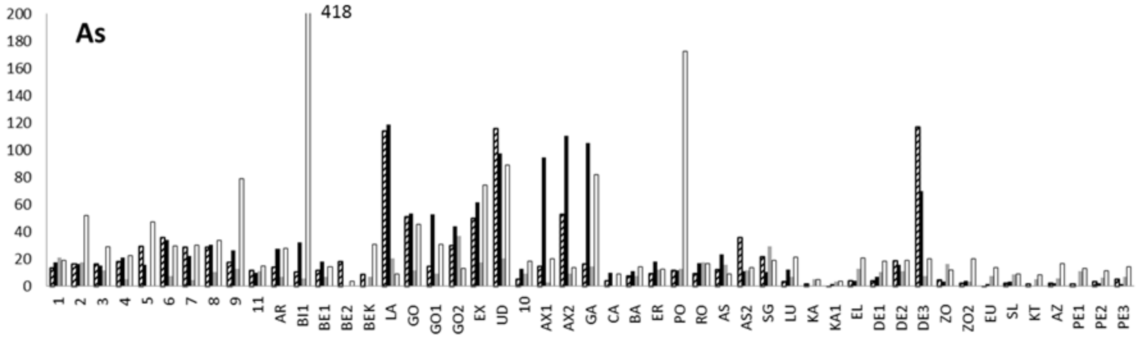
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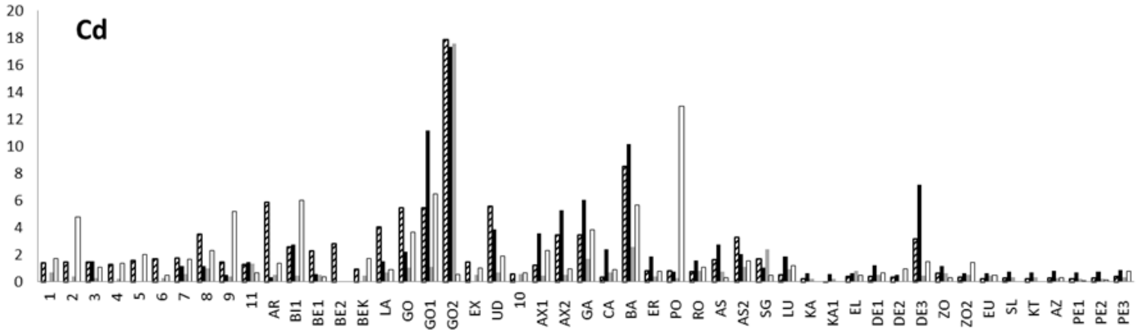
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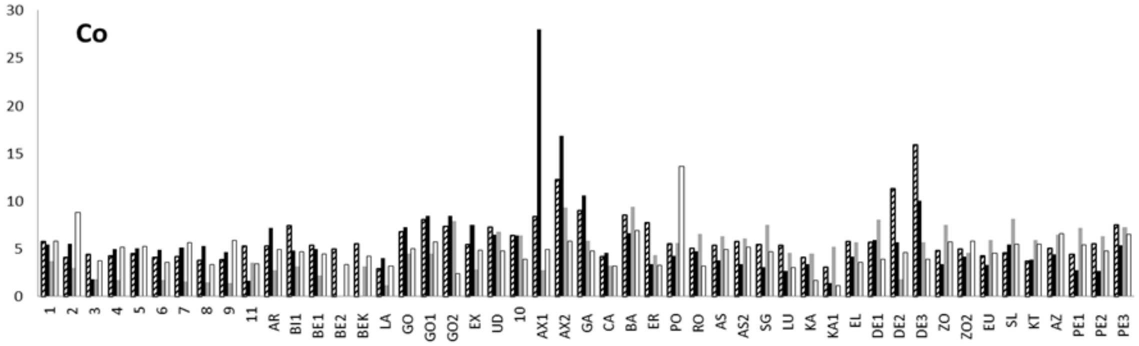
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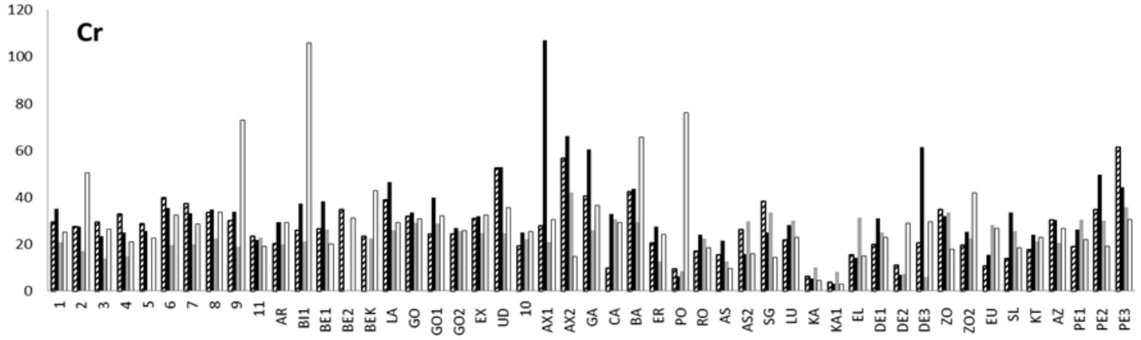
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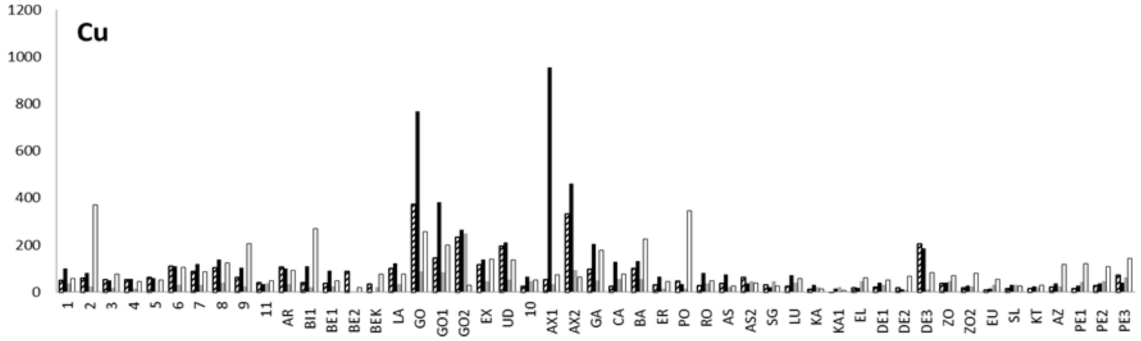
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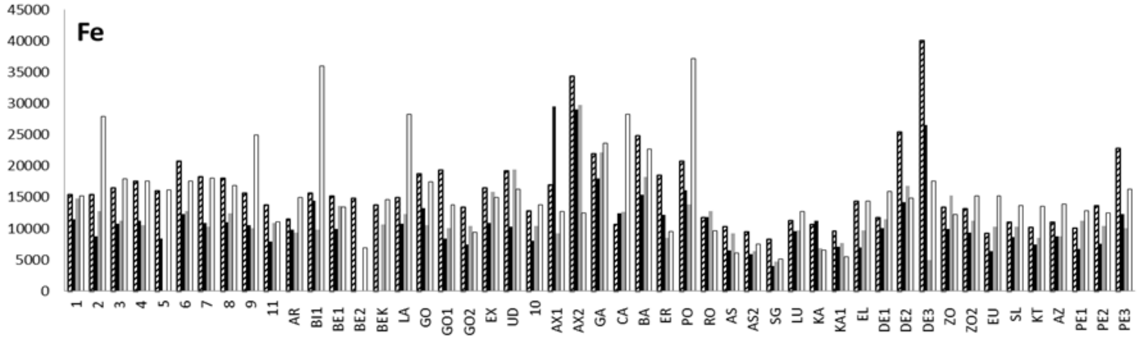
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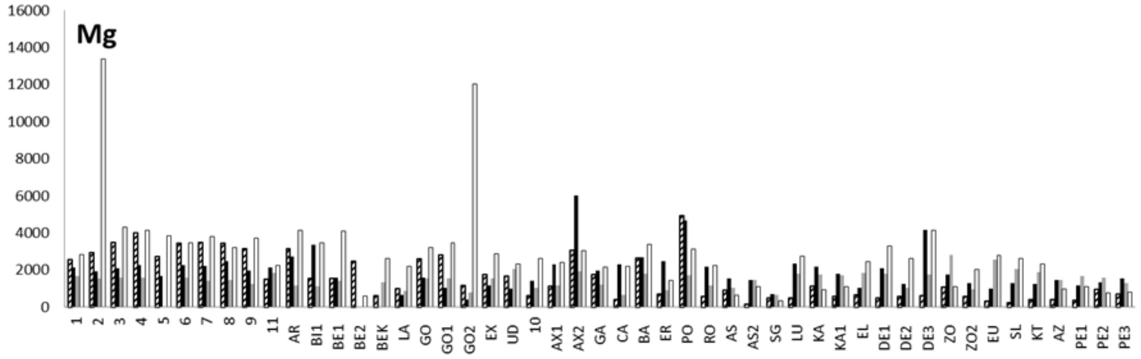
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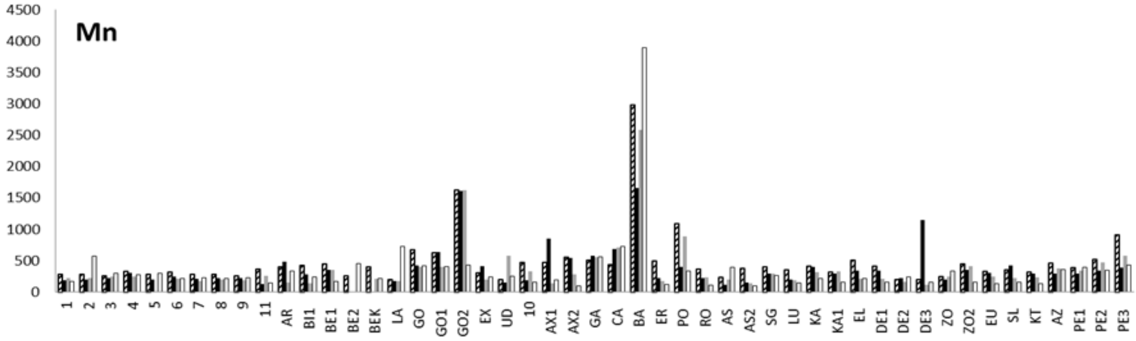
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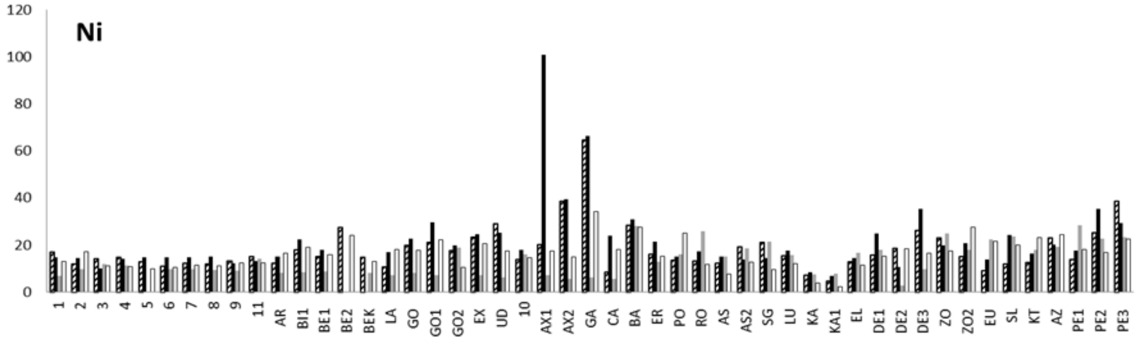
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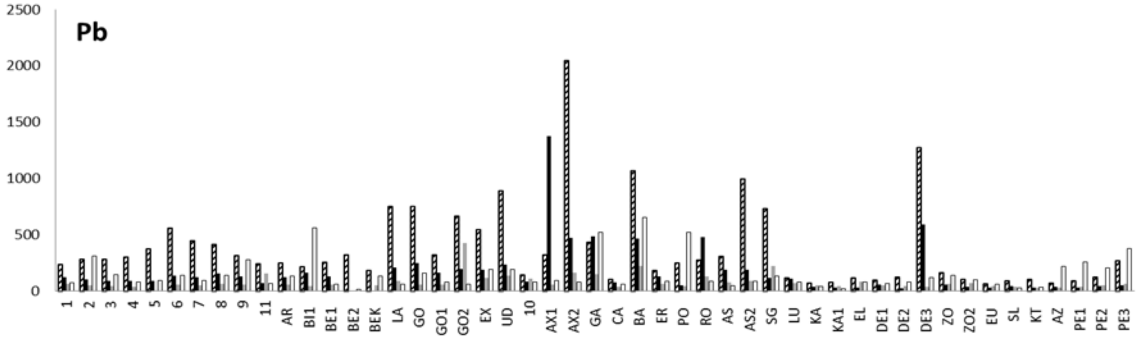
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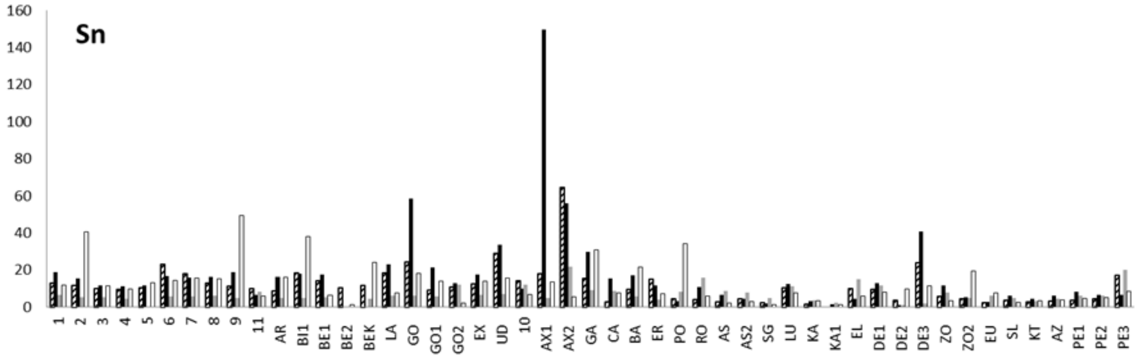
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1 2 3 4 5 6 7 8 9 11 AR BI1 BE1 BE2 BEK LA GO GO1 GO2 EX UD 10 AX1 AX2 GA CA BA ER PO RO AS AS2 SG LU KA KA1 EL DE1 DE2 DE3 ZO ZO2 EU SL KT AZ PE1 PE2 PE3

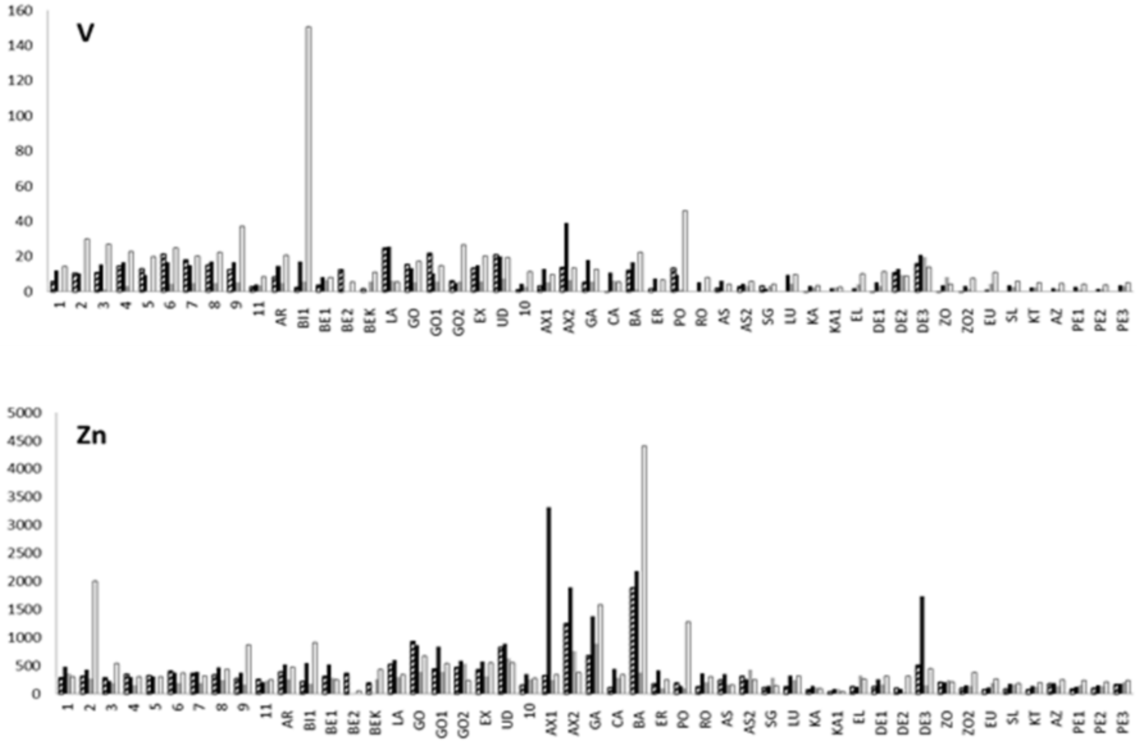


Figure 2. Element concentrations found in the sediments collected in 2009 (striped bar), 2010 (black bar), 2011(grey bar) and 2014 (white bar) sampling campaigns.

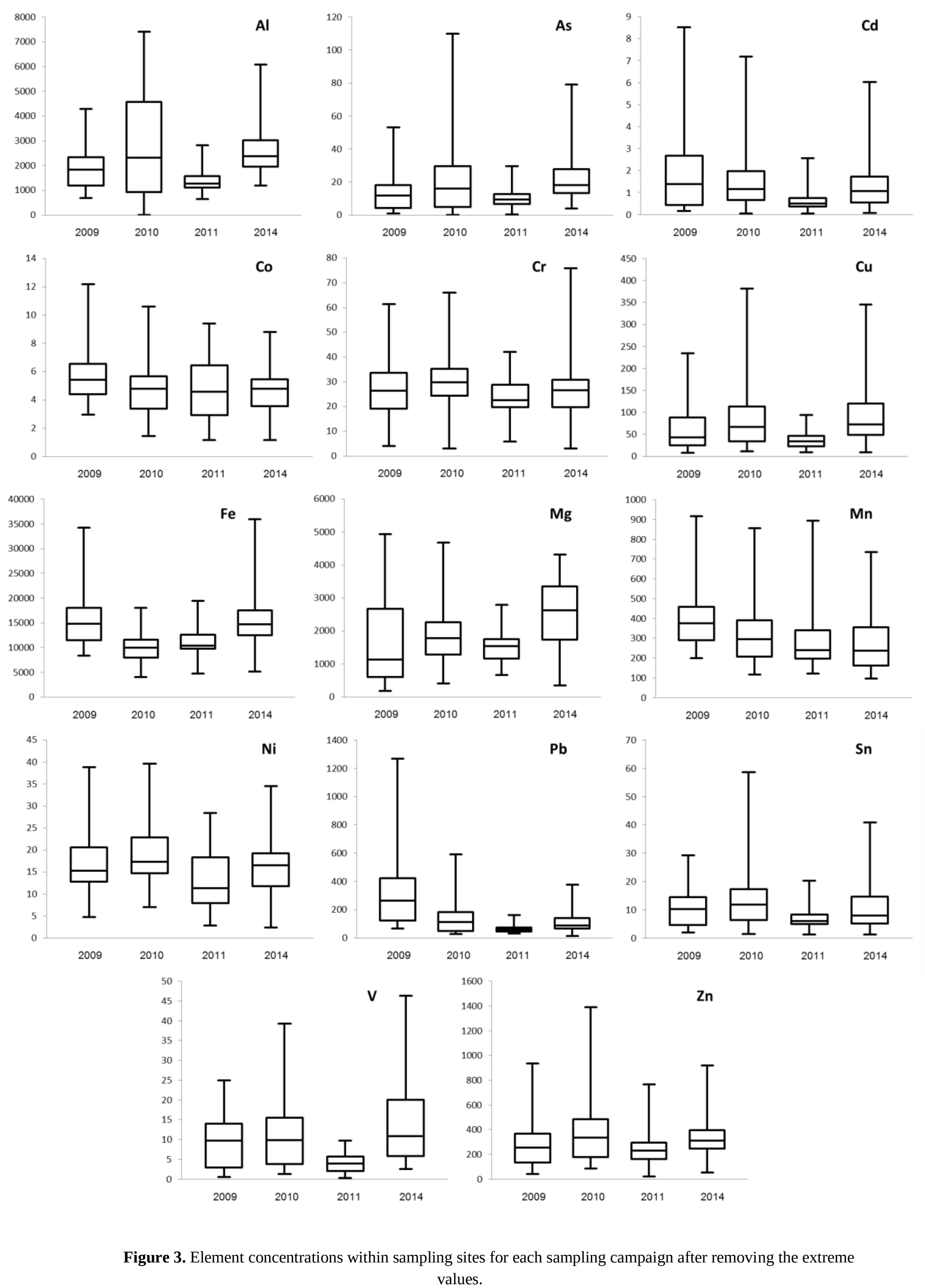


Figure 3. Element concentrations within sampling sites for each sampling campaign after removing the extreme values.

3.2. PRINCIPAL COMPONENT ANALYSIS: PATTERN RECOGNITION

Principal Component Analysis (PCA) of the datasets was separately performed for each sampling campaign by means of The Unscrambler Program (v. 9.2 Camo, Oslo, Norway). Each dataset consisted on a matrix with n rows (n: number of sampling sites) and m columns (m: number of elements for which concentration was measured in each sample) which was centred and scaled before PCA. Concentrations below the detection limit were replaced by half of the detection limit estimated for the element. PCA is a multivariate statistical technique able to discern patterns in large environmental datasets. PCA transforms the two dimension multivariate data array into a new data set, so that some of the new variables (principal components, PCs) are linear combinations of the original variables and explain most of the information contained in the original data (Jolliffe, 2002). The first component represents the maximum variation of the data set and corresponds to the direction explaining the maximum variance; the second PC corresponds to the direction, orthogonal to the first PC, explaining the maximum variance not explained by the first PC, and so on.

Models with 3 PCs were finally selected to explain the variability of the four datasets considered. For the 2009, 2010, 2011 and 2014 sampling campaigns, the models explained, respectively, 73, 82, 67 and 79% of the total variance of data. The scores and loading plots obtained in each case are shown in Figures 4-7. In general, PC1 discerned between sites with low and high metal concentrations, while PC2 and PC3 made a more qualitative difference among clusters according to the most influencing elements in each one. The results confirmed the general conclusions drawn from the direct observation of row data (Section 3.1). Very similar patterns were obtained for the 2009, 2010 and 2011 sampling campaigns, with high scores on PC1 for sites located in the area of influence of the Gobela and Galindo Rivers (GO, GO1, GO2, UD, AX1, AX2, GA, BA), and the channel of Deusto (DE3). The sediments from these sites are associated to high concentrations of elements such as As, Cd, Cu, Pb, Sn and Zn (with high loadings on PC1). In 2014, however, the sites with the highest scores on PC1 were 2, 9 and BI1 (in the Abra Bay), BA and GA (close to the effluent of the WWTP) and PO (dock of Portu). The metals with the highest loadings on PC1 this time were Pb, Cd, Sn, Cu, Cr, Fe, As, V and Zn.

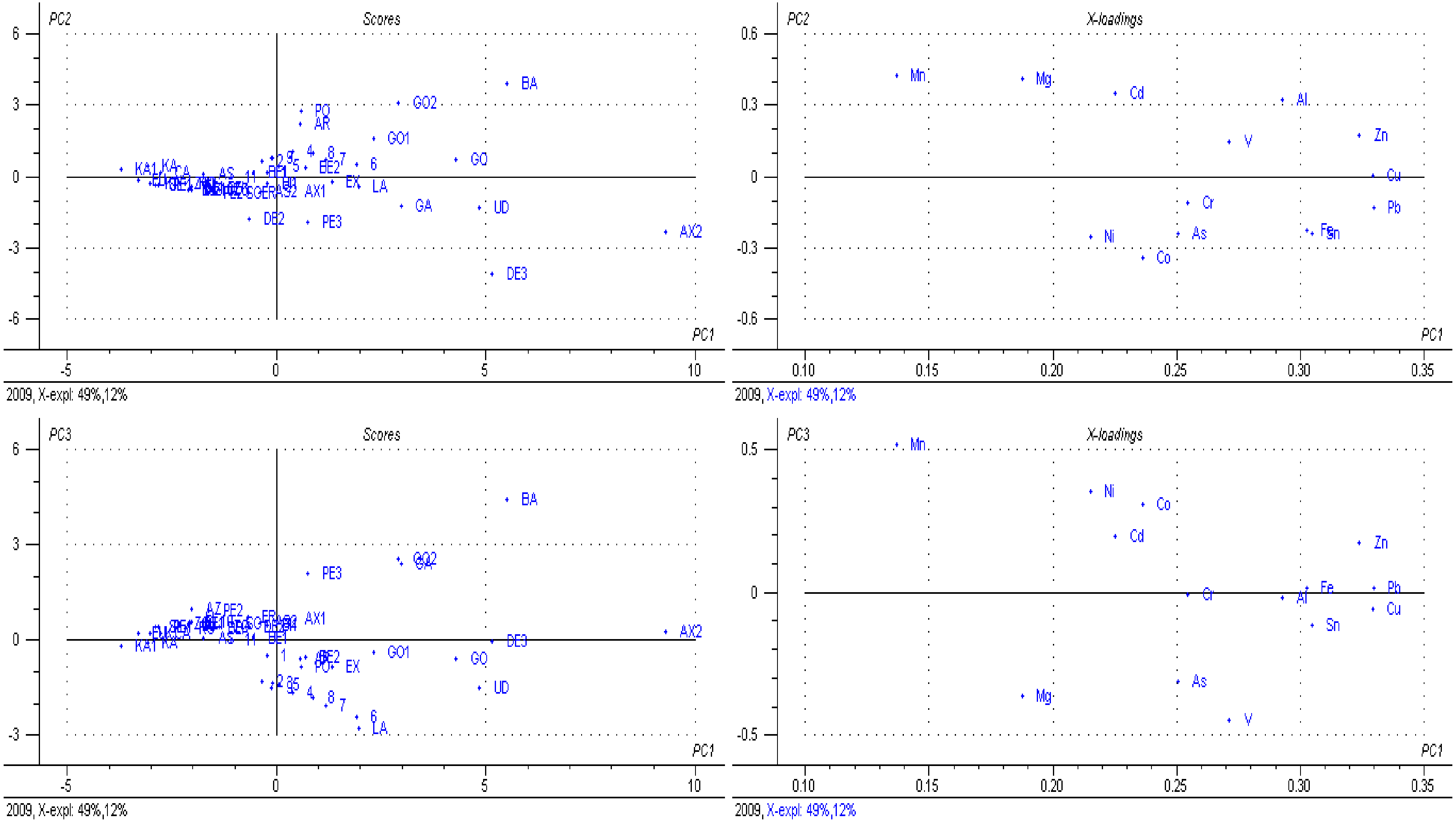


Figure 4. Scores and loadings plots (PC1-PC2 and PC1-PC3) obtained after PCA of the dataset corresponding to the 2009 sampling campaign.

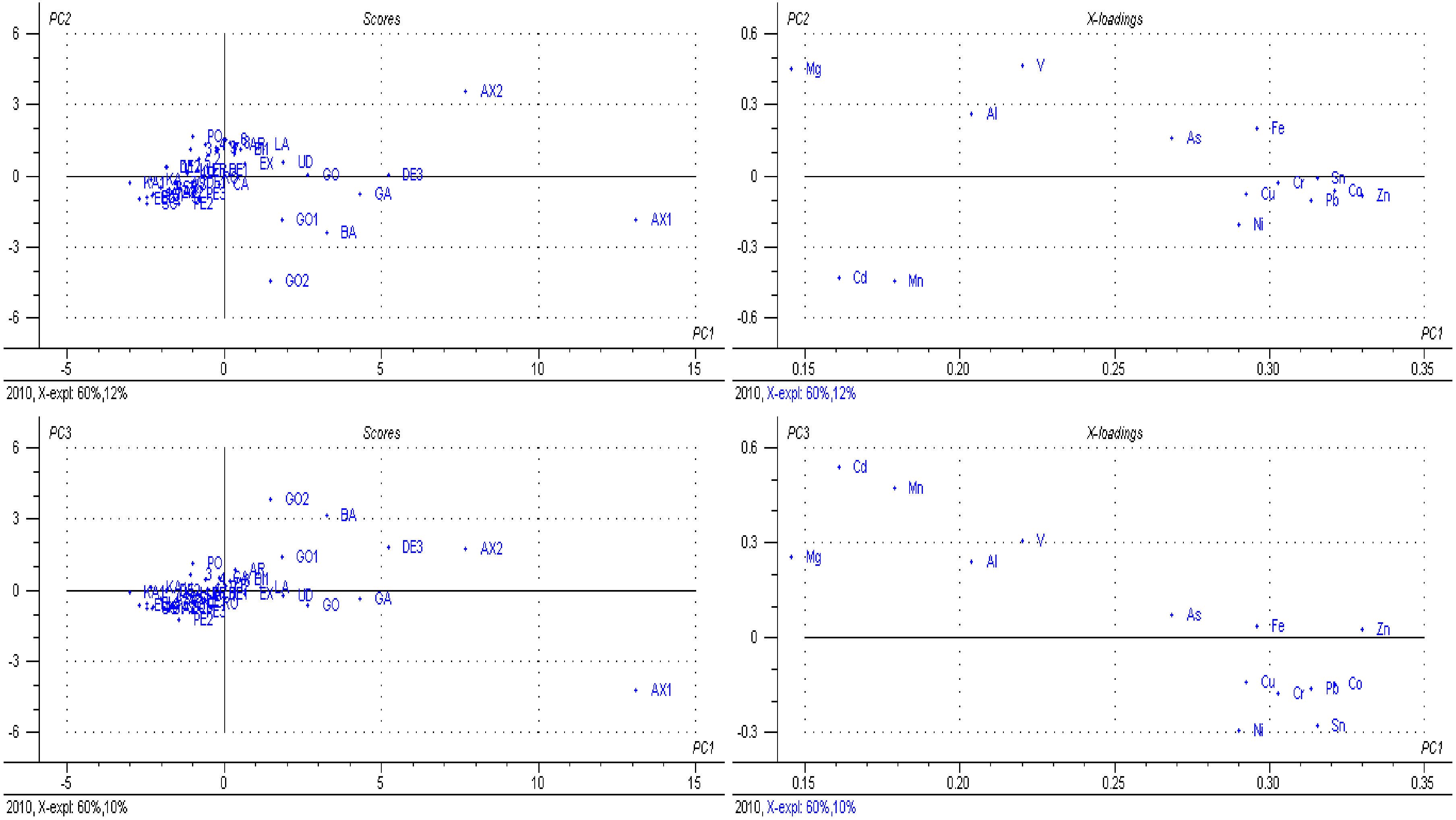


Figure 5. Scores and loadings plots (PC1-PC2 and PC1-PC3) obtained after PCA of the dataset corresponding to the 2010 sampling campaign.

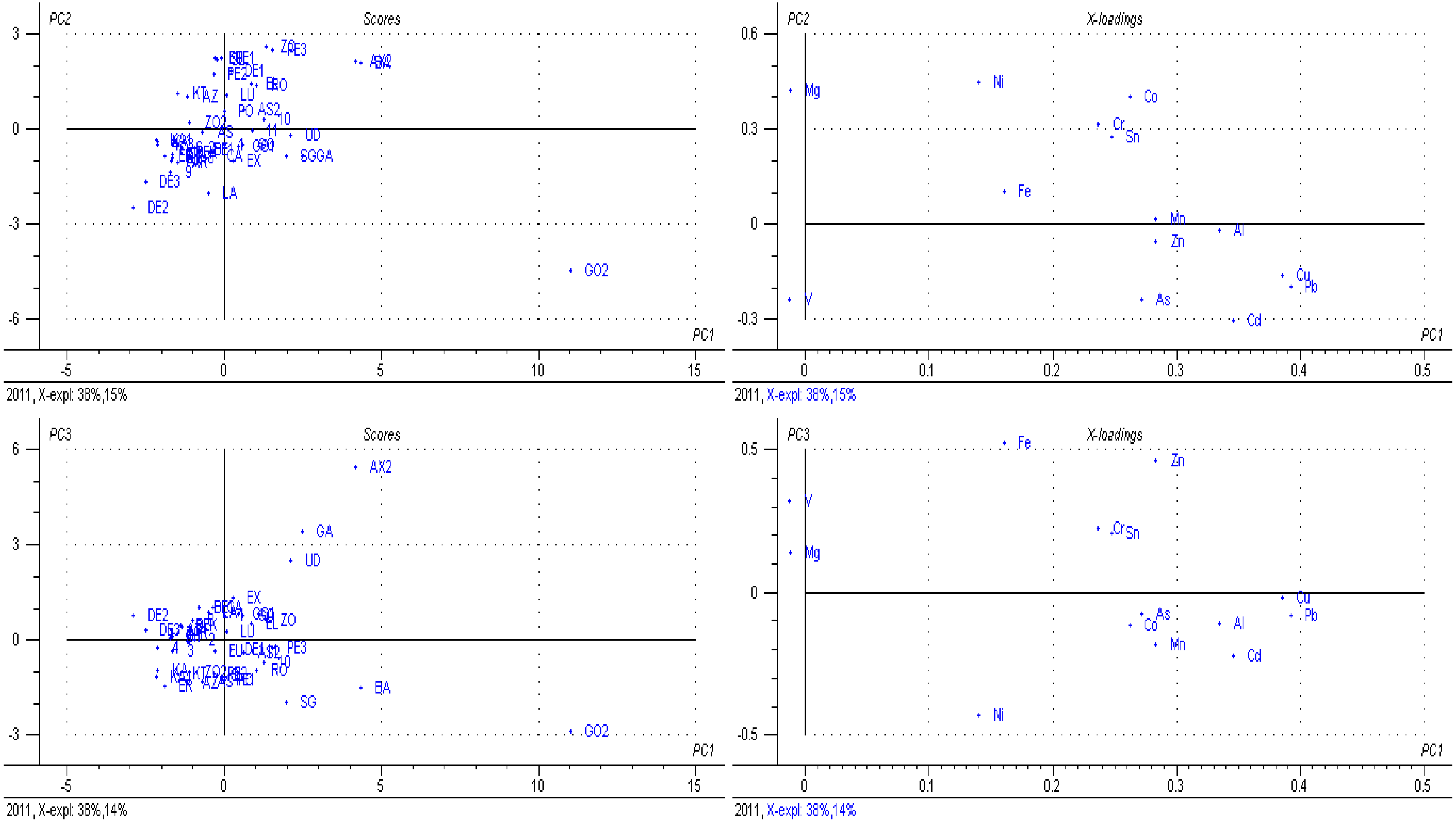


Figure 6. Scores and loadings plots (PC1-PC2 and PC1-PC3) obtained after PCA of the dataset corresponding to the 2011 sampling campaign.

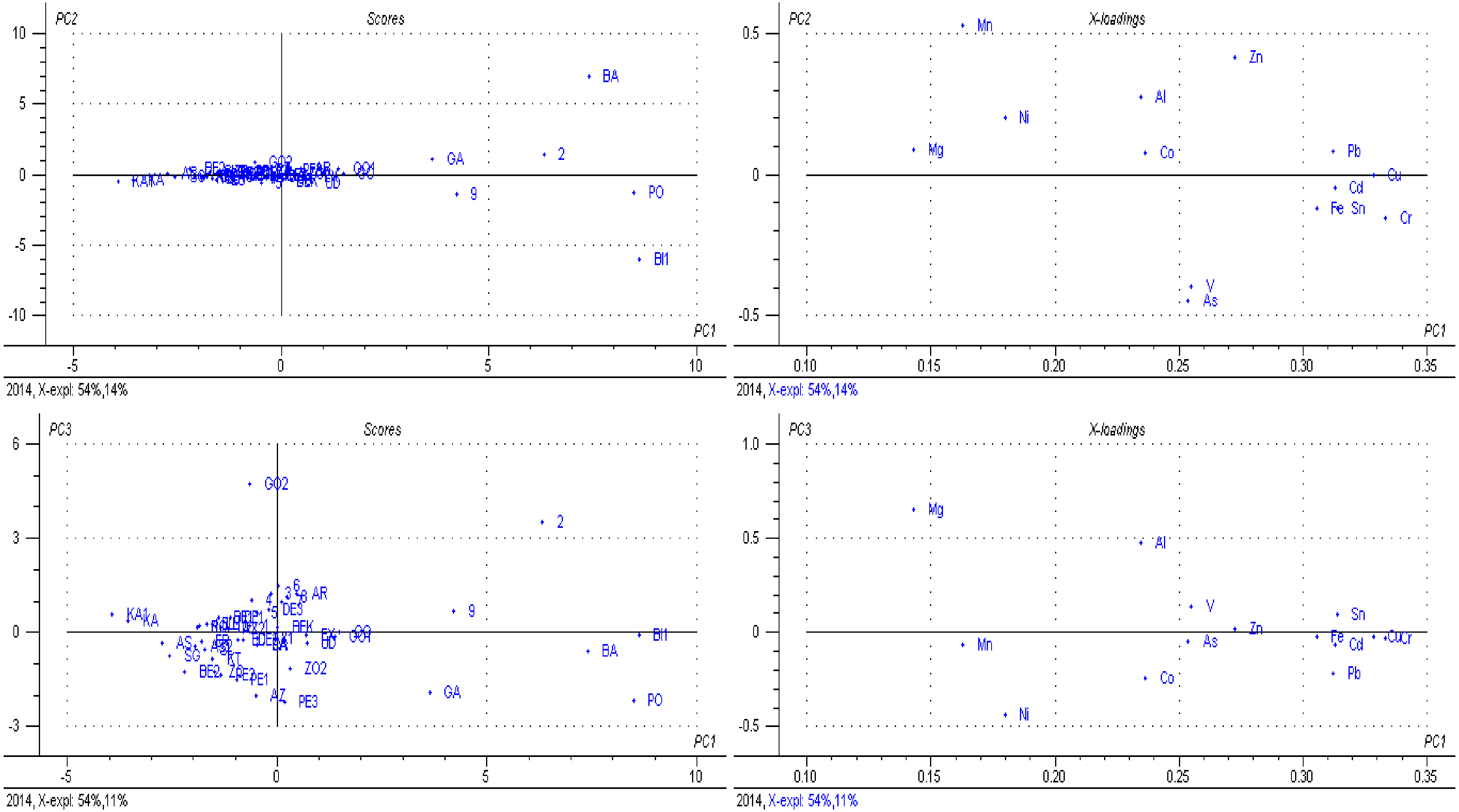


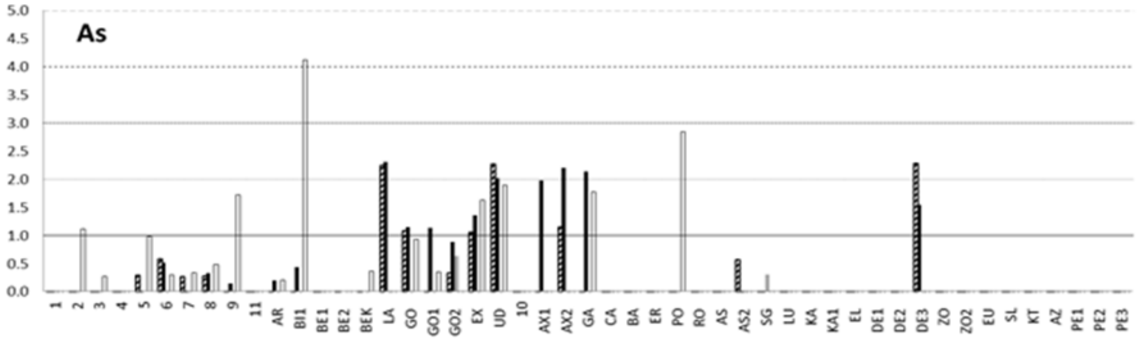
Figure 7. Scores and loadings plots (PC1-PC2 and PC1-PC3) obtained after PCA of the dataset corresponding to the 2014 sampling campaign.

3.3. GEOACCUMULATION INDEXES: ANTHROPOGENIC INPUT

Geoaccumulation indexes (I_{geo}) were also calculated according to Eq. 1 (Mueller, 1981) in order to estimate the contamination level of the sediments collected at each sampling site and each sampling campaign for each individual element,

$$I_{geo} = \log_2 \frac{c}{1.5 c_{bg}} \qquad \text{(Eq. 1)}$$

where c is the actual concentration of a given contaminant in the sediment, and c_{bg} is the background concentration estimated for that contaminant in the studied area. The I_{geo} can be used to estimate the magnitude of the anthropogenic impact in the area to be investigated. According to the scale established by Müller (Mueller, 1981), a sediment can be classified as non-contaminated ($I_{geo} < 1$), very slightly contaminated ($1 < I_{geo} < 2$), slightly contaminated ($2 < I_{geo} < 3$), moderately contaminated ($3 < I_{geo} < 4$), highly contaminated ($4 < I_{geo} < 5$), and very highly contaminated ($I_{geo} > 5$). The background values used in this study were estimated by Cearreta et al. (Cearreta et al., 2000), except for Cd which was estimated by Rodriguez et al. (Rodriguez et al., 2006). It is worth noting that the use of poorly estimated background concentrations in the calculation may lead to non-reliable conclusions. The values of I_{geo} obtained in this studied are summarised in Figure 8. The I_{geo} calculated for Cr and Fe were not plotted because negative values of I_{geo} were obtained in all the cases. This suggests that, regarding their relatively high concentrations in sediments, the origin of Cr and Fe in the estuary is mainly natural. The situation for Ni is very similar, with only one sediment collected at AX1 in 2010 with a I_{geo} value slightly higher than 1. The highest values of I_{geo} were obtained for Pb, Cd and Zn (with some $I_{geo} > 5$), Cu (with several I_{geo} between 3 and 5) and As (with several I_{geo} between 1 and 3, and one I_{geo} over 4 (BI1, 2014)). Only a few sites (GO2, BA and DE3) present sediments slightly contaminated with Mn.


As
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
8
9
11
AR
BI1
BE1
BE2
BEK
LA
GO
GO1
GO2
EX
UD
10
AX1
AX2
GA
CA
BA
ER
PO
RO
AS
AS2
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PE1
PE2
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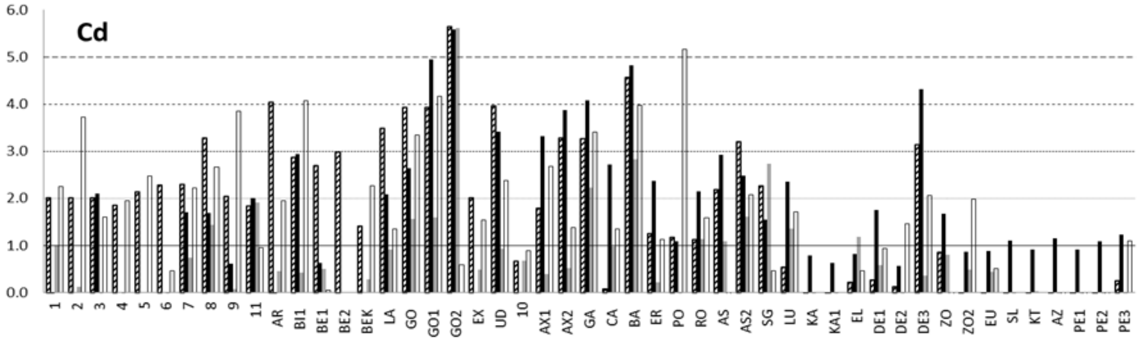
Cd
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1
2
3
4
5
6
7
8
9
11
AR
BI1
BE1
BE2
BEK
LA
GO
GO1
GO2
EX
UD
10
AX1
AX2
GA
CA
BA
ER
PO
RO
AS
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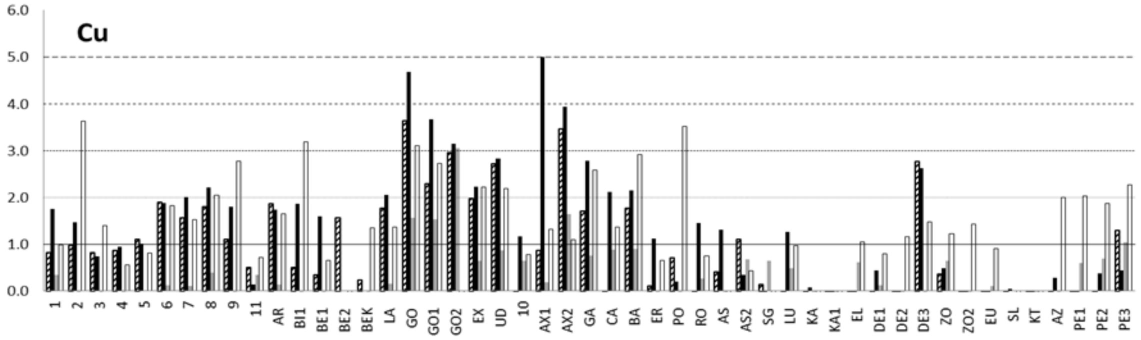
PE3


Cu
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1
2
3
4
5
6
7
8
9
11
AR
BI1
BE1
BE2
BEK
LA
GO
GO1
GO2
EX
UD
10
AX1
AX2
GA
CA
BA
ER
PO
RO
AS
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KT
AZ
PE1
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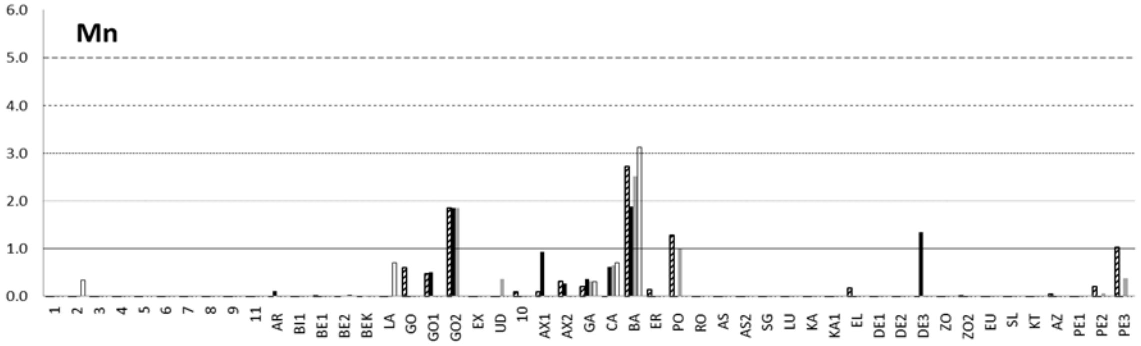
Mn
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1
2
3
4
5
6
7
8
9
11
AR
BI1
BE1
BE2
BEK
LA
GO
GO1
GO2
EX
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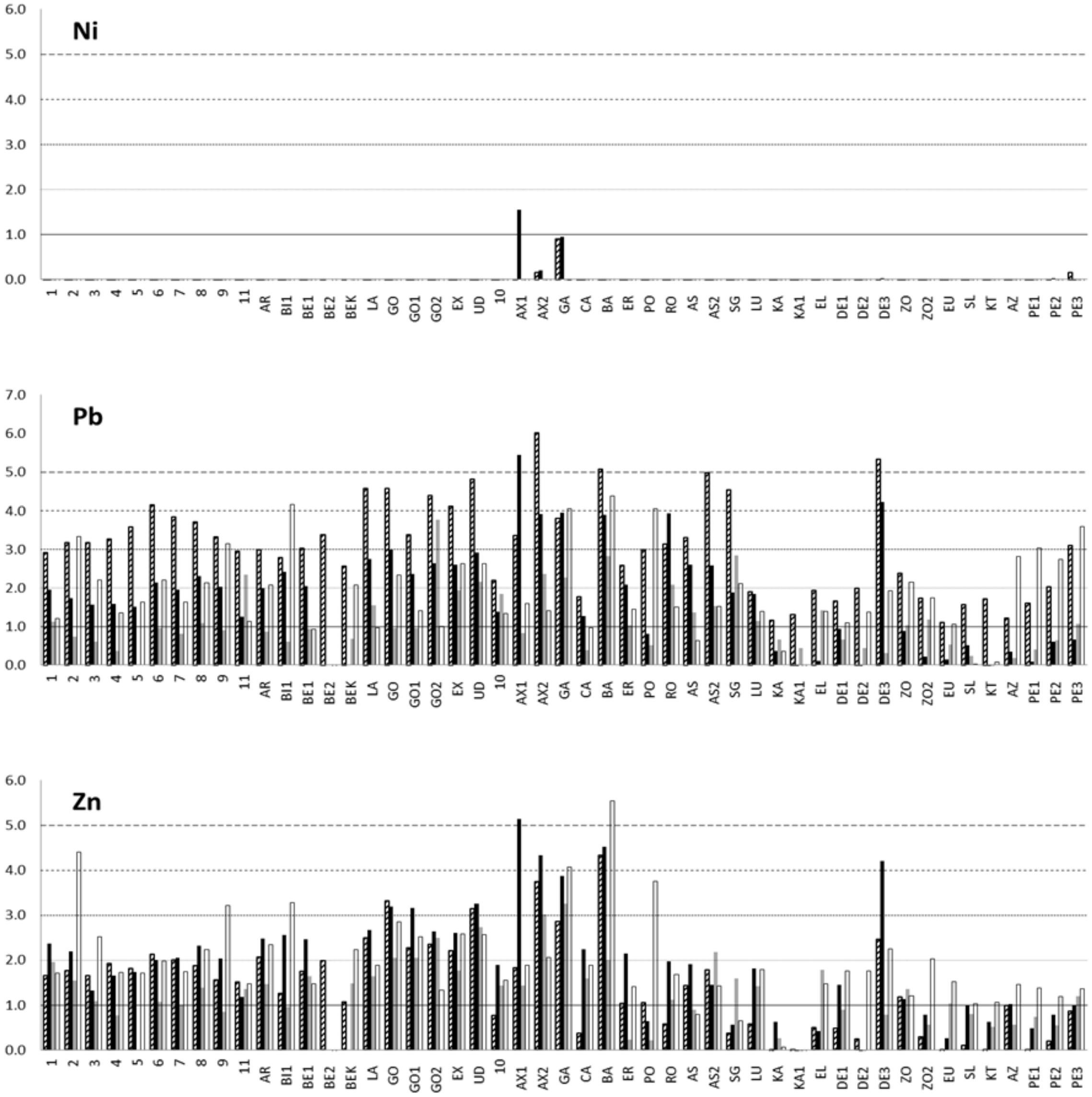

Figure 8. Geoaccumulation Indexes (I_{geo}) obtained for the sediments collected at different sampling sites in 2009 (striped bar), 2010 (black bar), 2011(grey bar) and 2014 (white bar). $I_{geo} < 1$: non-contaminated; $1 < I_{geo} < 2$: very slightly contaminated; $2 < I_{geo} < 3$: slightly contaminated; $3 < I_{geo} < 4$: moderately contaminated; $4 < I_{geo} < 5$: highly contaminated; $5 < I_{geo}$: very highly contaminated.

3.4 NORMALIZED AND WEIGHTED AVERAGE CONCENTRATIONS: IDENTIFICATION OF HOTSPOTS OF CONTAMINATION

The Normalised-and-Weighted Average Concentration (NWAC) is a cumulative index to sort samples (in this case, sediments) according to their content of selected contaminants. Samples are characterised by a single score in a relative scale from 0 to 10, the NWAC, which is calculated taking into account exclusively the concentration of contaminants freely selected by the user and present in the sample. More information about the NWACs and the methodology for their calculation can be found elsewhere (Gredilla et al., 2014). The calculation of the NWACs and their representation on a map according to a colour based code allow us identifying, in a very simple and intuitive way, the sampling sites of higher concern (hotspots of contamination) in the studied area.

The concentrations of the 14 elements measured in the samples were used to calculate the corresponding NWACs separately for each sampling campaign. A colour based map was produced by the Kriging interpolation method feeding the 3D Field Programme (v. 3.8.8.0, by Vladimir Galouchko) with the NAWC values obtained in each case (Figures 9-12). The difference between the geographical distribution of metals in 2014 and that in the rest of campaigns (2009, 2010 and 2011), already discussed in previous sections, becomes evident in Figures 9-12. Furthermore, the amount of hotspots substantially decreases from 2009 to 2011, with concomitant homogenisation of metal contamination within the estuary. Two hotspots of contamination remain in 2011, one upstream the Gobela River (GO2) and another one in the surroundings of the effluent of the WWTP (BA). In Figure 12, corresponding to 2014 campaign, the hotspot in Gobela has disappeared but, in turn, new hotspots of contamination have appeared in the Abra Bay (2, 9 and BI1) and the dock of Portu.

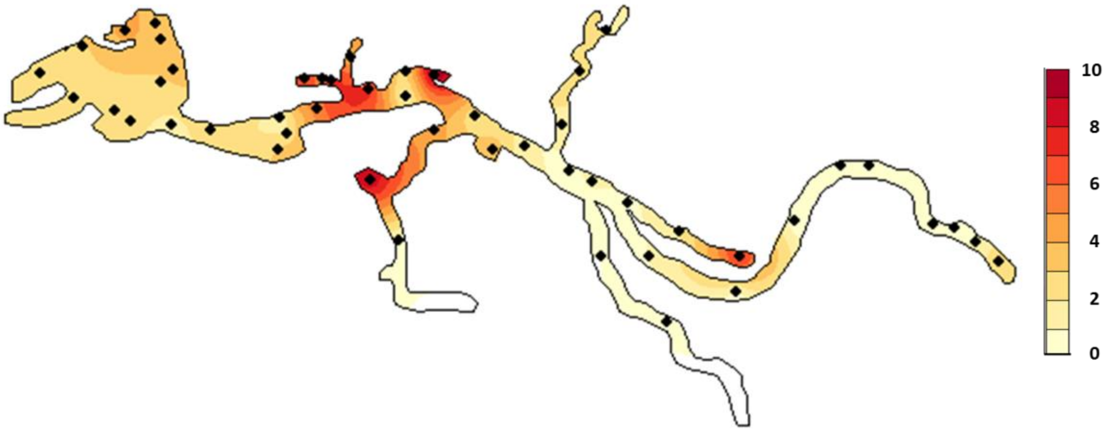

Figure 9. Hotspots of metal contamination in the estuary of the Nerbioi-Ibaizabal River according to the NWAC values calculated for the sediments collected in January 2009. The concentration of all the elements considered in this work were used in the calculation of the NWACs.

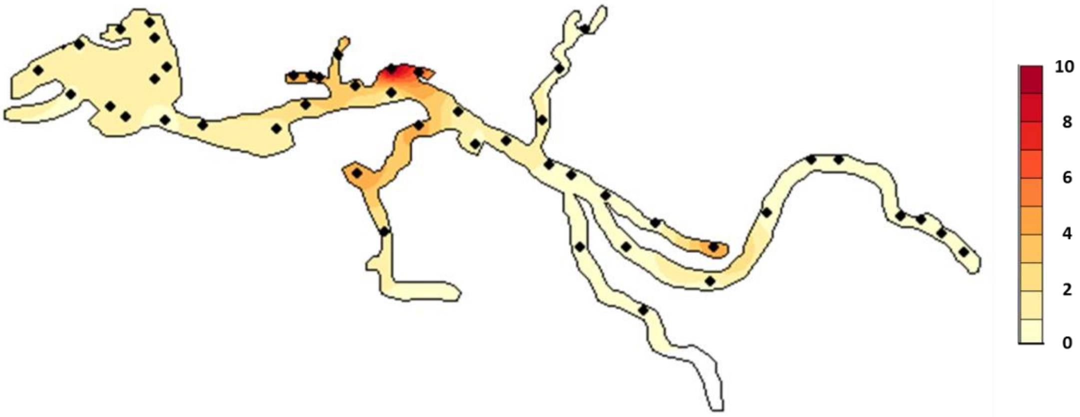

Figure 10. Hotspots of metal contamination in the estuary of the Nerbioi-Ibaizabal River according to the NWAC values calculated for the sediments collected in January 2010. The concentration of all the elements considered in this work were used in the calculation of the NWACs.

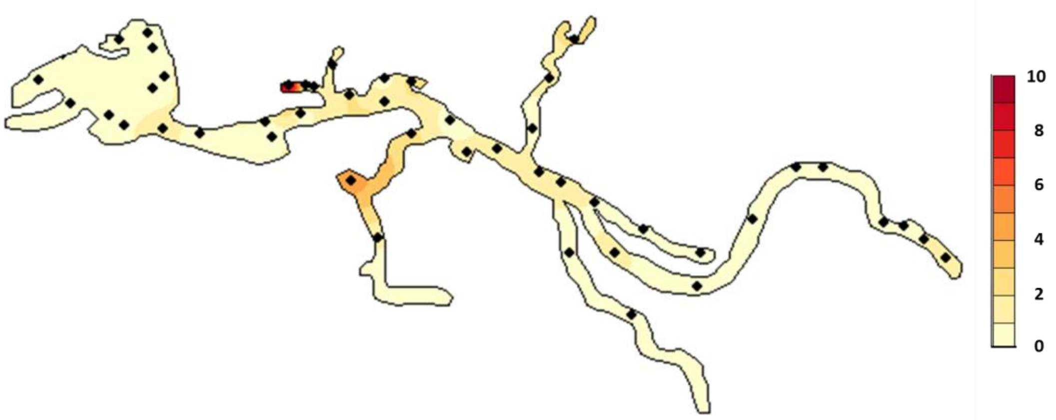

Figure 11. Hotspots of metal contamination in the estuary of the Nerbioi-Ibaizabal River according to the NWAC values calculated for the sediments collected in January 2011. The concentration of all the elements considered in this work were used in the calculation of the NWACs.

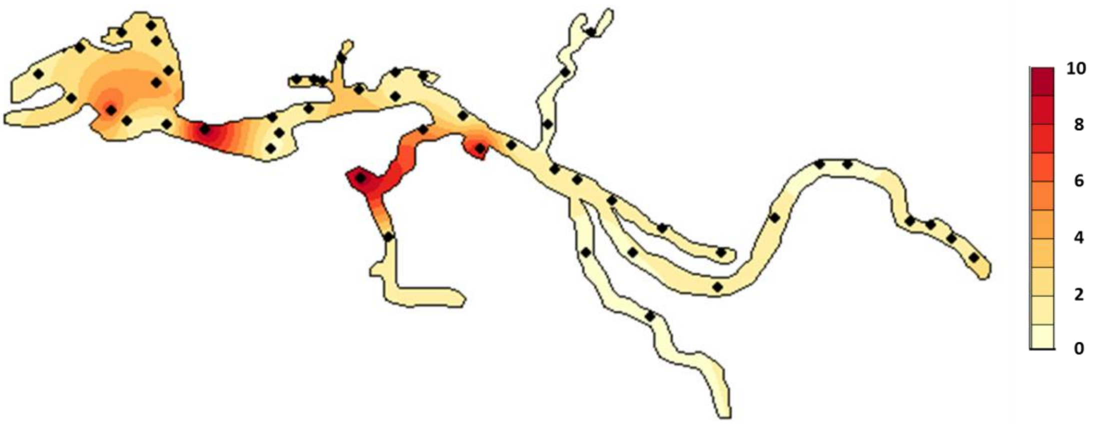

Figure 12. Hotspots of metal contamination in the estuary of the Nerbioi-Ibaizabal River according to the NWAC values calculated for the sediments collected in January 2014. The concentration of all the elements considered in this work were used in the calculation of the NWACs.

3.5 MEAN EFFECTS RANGE-MEDIAN QUOTIENTS: ESTIMATION OF TOXICITY

The mean Effects Range-Median quotients (mERMq) were also calculated according to Eq. 2 (Long et al., 2006) to estimate the toxicity associated to each individual sediment sample,

$$mERMq = \frac{1}{N}\sum_{i}^{N}\frac{c_i}{ERM_i} \qquad \text{(Eq. 2)}$$

where N is the amount of pollutants considered in the calculation, c_i is the actual concentration of the i pollutant in the sample and ERM_i is the concentration estimated for the i pollutant above which toxic effects are expected. The mERMq is a cumulative index that takes into account not only the concentration of selected pollutants in the sample, but also toxicological data obtained in the laboratory using model organisms under controlled conditions. For metals, values of ERM_i are only available in the literature for As, Cd, Cr, Cu, Pb, Ni, Hg and Zn. Other elements are considered to be non-toxic, or toxic only at extremely high concentrations. mERMq values in the range 0-0.1, 0.1-0.5, 0.5-1.5 and >1.5 indicate a probability of being toxic of 9% (non-toxic), 21% (slightly toxic), 49% (moderately toxic) and 76% (highly toxic), respectively (Rodriguez et al., 2006).

The mERMq values corresponding to the sediments collected in this work were calculated using the concentrations of As, Cd, Cr, Cu, Pb, Ni, and Zn measured in the samples. The results are shown in Figure 13. mERMqs higher than 1.5 (highly toxic sediments) were only found in the dock of Axpe (AX1, AX2) and close to the effluent of the WWTP (BA) in 2009 and 2010, as well as in BI1 (Abra Bay), BA and the dock of Portu (PO) in 2014. Sediments from the closed dock of Deusto (DE3) also showed mERMq values close to 1.5 in 2009 and 2010. All the sediments collected in 2011, except those from GO2 and GA, were slightly toxic or non-toxic.

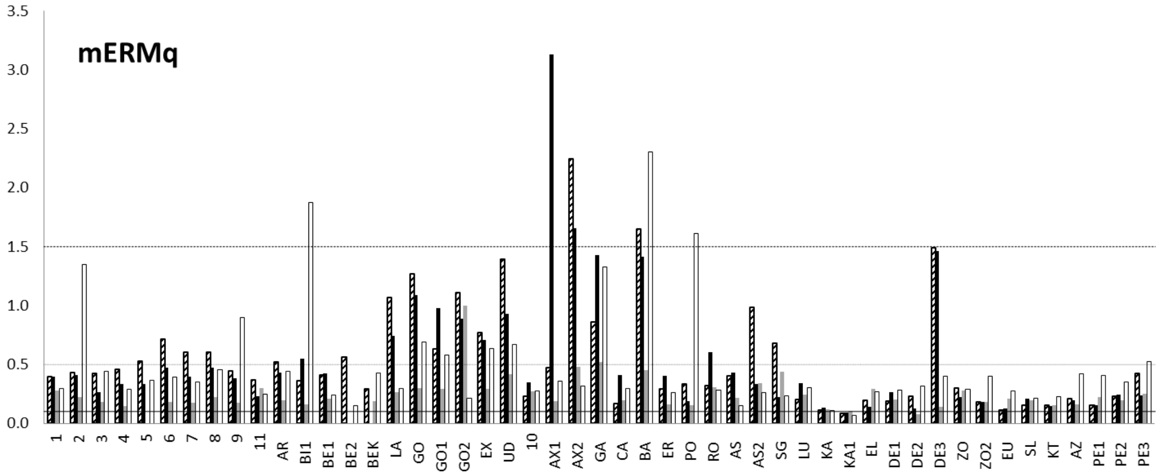

Figure 13. mean Effects Range-Median quotient (mERMq) values obtained for the sediments collected at different sampling sites in 2009 (striped bar), 2010 (black bar), 2011 (grey bar) and 2014 (white bar): 0-0.1 (non-toxic), 0.1-0.5 (slightly toxic), 0.5-1.5 (moderately toxic) and >1.5 (highly toxic).

4. CONCLUSIONS

The distribution of metals and metalloids in the sediments of the estuary of the Nerbioi-Ibaizabal estuary has substantially changed from 2009-2011 to 2014. The hotspots of metal pollution identified in 2009, 2010 and 2011 disappeared in 2014 and, in turn, new hotspots popped up close to the mouth of the estuary and in the dock of Portu. The works to construct in the Abra Bay a new place to dock for long tourist cruise liners may be partially responsible of this substantial change in the distribution of metals within the estuary. The surroundings of the effluent of the biggest waste water treatment plant operating in the area remains as a point of concern in 2014, probably due to punctual failures in the normal operation of the plant that result in direct release of substantial amounts of different metals to the river. The lowest metal concentrations were measured in sediments collected in 2011, probably as a result of a dilution effect caused by an increasing amount of fresh particulate matter that resulted from the heavy rains registered a few days before sampling. The origin of metals like Pb, Cd, Zn, Cu, As, Sn, V and Mn in the estuary is mainly anthropogenic, while Al, Co, Ni, Cr, Mg and Fe are probably natural. The presence of metals of anthropogenic origin results in moderately to highly toxic sediments in the hotspots of pollution. In January 2014, just a few months before the official beginning of the works to open the close channel of Deusto, the sediments upstream the point in which the Asua tributary joins with the main channel of the estuary presented low concentrations of all the elements considered, no important anthropogenic input of toxic metals and, consequently, a very slight possibility of being toxic.

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Chapter 10

HISTOPATHOLOGICAL ALTERATIONS IN AUTOCHTHONOUS OYSTERS DERIVED FROM THE OCCURRENCE OF METALS IN THE ESTUARY OF THE OKA RIVER (URDAIBAI, BASQUE COUNTRY)

CHAPTER 10

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ABSTRACT

Oysters have been frequently used as indicators of chemical pollution, specifically metal pollution, in estuaries. However, the question, *to which extent oysters are affected at histopathological levels by the presence of metals and metalloids in the surrounding environment?*, remains nowadays open to discussion. The estuary of the Oka River (Urdaibai, Basque Country) was selected in this work to investigate the possible correlation between the presence of metals in the estuary with the health status of autochthonous oysters. All the tidal part of the Oka River is included in the Reserve of the Biosphere of Urdaibai, so-declared by the UNESCO in 1984. Some polluting activities, however, are still operative in the area, resulting in considerable inputs of anthropogenic pollution to the estuary. Sediments and oysters were collected at seven different points of the estuary every three months from March 2010 to December 2011. The concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Sn, V and Zn were determined in all the samples. The results allowed us to draw conclusions on the geographical distribution of pollution in the estuary, and its evolution in time. In general, when high concentrations were obtained in sediments, low concentrations were obtained in oyster and *viceversa*. In addition, the gamete development, the presence of parasites and non-specific inflammations, the mean luminal radius to mean epithelial thickness (MLR/MET), the metallothionein content and the intralysosomal accumulation of metals (by autometallography) were also measured in oysters. In general, low to moderate concentrations of metals were found both in sediments and oysters. Accordingly, all the sediments were non-toxic or only slightly toxic, and the alterations observed in oysters at histological levels were not severe.

Keywords: Estuary; Oyster; Sediment; Metals; Urdaibai; Oka.

1. INTRODUCTION

Estuaries are areas of high concern due to their high biological productivity. At the same time, they use to be densely populated areas subjected to a great amount of potentially polluting activities like aquiculture, shipyard, industry and intensive agriculture and mining. Many pollutants of different origin and nature may consequently enter the estuary as a result of anthropogenic activity (Abrahim et al., 2007; Reboreda et al., 2008; Szava-Kovats, 2008). Metals and metalloids have been largely recognised as very toxic contaminants and their fate in estuaries has been investigated for decades (Pereira et al., 2015; Venkatramanan et al., 2014). The fate and reactivity of metals in estuaries highly depends on the physico-chemical conditions of the system. Metals can be stored in sediments, remobilised to the water column, adsorbed onto suspended particulates, associated to fulvic and humic acids or accumulated by living organisms in their tissues (Dekov et al., 1998; Franco et al., 2002). The extent of these processes is governed by a wide number of environmental variables. As estuaries are highly dynamic systems with a remarkable variability in time and space of parameters such as salinity, pH, dissolved oxygen, redox potential, amount of suspended particulate matter and organic carbon, the partition of metals within different compartments is also complex and difficult to predict (van Ryssen et al., 1999).

Sediments, water and molluscs like oysters are highly connected actors in estuaries. As oysters live in close contact with sediments and obtain food by filtering water, it becomes evident that the metal content in these three compartments must be highly interconnected. In fact, sediments and oysters have been frequently used to monitor metal pollution in estuarine waters (Fdez-Ortiz de Vallejuelo et al., 2014; Gredilla et al., 2015; Raposo et al., 2009). Both sediments and oysters show a high capacity to accumulate the low concentrations of trace elements usually found in water. Sediment is more conservative than water, as it accumulates historical data on processes within water bodies and the effect of anthropogenic factors on these processes. On the other hand, bioaccumulation and biomagnification processes may lead to extremely high concentrations of metals in oyster tissues.

The presence of metals in oyster tissues may result in histopathological alterations, metallothionein induction and accumulation in specific parts of the cell. The digestive gland of molluscs is a key organ in relation with accumulation, detoxification and elimination of xenobiotics including metals (Moore and Icarus Allen, 2002), and is the most studied organ in pollution monitoring programmes. Histopathological alterations are effect biomarkers that allow identifying pathological lesions in tissues induced by environmental contamination and disease (Aarab et al., 2011). The use of histopathological examination techniques in wild molluscs provides sensitive and useful indications of the overall health status of bivalves and can be used for the assessing of historical exposure, or effect, of a contaminant (Izagirre et al., 2014). Metallothioneins (MT) are cysteine-rich metal binding proteins and have been widely used as markers of metal contamination (Aldo Viarengo Bruno Burlando Francesco Dondero Anna Marro Rita, 1999) since they are involved in the detoxification of heavy metals, free radical scavenging, and in inflammatory responses (Gueguen et al., 2003). Changes in MT levels have been described after cadmium, copper, zinc, mercury or lead exposure in a great variety of aquatic organisms (Sheehan and Power, 1999). The presence of metals in selected cell-types can be visualized with the aid of histochemical techniques such as autometallography (AMG). This technique allows the localization of metal ions (as black silver deposits, BSD) in biological tissues and has been used to determine metal levels in aquatic molluscs (Soto et al., 2002). The use of cell and tissue level biomarkers can be influenced by the season and related changes such as gamete development, food availability, or general metabolic activity (Bocchetti and Regoli, 2006). Therefore, the understanding of natural variability of biomarkers is necessary in order to make correct interpretations of the obtained results.

In this work we have measured the concentration of selected metals and metalloids in sediments and oysters collected at different sites of the estuary of the Oka River (Urdaibai Reserve of the Biosphere, Basque Country) with the aim i) to investigate the occurrence and geographical distribution of metal pollution within the estuary, ii) to determine the overall health status of the estuary based on cell and tissue level biomarkers measured in oysters, iii) to check if the concentration of metals in sediments and oysters is correlated or not, and iv) to study if the existence of alterations in oysters depends or not on the presence of specific metals in their tissues or in sediments.

2. MATERIALS AND METHODS

2.1 STUDY AREA, SAMPLING AND SAMPLE TREATMENT

The area of Urdaibai (Bizkaia, Basque Country), including the estuary of the Oka River, was declared Reserve of the Biosphere by the UNESCO in 1984 (Fig.1). The estuary is generally accepted as a clean area and major pollution inputs have been related with urban discharges and dredging due to the presence of a shipyard since 1943 in Murueta (Bartolome et al., 2006). A remarkable industrial activity remains nowadays, however, in the surroundings of Gernika-Lumo (~17000 inhabitants), the most important town in the area. Leisure activities, including recreational boats and sport fishing, are also important. During summer time, traffic exhaust considerably increases due to the massive affluence of visitants to the beaches around. The presence of two marinas (in Sukarrieta and Laida) and a little port with a few middle-size fishing boats in Mundaka (~2000 inhabitants, located in the left bank close to the mouth of the estuary) also contribute as potential sources of contaminants. The concentrations of chemicals in sediments, fishes and molluscs from the estuary have been measured (Bustamante et al., 2012; Puy-Azurmendi et al., 2010; Zabaljauregui et al., 2007) and the existence of harmful effects on organisms derived from the presence of these chemicals have also been investigated (Orbea et al., 2002; Puy-Azurmendi et al., 2013; Uriarte and Villate, 2004). In general low metal pollution levels have been detected in oysters inhabiting the estuary and their overall health state has been described as fairly good.

Sediments and oysters were sampled at 7 sampling sites (Mundaka MK, Laida LA, Sukarrieta SU, Axpe AX, Kanala KA, Murueta MU and Arteaga AR) of the tidal part of the Oka River at low tide every three months from March 2010 to December 2011. Oysters from the December 2011 campaign were accidentally lost. The pH, temperature, electrical conductivity, redox potential and dissolved oxygen of water were measured *in situ* by means of a precalibrated YSI 556 multiparametric probe (YSI Environmental, Yellow Springs, Ohio). Salinity and total dissolved solids (TDS) were further estimated from electrical conductivity. The geographical location of the sampling sites together with their most outstanding features are summarised in table 1.

Figure 1. Sampling sites in the estuary of the Oka River (Urdaibai Reserve of the Biosphere): Mundaka MK, Laida LA, Sukarrieta SU, Axpe AX, Kanala KA, Murueta MU and Arteaga AR.

Table 1. Geographical location (Universal Transversal Mercator x and y coordinates, UTM 30T) and basic description of the sampling sites.

Identification	Code	X coordinate	Y coordinate	Characteristics
Mundaka	MU	524449	4806206	Inside a little port with fishing and recreational activity
Sukarrieta	SU	524784	4804410	Close to a marina
Axpe	AX	525127	4803205	Where a little tributary joins the main channel
Murueta	MU	525591	4801170	Close to a shipyard
Laida	LA	525878	4804747	Close to a marina
Kanala	KA	526065	4803243	No remarkable activity
Arteaga	AR	527238	4800249	No remarkable activity

About 250 g of surface sediment (upper 2-3 cm) were collected by hand at each site using latex gloves. Regarding oysters, 40 individuals (*Crassostrea gigas*, 8±2 cm longitudinal axe) were randomly collected by hand at each site in a ~10×10 m area. Sediment and oyster samples were transported to the laboratory in zip plastic bags inside a cool box. Twenty oysters were reserved for chemical analysis. The pretreatment and chemical analyses of the samples were carried out according to the procedures described in the Chapter 2, experimental. The concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Sn, V and Zn were measured in the sediment and oyster extracts by ICP/MS.

The NIST 1646a (estuary sediment) and SRM 2976 (mussel tissue) certified reference materials, all from the National Institute of Standards and Technology, were used for QC/QA purposes. Recoveries between 80% and 120% and repeatabilities below 5% in RSD were obtained in all the cases. The detection limits (LOD) estimated as the average plus three times the standard deviation obtained from the repetitive analysis of 8 independent procedural blanks were as follows for sediments and oyster, respectively: Al (LOD_{sed}: 0.76, LOD_{oys}: 0.23), As (LOD_{sed}: 0.36, LOD_{oys}: 0.12), Cd (LOD_{sed}: 0.11, LOD_{oys}: 0.063), Co (LOD_{sed}: 0.14, LOD_{oys}: 0.008), Cr (LOD_{sed}: 0.70, LOD_{oys}: 0.002), Cu (LOD_{sed}: 0.08, LOD_{oys}: 0.001), Fe (LOD_{sed}: 0.23, LOD_{oys}: 0.008), Mg (LOD_{sed}: 0.91, LOD_{oys}: 0.29), Mn (LOD_{sed}: 0.61, LOD_{oys}: 0.003), Ni (LOD_{sed}: 0.30, LOD_{oys}: 0.004), Pb (LOD_{sed}: 0.21, LOD_{oys}: 0.021), Sn (LOD_{sed}: 0.06, LOD_{oys}: 0.001), V (LOD_{sed}: 0.14, LOD_{oys}: 0.002) and Zn (LOD_{sed}: 1.1, LOD_{oys}: 0.18).

The other twenty oysters were used for biological measurements. A portion of the digestive gland/gonad complex of each oyster was dissected and fixed in formalin, dehydrated in a series of ethanol baths, paraffin embedded and sectioned at 3-5 µm thickness for histopathological examination and autometallography. Another portion of the digestive gland was dissected and frozen in liquid nitrogen for biochemical determination of MTs.

2.2. HISTOPATHOLOGICAL MEASUREMENTS

Oyster tissues were examined for health parameters related with reproductive and physiological conditions, inflammatory and non-specific pathologies and those associated with pathogen and parasite infections. The gamete developmental stage was determined according to Kim et al., 2006 (Kim et al., 2006). The presence of parasites and non-specific inflammation were scored as absent (0) or present (1) and then data was transformed into percentages. 10 individuals were examined from each site and season.

2.3. PLANIMETRICAL ANALYSES OF THE EPITHELIAL THICKNESS

In order to quantify the structure of the digestive tubules, a planimetric procedure was applied on hematoxilyn/eosin stained sections of digestive gland tissue. A total of 10 tubule profiles per individual were recorded in an image analysis system (Visilog 5.4 Noesis) attached to an Olympus BX50 light-microscope. Finally, the MLR (mean luminal radius) to MET (mean epithelial thickness) (MLR/MET) ratio was calculated (Cajaraville et al., 1992).

2.4. AUTOMETALLOGRAPHY

Intralysosomal accumulation of metals was determined in paraffin embedded sections following the autometallographical procedure (Danscher, 1984) modified by Soto, *et al.* (Soto et al., 1998). Paraffin sections were covered with a photographic emulsion (Ilford Nuclear Emulsion L4, Ilford, Mobberley, England) under safety light conditions. After drying for 45 min in total darkness, sections were rinsed in a developer bath (1:5, b/w Ultrathin Tetenal. Thetenal AG & Co, Norderstedt, Germany) for 15 min, rinsed in a stop bath (1% acetic acid) for 1 min, and finally rinsed in a fixative bath (1:10, b/w Agefix Agfa.

Agfa-Gevaert N.V., Mortsel, Belgium) for 10 min. Sections were mounted in Kaiser's glycerol gelatine (Merck). Metals were developed as black silver deposits (BSD) and the previously scheduled quantification could not be performed due to the special metal distribution in the digestive gland tissue.

2.5. METALLOTHIONEIN CONTENT

Metallothionein content was measured according to Viarengo et al. (Viarengo et al., 1997) and UNEP/RAMOGE (1999) in digestive gland of oysters stored at -80 °C. Reduced glutathione (GSH) was used as reference standard to estimate MT concentrations the results are expressed as µg MT/g tissue ww.

2.6. STATISTICS

Statistical analysis of the biological data was carried out by SPSS/PC+ statistical package (SPSS Inc., Microsoft Co.). For each variable studied, one-way ANOVA and subsequent Duncan's test for multiple comparisons between pairs of mean values were applied to detect significant ($p<0.05$) differences between experimental groups. Principal Component Analysis (PCA) of chemical data was performed by means of The Unscrambler Program (v. 9.2 Camo, Oslo, Norway). Concentrations below the detection limit were substituted by half of the detection limit. The dataset under study was centred and scaled before PCA.

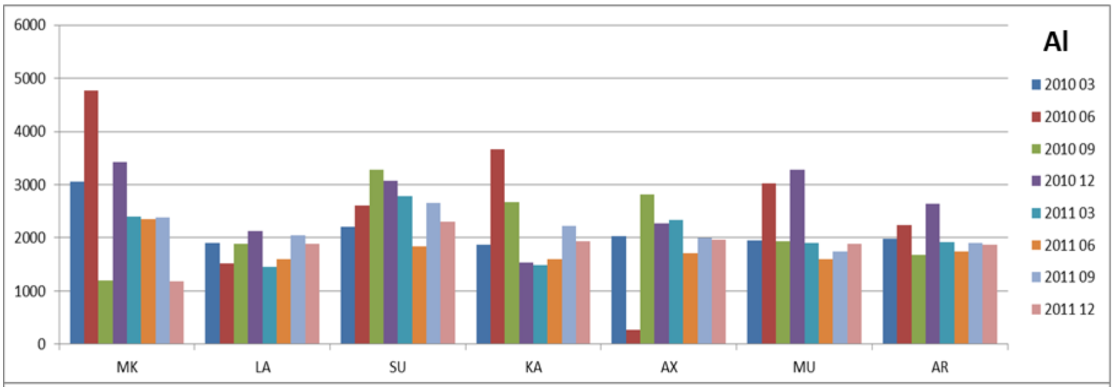
3. RESULTS

3.1. PHYSICAL-CHEMICAL PARAMETERS IN WATER

The average pH, electric conductivity, redox potential and amount of dissolved oxygen (%) measured in water in 2010 and 2011 were, respectively, 7.61±0.27-7.53±0.31, 18.26±17.71-40.66±10.15 mS/cm, 291±58-300±98 mV and 79±21-79±6 %. All these values are typical from clean estuaries and indicate well oxygenated waters. They did not change drastically from 2010 to 2011, except in the case of electrical conductivity. Salinity of water at the time of sampling was consequently higher in 2011 than in 2010.

3.2. CONCENTRATION OF METALS AND METALLOIDS IN SEDIMENTS

The concentrations found in sediments are summarised in Fig. 2. The concentration of Cd was below the detection limit in all the samples. Looking in detail at the results five different groups of elements with similar characteristics can be differentiated. The concentration of Mg in the sediments of Urdaibai is clearly influenced by the proximity to the ocean, with no clear seasonal trend and similar values in 2010 and 2011. Ni and Cr show a distinctive behaviour, with higher concentrations (especially Cr) in the most upstream part of the estuary (AR and MU), probably due to the industrial activity in the surroundings of Gernika and the shipyard in Murueta. The concentrations of these elements in sediments show no clear seasonal trend and they are rather similar in 2010 and 2011. As, Co and Fe present similar concentrations within sites without a clear seasonal trend, but with higher values in 2010 than in 2011 (very clear for Co and Fe, not so clear for As). Finally, the concentrations Al, Mn, Cu, Pb, Sn, V and Zn are systematically higher in sediments from MK and SU (very marked for Zn, Sn, and especially Cu, in MK) than from the rest of sites, and they are clearly higher in 2010 than in 2011. Higher concentration of these metals could be explained by the fishing activities in the port of Mundaka and the recreational uses of the marina in Sukarrieta. Al and Mn, however, do not show a clear seasonal trend while Cu, Pb, Sn, V and Zn systematically present higher concentrations in summer (March and June) than in winter (December and March). The concentrations of these last 5 elements are highly correlated ($c_{corr} > 0.6$) in sediments. It is to be highlighted that, except in the case of As (2011>2010) and Mg (2010~2011), the concentrations measured were higher in 2010 than in 2011.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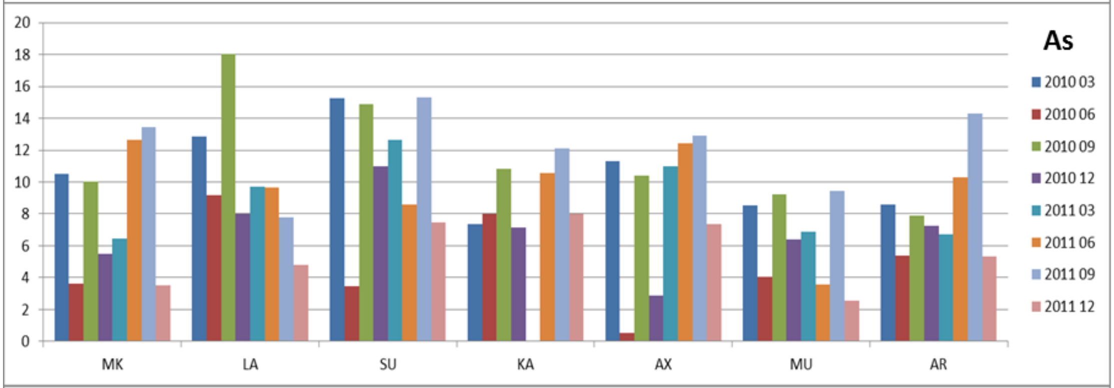
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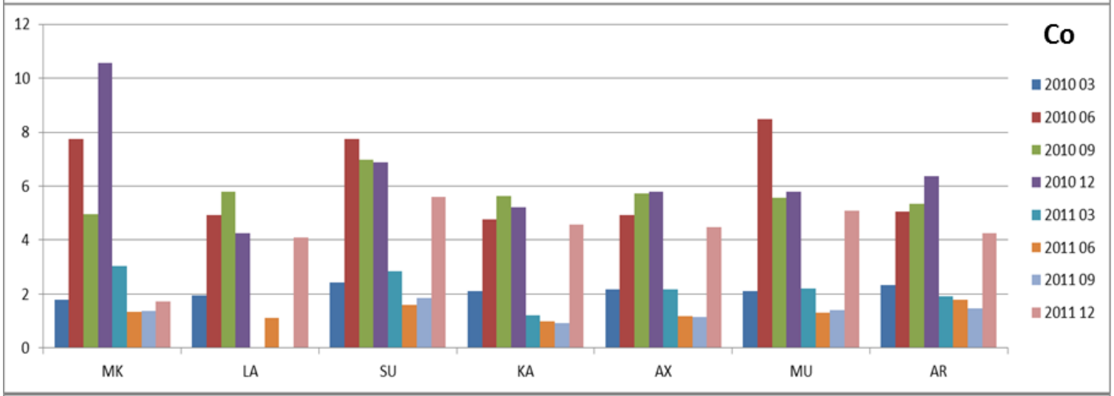
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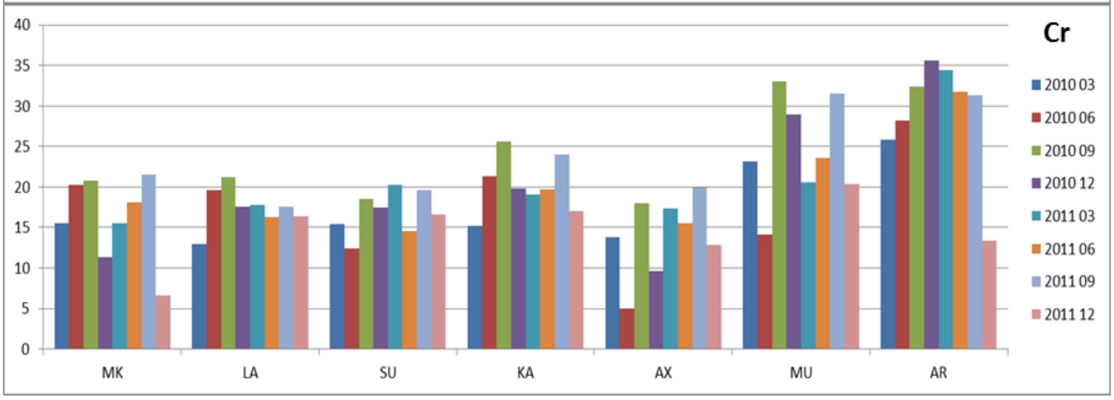
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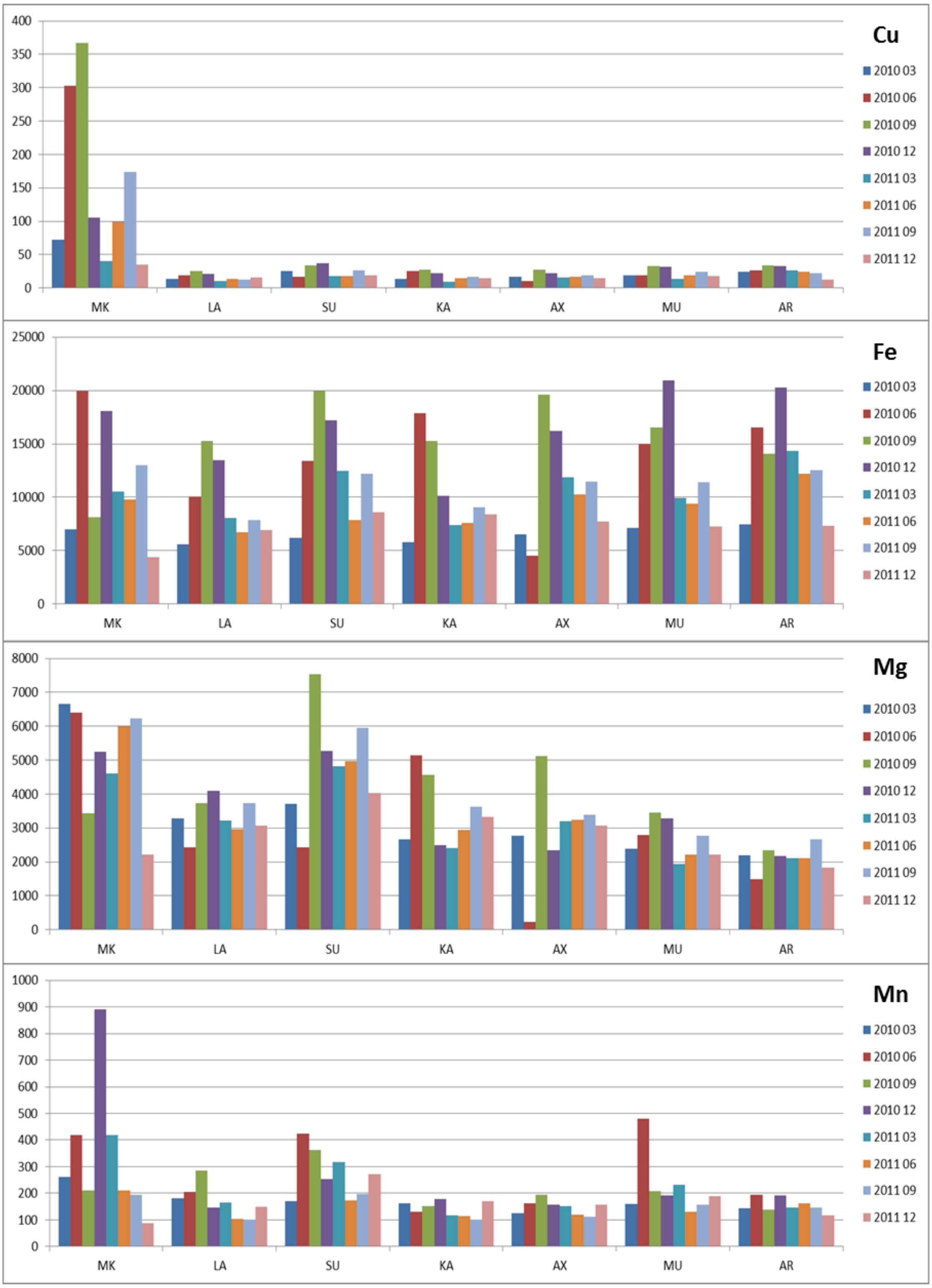
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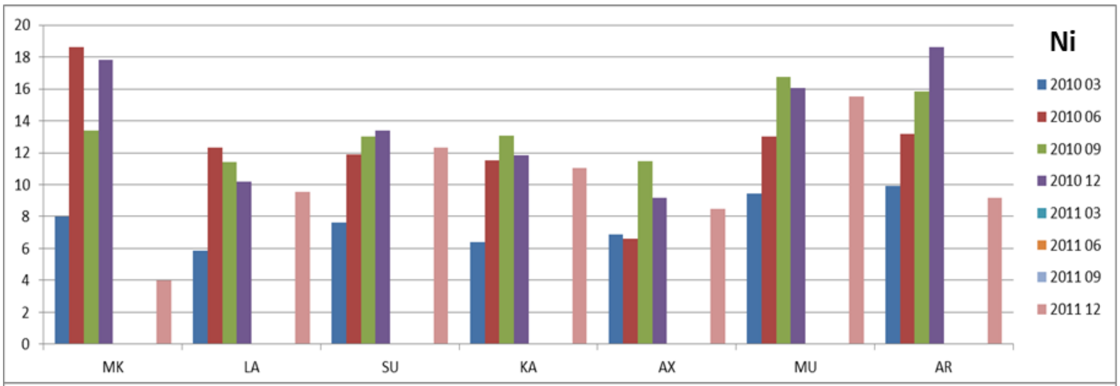
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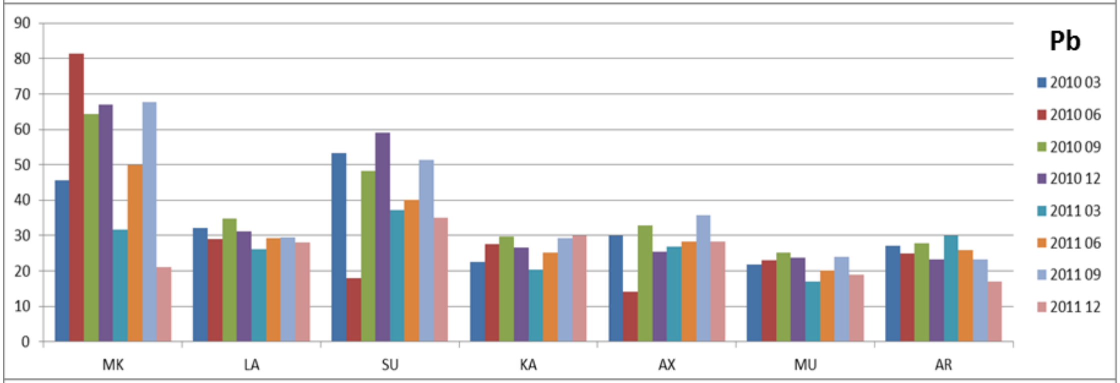
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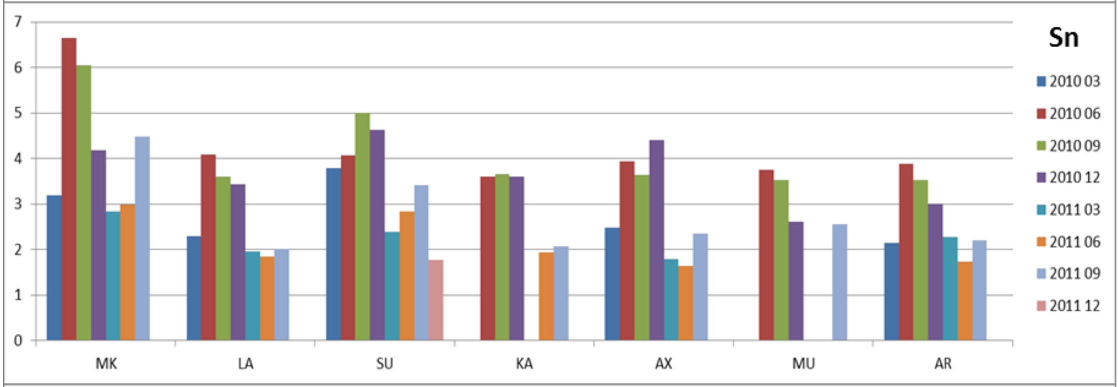
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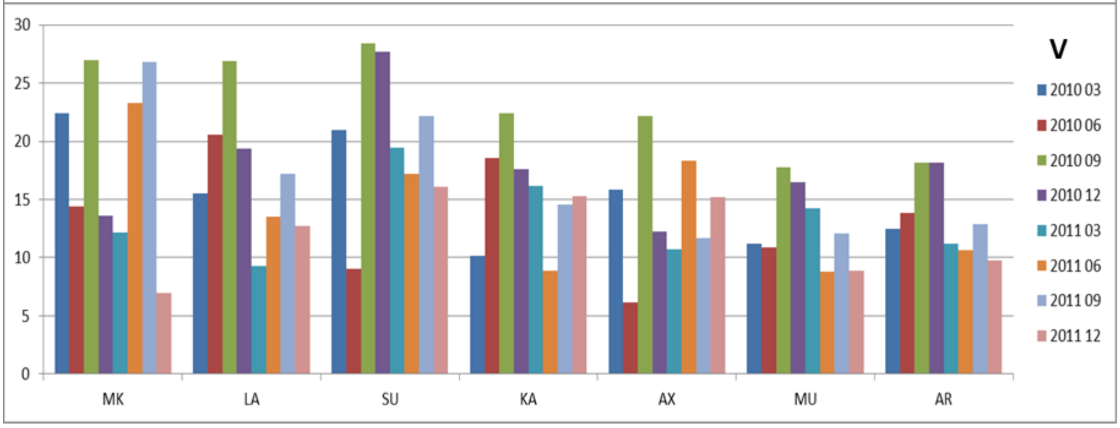
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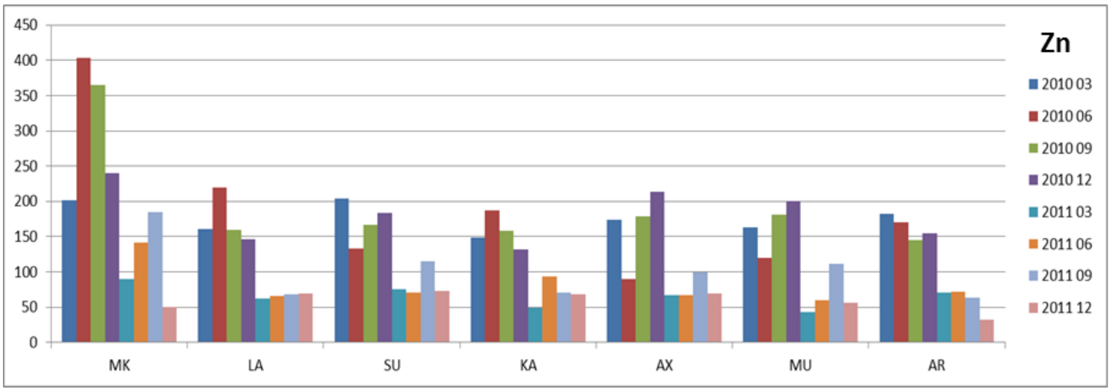


Figure 2. Metal concentrations (mg·kg^{-1}) measured in sediments collected at different sites and sampling campaigns in the estuary of the Oka River (Urdaibai Reserve of the Biosphere, Basque Country).

Principal Component Analysis (PCA) of the concentration data matrix of the sediments (56 rows, one per sampling site and sampling campaign; and 13 columns, one per element measured) was also carried out. Cd was removed from the data matrix because its concentration was below the detection limit in all the sites and campaigns. The data matrix was centred and scaled before analysis. The model finally selected explained 71% of the total variance using three PCs (PC1: 41%; PC2: 17%; PC3: 13%). The scores and loadings plots over the PC1-PC2 and PC1-PC3 can be seen in Fig. 3. The result highlights the clear difference between the concentrations measured in 2010 and 2011, and the distinctive behaviour of MK and SU sampling sites compared to the rest.

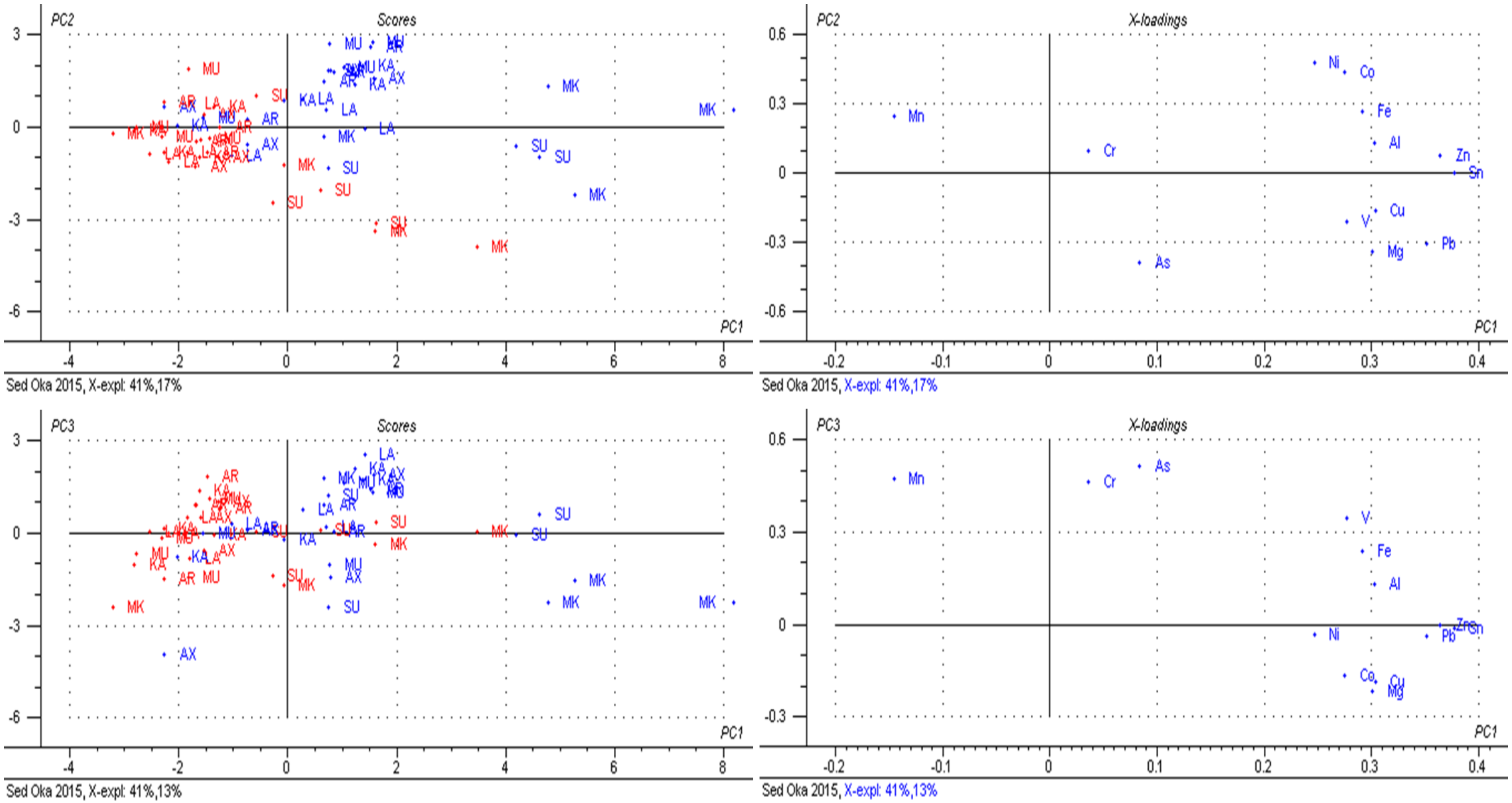


Figure 3. Scores and loadings plots obtained after PCA of the data matrix of the sediments. Samples from 2010 sampling campaigns are in blue, and those from 2011 campaigns are in red. MK: Mundaka, LA: Laida, SU: Sukarrieta, AX: Axpe, KA: Kanala, MU: Murueta, AR: Arteaga.

The mean Effects Range-Median quotients (mERMq) were also calculated according to Eq. 1 (Long et al., 2006) in order to estimate the toxicity associated to each individual sediment sample.

$$mERMq = \frac{1}{N}\sum_{i}^{N}\frac{c_i}{ERM_i} \qquad \text{(Eq. 1)}$$

where N is the amount of pollutants considered in the calculation, c_i is the actual concentration of the i pollutant and ERM_i is the concentration estimated for the i pollutant above which toxic effects are expected. The concentrations of As, Cd, Cr, Cu, Pb, Ni and Zn were considered in the calculation. The other elements are considered to be non-toxic, or toxic only at extremely high concentrations. mERMq values in the range 0-0.1, 0.1-0.5, 0.5-1.5, and >1.5 indicate a probability of being toxic of, 9% (non-toxic), 21% (slightly toxic), 49% (moderately toxic) and 76% (highly toxic), respectively. As it can be seen in Figure 4, all the sediments considered are non-toxic or slightly toxic. Sediments collected in 2010 are more toxic than those collected in 2011. The most toxic ones correspond to those sampled in MK. In general, higher toxicity was recorded in summer than in winter.

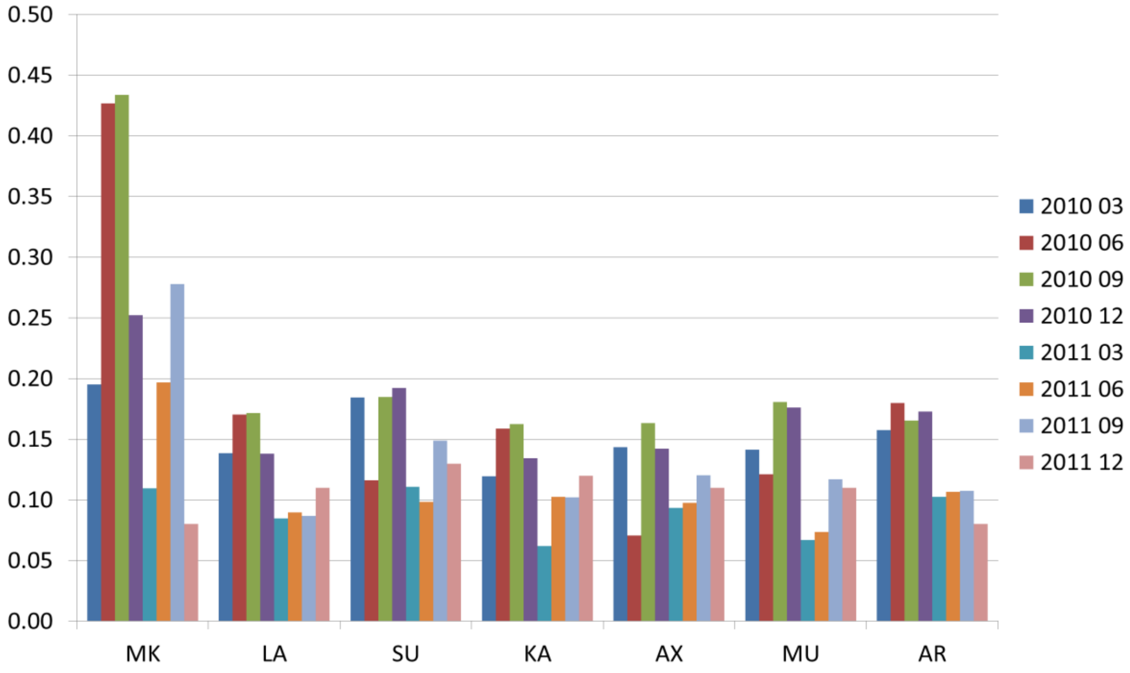
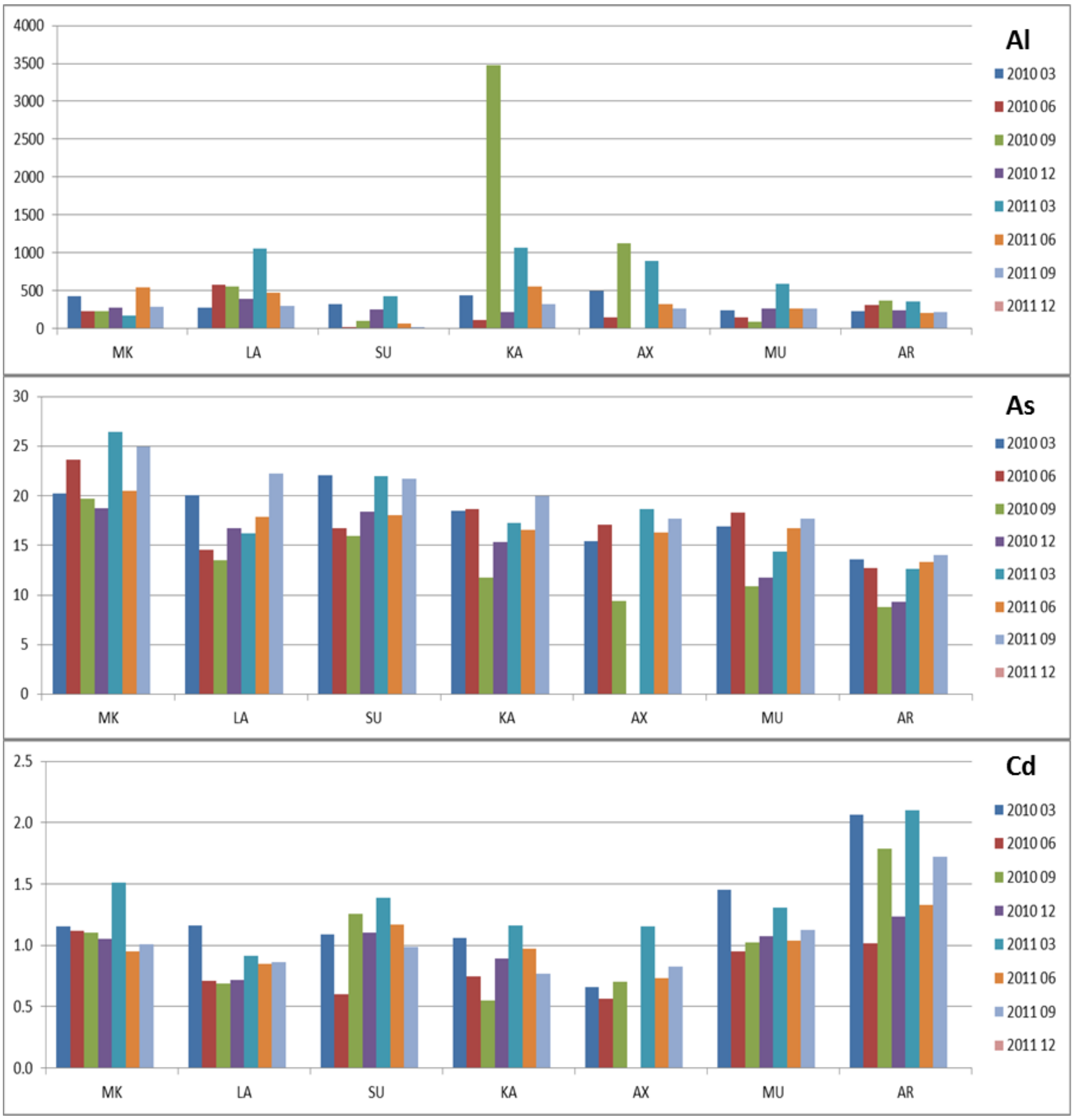


Figure 4. Toxicity (expressed as mERMq) of the sediments collected in the estuary of the Oka River at different sites and sampling campaigns. mERMq: 0-0.1: non-toxic; 0.1-0.5: slightly toxic; 0.5-1.5: moderately toxic; >1.5: highly toxic.

3.3. OYSTERS

3.3.1. CONCENTRATION OF METALS AND METALLOIDS IN OYSTERS

The metal concentrations found in oysters are summarised in Figure 5. Similar concentrations were found in oysters collected in the estuary in the 1990-2000 period (Franco et al., 2002) (except for As, 2.5 in (Franco et al., 2002) and 17.0 mg·Kg^{-1} in this work) and in 2002-2004 (Raposo et al., 2009). When differences between years (2010 vs. 2011) were found in metal concentrations (As, Cd, Co, Mn, Ni, Pb, Sn and Zn), those measured in samples from 2011 were always higher than those measured in oysters from 2010. The existence of seasonal trends in metals concentrations was not clear, but when detected (Cd, Cu, Fe, Mn, Ni, Pb and Zn), lower values were observed in summer than in winter, except for Mn. It is worth to remember that, for these two observations, the opposite behaviour was detected in the case of sediments. Concerning the spatial distribution of metals in oysters, Al, Co, Fe, Cr, and Mn, showed rather constant concentrations within sampling sites, only with slightly higher values in KA (Al, Co, Fe) and KA and MU (Cr, Mn). The concentrations of Cd and Ni were significantly higher in the upper part of the estuary (AR and MU), and those of As and Mg decreased with the distance to the ocean. Finally, Zn and Cu presented concentrations markedly higher in MK than in the rest of sites, and Pb, V and Sn both in Mk and KA. Specifically, it is to be highlighted the extremely high concentrations of Al, Co, Fe, Pb and V found in oysters collected at KA in September 2010. The concentrations of these elements (c_{corr}: 0.74-0.97), and those of Cu and Zn (c_{corr}: 0.92) were highly correlated.

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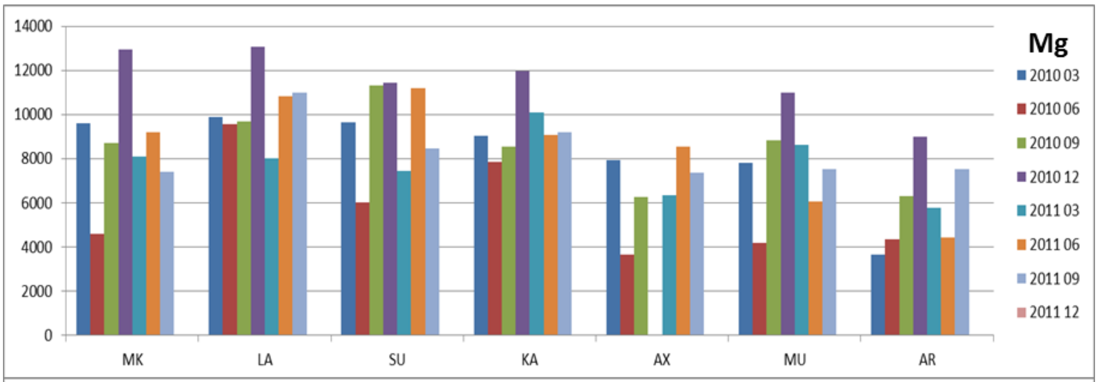
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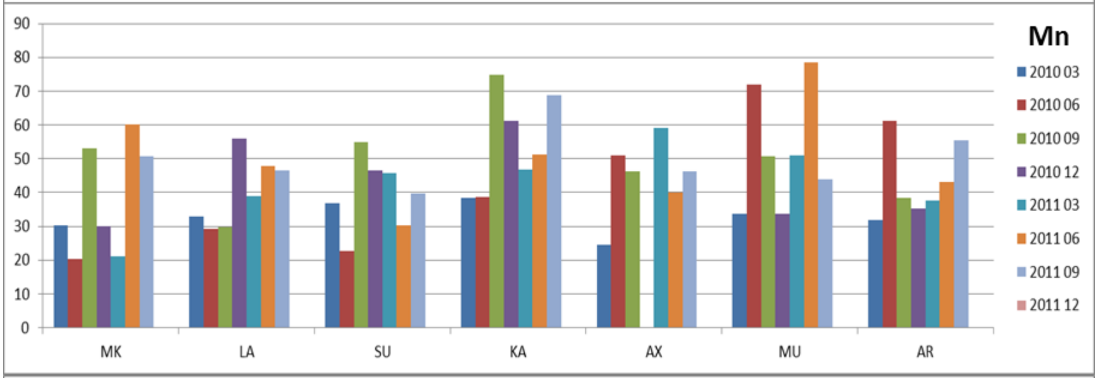
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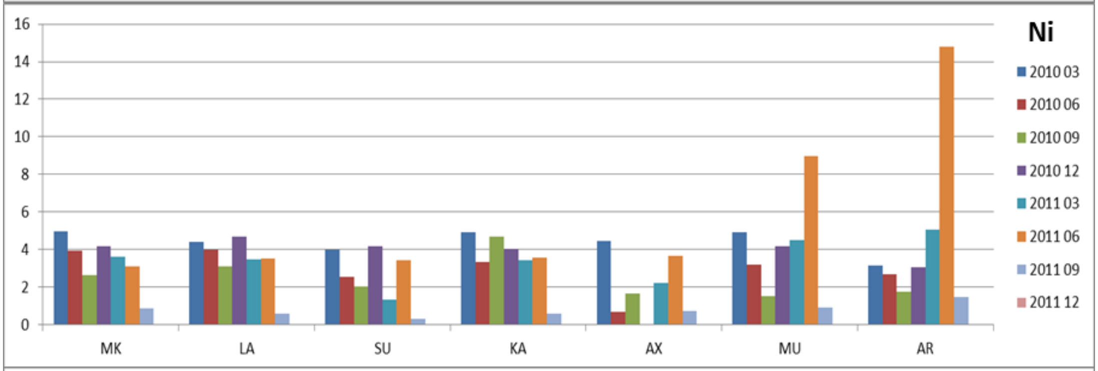
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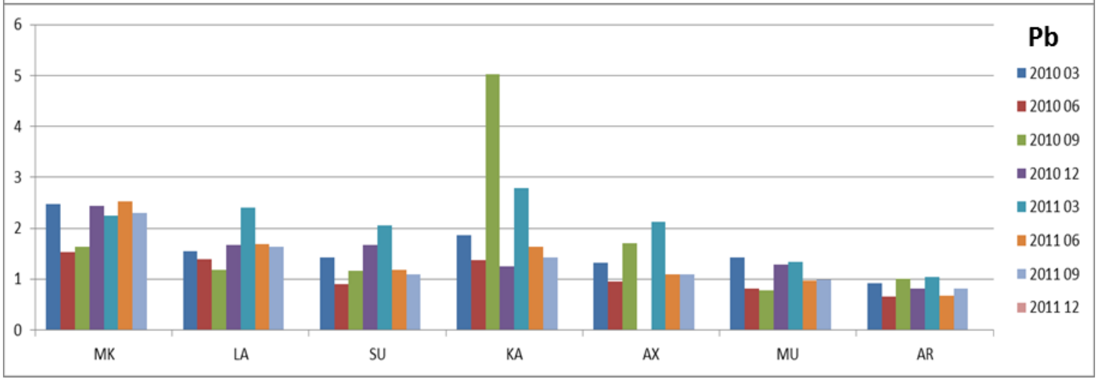
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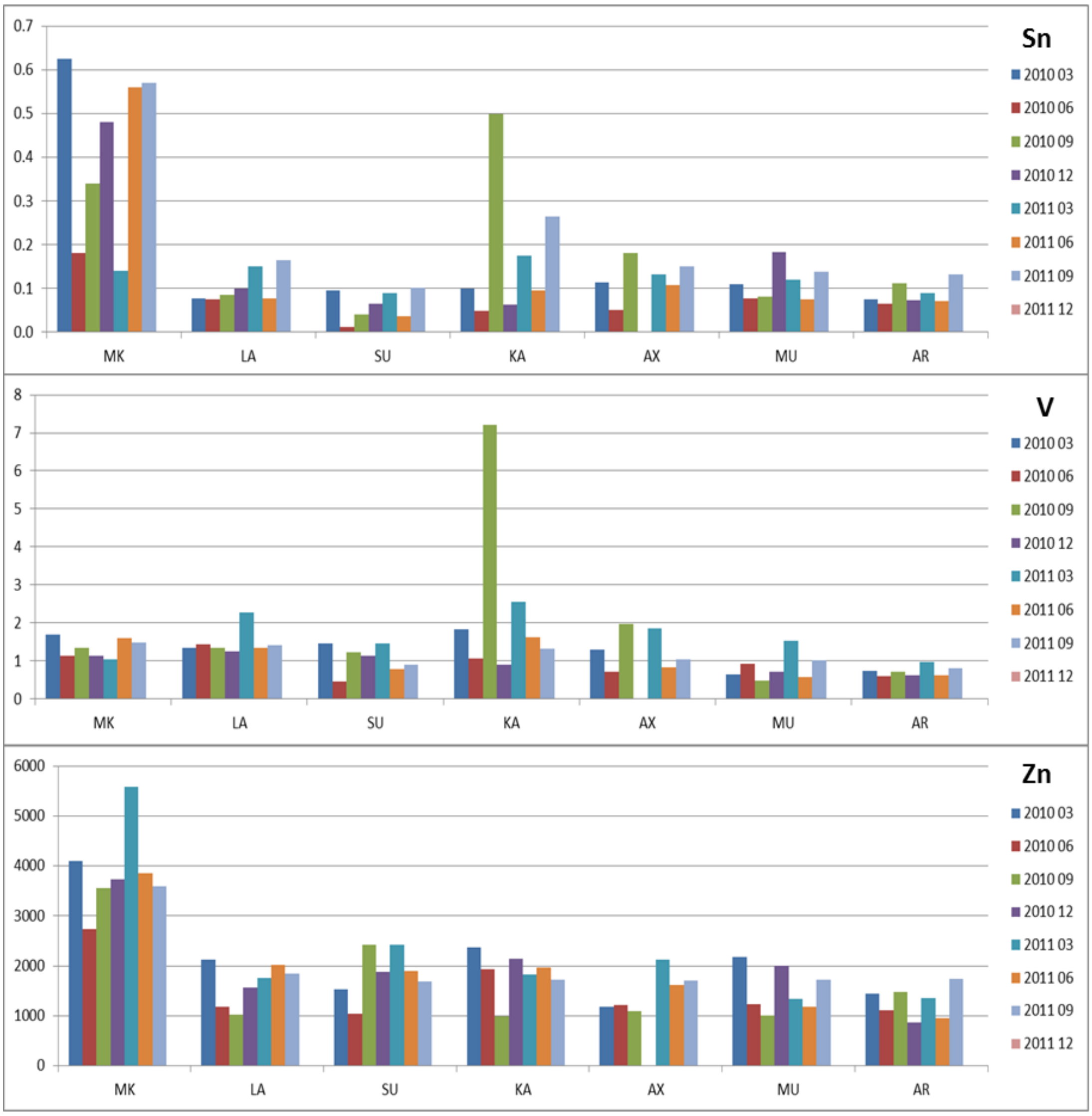

Figure 5. Metal concentrations (mg·kg^{-1}) measured in oysters collected at different sites and sampling campaigns in the estuary of the Oka River (Urdaibai Reserve of the Biosphere, Basque Country).

The data matrix of the oysters (49 rows, one per sampling site and sampling campaign; 14 columns, one per element considered) was subjected to Principal Component Analysis (PCA). The data matrix was centred and scaled before PCA. A final model with 3 PCs was finally selected to explain the variability of data. The model explained 70% of the total variance (PC1, 37%; PC2, 23%; PC3, 10%). The scores and loadings obtained are shown in Figure 6. Most of the samples are grouped together except those from the MK sampling site, which are characterised by high concentrations of As, Cu and Zn, and that collected at KA in September 2010, which presents high concentrations of Al, Cu, Fe, Pb and V. This is in good agreement with the information obtained after direct observation of data (Figure 5).

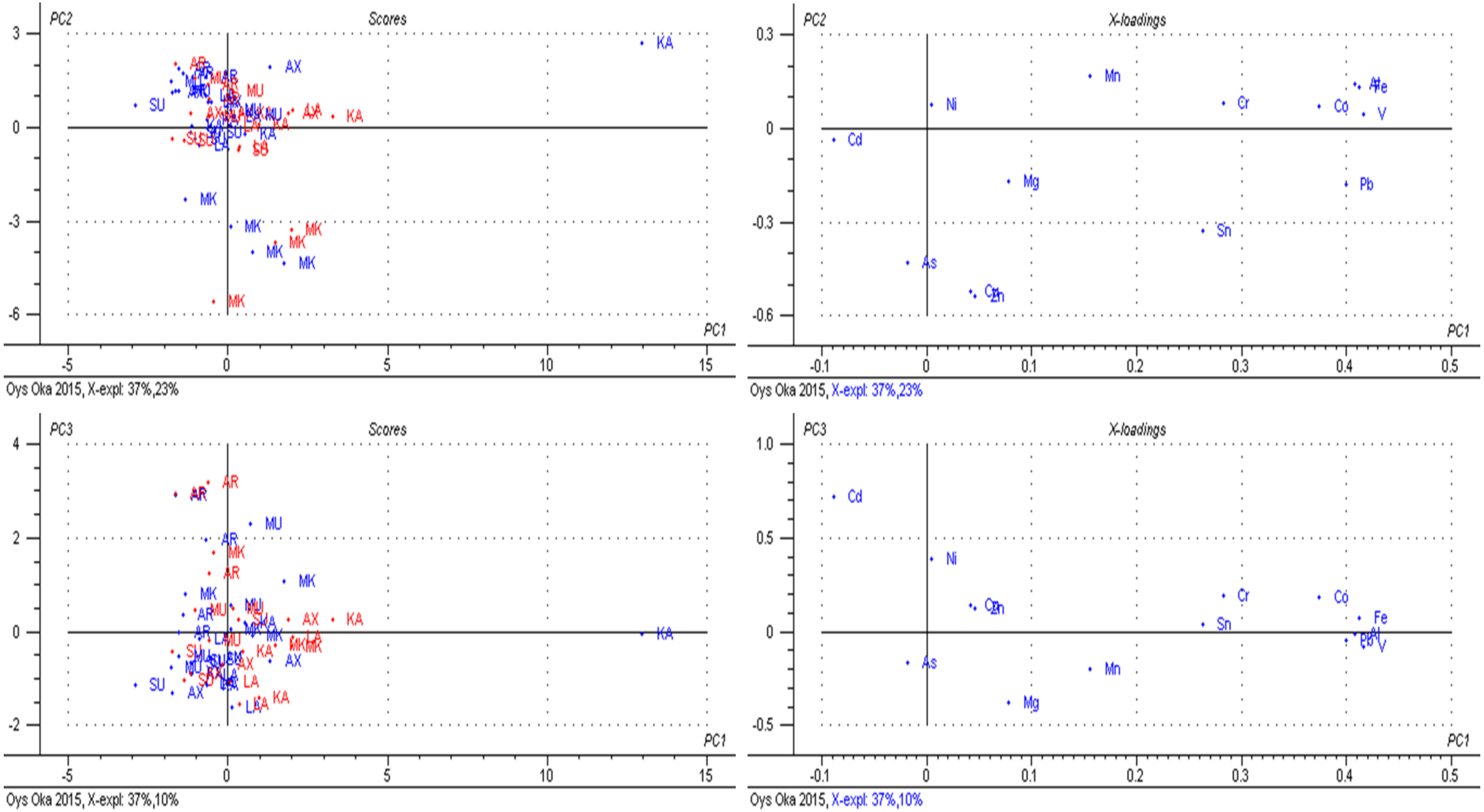

Figure 6. Scores and loadings plots obtained after PCA of the data matrix of the oysters. Samples from 2010 sampling campaigns are in blue, and those from 2011 campaigns are in red. MK: Mundaka, LA: Laida, SU: Sukarrieta, AX: Axpe, KA: Kanala, MU: Murueta, AR: Arteaga.

3.3.2. BIOLOGICAL MEASUREMENTS IN OYSTERS

3.3.2.1. GAMETE DEVELOPMENTAL STAGE

Gonad index values indicated clear seasonal variation in oysters with lower values in March and increasing values in June and September, with gonads in post-spawning stage in November. In general, few differences were recorded between sites

3.3.2.2. HISTOPATHOLOGICAL ALTERATIONS

Regarding inflammatory responses such as haemocytic infiltrations, inflammations and granulocytomes more differences were observed between the different seasons than between different sites (Figure 7). According to the results, oysters collected in March presented the highest prevalence of inflammatory responses with high prevalence of haemocytic infiltrations and inflammation and in the year 2011 also higher levels of granulocytomes were recorded. In the remaining sampling times lower levels of inflammatory responses were observed in oysters but a decrease (except in March) of these lesions are observed from the year 2010 to the year 2011. When different sites are compared, in general Axpe presented the highest levels of inflammatory responses both in 2010 and 2011. On the other hand, Mundaka presented very low prevalence in 2010.

Histopathological analyses also revealed the presence of some parasites within the digestive gland and gonad of oysters. In general gregarines such *Nematopsis*, some mitcrosporidian and digenean metacercariae and the copepod *Mytilicola intestinalis* were observed. However, measured prevalences and intensities were in general low (below 20% prevalence except for *Nematopsis*) and no clear seasonal trend was observed. Only in Sukarrieta metacercariare was regularly observed and slightly higher metacercarian presence was detected in March and June 2011.

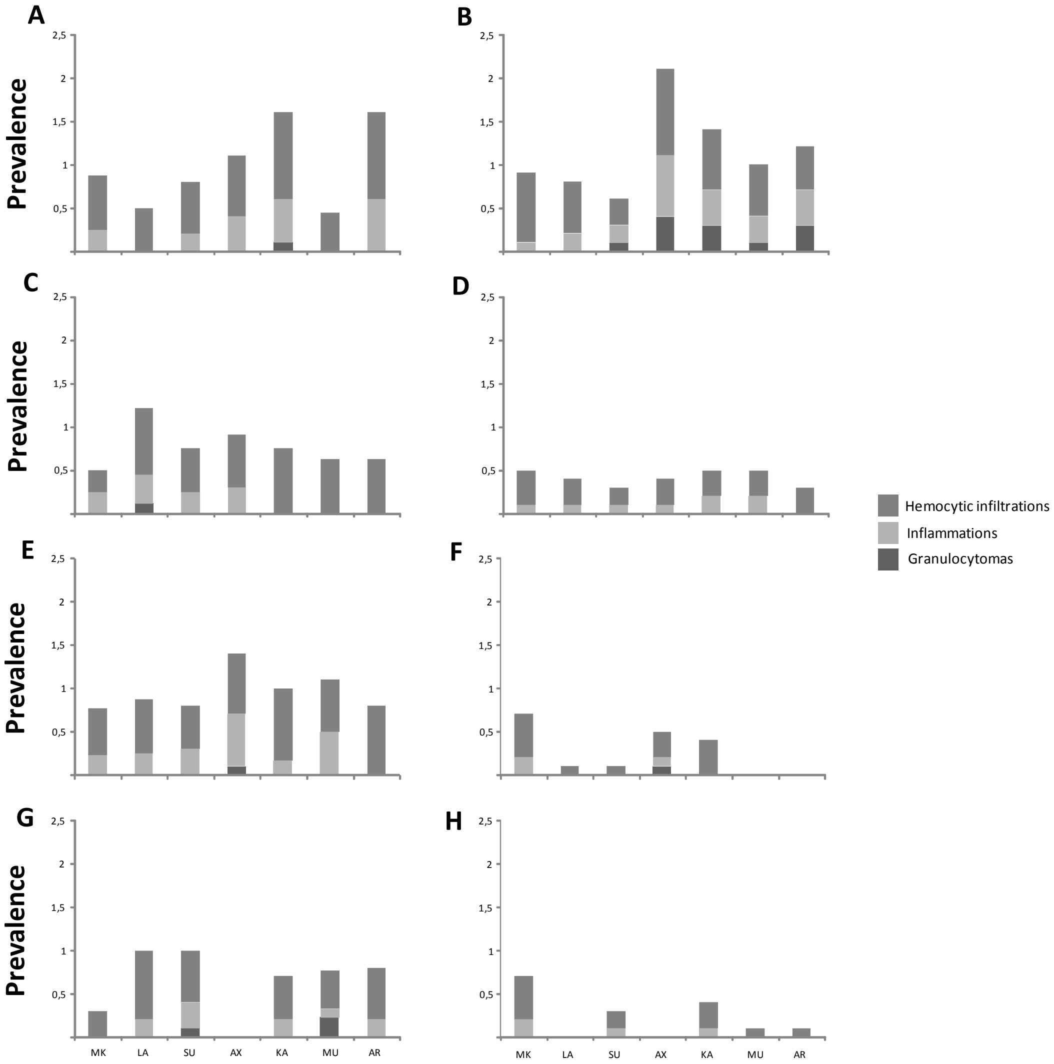

Figure 7. Prevalence of inflammatory responses (Granulocytomes, inflammations and heamocytic infiltrations) in the digestive gland/gonad of oysters collected in March (A), June (C), September (E) and December (G) 2010 and March (B), June (D), September (F) and December (H) 2011.

3.3.2.3. TISSUE LEVEL BIOMARKERS

Quantitative changes in the morphology of the digestive alveoli epithelium were determined after the MLR/MET measurement (Figure 8). In general few differences between sites are observed with only some significant differences recorded in March and June. On the other hand, more relevant differences are observed when comparing 2010 and 2011 years. In March very few differences are observed but in June, September and December, higher values (and also higher variability) are recorded in 2011 when comparing to 2010 with significant differences between the same site in different years. In general all values are under 2,0 indicating some affection but no strong levels of stress.

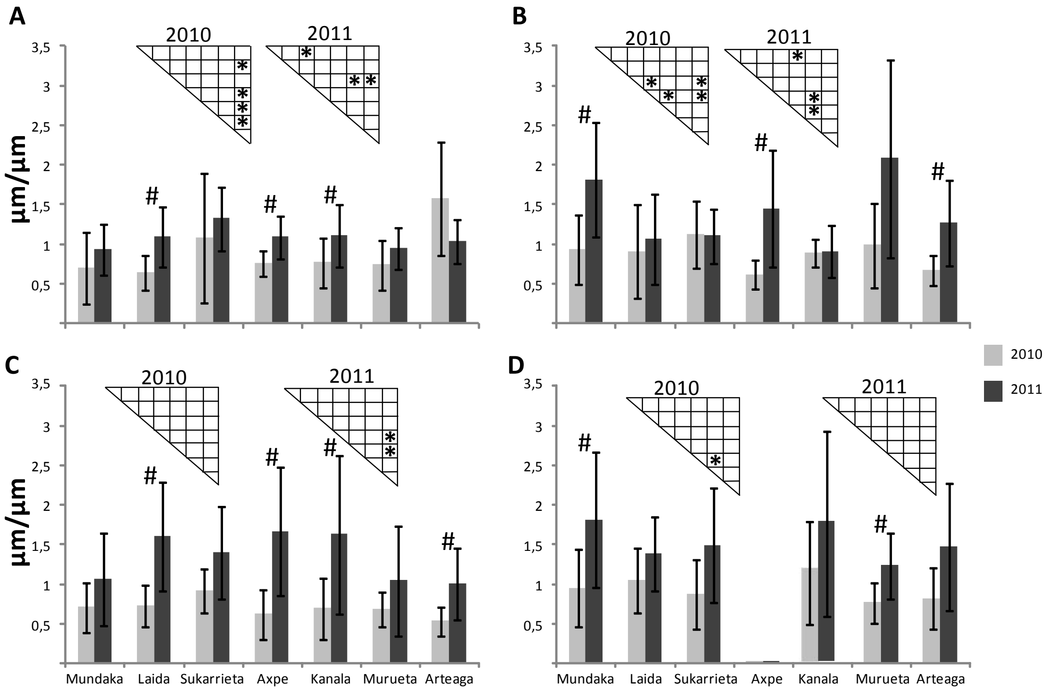

Figure 8. Mean luminal radius to Mean epithelial thickness ratio (MLR/MET) in oysters collected in March (A), June (B), September (C) and December (D) 2010 and 2011. Asterisks indicate differences between sites at p<0,05 level.# indicates differences between the same site at different season at p<0,05 level.

3.3.2.4. AUTOMETALLOGRAPHY

Autometallography revealed the presence of metals as black silver deposits (BSD) that are mainly accumulated in the haemocytes of the gills and the connective tissue surrounding the digestive gland and also in the basal layer of the digestive alveoli (Figure 9). Some metal accumulation was also observed in the apical part of the gills and within the digestive gland lysosomes. In order to quantify BSD extent values by means of image analysis, in general digestive cell lysosomes are selected for bivalves (Soto and Marigomez, 1997), but in the present work BSD in the digestive cells were only found in some sites and not in all the sampling campaigns the previously scheduled measurement was not performed. However, in general, oysters from Sukarrieta presented high amounts and staining intensity of BSD when comparing to the other sites. On the other hand, no clear pattern was observed regarding season of the year.

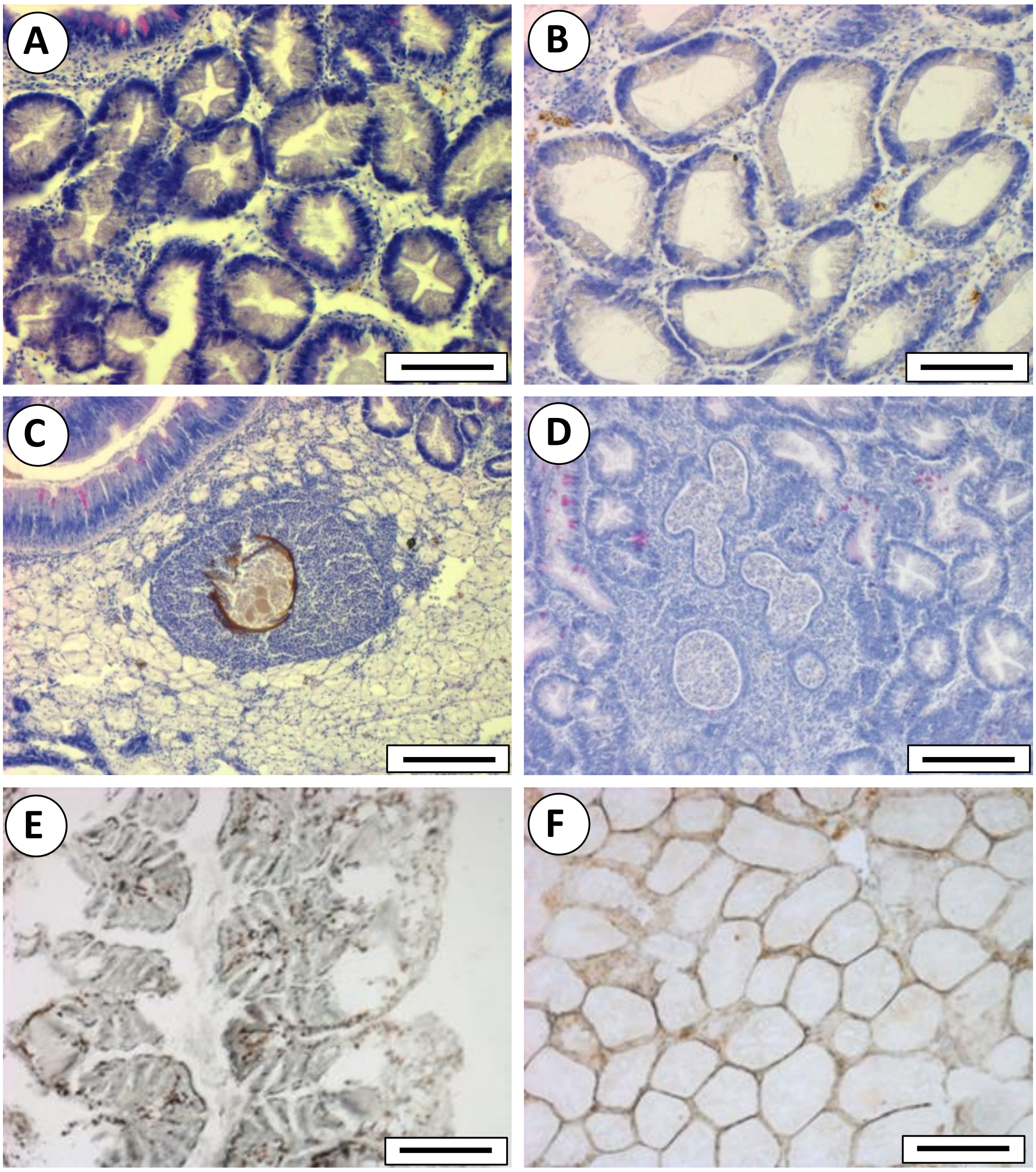

Figure 9. Paraffin sections of the digestive gland stained with hematoxilyn-eosin (A-D) and autometallography (E-F). A: oyster collected in Mundaka in September 2011. B: oyster collected in Kanala in December2011. Note the difference in the height of the epithelium. C: Metacercariae surrounded by haemocytes in Axpe in March 2011. D: Granulocytoma in oyster from Axpe in September 2011. E: Gills of oyster from Sukarrieta collected in March 2011. Note the heavy staining in the haemocytes and abfrontal zone of the gill epithelium. F: Digestive gland of oyster collected in March 2011 in Laida. Note the almost exclusive staining in the basal layer of the digestive gland. Scale bars: A and B= 100 µm. C-F=200 µm.

3.3.2.5. METALLOTHIONEIN INDUCTION

Metallothionein levels detected in Kanala and in some cases in Axpe were in general higher than in the other sampling sites although also high levels were detected in Laida (June 2010), Arteaga (June 2011) and Murueta (December 2011) (Figure 10). In general Mundaka is the station that presented lowest MT induction. Regarding season, similar levels were recorded in all seasons but values were lower in September. Finally, when comparing years

2010 and 2011 in March and June very few differences are observed between years; however, in September and December values observed in 2011 are significantly higher than values of 2010.

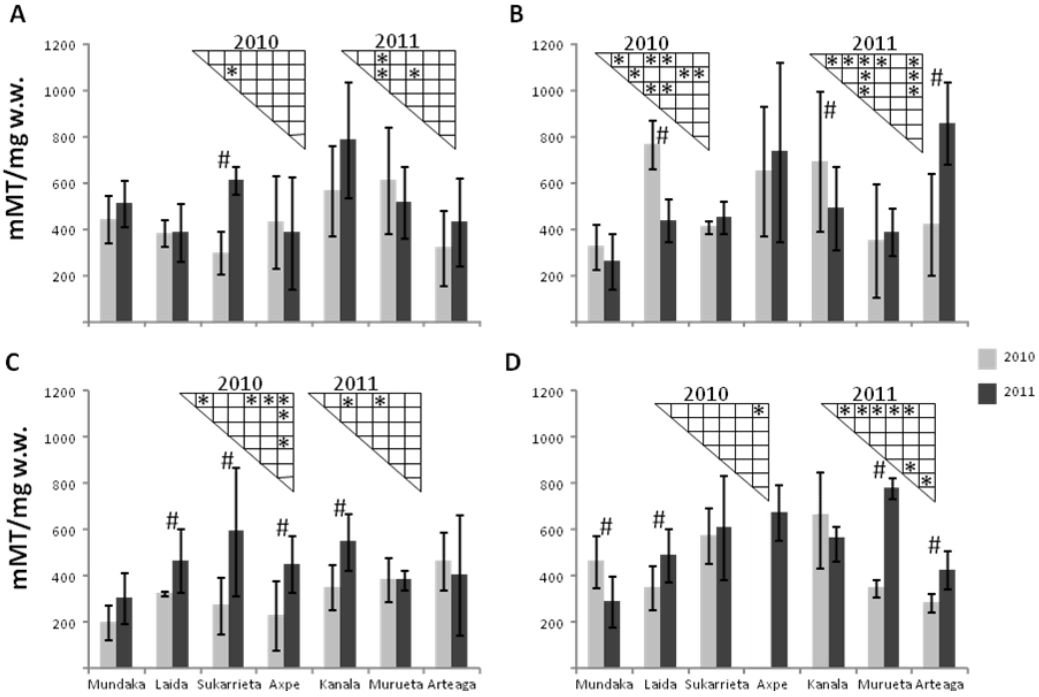

Figure 10. Metallothionein levels in oysters collected in March (A), June (B), September (C) and December (D) 2010 and 2011. Asterisks indicate differences between sites at p<0,05 level.# indicates differences between the same site at different season at p<0,05 level.

4. DISCUSSION

No clear correlation ($c_{corr} > 0.6$ or $c_{corr} < -0.6$) was identified between metal concentration in sediments, metal concentration in oysters and biological parameters measured in oysters. The low toxicity estimated for the sediments, however, is in good agreement with the low biological affection observed in oysters. The metal concentrations recorded in oysters were in general low to moderate (Kimbrough et al., 2008) and therefore the alteration observed at histological levels were not very severe with only some inflammatory responses and few increase in the metallothionein levels.

Interestingly, concerning metal concentrations opposite behaviours were observed in sediments and oysters, e. g., i) in general, the concentrations in oysters were higher in 2011 than in 2010, and those of sediments higher in 2010 than in 2011; ii) when seasonal trends were observed, maximum values in summer and minimum values in winter were measured in sediments, while oysters presented minimum values in summer and maximum values in winter. Similar seasonal trends in oysters from the estuary of the Oka River have already been observed in a previous work (Franco et al., 2002). This opposite behaviour indicates, as expected, a close relation between pollutant concentrations in sediments, oysters and the compartment in contact with both of them, water. Physicochemical characteristics of water (pH, redox potential, salinity, dissolved organic matter,…), which clearly depend on seasonal changes, highly affect the water-sediment interactions. Salinity, for example, influences directly the reactivity of trace elements in estuarine system, inducing desorption from sediments or from suspended matter to the water column (Fernández et al., 2008; Millward and Liu, 2003). As oysters feed by filtering water, when metal concentration is high in water (and consequently low in sediment), high metal concentration should be expected in oyster tissue.

The presence of inflammatory lesions such as haemocytic infiltrations has been related with different environmental stresses such as the presence of heavy metals (Rasmussen, 1986) and organic pollutants (Bignell et al., 2011). Presently, haemocytic infiltrations have been recorded all along the year in oysters from Urdaibai with higher prevalence observed in 2010 compared to 2011. However, the recorded haemocytic infiltration prevalences are lower than the ones measured in previous works in the area (Díez, 1996) and may be indicative of a general improvement of the environmental status of Urdaibai. It is also noticeable that the high haemocytic infiltration prevalence detected in general in Mundaka could be related with the higher As, Cu, Pb, Sn and Zn concentrations measured in oysters from this site and the response could be indicative of the stress that oysters of Mundaka are suffering due to the presence of As, Cu, Pb, Sn and Zn. Previous works in mussels have indicated augmented haemocytic infiltrations after 1 day of exposure to Cu (Katsumiti et al., 2013). Moreover other authors have linked the presence of high levels of haemocytic infiltrations with the presence of lipofuscin-like pigments in molluscs (Carballal et al., 1997) that could be produced due to oxidative stress produced by the higher concentrations of Cu and As measured in oysters from Mundaka. In this sense, the relatively high correlation coefficient (c_{corr} = 0.40) observed in other inflammatory lesion such as granulocytomes with the Cr concentration measured in oysters could be in relation with higher oxidative stress condition in oysters. The presence of granulocytomes has been reported in bivalves exposed to pollutants including heavy metals (Svardh and Johannesson, 2002). Presently, granulocytome prevalences are low except in March in most sites in 2011 and in Murueta in December 2010. No clear seasonal pattern has been described for granulocytomes (Bignell et al., 2008; Garmendia et al., 2011), however, is some cases these types of inflammatory responses have been linked with the presence of digenean metacercariae that also has been reported in March 2011. In any case,the levels of granulocytome prevalence detected in the Urdaibai estuary in oysters is similar to those detected in Mundaka in mussels from 2003 to 2006 (Garmendia et al., 2011) and could be considered as background levels of these inflammatory lesions in the area. The presence of pathogens has been very scarce in all the studied oysters and in general similar to that detected in oysters (Díez, 1996) and mussels (Garmendia et al., 2011) from the estuary of the Oka River in previous works.

Among the histopathological changes observed in molluscs the thinning or the presence of atrophic digestive epithelium has been linked with general stress situations including exposure to pollutants such as heavy metals and PAHs, or starvation (Couch, 1984; Garmendia et al., 2011; Rasmussen et al., 1983). Values of the MLR/MET ratio were in general higher in 2011 than in 2010 indicating some type of stress in oysters from 2011. This is in good agreement with chemical data, metal concentrations in oysters were also higher in 2011 than in 2010. It is noteworthy the close relationship between the MLR/MET index and the As concentration measured in oysters. Arsenic has been described as toxic metal that induces cell death by enhanced apoptosis or by increased levels of reactive oxygen species

(Boelsterli, 2007). Therefore a general stress response like the loss of digestive epithelial cells and the concomitant increase of the MLR/MET ratio could not be discarded since these type of responses have also been observed in mussels collected in the area (Cuevas et al., 2015). However, recorded values although higher than those recorded in previous works (Díez, 1996) are in general below or around 1,6 that has been considered as threshold levels in mussels from the area (Garmendia et al., 2011). Regarding epithelial thickness, some kind of seasonal trend was observed with lower values in March and September and higher in June and November (mainly in 2011). Note that similar seasonal trends were found in the concentration of several metals (Cd, Cu, Fe, Ni, Pb, V and Zn) in oysters. A similar trend has been observed in mussels from the Bay of Biscay after the Prestige oil spill and this seasonality has been linked with the recovery of the normal annual behaviour (Garmendia et al., 2011).

Autometallographical detection of metals presented some unexpected results. In general, the organs and cells most closely related with the metal distribution in molluscan tissues are the gills, the haemocytes and the digestive gland (Marigomez et al., 2002). In the present work, metals were localized in the glycocalix of the gills, haemocytes and basal layer of the digestive epithelium. In mussels, heavy metals have been localized in the mentioned areas but also in the abfrontal zone of the gills and within the lysosomes of the digestive cells (Soto et al., 1996). The different localization of metals made impossible to apply measurements of black silver deposits within the digestive cells as previously scheduled. However, this differential metal distribution within the tissues in oysters has been reported both in oysters exposed to silver (Martoja et al., 1988) and field experiments (present work). Unfortunately, Ag was not considered in this work.

Metallothioneins (MT) are low molecular weight and metal binding proteins that have been described in several marine organisms (Langston et al., 1998). Since MT have affinity to bind class metal ions such as Ag, Cd, Cu, Hg or Zn they have been widely used as metal exposure biomarkers (Roesijadi, 1992). Few differences have been observed in the present work among stations. Only in June some differences have been observed with higher values in Laida, Axpe and Kanala and lower in Murueta and Sukarrieta. It these two sampling sites, higher values were recorded in 2011 compared to 2010. The higher MT levels measured in Kanala could be related with the high Al, Co, Cr, and Fe concentrations measured in oysters. After this increase, MT levels seem to return to lower values in September. On the other hand, in general, higher MT values were recorded in 2011 than in 2010 indicating that the overall health status of oysters is better in 2010 than in 2011. This is again in good agreement with chemical data. Seasonal variation of MT levels in bivalves due to environmental conditions has been observed in previous works (Ivankovic et al., 2005; Riba et al., 2003). Other authors, however, have not detected seasonal trends in MT levels working specifically with oysters (Trombini et al., 2010), which is in good agreement with the results obtained in this work.

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Chapter 11

DEVELOPMENT OF AN EXTRACTION METHOD FOR THE ANALYSIS OF SILVER NANOPARTICLES IN SEDIMENTS

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ABSTRACT

Concern regarding the use and release of potentially toxic nanoparticles into the natural environment is increasing, at the same time as the analytical tools and methods are lacking which allow detection and identification of e.g. silver nanoparticles (AgNPs) in complex media, such as sediments. The aim of this research was therefore to develop a method to be applied to real sediment samples where AgNPs were suspected. Firstly, four AgNPs synthesis methods were evaluated, with regards to AgNPs stability, using UV-vis, particles size analysis and TEM analysis. The AgNPs synthesised by chemical reduction using $NaBH_4$ (0.002 M) and sodium dodecyl sulfate proved suitable. Secondly, ten potential extraction solutions were investigated, again investigating the effect of these solutions on AgNPs stability. The results proved NaOH (0.01 M) to be appropriate; having an average extraction efficiency of about 6% total Ag, and with minimal effect on AgNPs shape, size or stability. The method was applied on natural Hugli River sediment samples, using ultrafiltration to separate the dissolved from the non-dissolved fraction. Although large portions of Ag were retained by the ultrafilters, Single Particle Inductively Coupled Plasma Mass Spectrometry (spICP-MS) analysis showed that these were not present as NPs, but rather complexed with natural organic matter. However, the result is an important first step to developing a reliable method to determine AgNPs in environmental samples.

Keywords: Silver nanoparticles; Sediments; Stability; Extraction.

1. INTRODUCTION

Nanoparticles (NPs) are emerging contaminants and are defined as falling within a size range of 1-100 nm (Laborda et al., 2013). These materials have unique properties due to their high reactive surface area (compared to bulk material) and differ in behaviour and potential toxicity compared to dissolved ions of the same material (Luyts et al., 2013; Peretyazhko et al., 2014; Yu et al., 2013). Nanotechnology is attracting significant interest and investment from government, industry and academia and global production of NPs is estimated to thousands of tons annually (Karn et al., 2011). Consequently, the likelihood of environmental exposure to engineered NPs is increasing (Nowack and Bucheli, 2007).

Silver nanoparticles (AgNPs) are one of the most commonly produced NPs (Benn and Westerhoff, 2008). Due to their antimicrobial properties, AgNPs (Yu et al., 2013) are increasingly used in a variety of personal care products, sun screens, medical creams, textiles (especially sport wear), food storage containers as well as for water and waste water treatment (Fabrega et al., 2011; Liu et al., 2012; Nowack et al., 2009; Prathna et al., 2011). Consequently, AgNPs are directly released into the wider environment during product use and disposal and are also flushed into waste water, ultimately ending up in waste water treatment sludge or, if waste treatment is minimal or not effective, being released into rivers and finishing deposited on river sediments (Dobias and Bernier-Latmani, 2013; Kiser et al., 2010; Limbach et al., 2008).

There are concerns that the anti-microbial properties of AgNPs can negatively impact on human and ecosystem health (Wiesner et al., 2006). Currently, AgNPs toxicity, transport and fate in aquatic and terrestrial environments are poorly understood, which makes regulation of such materials extremely difficult (Aschberger et al., 2011; Baun et al., 2009).

Transport, fate, bioavailability and toxicity of AgNPs, is affected by a variety of factors, including NP size, shape, aggregation state, surface charge, surface area and concentration (Mitrano et al., 2012a; Mitrano et al., 2012b). The antimicrobial efficiency and consequent toxicity of AgNPs increase with decreasing nanoparticle and aggregate size (Morones et al., 2005; Song et al., 2009). The AgNPs synthesis method used also affects NP features, especially in regards to their stability (Tolaymat et al., 2010; Wojtysiak and Kudelski, 2012). Synthesis methods include chemical reduction (Solomon et al., 2007), radiolytic process (Shin et al., 2004), electrochemical method (Pal et al., 2007) and thermal decomposition (Maillard et al., 2002). However, the most widely used method is chemical reduction in which a metal precursor (ionic salt, *i.e.* $AgNO_3$) in an appropriate medium is reduced and stabilized by a surfactant which protects the particles from aggregation (Zambare et al., 2014). In formation of AgNPs by chemical reduction, the particle size, aggregation state and shape of the AgNPs are controlled by the initial $AgNO_3$ concentrations, reducing agent/$AgNO_3$ molar ratios and surfactant concentrations (Pillai and Kamat, 2004). The surrounding aquatic and terrestrial environment also affects the stability, transport and fate of AgNPs (Mackay Christopher et al., 2006; Wiesner et al., 2009), which are influenced by *e.g.* photodegradation, chemical oxidation, sulfidation (Lowry et al., 2012), dissolved oxygen, pH, ionic strength, electrolyte composition and the presence of natural organic matter (Peretyazhko et al., 2014; Yu et al., 2013). Whereas dissolved organic matter (DOM) generally stabilises NPs against aggregation and sedimentation (Hyung et al., 2007; Quik et al., 2010; von der Kammer et al., 2010), NPs in water with high ionic strength have showed faster sedimentation due to increased aggregation (Limbach et al., 2008). The presence of natural colloids also provide a surface area for potential deposition of NPs (Keller et al., 2010), while waste water biomass has been shown to greatly increase settling of AgNPs in sediments (Kiser et al., 2010).

A major technical challenge in environmental NP research is the low concentrations at which NPs are likely to be present in the environment. Moreover, NP *size* and *number* concentration information are required, parameters that are extremely challenging to determine in natural matrices. Most available methods, *e.g.* Scanning Electron Microscope (SEM), Transmission Electron Microscopy (TEM), Brunauer–Emmett–Teller (BET), Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM), are usually used to characterise synthesised NP material in relatively high concentrations and/or as a liquid. However, the relatively high detection limits of these techniques is a disadvantages regarding environmentally relevant analysis of NP concentration and size. Single-Particle Inductively Coupled Plasma Mass Spectrometry (spICP-MS) is able to determine both size and number concentration of NPs (Cornelis and Hasselloev, 2014; Laborda et al., 2014; Pace et al., 2011), at least down to Ag particle sizes of about 18 nm, with the assumption of spherical particles (Laborda et al., 2011). During spICP-MS, the dwell times are short enough to detect the short but strong ion bursts caused by ion clouds produced from NPs ionized in the plasma, provided that these bursts are somewhat more intense than the more continuous signal coming from dissolved ions from the same element (Laborda et al., 2013). Alternatively, the dissolved element mass concentration can also be distinguished from the fraction occurring as NPs or complexes using the ultrafiltration method, which is a single pass procedure to size select and to concentrate a target species using a series of membrane modules with pores ranging from 1 nm to 100 µm (Trefry et al., 2010). Both these methods will be used in the research presented in this paper.

The tendency of NPs to sediment presents additional analytical challenges, since the methods used for detecting elements in solid matrices (e.g. X-ray Diffraction or X-ray Fluorescence) do not have low enough spatial resolution to identify individual NPs and are not sensitive enough to detect the low concentrations that are likely to occur in environment samples. Typical methods used to extract trace metals from soils or sediments involve semi-total digestion using nitric or hydrochloric acid or total digestion with hydrofluoric acid, which would entirely dissolve any NPs present and make them indistinguishable from dissolved metals of the same element (Prathna et al., 2011; Yu et al., 2013). Therefore this study also drew from standard methods for “sequential extractions” of soils in order to quantify elements present in exchangeable fraction, organic phase, metals associated with iron and manganese oxides and finally in the mineral fraction. These use extraction media of increasing strength *i.e.* acetic acid, hydroxylammonium chloride, hydrogen peroxide with ammonium acetate and finally *aqua regia* or concentrated nitric acid (Rauret et al., 1999). However, these methods are only concerned with quantifying metal concentrations found in different phases, not with quantifying or identifying of particles associated with those phases. A standard method to extract NPs without dissolving or transforming the particles, does not currently exist, making both qualitative and quantitative analysis of NPs difficult in sediments (Lowry et al., 2012). The development of reliable extraction methods of AgNPs from complex media, such as sediment, is crucial in order to quantify and understand the transport and fate of AgNPs and to determine potential effects of AgNPs on the environment (Baalousha et al., 2013).

Therefore the aim of this study was to develop a reliable extraction method for AgNPs from sediment, followed by quantification using two complementary methods. The method was finally tested on natural Hugli River sediment samples and the extracts were analysed using spICP-MS for AgNPs.

2. MATERIALS AND METHODS

The following steps were performed: a) four AgNPs synthesis methods were evaluated to determine which one produced the most stable AgNPs b) the AgNPs produced were used to investigate the effect of ten different extraction solutions on AgNPs shape and size, c) the chosen extraction solution was applied on a real sediment sample to determine the extraction efficiency of the solution compared to total Ag concentration present. Then the extract was finally filtered and analysed for AgNPs by spICP-MS.

2.1. SYNTHESIS METHODS

Four synthesis methods of AgNPs were tested, all using silver nitrate ($AgNO_3$) 0.001 M (Fischer) as a precursor. An overview of the synthesis methods are given in Table 1. In Method 1, 30 mL of 0.002 M $NaBH_4$ (sodium borohydride) (Acros Organics) was cooled to 0°C in an ice bath under continuous stirring with a magnetic stirrer. 10 mL of $AgNO_3$ was added drop-wise, under continuous stirring. The solution was then removed from the stirrer to avoid aggregation of the AgNPs. In Method 2, $NaBH_4$ and sodium dodecyl sulfate (SDS, Acros Organics) (SDS: $AgNO_3$ weight ratio = 10) were mixed and stirred for 30 minutes. Again, 10 mL of $AgNO_3$ was added drop-wise while stirring continuously, this time without cooling. Once all the $AgNO_3$ had been added, the solution was stirred for a further hour. In Method 3, 50 mL $NaBH_4$ and SDS (SDS: $AgNO_3$ ratio = 10, w/w) were mixed and stirred for 30 minutes. 50 mL $AgNO_3$ was added drop-wise under continuous stirring. Once all $AgNO_3$ was added, the solution was stirred for a further hour. Finally, in Method 4, 50 mL $AgNO_3$ was heated to boiling, under continuous stirring. Trisodium citrate ($Na_3C_6H_5O_7$, 5 mL, 1%, Sigma Aldrich) was then added drop-wise. Once the solution turned yellow, it was removed and left to cool at room temperature.

Table 1. Overview of synthesis methods used, all using $AgNO_3$ as a precursor.

Reducing agent	Concentration, volume	Stabilising agent	Concentration
1. $NaBH_4$	0.002 M, 30 mL	-	-
2. $NaBH_4$	0.002 M, 30 mL	SDS	0.0019 M
3. $NaBH_4$	0.004 M, 50 mL	SDS	0.017 M
4. Trisodium citrate	1%, 5 mL	-	-

The stability of synthesised AgNPs was recorded using UV-Vis and particle size analysis (as described in section b) for 10 days after the synthesis: i) on the day of synthesis after 30 minutes, ii) after 2 days and iii) after 10 days. The $NaBH_4$ and $Na_3C_6H_5O_7$ were used as blank.

2.2. STABILITY TEST OF SYNTHESISED AgNPs OVER TIME

The stability with respect to time of the synthesised AgNPs was investigated using UV-Vis (Varian Cary 100 UV-Visible spectrophotometer), placing the AgNPs solution in a 10 mm quartz cuvette of Perkin Elmer. The Surface Plasmon Resonance (SPR) peak was monitored by scanning the UV-Vis spectra of the synthesised solutions from 200 to 600 nm. A Particle Size Analyser (Brookhaven 90 Plus/BI-MAS Multi Angle Particle Sizing) was used to measure the hydrodynamic diameter and polydispersity.

2.3. STABILITY TEST OF THE SYNTHESISED AgNPs IN DIFFERENT MEDIA

Ten different extraction solutions were selected with the aim to investigate the effect on AgNPs stability (*i.e.* size and shape). Ten mL of AgNPs solution (synthesised using Method 2) was diluted in 100 mL of extraction solution.

The following extraction solutions were selected for the experiments: de-ionised water (MilliQ, Millipore), dilute nitric acid (HNO_3) at pH 2 (Fisher Scientific), dilute hydrochloric acid (HCl) at pH 2 (Fisher Scientific), dilute sodium hydroxide (NaOH) at pH 12 (Fisher Scientific), sodium chloride (NaCl) solution 3.5% and 1.75% by weight (Fisher Scientific), magnesium chloride ($MgCl_2$) 1 M (Acros Organics), acetic acid (CH_3COOH) 0.11 M (Acros Organics), ammonium nitrate ($NH_4(NO_3)$) 1 M (Fisher Scientific) and finally ammonium hydroxide ($NH_4(OH)$) 25% in MilliQ water (Fisher Scientific).

AgNPs stability was investigated using UV-vis, particle size analysis and Transmission Electron Microscopy TEM (Philips CM120) over a period of 10 days: *i)* on the day of synthesis and addition of extraction solution, *ii)* after 2 days and *iii)* after 10 days.

To confirm the stability of AgNPs in the selected extraction media, a commercially available AgNPs standard solution (40 nm, BBI Solutions) was added to the extraction solution and analysed using a Dynamic Light Scattering DLS (Malvern Zetasizer).

2.4. STUDY AREA AND SEDIMENT SAMPLING

Sediment samples for the study were provided by Dr. Sarkar, the University of Calcutta, India, and were collected along the banks of the Hugli River, a major tributary of the Ganges River. The river is subject to a variety of anthropogenic contamination including municipal waste, untreated industrial effluents and boating activities as was described extensively by Sarkar et al., in (Sarkar et al., 2002; Sarkar et al., 2012) and concerns have been raised that *e.g.* the textile industries located along the river may be a source of AgNPs, with the potential environmental impact on the rich biodiversity in the Sundarban delta. There is indeed a lack of research on NPs in the Indian environment (Kumar et al., 2012), where this study provides a first attempt to detect and quantify AgNPs in estuarine sediment from India.

Eight surface sediment samples (0–2 cm), S_1-S_8 were collected from the river bank at low tide in July 2014 (Figure 1). Samples were transported to the laboratory in sterile plastic bags at 4ºC and oven dried at 48 ^{o}C for at least 48 hours. Samples were pulverized using an agate mortar and pestle, sieved through 63 µm metallic mesh and kept in the refrigerator at 4ºC in dark until analyses.

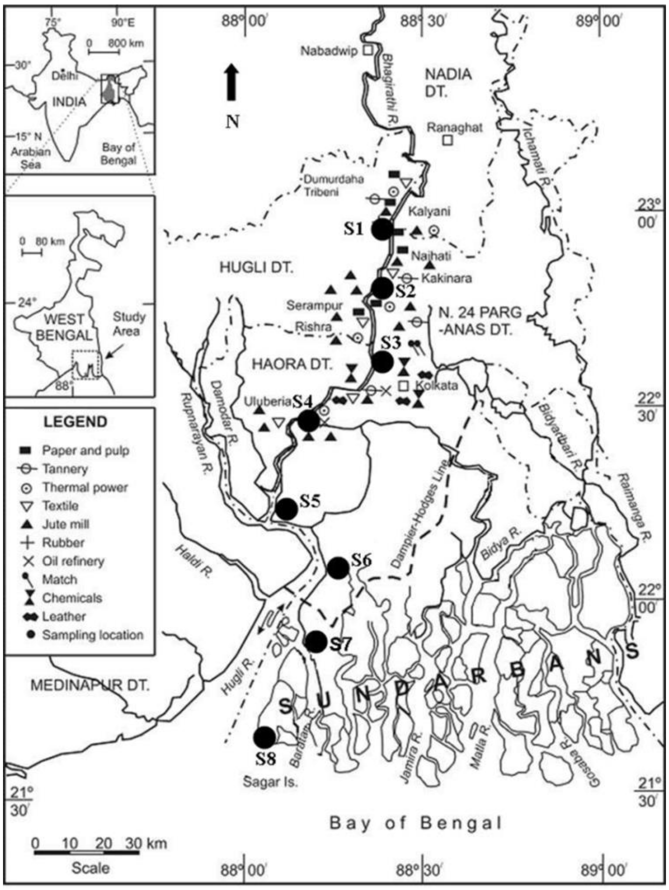

Figure 1. The Sundarban wetland in the Bengal region (India) and the sampling points. S1: Tribeni, S2: Barrackpore, S3: Babughat, S4: Budge Budge, S5: Nurpur, S6: Diamond Harbour, S7: Lot 8 and S8: Gangasagar. Courtesy of Dr. Sarkar, Calcutta University.

2.5. Ag EXTRACTION EFFICIENCY OF NaOH AND DISSOLVED OR PARTICULATE Ag DETERMINATION IN INDIAN SEDIMENTS.

The aims of these experiments were: *i)* to determine the Ag extraction efficiency of NaOH from sediment and *ii)* to determine presence and concentration of AgNPs concentration in the sediment samples from the Hugli River.

Firstly, the total Ag concentration in the sediment samples was determined. Triplicate samples (0.25 g) of sediment samples as well as a peat reference material (NIMT/UOE/FM/001) (Yafa et al., 2004) were oven dried (105°C), ashed (450°C) and digested using a microwave (CEM Mars 5) with concentrated HNO_3 (Aristar, VWR) (adapted US-EPA method 3051) (Yafa and Farmer, 2006). Following digestion the samples were gently evaporated to near-dryness (~ 1 cm^3) on a hot plate, added to a 25 mL volumetric flask (with three beaker washes) and diluted with nitric acid (2% v/v, VWR, Aristar). The diluted extracts were filtered through 11 μm filters (AshlessWhatman 45) into a polypropylene centrifuge tube (Fisher) and stored at 4°C prior to analysis with Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7500ce). The ICP-MS instrument was prepared by flushing it, using an alkaline wash solution (10 mg/L EDTA (as acid), 0.8 mg/L Triton X-100, 60 mg/L HN_4OH and 80 mg/L H_2O_2) to reduce Ag background levels (monitored using ^{107}Ag), which are likely to be high, due to the "stickiness" of Ag within the instrument. Moreover the concentrations of Ag in the sediments were very low, thereby requiring low Ag background. An added 30s wash with the alkaline solution was also inserted prior to the nitric acid rinse (2%, 30 s), between samples. Blank samples (2 % dilute nitric acid) were inserted every third sample, and a certified reference water (SRM 1640a or SRM 1643e, NIST) was analysed for a quality control.

Following the stability tests (section 2.3.), NaOH was selected for further experiments. To determine the NaOH extraction efficiency, 20 mL of NaOH 0.01 M was added to 2 g of sediment and this suspension was stirred for 24 h. The NaOH extracts were then filtered through 0.45 µm cellulose filters (treated with $CuNO_3$ 0.1 M to avoid Ag losses) (Cornelis et al., 2010). For the total Ag after NaOH extraction, 0.5 mL of filtered extract was digested using *aqua regia* (HCl/HNO_3, 3:1 volume ratio) in a microwave oven (Milestone Ethos 1600) and Ag after NaOH extraction was analysed by ICP-MS (Finnigan ELEMENT2).

Finally, the <1kDa fraction of the NaOH extracted samples was determined as a proxy for the dissolved (non-particulate) Ag concentration after ultrafiltration. The ultrafiltration filters (Vivaspin 20) were similarly treated with $CuNO_3$ 0.1 M dissolution. Then, 2 mL of the NaOH extract were ultra-filtrated and the filtered fraction was analysed in the ICPMS (Finnigan^{TM} ELEMENT2).

2.6. spICPMS ANALYSIS AFTER NaOH EXTRACTION

All NaOH extracts and filtered samples, standards and blanks were prepared in 0.1 % thiourea to minimize carry-over and minimize wash-out times (Chen et al., 2000). Five blanks (0.1% Thiourea and 0.01 M NaOH), six Ag and Au standards (1, 5, 10, 30, 100 and 1000 $ng \cdot L^{-1}$) for the Ag and Au calibration, Au 60 nm NIST reference material (10^4, 10^5 and 10^6 times diluted) for the nebulisation efficiency (Pace Heather et al., 2011) and finally NaOH extracted Indian sediments samples (100 times diluted) were measured in the ICP-MS (Finnigan^{TM} ELEMENT2) with 5 ms dwell-time. The signal deconvolution method was used to distinguish AgNPs from dissolved Ag^+ present in the sediment extracts (Cornelis and Hasselloev, 2014).

3. RESULTS AND DISSCUSION

3.1. EVALUATION OF AgNPs SYNTHESIS METHOD

As it has been mentioned, the stability of AgNPs produced using four different synthesis methods (Table 1) was investigated using UV-Vis and particle size analysis. The results from the UV-Vis are presented in Figure 2. The width of the UV-Vis peak around 400 nm is related to the particle size distribution, and the peak wavelength can be used to determine particle size (Bonsak, 2010). The SPR peak of the synthesised AgNPs is around 400 nm (Figure 2), which corresponds to AgNPs of around 12 nm (Solomon et al., 2007). The SPR peak of the AgNPs synthesised using Method 2 had a peak width at half the absorption maximum (PWHM) of about 60 nm. The AgNPs synthesised using Method 1, 3 and 4 had higher PWHM (Figure 2), suggesting a wider particle size distribution than those synthesised using Method 2 (Bonsak, 2010).

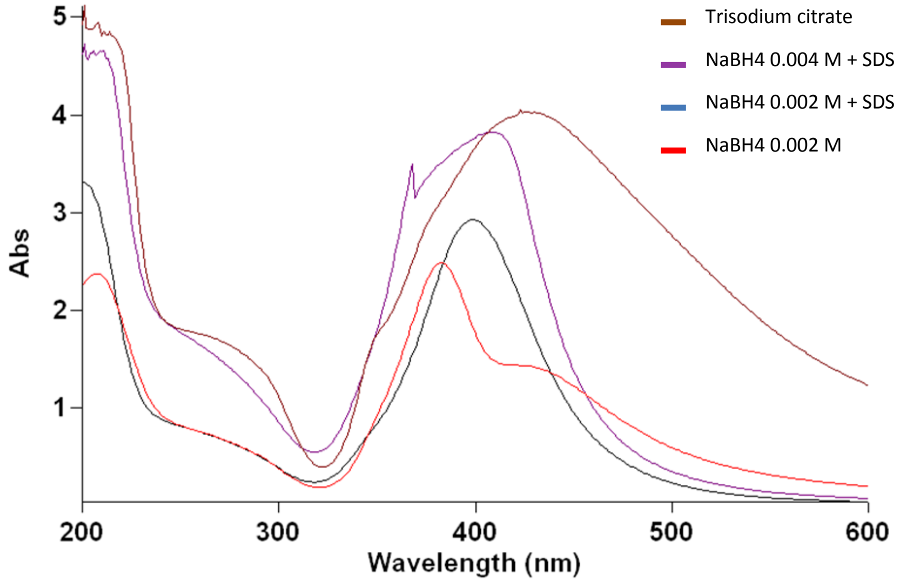

Figure 2. UV-Vis spectra of the AgNPs synthesised using methods: 1) SDS, $NaBH_4$ 0.002M, 2) SDS, $NaBH_4$ 0.002M SDS, 3) SDS, $NaBH_4$ 0.002M and 4) $Na_3C_6H_5O_7$.

The hydrodynamic diameters of the AgNPs synthesised using Methods 1-4, were 26.3, 24.0, 24.7 and 30.9 nm, respectively, showing that all methods used produced AgNPs of similar size on the day of synthesis. However, the polydispersity indices were 0.309, 0.204, 0.311 and 0.357 respectively, showing that the AgNPs synthesised with Method 2 were more monodisperse than the AgNPs produced using Methods 1, 3 and 4 as also suggested by the SPR peak values of the UV-Vis (Peretyazhko et al., 2014; Prathna et al., 2011).

The Z-average hydrodynamic diameter and the UV-VIS spectrum of AgNPs synthesised using Method 2 were measured daily for 10 days to confirm their stability over time (Table 2 and Table 3). The results, showing only a small increase in size as well as similar wavelength, indicated that the AgNPs were kinetically stable for at least 10 days. The stability of the AgNPs was confirmed by visual analysis using TEM (Figure 6.a), which suggest that the AgNPs synthesised were of about 10-20 nm diameter in core size.

Table 2. UV-Vis measurements for AgNPs synthesised using Method 2.

DAY 1		DAY 5		DAY 10	
W (nm)	I	W (nm)	I	W (nm)	I
399	2.926	398	2.904	395	2.833

*W: SPR wavelength; I: SPR intensity.

Table 3. Particles Size Analysis results using Method 2.

1 DAY		5 DAY		10 DAY	
D (nm)	P	D (nm)	P	D (nm)	P
24.0	0.204	26.6	0.278	27.0	0.285

*D: hydrodynamic diameter; P: polidispersity

3.2. EFFECT OF THE SOLUTION ON AgNPs SIZE AND STABILITY

The stability of AgNPs produced in the ten different potential extraction solutions was investigated using UV-Vis, particle size analysis and TEM in order to determine a suitable extraction solution for AgNPs from sediments.

The first step was to determine the effect of the extraction solution on AgNPs stability, measured primarily through hydrodynamic size and solubility (UV-Vis and particle size analysis). The UV-Vis results are displayed in Figure 3.

Untreated AgNPs show a SPR at 391 nm wavelength over the 10 days (Figure 3). The SPR intensities of the synthesised AgNP, were 3.241 on the first day, 3.254 on the second day and 3.257 on the tenth day (Figure 4), showing the kinetic stability of synthesised AgNPs over a 10-day period.

When mixed with HNO_3, NaCl 3.5% or 1.75% or in $NH_4(OH)$ (Figure 3), the AgNPs did not give rise to a SPR peak, which suggests that the AgNPs were dissolved or aggregated, resulting in a wide particle size distribution.

When mixed with $MgCl_2$ medium, the AgNPs aggregated showing a SPR at 407 nm relative to the 391 nm SPR wavelength of the synthesised (non-aggregated) AgNPs (Baalousha et al., 2013).

The AgNPs were not kinetically stable beyond the first day of mixing with HCl or NH_4 (NO_3) media, as demonstrated by the increase of wavelengths from 391 to 399 and 402 nm, respectively, on the second day. Furthermore the SPR intensities decreased to 0.019 and 0.068. The AgNPs remaining in dissolution increased in size because of Ostwald Ripening, increasing the particle size distribution. In fact, no SPR was recorded for the AgNPs solutions in HCl and $NH_4(NO_3)$, on the tenth day (Peretyazhko et al., 2014).

The SPR values indicate that the AgNPs in deionized water and in NaOH remain kinetically stable from the first day to the second day (Figure 3). AgNPs in acetic acid showed an increase in SPR wavelength from 387 nm on day 1 to 389 nm on day 2 (Figure 3). The intensity of the SPR in the case of acetic acid for the first day was 0.117, compared to 0.287 and 0.288 in the cases of deionized water and NaOH respectively (Figure 4), showing that the synthesised AgNPs were kinetically more stable in deionized water and NaOH media than in acetic acid.

Since only the AgNPs that were left untreated or the ones that were treated with deionised water, acetic acid or NaOH remained reasonably stable, these were monitored for 10 days using UV-vis, particle size analysis and by visual inspection using TEM. After 10 days of mixing the AgNPs remained kinetically stable when mixed with deionized water and NaOH. For AgNPs in deionised water the SPR increased from 394 nm on the day of synthesis to 397 nm on the tenth day, while for the AgNPs in NaOH, the SPR wavelength remained at 395 nm over the 10 day period (Figure 3). However, for AgNPs in deionized water the intensity of the SPR decreased from 0.287 to 0.181, indicating dissolution of the AgNPs (Figure 4). For AgNPs in NaOH, the SPR intensity varied slightly from 0.288 to 0.284, demonstrating that the AgNPs were kinetically stable in NaOH. When mixing AgNPs with acetic acid, the AgNPs did not give rise to a SPR peak after 10 days, indicating that most of the AgNPs were dissolved.

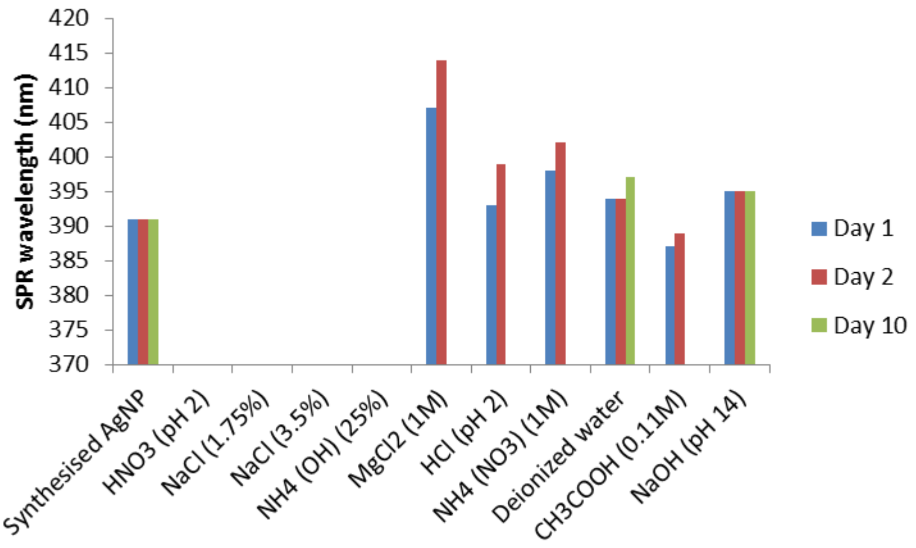

Figure 3. UV-Vis SPR wavelength AgNPs in different media, over time (1, 2 and 10 days).

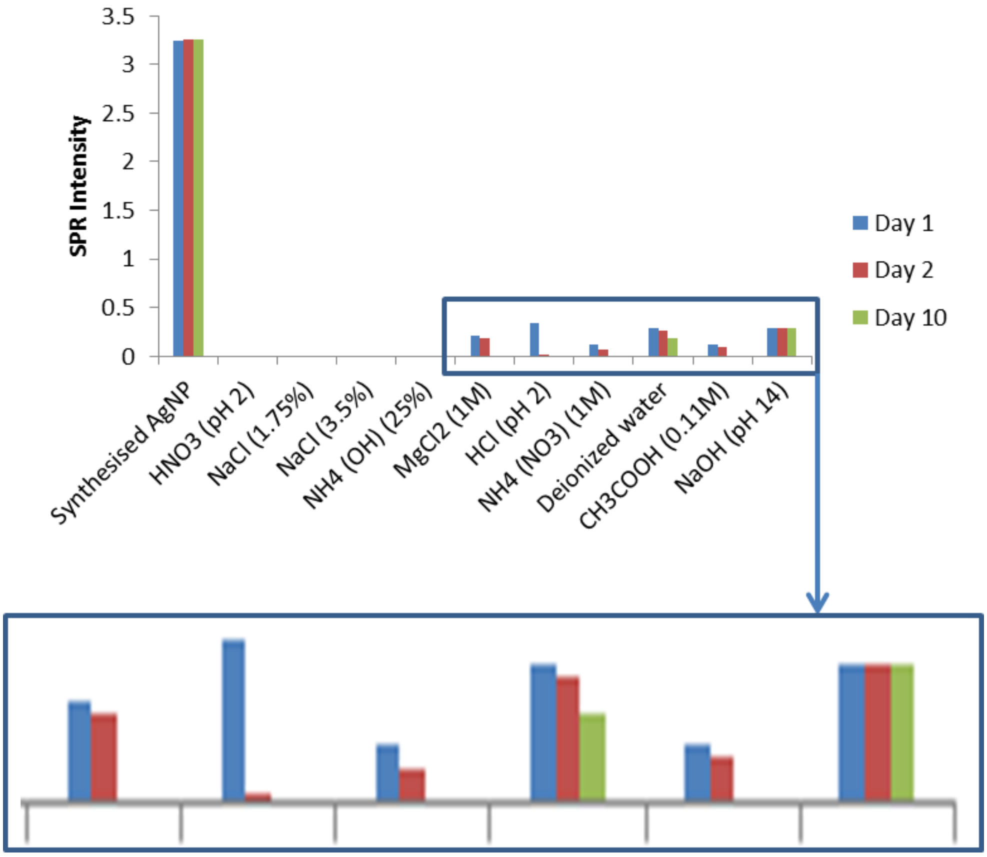

Figure 4. UV-Vis SPR intensities for the stability test of the AgNPs in different mediums and over time (1, 2 and 10 days).

The results from the particles size analysis for untreated AgNPs and AgNPs treated with NaOH, acetic acid and deionised water are displayed in Figure 5. The results showed that the Z-average hydrodynamic diameter (d) of the untreated AgNPs was 35.1 nm the first day, 36.6 nm on the second day and 36.9 nm the tenth day (Figure 5). AgNPs in acetic acid, however, showed a large increase in *d* from the first day to the second, namely from 33.3 to 40 nm (Figure 5). While after 10 days the *d* value of the AgNPs in acetic acid increased to

103 nm. For AgNPs in deionized water, the *d* increased from 41.7 nm of the first day to 53.6 nm in the tenth day and in the case of AgNPs in NaOH, *d* decreased from 47.8 to 44.8 nm, remaining kinetically more stable in NaOH media than in deionized water. These results were confirmed by the TEM images, which after 10 days showed that the AgNPs in NaOH retained their shape and size (Figure 6.b), while the AgNPs in acetic acid lost the spherical shape already on the first day after mixing, (Figure 6.c). After 10 days in acetic acid, the AgNPs had aggregated. The TEM images of AgNPs in deionised water confirmed that AgNPs dissolved within 10 days in deionized water as was indicated by the UV-Vis results.

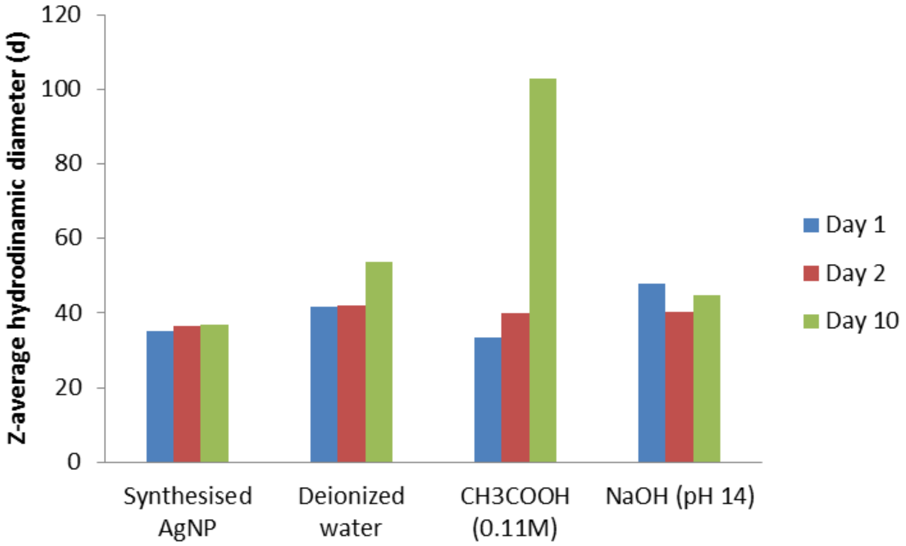

Figure 5. Particles size analysis of the AgNPs in different mediums over 10 days.

To confirm the stability of AgNPs in NaOH solutions, standard AgNPs from BBI solutions (40 nm) were added to NaOH and measured using DLS. According to the DLS results, the AgNPs remain kinetically stable in size in deionized water and in NaOH. The hydrodynamic diameter of the 40 nm AgNPs in the BBI standard were determined to 50.64 nm. After 2 days of mixing of the 40 nm AgNPs BBI standard in deionized water and in NaOH, the hydrodynamic diameters were 55.35 nm and 52.91 nm respectively, showing that the AgNPs size remained stable in both solutions.

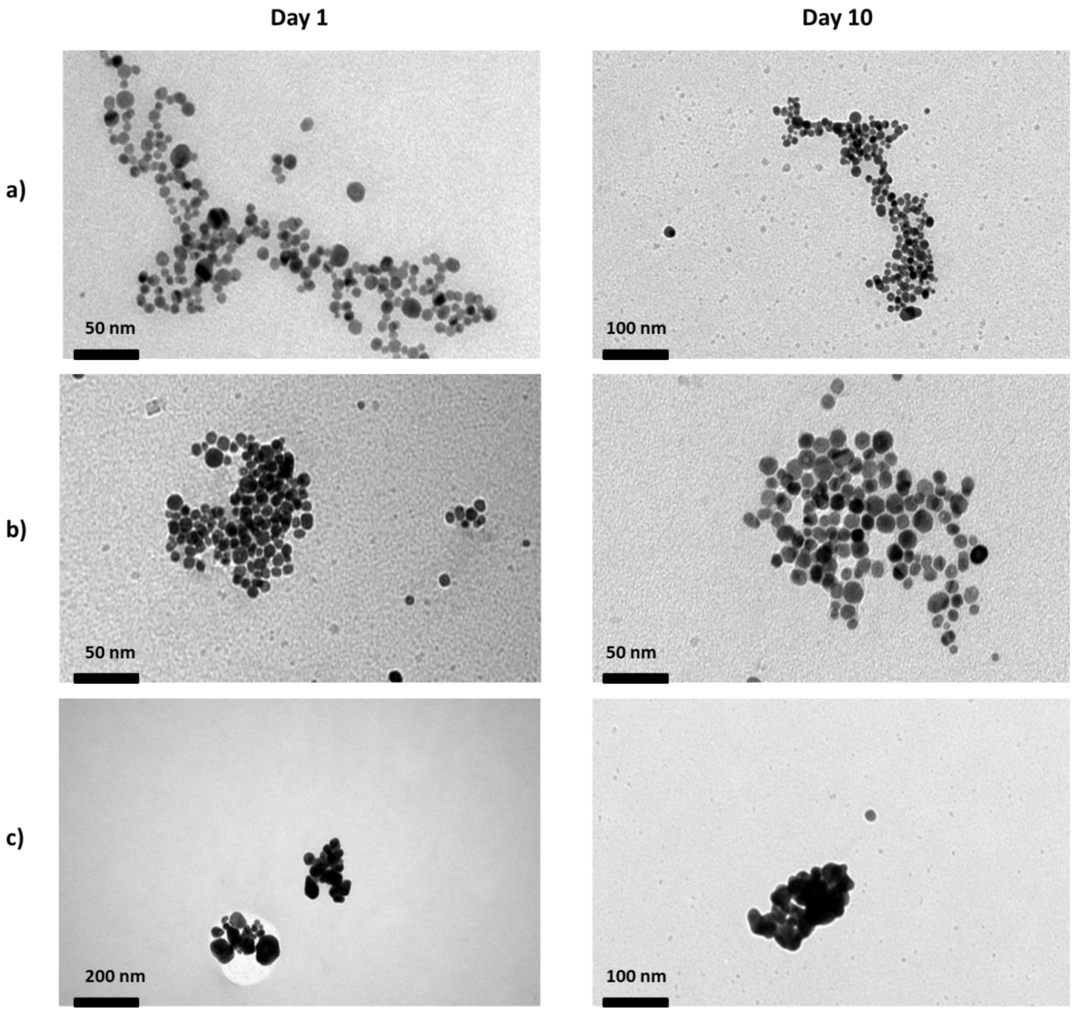

Figure 6. TEM images of the AgNPs 1 day and 10 days after mixing the AgNPs in the following mediums: a) untreated, b) NaOH 001 M and c) acetic acid 0.11M.

3.3. NaOH Ag EXTRACTION EFFICIENCY FOR REAL SEDIMENT SAMPLES

Sediment samples were processed and analysed for total Ag (determined by acid digestion) as well as Ag extracted using NaOH. The results are displayed in Table 4, together with the calculated Ag extraction efficiency using NaOH. The average NaOH Ag extraction efficiency was 6.78 ± 3.85 %. S4 seems to be the sampling point with the highest total Ag concentration and total Ag after NaOH extraction, although the extraction efficiency for this sample was the lowest. NaOH showed lower extraction efficiencies in upstream sampling sites than in downstream ones.

As seen in Table 4, upstream sampling sites had somewhat higher total Ag concentrations than downstream sampling sites. This could be due to close proximity to sources of Ag, but also to the lower water salinities of the upstream sampling sites than downstream ones, since downstream sampling sites are closer to coastal waters. Salinity could affect in the sediments metals desorption processes, dissolving metals from the sediments to the water mediums and contributing to decrease the metals concentrations in the sediments (He et al., 2006). Cations, e.g. Na^+ and K^+ present in saline water, can exchange metallic elements placed in sediments metal adsorption sites and Cl^- anions can create metals dissolved complexes (Comans and Van Dijk, 1988; Paalman et al., 1994), contributing to decrease the metal concentration found in the sediments.

Table 4. Sampling site water salinity (g·L^{-1}), sediments organic carbon (%), total Ag (µg·kg^{-1}), Ag in NaOH extraction (µg·kg^{-1}) and Ag extraction efficiency (%) of the NaOH (n=3).

Samples	Salinity (g·L^{-1})	Organic carbon (%)	TOTAL Ag (µg·kg^{-1})	Ag (NaOH 0.01M) (µg·kg^{-1})	Extraction efficiency (%)
S1	0.146	0.68	140 ± 10	8.58 ± 0.56	6.13
S2	0.211	0.71	100 ± 20	3.99 ± 0.38	3.99
S3	1.824	0.62	60 ± 10	2.11 ± 0.35	3.52
S4	4.011	0.91	870 ± 140	8.54 ± 2.15	0.98
S5	3.957	0.70	70 ± 4	7.90 ± 1.02	11.29
S6	7.827	0.56	50 ± 10	5.91 ± 1.23	11.82
S7	12.24	0.47	66 ± 29	5.97 ± 0.85	9.05
S8	12.88	0.52	41 ± 16	3.07 ± 0.20	7.49
Average	-	-	-	-	**6.78 ± 3.85**

To separate the dissolved Ag fraction from particulate or complexed material, the NaOH extracts were filtered with a 1 kDa ultra-filter, and the resulting Ag concentration in filtrate and retentate for each sediment sample are displayed in Figure 7. The retentate Ag in the >1kDa ultrafiltration was likely to be present as NPs or as complexed Ag. The retentates of S4 and S1 sediment extracts contained the highest Ag concentrations compared to the other samples (Figure 7). The difference between total Ag extracted by NaOH and dissolved Ag in S4 was the highest, (7.36 µg·L^{-1}), followed by S1 (6.54 µg·L^{-1}). This shows that S4 and S1 were the sampling points with the highest concentrations of easily available particulate or complexed Ag. In order to determine whether the Ag was present as NPs or rather as complexed Ag, spICP-MS was performed of the solutions, as described in the following section.

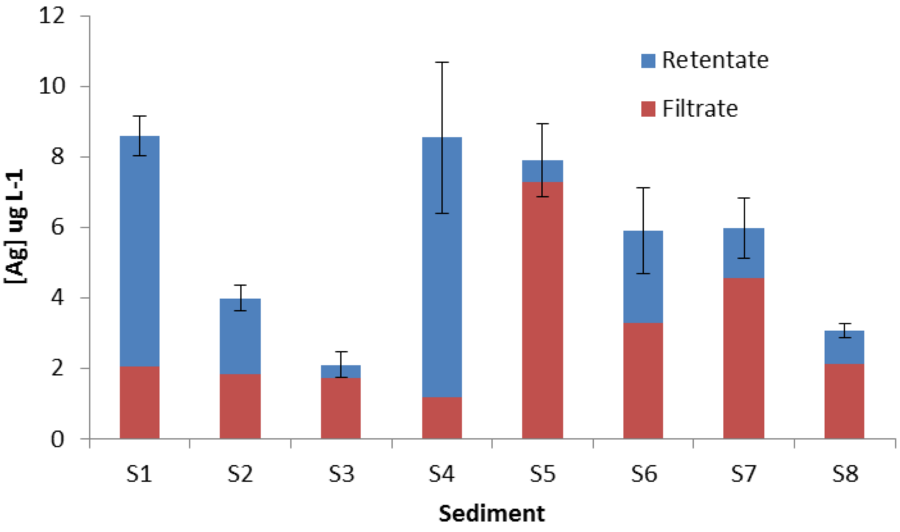

Figure 7. Dissolved (filtrate) and particulate or complexed (retentate) Ag results after NaOH extraction and ultrafiltration (1kDa) (n=3).

3.4. spICPMS ANALYSIS OF NaOH EXTRACTION SOLUTION

The processed particle size distribution results of the spICP-MS are displayed in Table 5. A correlation analysis between the total AgNPs mass concentration of the spICP-MS and total mass of retentate Ag after NaOH extraction and filtration (retentate Figure 7) was carried out in order to determine whether there is a correlation between both variables, which would indicate the presence of NPs. However, no significant correlation (95% confidence level) was found. Instead, a much better correlation with a Pearson coefficient of 0.89 (95% confidence level) between the dissolved Ag after NaOH extraction (Figure 7) and the spICP-MS total AgNPs mass concentration (Table 5) was found, suggesting that the spICP-MS was only picking up false positives of the dissolved Ag and that the retentate Ag was not in the form of AgNPs. However, the results from the ultrafiltration suggest that the Ag was present in a form that allows retention by the ultra-filters, e.g. as elemental Ag bound in complexes.

Table 5. Ag particle size distribution in sediments measured by spICP-MS.

Samples	AgNPs number concentration Nº·L^{-1}	AgNPs mass concentration ng·L^{-1}
S1	793	0.05
S2	1386	0.05
S3	1351	0.04
S4	1457	0.02
S5	2682	0.21
S6	1734	0.13
S7	848	0.06
S8	980	0.03

It is known that Ag easily forms complexes with dissolved organic matter and thiol groups (Hedberg et al., 2015; Ho et al., 2010; Liu and Hurt, 2010). In fact, the NaOH used in this study for the Ag extraction, is also used in e.g. soil studies to extract organic matter from inorganic fractions (Chang et al., 2014; Yang et al., 2013). In general, ultrafiltration can retain at least 20-30% of dissolved organic matter (Guo and Santschi, 2007; Quik, 2013), which, if the Ag is complexed with the organic matter, could explain the Ag retention by the ultra-filters. Furthermore, the correlation analysis between the organic carbon contents of the sediment samples (Table 4) and particulate or complexed Ag fraction of the ultrafiltration experiment (Figure 7) shows a 0.66 Pearson coefficient, indicating that the Ag retained in the ultra-filters is not likely to be AgNPs as spICPMS analysis results indicate, but rather Ag complexed with dissolved organic matter. That is why downstream sampling sites which were the sampling sites with lower organic carbon contents, showed higher dissolved Ag fraction than upstream sampling sites (Figure 7).

4. CONCLUSIONS

The most kinetically stable AgNPs were synthesised using $NaBH_4$ (0.002 M) and sodium dodecyl sulfate as stabilizer. The experiments suggested NaOH as a suitable extraction solution for AgNPs, since AgNPs shape and size remained intact even after a period of 10 days in NaOH. Comparing to total Ag present in sediment, NaOH extracted an average of 6.78 ± 3.85 % and up to 11 % of the Ag present. Out of the sediment samples analysed, S1 (Tribeni) and S4 (Budge Budge) contained the highest total Ag concentration. However, even though spICP-MS is currently the most sensitive method available to analyse NPs in aqueous samples, it was not able to detect any AgNPs in extracts from Hugli River sediments, despite the likely presence in these. Experiments using ultrafiltration and spICP-MS showed that the Ag was not present as NP but rather complexed with dissolved organic matter.

The NP extraction methods will be evaluated further using natural sediment samples, while methods using spICP-MS will be developed further to analyse NP at concentrations likely to be present in the natural environment.

The research and method development addresses a crucial gap in water and environmental analysis of NPs, where fundamental methods and data for the understanding of the consequences of nanoparticle pollutions is urgently needed in order to understand the transport, fate and toxicology of NPs in the natural water environment. This information is needed to inform policy makers for setting of guidelines and regulations regarding NP pollution.

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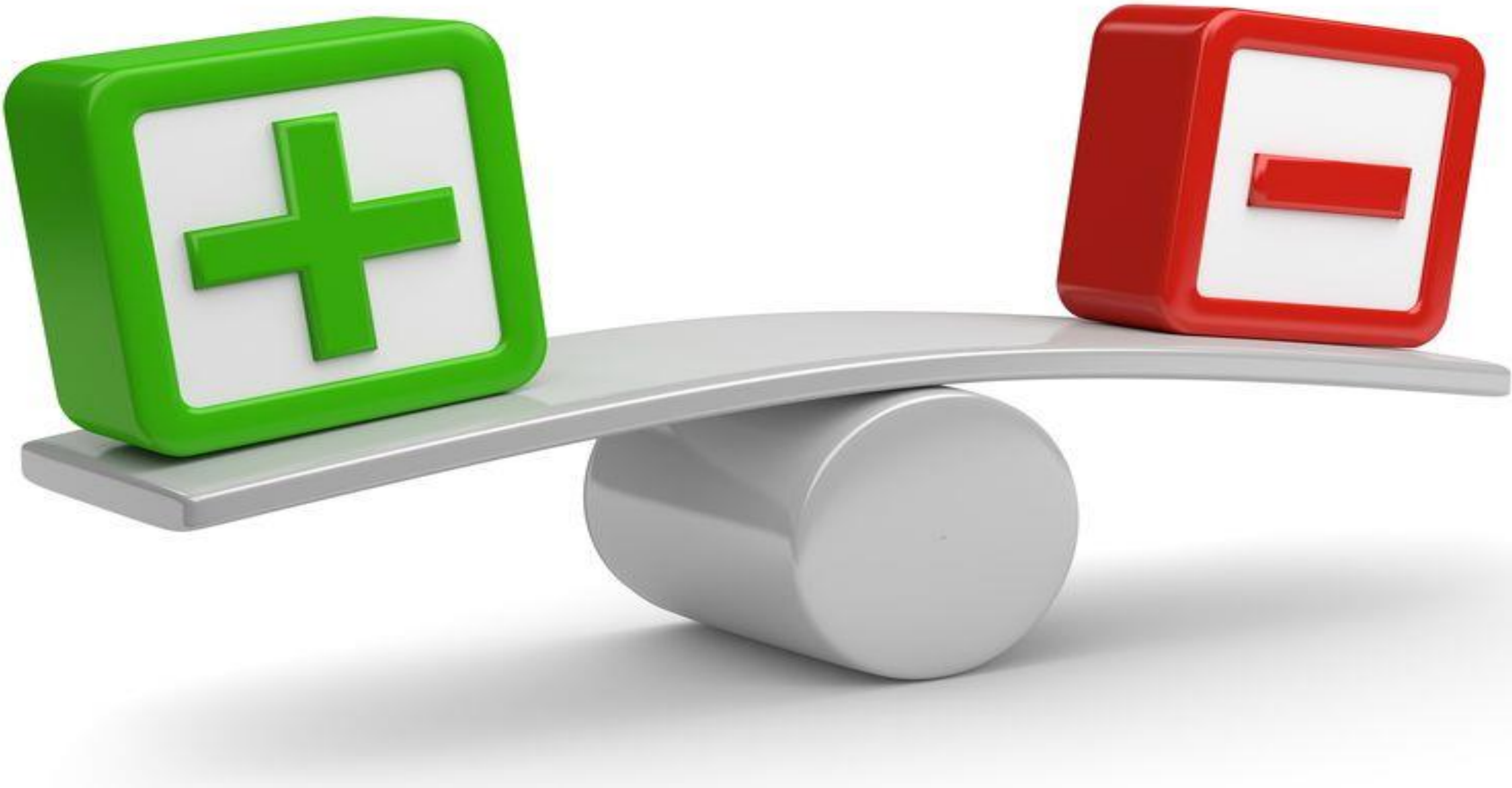
Chapter 12

GENERAL CONCLUSIONS



CHAPTER 12

GENERAL CONCLUSIONS

In the last decades, environmental pollution has been considered and still is being considered one of the greatest problems that the world and human beings are facing. This problem is constantly increasing due to the life style that human beings are carrying out and several environmental compartments are being directly or indirectly affected by pollution. Two of the main compartments affected are water and atmosphere. Our research group has been investigating the occurrence of toxic chemicals, and particularly, metal and metalloids in Basque estuaries for a long time using sediments samples. About five years ago we started also working in estuaries of other world regions and other environments like forests and high altitude lakes. Therefore, and taking into account the importance that any kind of environmental issue has nowadays in our Society, and the threatens of the uncontrolled increase of pollution, the main objectives of this Thesis were to study the geographical distribution of metal and metalloid contamination in selected estuarine and high altitude mountain environments and its evolution in time, to make hypothesis about their possible source, to compare the efficiency of different indicators to monitor metal and metalloid contamination in these environments, and to study the toxicological implications that the presence of metals and metalloids in the selected environments has on the organisms living in those environments.

Concerning high altitude mountain ranges, it can be concluded that even in remote areas as the Pyrenees, significant effects of the human activities can be observed and demonstrated using lake sediments and bioindicators as sentinels of pollution. Pollutants can reach these areas transported by the wind, which can airborne metallic contamination to long distances far from the contamination source. The highly industrialized areas of the Basque Coast and Iruñea, the mining activities carried out long time ago in the area, the combustion of fossil fuel over recent years and finally the road traffic in and in the surroundings of the Pyrenees can be mentioned among the possible contamination sources in the Irati Forest. Furthermore, lake sediments core analysis demonstrated that problems concerning metallic pollution started long time ago. On the other hand, beech leaves, lichens and mosses have shown their ability to be used in a complementary way as indicators of atmospheric metal contamination in monitoring exercises, but it should be taken into account that they provide information not representative of the same period of time due to their different longevities.

Regarding estuarine compartments, the analysis of water and sediments from the Tubarão River (Santa Catarina, Brazil), which is located in an area seriously affected by coal mining activities, urban discharges, thermoelectric plant, farming and livestock activities, showed that after typical tropical strong rainfall events, the mobility and distribution of metal and metalloids in the water column and sediments can be modified due to the changes in the hydrodynamic conditions of the estuary. Thus, remobilisation of sediments and the consequent transport downstream of those sediments was demonstrated.

For the Cávado estuary (Esposende, Portugal), which is located in a relative clean area with just agricultural and recreational activities, the highest concentrations of toxic metals were identified in the upstream sampling sites of the estuary but, in all the cases, with concentrations similar to those of the background values. Two sampling sites constitute the exception for Ni and Cr. However, the concentrations measured in those sediments did not represent a toxicological threat for the living organisms of the estuary. Furthermore, comparison with previous works carried out in surface sediments of the same area demonstrated that metallic contamination in the Cávado estuary is decreasing with time.

When it comes to the estuary of the Nerbioi-Ibaizabal River, similar metallic geographical distribution were detected in 2009, 2010 and 2011 sampling campaigns, but the situation changes drastically in 2014. New hotspots were identified close to the mouth of the estuary and in the dock of Portu, probably due to the construction in the Abra Bay of a new place to dock for long tourist cruise liners. The surroundings of the biggest waste water treatment plant operating in the area remains as a point of high concern in all the sampling campaigns. The origin of metals like Pb, Cd, Zn, Cu, As, Sn, V and Mn in the estuary is mainly anthropogenic, while Al, Co, Ni, Cr, Mg and Fe are probably natural. Finally, the occurrence of metals of anthropogenic origin results in moderately to highly toxic sediments in the hotspots of pollution. It has also to be mentioned, that one of the aims of this work was to characterise the estuary of the Nerbioi-Ibaizabal River in terms of metal pollution, in order to make feasible a future quantification of the effects derived from the Zorrozaurre island actuation, which is being carried out nowadays, with tones of sediments mobilised in the surroundings of the channel of Deusto.

For the estuary of the Oka River, it was concluded that the low toxicity estimated for the sediments was in good agreement with the low biological affections observed in oysters. Just some inflammatory responses as haemocytic infiltrations and few increases in the metallothionein levels were observed in autochthonous oysters of the estuary. Furthermore, the metal concentrations recorded in oysters were from low to moderate and, accordingly, the recorded haemocytic infiltration prevalences were lower than those measured in previous works. The values of the MLR/MET ratio were in general higher in 2011 than in 2010 indicating some type of stress in oysters from 2011, which was in good agreement with the observed higher metal concentrations in oysters of 2011 than in oysters of 2010. The results suggest a general improvement of the environmental status of the estuary of Urdaibai with time.

Finally, it was concluded that NaOH solutions are suitable for the extraction of silver nanoparticles (AgNPs) from sediments, provided this reagent did not affect the stability of the nanoparticles. However, even though spICP-MS is currently the most sensitive method available to analyse NPs in aqueous samples, it was not able to detect any AgNPs in NaOH extracts of sediments from the Hugli River. Experiments using ultrafiltration and spICP-MS showed that Ag was not present as NP but rather complexed with dissolved organic matter. Something to be done in the future is to analyse sediments which have been previously spiked with AgNP. A confirmation of the presence of nanoparticles in the extract would confirm that NaOH is actually able to extract nanoparticles from sediments and allow their analysis by spICP-MS.

SCIENTIFIC PUBLICATIONS

Some of the results described in this PhD Thesis have already been published (or are already submitted or are currently in preparation) as original research papers in international scientific journals:

- Gredilla, A., Stoichev, T., Fdez-Ortiz de Vallejuelo, S., Rodriguez-Iruretagoiena, A., de Morais, P., Arana, G., de Diego, A. and Madariaga, J.M., 2015. Spatial distribution of some trace and major elements in sediments of the Cávado estuary (Esposende, Portugal). Marine Pollution Bulletin: DOI: 10.1016/j.marpolbul.2015.07.040.

- Rodriguez-Iruretagoiena, A., Elejoste, N., Gredilla, A., Fdez-Ortiz de Vallejuelo, S., Arana, G., Madariaga, J.M. and de Diego, A. Occurrence and geographical distribution of metals and metalloids in sediments of the Nerbioi-Ibaizabal estuary (Bilbao, Basque Country). Submitted to Marine Chemistry.

- Rodriguez-Iruretagoiena, A., Fdez- Ortiz de Vallejuelo, S., Silva, L.F.O., de Diego, A. and Madariaga, J.M. Effects of a tropical storm in a polluted estuary: Tubarão river as a case of study. In preparation.

- Rodriguez-Iruretagoiena, A., Bustamante, J., Fdez-Ortiz de Vallejuelo, S., Arana, G., Amouroux, D., Santamaria, J.M., Elustondo, D., de Diego, A. and Madariaga J.M. Mosses, lichens and beech leaves as bioindicators of atmospheric metallic pollution: a case study, the Irati Forest (Western Pyrenees). In preparation.

- Rodriguez-Iruretagoiena, A.,Rementeria, A., Zaldibar, B., Soto, M., Fdez-Ortiz de Vallejuelo, S., Gredilla,A. and de Diego, A. Histopathological alterations in autochthonous oysters derived from the occurrence of metals in the estuary of the Oka River (Urdaibai, Basque Country). In preparation.

- Rodriguez-Iruretagoiena, A., Graham, M., Cornelis, G., Fdez-Ortiz de Vallejuelo, S., de Diego, A. and Schulte-Herbrüggen, H. Development of an extraction method for the analysis of silver nanoparticles in sediments. In preparation.

- Rodriguez-Iruretagoiena, A., Gredilla, A., Fdez-Ortiz de Vallejuelo, S., Arana, G., Madariaga, J.M., Auguet, J.C., Gonzalez, A., Camarero, L. and de Diego, A. Sediments from high altitude Pyrenean lakes as indicators of metallic pollution. In preparation.

Part of the results of the PhD Thesis has also been presented in congresses and workshops as written or oral communications:

- Rodriguez-Iruretagoiena, A., Elejoste, N., Gredilla, A., Fdez-Ortiz de Vallejuelo, S., Arana, G., Madariaga, J.M. and de Diego, A. Historical records and geographical distribution of metallic pollution in sediments of the Nerbioi-Ibaizabal River (Bilbao, Basque Country), 13th International Estuarine Biogeochemistry Symposium (IEBS), Bordeaux, France, June 7-10 (2015), (ORAL PRESENTATION).

- Rodriguez-Iruretagoiena, A., Gredilla, A., Fdez-Ortiz de Vallejuelo, S., Arana, G., Madariaga, J.M., Auguet, J.C., Gonzalez, A., Camarero, L. and de Diego, A. Pirinioetako altuera handiko lakuetako sedimentuak, inguruko kutsadura metalikoaren adierazle, Nazioarteko ikerketa euskaraz (IkerGazte), Durango, Basque Country, May 13-15 (2015), (ORAL PRESENTATION).

- Rodriguez-Iruretagoiena, A., Gredilla, A., Bustamante, J., Martinez-Arkarazo, I., Fdez-Ortiz de Vallejuelo, S., Arana, G., de Diego, A. and Madariaga, J.M. Geographical distribution and evolution in time of metal pollution in sediments, oyster and water from the estuary of the Oka River (Bay of Biscay, Basque Country), Environmetal&Pyrenees International Conference (EPIC), Iruñea, Basque Country, October 19-21 (2011), (POSTER PRESENTATION).

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Environmental pollution is considered one of the greatest problems that the world is facing nowadays. Amongst others, some metals and metalloids are chemicals able to cause serious problems to the environment. The anthropogenic inputs of metals and metalloids to the environment have grown constantly since the industrial revolution. Metals and metalloids are no degradable substances and consequently highly durable in the environment. Their toxicity highly depends on their concentration and speciation in each specific environment.

In this dissertation, the occurrence, geographical distribution and evolution in time of metals and metalloids was investigated in selected environments. On the one hand, atmospheric metal contamination was studied in different Pyrenean ecosystems, namely lakes and forests. Surface sediments and sediment cores of several high altitude remote lakes were collected and analysed to, respectively, describe the geographical distribution and track the historical records of metallic pollution in the Pyrenees. In addition, the efficiency of bioindicators such as beech leaves, lichens and mosses to monitor atmospheric metal pollution in the Irati Forest was checked. On the other hand, some characteristic estuaries located in different parts of the world were also investigated. The estuaries selected were those of the Tubarão River (Santa Catarina Region, Brazil), the Cávado River (Esposende, Portugal), the Nerbioi-Ibaizabal and the Oka Rivers (both in the Basque Country) and finally the Hugli River, one of the main tributaries of Ganges River (West Bengal, India). Sediments from those estuaries were analysed to describe the geographical distribution and evolution in time of metallic contamination in each studied area. In some cases, water samples and autochthonous oysters were also analysed in order to carry out a more complete assessment of the toxicity associated to the presence of toxic elements. In the case of the estuary of the Hugli River, in addition, a new analytical strategy for the analysis of emerging contaminants like silver nanoparticles in sediments was developed.