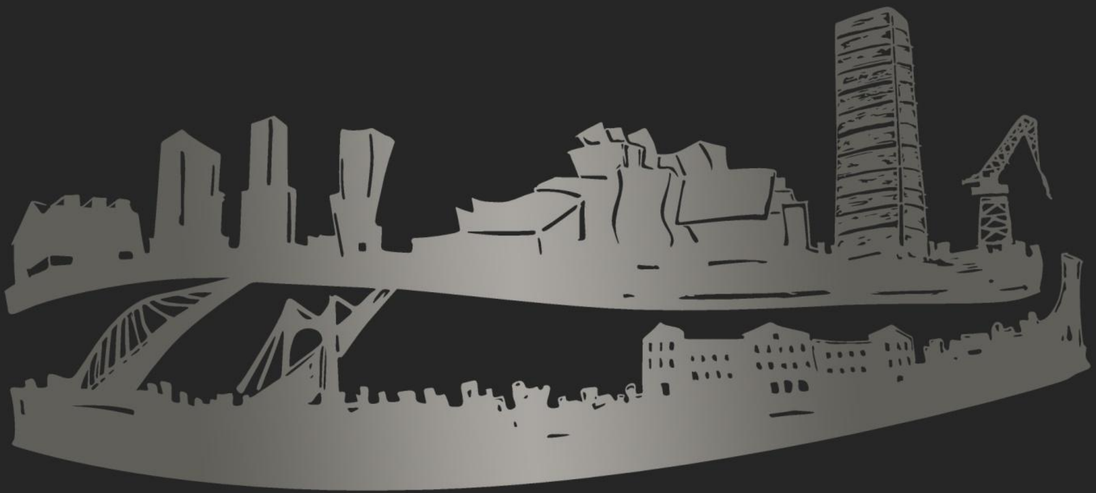
Enhanced analytical methodologies for environmental studies of metal pollution in rivers and estuaries

Unai Villanueva Saracho

February 2016



eman ta zabal zazu

ZTF-**FCT**
Zientzia eta Teknologia Fakultatea
Facultad de Ciencia y Tecnología

Kimika Analitikoa Saila
Dpto. de Química Analítica

Universidad del País Vasco
Euskal Herriko Unibertsitatea

Enhanced analytical methodologies for environmental studies of metal pollution in rivers and estuaries

Memoria para optar al grado de
Doctor en Ciencias Químicas

Unai Villanueva Saracho

Febrero 2016

Quiero agradecer a la universidad del País Vasco la oportunidad de haber desarrollado mi formación doctoral con el amparo de la beca de colaboración "Diseño de procesos de tratamiento de residuos con valorización de los mismos" y con el contrato de personal investigador en formación en prácticas entre los años 2004 y 2007.

El presente trabajo se ha realizado dentro de los proyectos de investigación estratégica Investigación estratégica para la protección y recuperación de la salud y calidad del recurso suelo (BERRILUR), Programas ETORTEK, refs. IE06-179 y IE09-24.

Asimismo, quiero agradecer a mis directores Juan Manuel Madariaga y Juan Carlos Raposo, por su ayuda y sobre todo por su infinita paciencia hasta llegar a este momento.

No puedo sino expresar también mi más profunda admiración a todos los miembros del departamento por su dedicación a la enseñanza y a la investigación, en especial a Luis, Juanma, Alberto, Nestor, Irantzu, Olatz, Gorka, Aresatz, Marian, Kepa, Mirari y por supuesto, a Antonio.

Durante todo este tiempo, han pasado por mi vida un montón de personas a las que quiero agradecer su apoyo científico y moral. Es posible que me deje a alguno, pero todos sabéis cuanto os agradezco todo lo que habéis hecho por mí.

Del departamento a: Eukeni, Jagoba, Txesko, Juancar, Joana, Sandra, Pati, Etxe, Alfredo, Josean, Ailette, Asier, Ibone, Jone, Mireia, Maite, Haizea, Ekhine, Oihana, Silvia, Laura, Naiara, Ainara, Gorka, Juanito, Mariluz, Nerea, Nuri, Oscar y a todos los que no os nombro, pero que ahora mismo estáis luchando para sacar la tesis adelante…

De Agilent a: Carmen, Eukeni, Marcos…

De Intertek a: Ainara, Aitor, Andrea, Ángel, Eduardo, Elena, Emilio, Erika, Eva, Goio, Gorka, Humberto, Iara, Imanol, Iñaki, Javi, Josu, Julian, Laura, Lidia, Lino, Maite, Marta, Mikel, Miriam, Nerea, Nuria, One, Pili, Unai, Ziortza.

De la cuadrilla a: Asier, Iker E., Iker M., Iker O., Laura, Javier, Jorge, Marta, Fernan, Laura, Rubén, Amaia, Sabin y por supuesto, a Ainhize.

Y por supuesto, a los de casa: a mi padre César que me ha enseñado todo y me ha hecho como soy (espero que estés orgulloso de mi), a mi madre Mª Cristina, ojalá pudieras verlo (sabes que cambiaría todo por un último baile contigo), a Maitane que me quiere (y que sorprendentemente me aguanta), a Edurne que me apoya en todo lo que hago, a Mirian que siempre que puede está dispuesta a echar un cable, a Patxo que se le echa de menos (todavía recuerdo lo primero que me dijo) y a Puri que se desvela por todos y a la que considero como una segunda madre. Todo esto es gracias a vosotros y sin vuestra ayuda, nada tendría sentido. Os quiero.

Unai

Resumen

Durante décadas, el desarrollo industrial ha mejorado la calidad de vida de los seres humanos facilitando el trabajo y proporcionando nuevos materiales entre otras ventajas. Este desarrollo industrial y sobre todo, la industria química en general, producen una gran cantidad de contaminantes que de una u otra forma, pueden alterar el medio ambiente y los procesos que se dan en el ecosistema.

El estudio analítico del ecosistema o del entorno natural es un indicativo de la calidad de propio entorno frente a esa contaminación producida y acumulada. Además, las zonas habituales de ocupación son aquellas cercanas a fuentes naturales de agua debido a la necesidad intrínseca de este recurso natural. Es por esto que ríos y zonas costeras como los estuarios son lugares de mucha actividad industrial y humana, y por tanto, son zonas de con mayor impacto, con mayor aporte de materiales antropogénicos y de contaminantes al medio.

El estudio de la acumulación de metales en el medio ambiente y su impacto en los ecosistemas es un objetivo relevante en distintos campos de investigación. Desde un punto de vista químico, y más concretamente analítico, el estudio de la acumulación de metales se realiza por medio de métodos cuantitativos que permiten determinar la cantidad total de los metales en varios compartimentos del medio natural, como la concentración total en agua, la concentración total extraíble en sedimento y la

concentración total en diversos organismos centinelas, que por su naturaleza, tienen a acumularlos.

En este marco de trabajo, el proyecto de esta tesis doctoral se basa en el **desarrollo de nuevas metodologías o mejoras de las ya existentes para el análisis de metales en los compartimentos de los ríos y estuarios.** Para el desarrollo del trabajo experimental, se han seleccionado diferentes lugares de muestreo repartidos por toda la geografía del País Vasco, distribuyéndose en cuatro ríos de la provincia vizcaína (Nerbioi, Ibaizabal, Oka y Kadagua), tres ríos de la provincia guipuzcoana (Deba, Urola y Estanda) y uno de la provincia alavesa (Zadorra), además de el estuario del Nerbioi-Ibaizabal (Arriluze, Puente Colgante, Las Arenas, Agra, Galindo, Lamiako, Udondo, Asua, Gobela, Zorroza, San Inazio y Alde Zaharra).

El análisis elemental en cada compartimento proporciona, en la mayoría de los casos, información suficiente para determinar la concentración total de metales. Sin embargo, a menudo se requiere obtener información molecular que permita discernir las distintas especies en las que se encuentran dichos metales, ya que la potencialidad tóxica para el medio-ambiente puede variar. Una técnica analítica idónea para la diferenciación entre las especies químicas es la espectroscopia Raman. A pesar de que una de las ventajas más relevantes de esta técnica se basa en la posibilidad de realizar análisis directamente y sin pre-tratamiento de muestra, la detección de los compuestos de interés queda supeditada a las características de la matriz.

En el caso concreto de los sedimentos, en esta tesis doctoral se ha desarrollado un método de lavado del sedimento previo que facilita el análisis directo, disminuyendo las interferencias normalmente observadas por la propia matriz (fluorescencia debida a la presencia de altas cantidades de arcilla y materia orgánica), sin alteración de las especies químicas. Además, el uso de un microscopio acoplado a un espectrómetro Raman permite el micro-análisis en distintas zonas del sedimento y la detección de las distintas especies con mayor resolución.

Este método de lavado, consiste en lavar el sedimento una vez seco y tamizado con 10 ml de acetona durante 30 minutos en un baño de ultrasonidos. Tras el tratamiento, el sedimento se filtra y se seca al aire. Este procedimiento permite identificar las distintas especies químicas presentes en los sedimentos, y se han podido distinguir especies naturales y antropogénicas, que pueden ser indicadores de las zonas más afectadas.

Tal y como se ha mencionado anteriormente, la potencialidad tóxica de los sedimentos se evalúa frecuentemente mediante la determinación de la concentración metálica extraíble con tratamientos ácidos. Sin embargo, el análisis de los sedimentos de un modo aislado, sin tener en cuenta por un lado los procesos físico-químicos que se pueden dar en las interfases agua/sedimento y por otro las características fisicoquímicas de ambas matrices, pueden llevar a conclusiones erróneas. Por ello, en esta tesis doctoral, se estudió la posibilidad de mejorar la metodología para la evaluación de la movilidad de las especies metálicas entre los compartimentos agua/sedimento. Hasta el momento, la mayoría de los análisis de contaminación metálica en sedimento se realizan por métodos estandarizados que proporcionan información sobre la cantidad total de metal extraíble o, mediante métodos de extracción secuenciales, proporcionando la cantidad de contaminantes metálicos que son capaces de extraer éstas disoluciones aplicadas secuencialmente al sedimento. Todos estos métodos emplean unas disoluciones sintéticas preparadas en el laboratorio con diferente capacidad extractante. Uno de los métodos más empleados es la norma DIN 19730, que mediante una agitación durante dos horas con una disolución de NH_4NO_3, emula el potencial de disponibilidad de la contaminación metálica del sedimento a la fase acuosa. Sin embargo, el empleo de este tipo de disoluciones puede reflejar un escenario con condiciones muy dispares a las que se dan en los sistemas estuáricos y fluviales, y por lo tanto, unas conclusiones alejadas a la realidad. Es por ello que, en esta tesis se propone la modificación del método DIN 19730 sustituyendo la disolución de NH_4NO_3 por el empleo del mismo agua de río en contacto con el sedimento.

Para la evaluación de la capacidad de la metodología, se han seleccionado varios ríos del País Vasco y se ha ensayado la movilidad del sedimento con el propio agua, tal y como se propone en la nueva metodología, y según la norma DIN 19730. Los resultados obtenidos indican que el potencial de movilización encontrado según la norma DIN 19730 es siempre mayor. La mayor movilización de los metales utilizando las condiciones propuestas por la norma puede estar atribuida a la formación de amino-complejos que favorecen su movilidad. De esta forma, se indica que los valores de movilidad encontrados con la metodología propuesta (empleando el propio agua en contacto con el sedimento), tiene un valor más cercano al valor real de movilidad que se produce naturalmente.

Siguiendo un esquema de trabajo similar, la metodología de movilidad se extendió al estudio del sistema estuárico, con mayor influencia mareal.

Al igual que en el sistema fluvial, los ensayos de movilidad realizados en el sistema estuárico (sedimentos en contacto con agua de estuario de distinta salinidad) indicaron que el empleo de los reactivos propuestos en la norma indican unos valores de movilidad superiores a los que acaecen en la realidad. La modelización química mediante herramientas informáticas que permiten tener en cuenta la formación de las especies químicas a nivel termodinámico en las condiciones reales, pone de manifiesto el hecho de que la presencia o aporte de un agente externo a las especies químicas que naturalmente se encuentran en el medio, puede alterar el comportamiento natural de las especies.

Con el objetivo de ahondar más en el comportamiento de los metales en las condiciones estuarícas (marea alta y baja) y fluviales, se ha realizado un estudio de especiación termodinámica de los compuestos de arsénico presentes en la naturaleza y su comportamiento.

La modelización termodinámica requiere, en primer lugar, la determinación de las constantes de equilibrio de formación de las distintas especies de arsénico. Estas se han calculado empleando la metodología modificada de Bromley para el cálculo de los coeficientes de actividad de cada especie y utilizando la fuerza iónica calculada teniendo en cuenta las especies mayoritarias del sistema. A partir de estos modelados termodinámicos, se pudo concluir que el comportamiento del arsénico en los ríos está condicionado por la cantidad de hierro y calcio que hay en el sistema. Sin embargo, para las condiciones de estuario, son el calcio y el magnesio los que regulan el comportamiento del arsénico favoreciendo su deposición en el sedimento en condiciones de marea baja o su redisolución en condiciones de marea alta. La presencia de las especies químicas propuestas en los distintos modelos termodinámicos se pudo corroborar mediante el análisis de los sedimentos mediante espectroscopia Raman, avalando la metodología como una herramienta muy útil con diversas aplicaciones.

Además de la especiación, la materia orgánica presente en los distintos compartimentos medioambientales juega un papel importante a la hora de estimar la movilidad de los metales. Las sustancias húmicas se consideran compuestos complejos y reactivos que afectan a la movilidad de los metales en la interfase agua – sedimento. Según la bibliografía consultada, estas sustancias son complejantes de diversas sustancias contaminantes, como por ejemplo los metales y es claro su papel en la biodisponibilidad de los metales para los seres vivos. Teniendo en cuenta la importante influencia que presenta la materia orgánica en los sistemas naturales, se ha propuesto una nueva metodología para la cuantificación de las sustancias húmicas mayoritarias, el ácido húmico y el ácido fúlvico.

Hasta el momento, no existe una metodología rápida para su análisis cuantitativo, siendo necesarios procesos tediosos o extracciones sucesivas. En este trabajo se plantea una metodología nueva basada en el ultrasonidos focalizado y posterior análisis mediante espectroscopia ultravioleta-visible permite la cuantificación de los ácidos húmicos y fúlvicos en sedimento. Para garantizar unas condiciones óptimas de análisis, el trabajo detalla la optimización exhaustiva de todos los procesos utilizados.

Esta nueva metodología se aplicó al análisis de sedimentos y aguas muestreadas en diversos puntos del estuario del Nerbioi-Ibaizabal. Los análisis revelaron la variabilidad de

dichos en función de la época del año, con máximos de concentración en meses de invierno y mínimos en verano, así como en función de los parámetros fisicoquímicos del agua (la salinidad/conductividad del agua y el potencial de oxido-reducción) y del sedimento. Su distribución puede ayudar a identificar el comportamiento de los puntos de muestreo, definiéndose dos tipos de comportamiento, uno de tipo "afluente" y otro de tipo "curso principal", siendo más favorecido en el primero la acumulación y en el segundo la movilización del sedimento al agua.

Con respecto a la tendencia de estas sustancias con respecto a la movilidad empleando el anterior método descrito, se observa una mayor deposición en marea baja y una mayor solubilización en marea alta. Este comportamiento está relacionado con los datos de movilidad encontrados en los metales analizados.

Finalmente, una vez analizados varios compartimentos de los sistemas fluviales y estuáricos, cabe destacar que el análisis de la concentración de metales en ciertos organismos que viven dentro de los compartimentos analizados en esta tesis doctoral, es un tema en alza dentro de las investigaciones que se llevan a cabo como evaluación de la biodisponibilidad de los metales u otros contaminantes.

Los organismos centinela, como los mejillones, se consideran marcadores adecuados para evaluar de una manera relativamente rápida y sencilla el estado de determinados escenarios medioambientales. Estos organismos centinela pueden acumular los contaminantes presentes en el agua por filtración, y por lo tanto, son considerados muy adecuados para la evaluación de la biodisponibilidad. Sin embargo, el análisis de este tipo de matrices biológicas es problemático debido a la presencia de materia orgánica que produce fluctuaciones y aumenta la imprecisión de las medidas analíticas.

Para evitar esta problemática, se ha diseñado una nueva metodología de limpieza que consiste en el lavado con 5 ml de diclorometano durante 5 minutos del tejido de mejillón previamente seco y molido. Este método ha facilitado que en la etapa de extracción ácida posterior (también optimizada), se genere una cantidad mucho menor de sustancias

orgánicas que interfieren en la medida de los metales. Se ha comprobado que la precisión de las medidas mejora sustancialmente, además de reducirse considerablemente el mantenimiento necesario por la contaminación de estos subproductos orgánicos.

En resumen, las metodologías presentadas en esta tesis doctoral pretenden ser de aplicabilidad en los estudios medioambientales enfocados a la contaminación metálica, de tal manera que este tipo de estudios no se realice únicamente en grandes entidades y organizaciones de investigación sino también en empresas e industrias. A pesar de que la evaluación completa de un sistema medioambiental es complicado, el desarrollo de métodos analíticos que simulen escenarios lo más reales posibles así como la interpretación y contextualización de los datos analíticos por un equipo de trabajo multidisciplinar permite satisfacer en mayor o menor grado las necesidades planteadas desde las diversas áreas científicas.

Index

1. Introduction

1. Introduction

Chemistry is related to all human beings surroundings. During all mankind evolution some advances have been carried out, but the biggest were those related to industrial and technological advances occurred mainly from XVIII to XX centuries. Concretely, after the Second World War, modern society evolved through a technological rush that looked as if the progress was unlimited, with chemical industries delivering new products at an imposing producing rate. The industrial revolution went on in parallel to the economy growth but, unfortunately, it caused increased and widespread pollutant emissions to the environment. Although it was thought that this problem was limited to the closest area of the emission sources, scientific works proved the increasing of large-scale contamination scenarios. The specific case of methylmercury poisoning in Minamata (Japan) in 1961 was an historical example of this large-scale contamination: scientists discovered that, even in the open ocean, high concentrations of mercury were found bioaccumulated in fishes caught worldwide.

Nowadays, although emissions are more regulated, there is always something related to chemical processes everywhere it can be looked such as refinery processes, food industry, textile industry, steelworks, pharmaceutical industries – to name a few – where fuels, polymers, clothes, food, drugs and personal care products are processed.

During these industrial processes, some side-products are always obtained as wastes when trying to get some goods. Moreover, not only side-products, but also, chemicals without a non valuable profit are discarded to the environment. Probably, due to the still unknown possible toxic effects of those discarded products or to the absence of specific legislation, the main dumps of those products have been accumulated in environmental bodies (mainly soil, sediments, sludge and water). As a consequence, the disturbance and acceleration of naturally occurring geochemical cycles of such compounds can pose risks to human health and the ecosystem living beings by direct contact with contaminated matrixes, intrusion in the food chain and drinking of contaminated water, among others.

The awareness of the potential toxicity of certain chemicals resulted in a call to regulate their presence in the environmental bodies. The application of some long-term monitoring programs during several decades (see Table 1.1, [Roose et al., 2005]) revealed the ubiquitous presence of trace organic microcontaminants and metals in all compartments of the environment, being necessary their continuous monitorization.

Table 1.1. *Overview of long-term monitoring programmes including contaminants and matrices.*

Monitoring programme	***Start Date***	***Monitored compounds***	***Environmental sample***
AMAP	1978	HM, PCBs, PAHs, OCPs	Biota, sediment, water
OSPAR	1978	HM, PCBs, PAHs, OCPs, OTins	Biota, sediment
HELCOM	1979	HM, PCBs, PAHs, OCPs, OTins	Biota, sediment
NS&T	1986	HM, PCBs, PAHs, OCPs	Biota, sediment

AMAP: Arctic Monitoring and Assessment Programme; HELCOM: The Baltic marine environment protection commission; HM: Heavy metals; NS&T: National Status and Trends programme; OCPs: Organochlorine pesticides; OSPAR: The North-East Atlantic marine environment protection convention; OTins: Organotins; PAHs: Polycyclic aromatic hydrocarbons, PCBs: Polychlorinated biphenyls.

1.1. Environment

Human is changing the Earth as it was cited in an article published some years ago in Science: *"Between one-third and one half of the land surface has been transformed by human action; the CO_2 concentration in the atmosphere has increased by nearly 30% since the beginning of the Industrial Revolution; more than half of all accessible surface freshwater is put out to use by humanity....The rates, kinds and combinations of changes occurring now are different from those at any other time in history; we are changing Earth more rapidly than we are understanding it. The world is in our hands and how we handle it will determine its composition and dynamics and our fate* [Vitousek et al., 1997]."

The interaction between humans and ecosystem points out the differences between Nature and the environment [Sagoff, 1994]: *"Nature, which includes the Earth's evolutionary and ecological heritage, is the object of religious, aesthetic and cultural contemplation. The environment, in contrast, is a concept of recent origin. It is the subject of economic and biological sciences that attempt to predict, control, and "price" the flows of materials and resources in order to maximize the long-run benefits nature offers man".* Consequently, it can be said that human beings can pose stress on the environment by promoting changes to the atmosphere, hydrosphere, lithosphere and biosphere, and hence, alter the natural evolution of nature.

Humans have interacted with their ecosystem for thousands of years, changing their surroundings based on their needs and capacities. Most of sites occupied by humans are close to water sources, warm, with intermediate relative humidity and fertile soils. Thus, human settlings are usually near to water areas such as rivers, estuaries and coastal locations which are plenty of natural resources, satisfying economic necessities and being intensely exploited for centuries. It is already known that the degradation of ecosystems and pollution problems are directly related to the increasing of population density [Adams et al., 2005]. As an example, Figure 1.1 shows the population in the Basque Country, where the higher number of inhabitants is often found close to river or coastal zones.

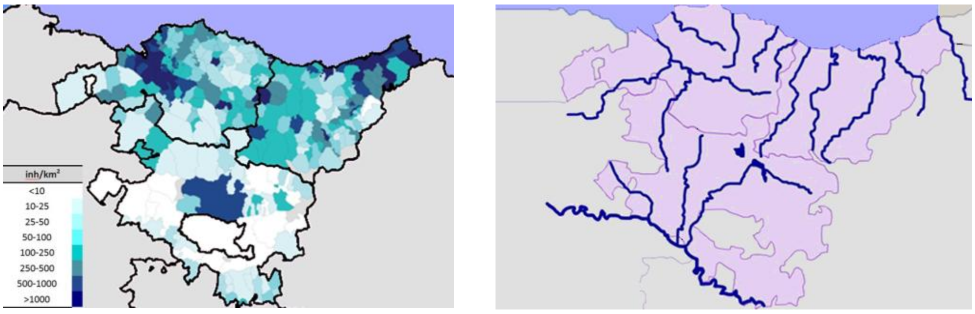


Figure 1.1. *Density of inhabitants in the Basque Country and location of the main rivers from the Basque Country (data taken from census 2009 [Ruiz, 2009]).*

In this scenario, the scientific works focus on different environmental studies assessing the impacts as well as their possible long-term consequences in this specific zone [Bartolome et al., 2010; Marigomez et al., 2013; Fernandez et al., 2008; Sanz et al., 2004; Moros et al., 2009; Fdez-Ortiz de Vallejuelo et al., 2010].

Taking into account the previous definitions and discussions, environmental concerning studies have being nowadays extended over any process that would affect this well-being, whether it is physical, chemical or biological; since the environment is not a static system with an important natural self-regulation capacity (this is, maintaining the consistency of its natural functions and dampening the changes occurring in it). However, this natural capacity can be exceeded by changes resulting from anthropogenic generated pollution, producing environmental degradation and deterioration of biotic and abiotic systems.

1.2. Presence of contaminants in the environment

As it has been stated previously, it is known that chemical and pharmaceutical industry products are considered as the new type of environmental pollution from the end of the last century, since their continuous presence in the environment is producing health risks for humans [Clarke, 2011]. Nevertheless, the presence of metals in all environmental matrixes is still being a concern.

Metals[1] are naturally occurring in aquatic systems (concentrations up to $\mu g \cdot l^{-1}$ in surface waters), sediments (concentrations up to $\mu g \cdot g^{-1}$ or $mg \cdot g^{-1}$ levels) and biota (concentrations up to $ng \cdot g^{-1}$ or $\mu g \cdot g^{-1}$). Silicon, aluminum, iron, calcium, sodium, potassium, magnesium and titanium are the major elements that are naturally occurring on the Earth's surface [Giddings, 1973]. Among them, Al, Fe, Mn, V and Ba are the elements coming from the rock erosion that reach surface and underground waters at larger quantities. Volcanic eruptions are another natural source of inorganic elements to the environment being As, Sb, Hg, Cu, Pb, Se and Zn the most emitted. Sea aerosols, forest fires, biogenic emissions and natural hydrocarbon seeps are also considered as natural sources [Garrett, 2000].

Nowadays, the presence of these elements has increased in the environment as a result of human activities. This legacy of contamination is currently worsen via direct inputs that are often the consequence of the increasing urbanization and discharges of domestic effluents (Al, Cu, Fe, Ni, Pb and Zn), industrial waste management (Cd, Cu, Fe, Pb, Mn, Ni and Zn), mining (As, Cu, Cd, Pb, Hg and Mn) and agricultural waste products (Pb, Cd and Cu) as well as surface runoff, soil erosion and mobilization of contaminated sediments [Scancar et al., 2000]. Table 1.2 summarizes some applications in which these elements are often used.

Trace elements are persistent in the environment and are potentially bioavailable, bioaccumulable and toxic[2] to aquatic biota at high-medium concentration levels [Wood et al., 2012]. All the contaminants spilled to environment suffer some processes until final setting and accumulation such as retention, precipitation, adsorption and movement among phases being able to change their oxidation state and becoming more dangerous than the original emission product. Consequently, and taking into account that metals can be present in various forms (i.e., as elements, as non-mobilizable compounds, as

---

[1] *They may be subdivided into: (i) transition metals (Co, Cu, Fe, Mn) which are essential to metabolism at low concentrations but may be toxic at high concentrations; (ii) metalloids (As, Cd, Pb, Hg, Se, Sn) which are toxic even at low concentrations. All of them will be referred to metals thereafter.*

[2] *An approximate order of decreasing toxicity is as follows: Hg > Cd > Cu > Zn > Ni > Pb > Cr > Al > Co.*

mobilizable compounds, etc.), the chemical form wherein they occur as well as the concentration level is decisive to understand the criticism of the contamination [Gredilla et al., 2014].

Table 1.2. *Most important applications of metals and metalloids considered in this work [USGS, 2015].*

Metal	***Most important applications***
Fe	98 % is forged into steel, construction, transportation, machinery, cans, containers.
Al	Transportation, packaging, construction, consumer durables, electrical transmission lines, machinery.
Cu	Power transmission and generation, wiring, telecommunication, electrical and electronic products, industrial machinery, consumer and general products.
Zn	Coating, galvanizing, alloys, construction materials, stabilizers, pigments, micronutrients.
Pb	Industrial and transport, batteries, ammunition, lead sheet, pigments, stabilizers.
Cr	Stainless steel and non-ferrous alloys, plating of metals, pigments, leather processing, catalysts, surface treatments and refractories.
Ni	Stainless and ferrous and non-ferrous alloys, super-alloys, electroplating, transportation, construction, machinery, electrical equipment, household appliances.
Sn	Coatings, alloys, solder.
Hg	Dentistry, switches, lighting, industrial applications such as production process of PVC.
Cd	Batteries, pigments, coatings, stabilizer.
As	Wood preservative, pesticide.
Mo	Alloying agent in steel, cast iron, super-alloys, also added to chromium, manganese, nickel or other alloy metals, catalysts, lubricants, and pigments.
Co	Rechargeable battery electrodes, super-alloys, airbags, catalysts, cemented carbides (also called hard metals), diamond tools, wear-resistant alloys, drying agents for paints, varnishes, inks, dyes and pigments, ground coats for porcelain enamels, high speed steels, magnetic recording media, magnets, steel-belted radial tires.

1.2.1. Metals in estuary, rivers, sediments and biota

In recent times, estuarine and coastal marine ecosystem have suffered increasing degradation caused by multiple stressors including toxic chemical pollutants, excess of nutrient inputs, hypoxia, turbidity and suspended sediments [Adams, 2005]. Within this scenario, Borja et al. [Borja et al., 2010] reviewed and evaluated some medium- and long-tem recovery of estuarine and coastal ecosystems in order to assess the patterns, rates and effectiveness of different estuarine restoration processes such as the Nerbioi-Ibaizabal estuary.

In general terms, estuaries are defined as water bodies that connect land and ocean and extend from fully marine conditions to the effective limit of tidal influence and where seawater is diluted by freshwater inflow [Hobbie, 2000]. These environments have been strategic occupation zones for human and diverse biological species, but they have been also used as waste disposals, which have contributed to their deterioration. Chon et al. [Chon et al., 2010] have reviewed the presence of substances at different source points (industrial wastes, effluents of waste water treatment plants, agricultural activities and sediments) in order to assess their contribution to the chemical distribution in estuaries.

Estuarine system quality is progressively improving as a result of the remedial actions implemented over the past 20 years, but metals still remain embedded within different estuarine compartments. Anyway, the good chemical quality of water bodies depends on both the surface waters and the underlying sediments [Dueri et al., 2008]. Hence, some results of research works suggest that the overall chemical quality should be assessed analyzing both water and sediments [Tueros et al., 2009]. In addition, the assessing of quality status as well as bioavailabity of contaminants in estuaries is widely performed by the analysis of sentinel organisms such as mussels [Bartolome et al., 2010; Marigomez et al., 2013].

A very high percentage (almost 90%) of metals is adsorbed to suspended particulate matter or colloids [Bustamante et al., 2012]. Afterwards, they can be settled into sediments through flocculation, incorporation into the lattice structure of minerals (e.g., Fe-Mn oxides) and precipitation by forming insoluble fractionation (such as metal sulfides [Zhang et al., 2014]). This is the reason why sediments are widely considered as natural sink of contaminants, where elements tend to distribute between the sediment and interstitial water. They are supposed to be a more reliable matrix to determine the source and partitioning dynamics of metals than the overlying water column, due to the discontinuity and fluctuations of the water [Alonso Castillo et al., 2013]. Consequently, sediments have been used to monitor the concentration of metals in estuaries worldwide [Bustamante et al., 2012]. Extractable metal concentrations of sediments analyzed in Europe were compiled by the Fraunhofer-Institut[3] and are presented in Table 1.3 [Chon et al., 2010].

Table 1.3. *Extractable metal concentration (µg·g^{-1}) in sediments analyzed in Europe.*

Metal	*Median (µg·g^{-1})*	*Mean (µg·g^{-1})*	*SD (of mean value)*	*Number of samples*
As	8	14	19	2708
Cd	2	12	91	2873
Cr	50	73	29	3409
Cu	38	127	1269	3153
Pb	60	140	266	3091
Hg	1	2	3	2735
Ni	27	32	7	2909
Zn	219	565	639	2833

In non-contaminated sediments, metals mainly occur as silicates and primary minerals with limited mobility constituting the bottom mud. When polluted, the form and distribution of metals in sediments is altered. In such cases, metals can interact with the

---

[3] *Fraunhofer-Institut, Revised Proposal for a List of Priority Substances in the Context of the Water Framework Directive (Combined monitoring based and modeling based priority setting, COMMPS Procedure): Final Report (Declaration ref.: 98/788/3040/DEB/E1), http://ec.europa.eu/environment/water/water-framework/preparation_priority_list.html. Last access, October, 2015).*

components of the complex environmental compartments. In the case of sediments, metals can interact with organic substances, Fe-Mn oxides, carbonates, sulphates, sulphides and clay minerals through different binding mechanisms [Gleyzes et al., 2002].

Bottom sediments contain up to five times the concentration of metals found in overlying waters. Resuspension processes of metals sorbed into sediment caused by strong tidal currents, dredging operations or during storms may release a significant amount of metals into the water column. This desorption of contaminants from particulate phases can cause secondary contamination in water bodies, biota and ecosystem.

Finally, and in order to assess metal fate in estuarine systems, numerical model studies of the fate and transport of metals in the estuarine compartments have been successfully developed and applied to water and sediment samples [Wu et al., 2005]. Even more, environmental studies have systematically included the monitoring of metals in biota and living organisms as an important indicative of the quality state using sentinel organisms collected over different estuaries and coastal regions [Fung et al., 2004; Bartolome et al., 2010; Marigomez et al., 2013; Spada et al., 2013].

1.3. The effect of water physicochemical and sediment geochemical properties on metals distribution and bioavailability

The potential harmful effects of contaminants are mainly related to their distribution, bioavailability and biodisponibility to the environmental compartments and organisms. As stated some years ago [Kennish, 1997], the distribution of metals in environmental waters and sediments mainly depends on:

- The mobilization of metals through solubilization, diffusion, transport and precipitation processes and their chemical speciation.

- The major sediment solid components to which metals are preferentially bound such as, particulate organic matter, iron and manganese oxyhydroxides and sulphides.
- The influence of physical and chemical parameters of environmental water bodies, such as salinity, redox potential, pH and ligand complexes.
- The competition between metals for uptake sites in organisms.
- The sediment texture, mineralogical composition and physicochemical transport.

The consequence of such interactions is a heterogeneous metal distribution, turning the estuary in a very complex system to be studied. Those parameters will be explained below as they are the basis to understand metal occurrence in estuarine environments. In this framework, Zhang et al. [Zhang et al., 2014] and de Souza Machado et al. [de Souza Machado et al., 2016] have recently published interesting reviews about the effects of geochemical parameters of estuarine sediments and waters on metal bioavailability and presence in aquatic living organisms. The processes that can affect the metal distribution are illustrated in Figure 1.2.

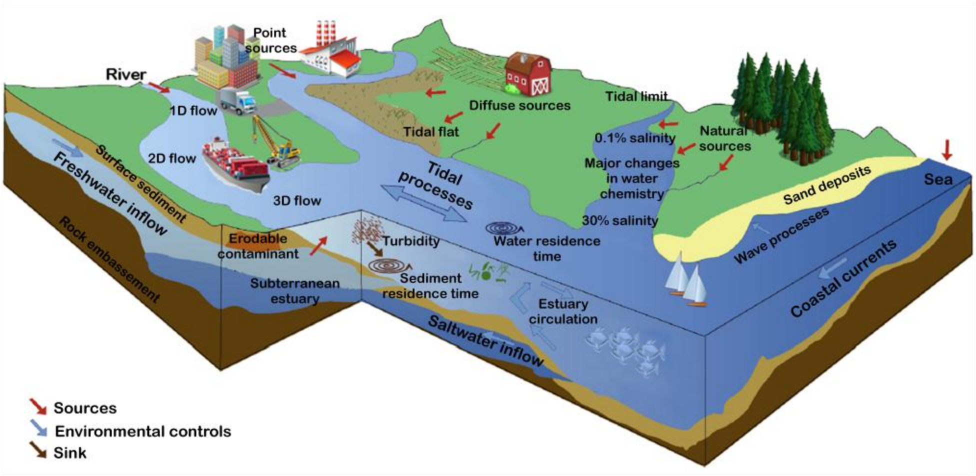


Figure 1.2. *Illustration of estuarine environment where physical, chemical and biological processes are shown. Modified from [de Souza Machado et al., 2016].*

1.3.1. Aqueous phase characteristics

Although the distribution of metals along the estuaries can be conservative, the interaction of environmental gradients (e.g., water salinity, density, flow velocity and suspended matter composition) and metal sources often yields in a non-conservative distribution of metals. Several works have demonstrated this non-linear distribution for Cd, Cu and Pb, which showed different partitioning coefficients under different estuarine conditions [Benoit et al., 1994; Spencer et al., 2002].

Water salinity is one of the main estuarine characteristics that brings great changes in water chemistry and ionic strength, resulting in different metal mobilization and, hence, a non-linear distribution of metals [Benoit et al., 1994]. Differences in salinity promote water density gradients (e.g., mixture of barotropic and baroclinic water flows), which are often enough to promote turbulent mixing processes and metal solubilization [Kundu et al., 2004]. Metal mobilization from sediments and suspended particulate matter increases with increasing salinity due to (i) complexation of metals with chloride and sulphate forming soluble inorganic complexes [de Souza Machado et al., 2016], and (ii) competition of major cations (e.g., Na, Ca, Mg) of saltwater for the sorption sites, displacing the sorbed metals in suspended organic particulate matter [Du Laing et al., 2009].

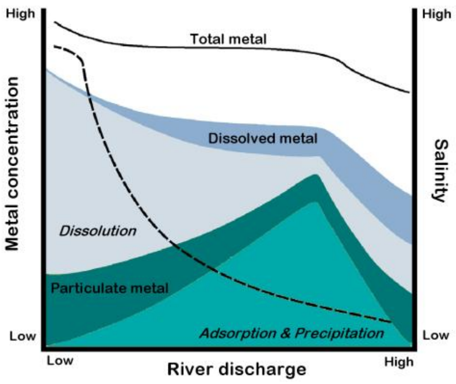


Figure 1.3. *Conceptual river discharge effects on particulate, dissolved and total metal concentration according to river discharge and salinity. It has been modified from [de Souza Machado et al., 2016].*

Figure 1.3 shows a conceptual model for metal mobilization according to salinity and river discharge. River discharge is another important variable in estuarine systems since it affects mixing processes, salinity gradients and the input of dissolved organic matter and suspended particulate material [Couceiro et al., 2009]. According to the results illustrated in the diagram, under low fluvial discharge (e.g., higher salinity) higher desorption of metals from sediments are expected. Contrarily, increasing river discharge implies more quantity of suspended material that intensifies metal adsorption and precipitation processes onto particulate material. In this situation, a maximum concentration of metals accumulated in sediments and minimum dissolved metal in water is expected. However, when even higher fluvial discharges occur (or in river systems) the influence of river water is larger throughout the estuary, so that the tidal processes are diminished. In this scenario, the presence of coarse material with lower adsorptive capacity is incremented so less metal adsorption processes are expected. The higher river turbulence promotes also the solubilization of some metal precipitates [Förstner and Wittmann, 1979].

Salinity also affects the water pH buffering capacity. The presence of higher concentration of carbonate ions in seawater flow enhances water buffering capacity so as to the pH values are increased up to 8.3 along the estuary in the seaward direction [de Souza Machado et al., 2016]. Hence, although the influence of water pH on metal behavior is expected to be greater at lower salinity conditions, pH is a very important parameter to understand metal speciation and mobility.

Broadly speaking, high water pH values cause adsorption and precipitation processes while low water pH values can weaken the metal-particulate material interaction, yielding in a lower retention of metals [Zhang et al., 2014]. These lower values are mainly detected at the bottom of the water column. Low pH values can reduce the negative charge of organic matter (mainly humic substances), clay particles and Fe-Mn-Al oxides and also tend to solubilize sulfide compounds causing a secondary release of metals [Du Laing et al., 2009]. Contrarily at higher pH values, metallic cations tend to precipitate as insoluble and stable hydroxides, carbonates, phosphates and sulphates [Hou et al., 2013].

Finally, it is widely assumed that oxidative-reduction potential (ORP) also controls the mobility of trace metals [De Jonge et al., 2012]. In freshwater saturated sediments, metal mobility is strongly determined by metal-sulphide interaction, metal-Mn-Fe oxide coprecipitation and metal-organic matter interaction. Under specific redox conditions, such compounds promote the precipitation of metals onto sediments or contrarily metal release from sediment surface. The sediments can be stratified into three main parts according to the oxygen content: (i) the oxic region where oxygen reduction occurs, (ii) the suboxic region controlled by the oxidation of nitrate, manganese and iron, and (iii) the anoxic region where sulfates are mainly reduced [Zhang et al., 2014]. Consequently, under aerobic conditions, Fe-Mn oxides can act as a sink of dissolved metals [Lu et al., 2014]. Under more reductive conditions, both Fe and Mn can be reduced and some metals (e.g., Co, Ni, Cr, Cd, Cu, Pb and Zn) can be released to water bodies promoting its mobilization [Zwolsman et al., 1993]. Contrarily, sulphate can be reduced to sulphide phases at very strong reducing conditions which can complex dissolved metals decreasing their mobility [Lu et al., 2014].

1.3.2. Solid phase characteristics

Metals' behavior in the aquatic environment is highly influenced by adsorption processes onto organic and inorganic particles. Dissolved metals may be transported through the water column via transport and dispersion processes, while metals adsorbed to particulate material can be settled out [Bourg, 1987]. Factors affecting water stratification have also influence in sedimentation, precipitation and flocculation of suspended particles [Förstner and Wittmann, 1979]. Moreover, mobilization as well as the residence time of particulate metal in water is highly influenced by the turbidity, which promotes enlarged sub-surface contaminated deposits [de Souza Machado et al., 2016].

The Fe-Mn oxides, acid volatile sulfides, and specially, organic matter show a high capacity to wrap up metals [Zhang et al., 2014]. The influence of each type of material will be highly dependent on redox conditions. For example, under anaerobic conditions, and in deeper layers of sediments (> 20 cm depth) acid volatile sulfides (AVS, mainly iron and manganese sulfides) can play an important role in metal mobilization [Hou et al., 2013]. Under these conditions, sulfate reductive bacteria can promote the reduction of sulfates leading the formation of AVS species [De Jonge et al., 2012]. These reactive sulfide species are thought to be important compounds for metal binding and partitioning under anoxic conditions. Among them, Fe and Mn sulfides constitute an important part of AVS-metal precipitations in sediments [Burton et al., 2006]. The generation of AVS and its reactivity with trace metals is shown in Figure 1.4. As an example, based on this mechanism, Zn and Cu can be accumulated as authigenic sulfide minerals or form discrete sulfide mineral deposits such as covellite (CuS) or sphalerite (ZnS) [Martin et al., 2003].

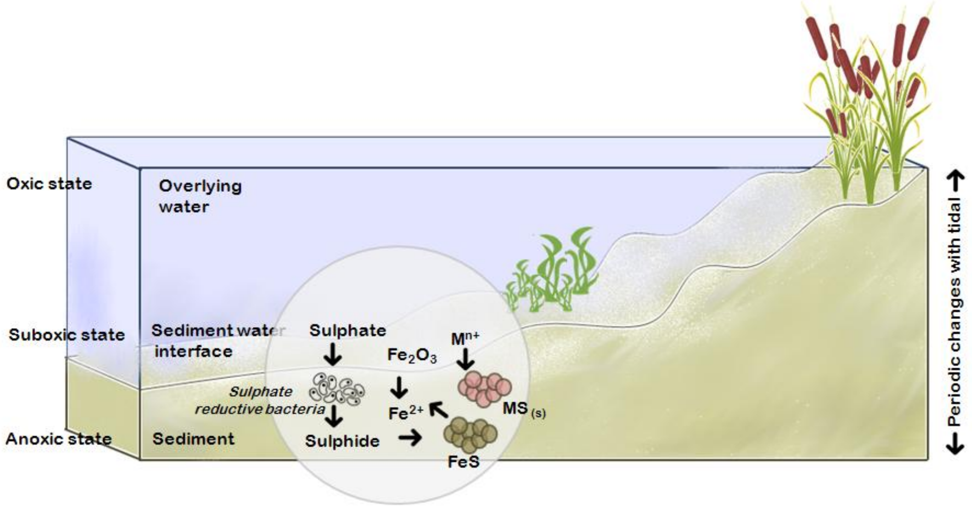


Figure 1.4. *The mechanism of formation of FeS and reactivity with metal ions in water bodies.*

Under more oxic conditions, metals can be complexed by the organic matter present in water bodies acting as a vehicle for metal mobilization through water cycles or, contrarily, be trapped and deposited onto the surface of the sediment when the conditions of turbulence and estuarine water flow are appropriate. Soluble organic matter, often referred as total organic carbon (TOC), is considered as an important reservoir phase for metal-binding in oxidized sediments [Besser et al., 2003].

Organic matter is formed by the degradation of plants, animal detritus and artificial organic materials that arrive to water systems [Fernandes et al., 2011]. It is a complex and heterogeneous component in water and sediments which is further decomposed, via various diagenetic processes, to fulvic and humic acids, kerogen and black carbonaceous material. Its distribution diminishes from the upper part of the sediment core to the deeper part, where the lowest abundances of organic matter are expected [Fernandes et al., 2011]. Among them, humic substances are considered the main metal binding materials in estuarine and coastal marine sediments, in such a way that sediments with greater humus concentrations will have higher metal contents [Besser 2003; Fernandes et al., 2011]. Since soluble organic matter is considered to be one of the main geo-sorbent materials, its characterization has been outlined in many research works in order to assess the fate of metals in sediments as well as to propose remediation strategies.

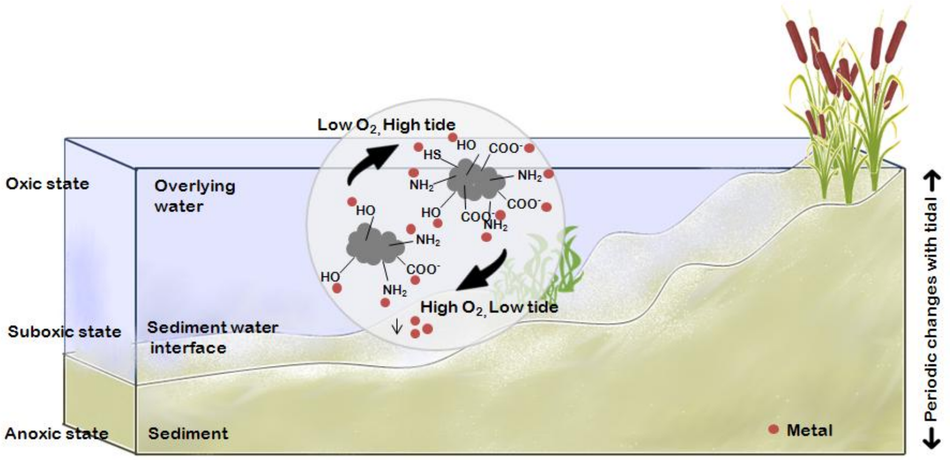


Figure 1.5. *Organic matter originated from different aquatic plants and organisms forms chelates solubilized metals via organic ligands (carboxilates, amines, hydroxiles, etc.). Organic matter can also suffer redox cycles in inter-tidal zones.*

The uptake of metals by organic matter is subjected to many chemical and physical factors. Low to medium molecular weight organic matter can act as dissolved ligand of metals to form soluble metal complexes (e.g., increases of 25% in the organic matter content can raise metal concentration in water bodies up to 200 times [Du Laing et al., 2009]). Contrarily, larger organic matter macromolecules can reduce metals' availability through flocculation processes. Besides, the oxidation state of organic matter can also alter the metal behavior in the aquatic system [Klüpfel et al., 2014] (see Figure 1.5). Under reductive conditions, organic matter could chelate metals and promote its mobilization. However, oxidized organic matter shows lower binding capacity and metals will be susceptible for Fe-Mn-oxi-precipitation [de Souza Machado et al., 2016].

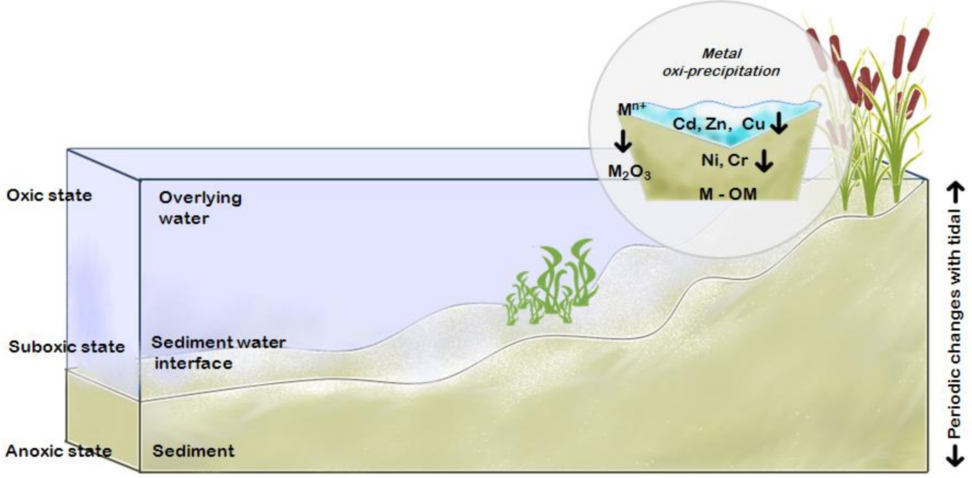


Figure 1.6. *Metal precipitation favoured by Mn-Fe oxyhydroxides.*

Finally, sediment mineralogical composition and texture can also affect metal distribution and accumulation. In this context, silt and clay minerals have great influence on the transport and storage of metals along estuarine and fluvial sediments. Clays are mainly composed by montmorillonite ($(Na,Ca)_{0.33}(Al,Mg)_2(Si_4O_{10})(OH)_2 \cdot nH_2O$) which is accepted as exceptional natural adsorbent due to its surface- and ion-exchange-sites [Saeedi et al., 2013]. Fe and Mn oxyhydroxides are the main components of authigenic minerals and due to their large surface area they can act as sorbents of some metals in oxic interfacial sediments [Fernandes et al., 2011] (see Figure 1.6).

Besides the composition of particles, the size of particulate matter is also a parameter to be considered in metal adsorption and accumulation processes. In this sense, it is widely proved that fine-grains of sediments (< 63 µm) are the most important ones in such processes in comparison to coarser particles, due to their larger surface area and higher cation exchange capacity [Zhang et al., 2014]. These fine particles (such as clay) are often associated with organic matter and exist in the form of organic-mineral complexes, so as to they are capable to adsorb active metals from the water body and settle them out in sediments surface.

Metals adsorbed to bottom sediments can be released to aquatic bodies through resuspension or desorption processes. All the parameters explained above will influence again in the metal distribution process. In estuarine environments, where tidal flats are especially relevant, in-situ precipitated metals are constantly remobilized by interaction with organic matter, water level, redox conditions and water chemistry changes [Förstner and Wittmann, 1979]. Within this scenario, the study of many of the parameters affecting the metal distribution in different environmental compartments is often required for a better understanding of metal toxicity.

1.3.3. Estuarine biological relationship with contaminants

As stated before, estuaries are a particular ecosystem where freshwater joins with sea water, with an increased input of organic matter coming from the pulled material from the whole river course, getting deposited through the course [de Souza Machado et al., 2016]. The nearness to the sea gives an important characteristic which is the salinity change as well as the flow change and material returning. This situation becomes estuaries as probably the richest water/ground system concerning to natural resources.

Living beings grow easily in this locations becoming the estuary as an important habitat for countless species.

Living organisms, interact with the different estuary compartments, mainly water, finding secure locations, mating and growing new generations, feeding from the resources they can find. This interaction can lead to an important incorporation of pollutants to living being bodies through absorption, contact or intake causing different problems depending on the contaminant [Bryan and Langston, 1992].

In Figure 1.7, there is shown the metal absorption in organic matter particulates which travel through the water flow and are deposited in the sediment, being available for living organisms uptake, such as fishes, mussels, seabirds, plants and vertebrates among others.

In the riverbeds, deposited metals are absorbed by plant roots being incorporated to their bodies and in higher vertebrates which feed from the plants or fishes. Dead bodies from plants or animals, return to the aquatic system after decomposition, incorporating organic matter as well.

In water and sediment bodies, as stated in 1.3.2, diverse processes occur, being the metals distributed between aquatic phase and solid phase. Fishes and mollusks such as mussels, filter and feed from the substances they can find in the water, incorporating the contaminants into their bodies. Thus, the contaminant uptake depends on the amount of pollutant present in the water, the capacity of the water of dissolving deposited contaminants in the sediment, the turbulence and the amount of contaminated articulates pulled by the flow and of course, the uptake capacity of the organisms [Björk, 1995]. In this aspect it is remarkable that some organisms such as mussels can filter up to 30 $ml \cdot min^{-1}$ of water [Clausen and Riisgård, 1996].

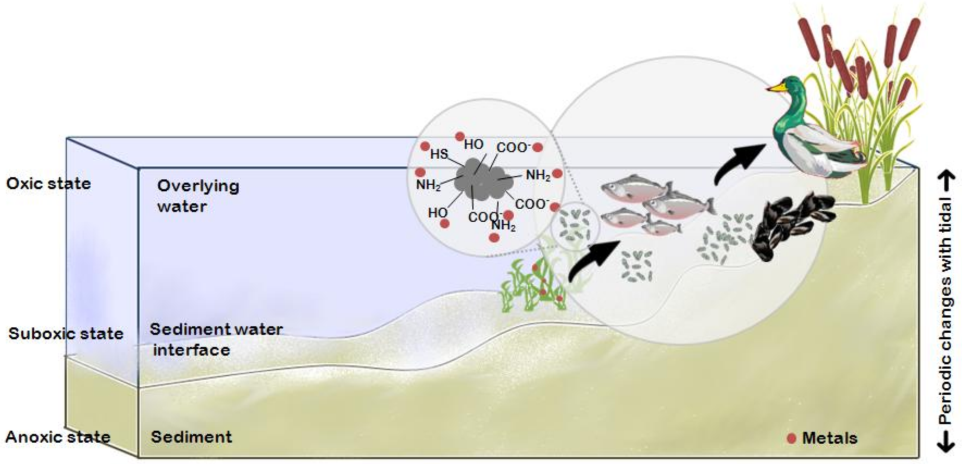


Figure 1.7. *Simplified biogeochemical cycle of contaminants in an estuary.*

1.4. Monitoring metallic contamination in aquatic environments: European legislation

Fluvial and estuarine environments are the major, if not the primary, contributor to the evolution of the environment. Due to the awareness of large contamination scenarios in aquatic systems, the E.U. Water Framework Directive was established in October 2000 (Directive 2000/60/EC of the European Parliament) in order to restore the quality of surface and ground waters (rivers, lakes, transitional waters, and coastal waters). It aims to prevent deterioration of water quality and to withdraw the concentration of contaminants at catchment levels [Chon et al., 2010]. Concretely, the directive makes specific emphasis on the monitoring of the status of the water as well as requests to all members of the European Union the production and implementation of management plans in order to prevent deterioration of water bodies.

After the goals marked by WFD, a series of European directives with special focus on water bodies were approved (e.g., EU, Decision 2455/2001/EC; EU, Directive 2008/105/EC; EU, Directive 2009/90/EC). In a first attempt, the first list of 33 substances that must be prioritized was set out [EU, Decision 2455/2001/EC]. In the case of metals, lead, nickel and their related compounds are listed as priority hazardous[4] substances whereas cadmium, mercury, tin and their related compounds are listed as priority substances[5] [EU, Directive 2013/39/EU]. According to WFD, the whole characterization of water body must be conducted following several monitoring programs [EU, Directive 2009/90/EC] in order to determine the water body state taking into account the Ecological Quality Ratios (EQRs) and Environmental Quality Standards (EQSs) [EU, Directive 2008/105/EC]. The EQS values of metals considered as priority and hazardous compounds are shown in Table 1.4.

Table 1.4. *The EQS values of metals (dissolved concentrations, ($\mu g \cdot l^{-1}$)) listed as priority substances.*

Metal	***Inland surface waters(a)***		***Other surface waters(b)***	
	Annual average	***Maximum allowable concentration***	***Annual average***	***Maximum allowable concentration***
Cd	< 0.08–0.25	< 0.45-1.5 (c)	0.2	< 0.45-1.5 (c)
Hg	0.05	0.07	0.05	0.07
Pb	7.2	--	7.2	--
Ni	20	--	20	--

(a) Rivers, lakes and related artificial or heavily modified water bodies; (b) Transitional, coastal and territorial waters; (c) The range depends on the hardness ($CaCO_3$) of surface waters.

In order to fulfill the requirements marked by the WFD, many improving actions have been conducted from the 1990s [Bald et al., 2005]. Among these actions, for example, waste waters were treated in wastewater treatment plants (WWTPs) before being emitted to transitional and coastal water bodies and some industrial activities close to the rivers were closed. Moreover, in an attempt to take decisions at regional level of water management, the Basque Water Agency was constituted in 1998. Most of the research

---

[4] *Priority hazardous substances – the group of substances that is required to be controlled for pull discharges, emissions and losses out.*

[5] *Priority substances – the group of substances that removed or phased out of discharges, emissions and losses by 2020.*

works were carried out to assess the “good status” of the water bodies of the Basque Country showing an improvement of the water quality, but being really complicated to restore original water conditions [UNESCO 2009].

Regarding sediments and, taking into account their characteristics to both accumulate contaminants from water bodies and release contaminants to aquatic media; it is very surprising that there is not a specific organism defining threshold values for contaminants. In fact, the directive just refers very few times to sediments and biota compartments [Borja et al., 2004]. In this scenario, there are many organisms treating to establish some values, methodologies or measurement protocols (i.e. total concentration vs mobilized concentration) as is the case of NOAA (National Oceanic and Atmospheric Administration in USA), CEDEX (Centro de Estudios y Experimentación de Obras Públicas, Spain), IHOBE (Environmental Management Public society, Basque Country) and CEQG (Canadian Environmental Quality Guidelines, Canada). However, all of them refer to soil. Since it is widely assumed that sediments give reliable information about contamination scenarios, recently, great efforts are being done in order to establish natural background levels [Gredilla et al., 2014]. Based on those background values, an area's contamination level can be estimated using biogeoacummulation indexes [Mueller, 1981].

In the case of aquatic organisms, there is only a commission regulation [EU, Regulation 2006/1881/EC] setting maximum levels for certain contaminants in foodstuffs. In the case of using this animals (foodstuff) contaminant concentration or for another use not being intake (i.e., monitorization of the aquatic media), there is no regulation or threshold value from any European official organism. When referring to bivalves, as per their water filtration ability and being used by several researches as a sentinel organism found in estuaries, the maximum limits as per foodstuff are 1.5 $mg \cdot kg^{-1}$ and 1.0 $mg \cdot kg^{-1}$ for lead and cadmium, respectively. These values are always referring to fresh basis, not dried and at any case the humidity of the animal must be taken into account in order to obtain a comparison with the limit values.

1.5. Analysis of metals in water, sediment and biota samples

The determination of the metal content in environmental solid and liquid matrices (soils, rocks, organic materials, etc.) requires the selection of suitable analytical methodologies including appropriate protocols for sampling, sample digestion when required and the application of sensitive and selective analytical techniques to obtain exhaustive chemical information in the shortest time possible.

1.5.1. Water, sediment and biota pre-treatment procedures for metal determination

Performing an appropriate sampling procedure is essential to get representative data. This involves not only an adequate sampling plan but also using suitable and clean sampling containers and sample stabilizing procedures.

When water samples must be analyzed, the use of plastic bottles (mainly polytetrafluoroehylene or polypropylene) is preferred in order to: (i) avoid the cross contamination of Si and Na released from glass bottles or (ii) to minimize the adsorption of metals onto glass bottle walls. Although no filtration of water samples is recommended when total metal concentration must be determined, a filtration step through 0.45 μm filters is required for the analysis of the dissolved metal fraction. The acidification of water samples (add HNO_3 to get pH 1-2) is also recommended once the water samples are filtered because acidic media minimizes metal reactivity and stops possible biological activity. Water samples do not require any special preconcentration step for the analysis of metal fraction so it is usually maintained at 4°C until analysis, which is often performed within 48 hours.

The analysis of metal content preserved in surface sediments (up to 4 cm in depth) reveals information about recent contamination events. When it is possible (e.g. sediment exposed to the air, often at low tide events in estuarine environments), sediments are directly collected by hand using gloves and plastic spatulas. As in the case of water samples, and in order to avoid possible cross-contamination, the use of plastic material is highly advised. The water fraction (> 90%) contained in sediment samples is often eliminated using freeze-drying procedures. To this aim, sediments are lyophilized (i.e., first frozen and then kept at -50°C under vacuum (0.01 – 0.05 Torr) for approximately 48 hours until water is eliminated by sublimation without losing any target analyte). This process can take more time depending on the quantity of sample to be lyophilized and the water content. The advantage of this procedure is that no particle aggregation occurs during the sample drying step and so, sample can be divided in different particle sizes by sieving afterwards.

Unlike metal analysis in water samples, metal extraction from the sediment is often required prior to its analysis. The selection of proper pre-treatment schemes for contaminated samples, including leaching of strongly bound compounds, is crucial, considered to be the most tedious step of the analytical process and where more error sources appear. Strong acid digestion with oxidizing agents (HNO_3, $HClO_4$ and H_2O_2) or their mixtures is probably the most commonly used decomposition technique for the determination of acid extractable metal concentration in sediments [Ongeri et al., 2014]. Alkaline fusion is also traditionally used to digest geological samples for the analysis of major elements and to decompose substances resistant to acid attack such as silicates, cassiterite (SnO_2), rutile (TiO_2) or spinel ($MgAl_2O_4$). Its decomposition effect is mainly based on the use of high fusion temperature (> 1000 °C) together with strong fusion agents (i.e. lithium metaborate). One of the drawbacks of the use of alkaline fusions is the high amount of the total dissolved solids that are present in the final solution (< 0.2% for ICP-MS); a fact that restricts the number of trace elements quantifiable by fusion preparations. Therefore, the method of dissolution by acid digestion is usually preferred to fusion.

Besides open vessel digestion and alkaline fusion, which are still in use for geological materials, nowadays faster digestion techniques that bring better recoveries and minimize cross-contamination are in vogue. Some methodologies helpful for speeding up and simplifying sample pre-treatment are those based on ultrasound energy, such as ultrasound baths and focused ultrasound extractions [Fdez-Ortiz de Vallejuelo et al., 2009]. However, the high amount of residual matrix compounds and/or the high organic carbon content in sample extracts treated by ultrasound makes the quantification of metals difficult [Väisänen et al., 2002]. Microwave digestions, however, have proved to be an alternative approach for digestion of complex matrices owing to advantages such as reduction in analysis time, decreased contamination, lower reagent and sample need, low loss of volatile species and improved operator safety. The microwave oven besides heating the contents to a high temperature and controlled pressure also helps in preventing analyte losses due to the use of closed vessel providing more reproducible results than the conventional methods. The use of microwave ovens for the decomposition of organic and inorganic samples was first introduced in 1975 [Abu-Samra et al., 1975] and became widely accepted as the best choice for decomposition of a large variety of environmental matrices, such as silicate rock [EPA Method 3052], soils, sludges and sediments [EPA Method 3051; ASTM D5258-92].

Metal concentration levels determined in such ways give an idea of the maximum potentially soluble content of metals, this is, a measure of the highest potential hazard that could occur in long term exposure or in extreme environmental conditions. Regarding the mobility and the ecotoxicological significance of metals in sediments, however, the possible risk is determined by their solid – solution partitioning rather than the total metal content itself [Sauve et al., 2000].

In this framework, the use of selective leaching extraction or sequential extractions have shown to be outstanding methods to infer information about the origin, mode of occurrence, biological and physicochemical availability and mobility of metals. In a few words, sequential extraction schemes make use of suitable reagents (un-buffered salts, weak and strong acids, reducing and oxidizing agents) which are applied in a given order to the sediment sample to obtain successive fractions with higher to lower mobility, specifically: (i) water soluble fraction, (ii) exchangeable fraction, (iii) acid soluble fraction, (iv) reducible fraction and (v) oxidizable fraction. The best known and most widely sequential methods are those proposed by BCR and Tessier [Rauret et al., 1999; Tessier et al., 1979].

When speaking about biota samples, more specifically about mussel samples, it is imperative to clean (remove impurities, shell and debeard), select by size, remove dead specimens and frozen as fast as it is possible in order to diminish the losses of analytes and contaminations. As in the case of sediments, freeze-drying is the best method in order to obtain representative dried dusty samples from the gathered mussels after their grinding.

Metals extraction in biota becomes a more-friendly extraction method because, in contrast with the sediment extraction, the complete digestion of the samples is easily performed using less aggressive solvents. The matter is that the organic material to be dissolved responds more actively to the oxidant acid mixtures than the sediment does. The bigger problem is to avoid overpressures and losses during the extraction of the dry materials in case of using microwave assisted extractions due to the CO_2 formation while the organic matter is being oxidized [Bartolome et al., 2010]. The concentration of metals expected in biota samples is less than those found in sediment samples, and hence, an adequate concentration and mixture of acids must be used in order to ease the sample digestion but minimizing the dilution of the extraction leachate. Other extraction methods such as ultrasonic assisted extraction are also published in the literature [Balarama Krishna and Arunachalam, 2004; Manutsewee et al., 2007].

1.5.2. Metal analysis in water, sediments and biota

The determination of metals in water samples is quite easy in comparison to sediment or biota samples. Analytically, sediment and biota are challenging matrices for many instrumental techniques since they present low concentrations of most of the inorganic elements and a tricky siliceous matrix or an organic enriched compounds matrix respectively. However, once sediment and biota samples are treated, the steps to be conducted are similar to those necessary for water samples.

The instrumental analysis following digestion procedures has lately evolved from mono-elemental spectroscopic techniques (i.e., Flame Atomic Absorption Spectroscopy (FAAS) and Graphite Furnace Atomic Absorption Spectroscopy (GFAAS) [Bettinelli et al., 2000]) to multi-elemental ones. These last, based on plasma spectrometry, allow the analysis of a large number of analytes in a reasonably short time and achieve a wider sample characterization. The successful use of these techniques is subjected to various conditions: good detection limits, linearity of calibration curves, compatibility with different acid mixtures for the sample dissolution and low sensitivity to matrix effects.

The application of inductively coupled plasma spectrometry to environmental samples has revealed a range of new working lines. Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) achieves not only better detection limits but also is more sensitive than Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES). Thus, ICP-MS is often regarded as one of the most suitable analytical techniques for elemental analysis. ICP-MS is, therefore, particularly appropriate for the determination of metals owing to the low detection limits (low pg levels), wide dynamic range, possibility of accurate multi-element analysis, the capability of measuring isotopic ratios, speciation capability when coupling a chromatographic device (i.e. liquid chromatography or capillary electrophoresis) and the possibility to be implemented in both solution and direct solid modes (i.e., Laser Ablation (LA-ICP-MS)). While the later mode may be an effective and powerful tool in many environmental applications [Kot et al., 2000], its performance for high precision multi-element trace and ultra-trace metal analysis is often lower to that of solution based on ICP-MS. Due to the low metal limit expected in biota matrix, this technique is the most used, despite of the fact that other spectroscopic techniques can be also used as GFAAS [Balarama Krishna and Arunachalam, 2004; Manutsewee et al., 2007].

The next analytical challenge could be, therefore, the capability to perform direct analysis without the need for digestion that involves lengthy processes. In this sense, methods involving interactions between electron beams and X-rays with solid samples such as X-Ray Fluorescence (XRF) and Particle Induced X-ray Emission (PIXE) [Tsuji et al., 2011], Scanning Electron Microscopy coupled to Energy Dispersive X-ray Spectroscopy (SEM-EDS) [Panfili et al., 2005], Laser Induced Breakdown Spectroscopy (LIBS) [Harmon et al., 2013] or Instrumental Neutron Activation Analysis (INAA) [Frontasyeva, 2011] have provided to be powerful techniques for quantitative analyses because they are non-destructive, multi-elemental, highly sensitive and they do not require special sample preparation. Among all of them, XRF is the cheapest and simplest analytical technique which is well suited for the investigations that involve bulk chemical analyses of major (Ti, Al, Fe, Mn, Mg, Ca) and trace elements (concentrations > 1 $\mu g \cdot g^{-1}$: Ba, Co, Cr, Cu, Sr, U, V, Zn) in soils and sediments. Besides, in practice, most commercially available instruments are very limited in their ability to precisely and accurately measure the abundances of elements with $Z<11$ in most of the natural earth materials.

Despite the large advantages provided by these techniques, one of the main drawbacks of direct measurements is the fact that quantitative studies are very limited and with higher detection limits (at $\mu g \cdot g^{-1}$ levels) than those obtained by ICP-MS for the dissolved trace elements. Thus, since the quantification is largely influenced by peak overlapping and inter-elemental matrix effect, these non-destructive techniques have been used as semi-quantitative approaches [Ge et al. 2005]. These direct methodologies are more and more used in several environmental application fields and the next analytical challenge involves assessing their potential to perform quantitative in-situ analyses [Shuttleworth et al., 2014].

In contrast to conventional analytical techniques used for the determination of metals in sediment analysis, vibrational spectroscopy combined with chemometrics can provide unique tools for the direct and simultaneous determination of many parameters in sediment samples based on infrared and Raman measurements, being employed for infrared analysis both, near infrared (NIR) and mid infrared (MIR) [Armenta et al., 2014].

Raman spectroscopy enables to differentiate between minerals of the same structural type, to distinguish polymorphic modifications, to discriminate between different hydration stages or even to detect impurities or inclusions in minerals. In fact, it is considered to be almost as powerful as X-ray diffraction for mineralogy but without the requirement for powdering the sample or being crystalline [Ellery et al., 2004]. Besides, in contrast to other vibrational techniques, the water signature is small enough in Raman so that it is applicable to untreated field-fresh material. Each group of oxyanionic minerals—carbonates, sulfates, nitrates, phosphates, arsenates and silicates—and most oxides, sulfides and hydroxides have characteristic Raman signatures [Goienaga et al., 2011]. Raman spectra can be used not only for mineral identification but also to provide semi-quantitative to quantitative determination of mineral proportions and some information about mineral compositions [Dorfer et al., 2010]. Finally, metal speciation by Raman spectroscopy has made possible the evaluation of metal mobility and potential bioavailability of hazardous compounds in urban soils and sediments [Carrero et al., 2012].

1.6. Rivers and Estuaries in the Basque Country

Most of the coastal areas, estuaries and river catchments have been historically affected by urban and industrial wastewaters and Zn, Pb and Fe mineral ores [Belzunce et al., 2001; Cearreta et al., 2004; Borja et al., 2008]. Consequently, although aquatic bodies in Basque Country are somewhat recovering their original “good” status quality, the region still supports a high number of anthropogenic pressures and impacts [Borja et al., 2006]. Among the main pressures identified in the Basque Country it must be outlined urban and industrial discharges (e.g., increases in organic matter and oxygen consumption) and hydromorphological pressures (e.g. construction of dykes and ports, dredging, sediment disposal and land reclamation). However, positive actions have been also taken including removal of discharges and implementation of water treatment programmes at catchment and estuarine levels [Borja et al., 2006; Tueros et al., 2009].

1.6.1. Rivers

Bizkaia, which is exceptionally conditioned by physical factors, brings together almost all of its industrial potential in a narrow zone extended from the headwaters of the river Ibaizabal (Elorrio, Zaldibar, Abadiano, etc.) to the western end of the left bank of the Bilbao estuary (Muskiz, Abanto and Zierbena, Santurtzi, Ortuella) including the two tributaries that extend from the bottom of the valley Kadagua (Balmaseda, Zalla, Güeñes) and Nerbioi (Arrigorriaga, Arrankudiaga, Miraballes, etc.) including some towns in Alava also drained by this river (Okendo, Llodio, Amurrio). The Oka river that ends in the estuary of Urdaibai (Urdaibai is an UNESCO biosphere reserve), crosses also some industrial municipalities (Gernika, Forua and Murueta).

On the other hand, Gipuzkoa shows a particular river location model. The most important structural feature is the high dispersion of industrial assets across most of the province. Indeed, except in Donostia and Hondarribi and in other small towns, the industrial activity is located out of the city and disseminated through all the territory. The major industrial activity is placed close to the upper and middle Deba river (Mondragon, Bergara, Eibar, Elgoibar), constituting the two main industrial areas of the province as they absorb 30% and 27% of total secondary employment, respectively. In a lesser extent, the regions of Goierri and Middle Oria (including the centers of Beasain and Tolosa), and the Valley of Urola (mainly Zumarraga and Azpeitia), contribute about 15% of the industry in the zone.

1.6.2. The Nerbioi-Ibaizabal Estuary

In Basque Country there are two differentiated estuaries that have been studied for a long time. These are Urdaibai and Nerbioi-Ibaizabal estuaries. The Urdaibai estuary is within a natural region that was declared a Biophere's Reserve by UNESCO in 1984 and it is considered relatively unpolluted area [Puy-Azurmendi et al., 2006]. On the contrary, the estuary of Nerbioi-Ibaizabal has been the most important industrial and commercial area in the Basque Country. This estuary is located in the south-east of the Bay of Biscay (43° 20'N, 3° 1'W) (see Figure 1.8). Both rivers (e.g. Nerbioi and Ibaizabal) meet in Basauri and the confluence of both rivers flows down the estuary where water input of another four tributaries occurs: Kadagua (27 %) and Galindo (4 %) rivers on the west bank of the estuary and Asua (0.7 %) and Gobela (0.3 %) on the east bank of the estuary (see Figure 1.8).

The awareness of health-status of this estuary has promoted several monitoring programs in the last decade [Fdez-Ortiz de Vallejuelo et al., 2010; Gredilla et al., 2013]. The stress suffered by this estuary for years is widely documented in the literature and also the actual restoration effectiveness [Borja et al., 2010].

Based on the historical human activities in the Nerbioi-Ibaizabal estuary, the main stress imposed by human activities will be afterwards mentioned. During the last 150 years, the hydrogeomorphology of the estuary has been dramatically changed in order to establish urban, port and industrial settlements [Cearreta et al., 2004]. In the mid-nineteenth century, and coinciding with an increment of population density, industrial activity encouraged the economy of the location.

Figure 1.8. *Geographical location of the Nerbioi-Ibaizabal estuary and the main tributaries. (Map taken from Google Earth, October 2015).*

The main activities were related to the explotation of iron ore. Consequently, in the last century the estuary has received many wastes from different origin (e.g. industrial effluents containing toxic products released from chemical, iron-steel and paper industries), leading to a degradation of the water body [Belzunce et al., 2001]. In an attempt to restore the estuary (e.g., reduction of metal concentration and organic matter and increase the concentration of dissolved oxygen), the implementation of wastewater treatment plant was accepted in 1980. The actions taken afterwards, such as the closure of main iron and industries in 1995 and the start-up of a secondary treatment plant in 2001 has derived in an ongoing improvement of the estuary [Borja et al., 2010].

1.7. Hope on environmental scientific researches and environment

As stated before, environmental research works are often focused on the analysis and/or behavior assessment of contaminants found out into an environmental system. Different environmental compartments tend to end-up accumulating a huge variety of contaminants resulting from human activities, such as industry or mining. Although the occurrence of many priority and emerging organic pollutants has gained the attention of many researchers, pollution by metals has been considered a world level problem since the early seventies. As a result of the scientific background get in the past few decades, the potential harmful effects of metals have been regulated and monitored all over the world.

Regarding environment, locally understood, it is similar to a big beaker in which thousands of reactions and equilibriums are occurring at the same time, related among them, in a big multidimensional equilibrium system. Any changing parameter will affect the whole system in a different manner even if it is only a little temperature variation. Thus, it is very difficult to reach real conclusions studying only a small part of the whole system and/or the variables in a separate way. In this regard, every environmental system behaves differently from the others as the parameters or variables defining each ecosystem are different from one location to another, and also, the same location can change its behavior depending, for example, the weather and the physicochemical parameters. In this sense, the obtained contaminant amount data is just another variable that needs to be taken into account for the whole system with all the variables.

An adequate assessment of metal occurrence in the environment should involve not only the metal amount investigation and the monitorization itself in all the environmental bodies, but also the whole characterization of the matrix as well as the assessment of the multiple-variables affecting the system. Besides, most of the works involving the study of biogeochemical processes of metals and on their concentration do not take into account the specific geochemical characteristics of each environmental system and focus only in the total amount of the contaminants in a particular system body (water, sediment or biota).

Researchers and official organisms should bear in mind that the ecosystem is a part of the human life, interacting with the environment as any other constituent. Pollution information about environment should be as complete as it can. Other point that should be taken in mind is the legislation on each environmental body that could help to responsible organisms to take decisions over the environment in order to maintain all the compartments of the ecosystem with the minimum amount of pollutants.

To include all these aspects in the daily work about environmental assessment studies performed by environmental consultants or researchers, new methodologies are required to be developed or improved in order to include better information relating the current total metal concentration levels in water, sediment and biota.

1.8. References

[Abu-Samra et al., 1975] Abu-Samra A, Morris JS, Koirtyohann SR. *Wet ashing of some biological samples in a microwave oven.* Anal. Chem. 1975; 47, 1475 – 1477.

[Adams, 2005] Adams SM. *Assessing cause and effect of multiple stressors on marine systems.* Mar. Pollut. Bull. 2005; 51, 649 – 657.

[Alonso Castillo et al, 2013] Alonso Castillo ML, Sanchez-Trujillo I, Vereda Alonso E, García de Torres A, Cano Pavon JM. *Bioavailability of heavy metals in water and sediments from a typical Mediterranean Bay.* Mar. Pollut. Bull. 2013; 76, 427 – 434.

[Armenta et al., 2014] Armenta S, de la Guardia M. *Vibrational spectroscopy in soil and sediment analysis.* Trend. Environm. Anal. Chem. 2014; 2, 43 – 52.

[ASTM D5258-92] *Standard practice for acid extraction of elements from sediments using closed vessel microwave heating.* The American Society for Testing and Materials, Philadelphia, 1992.

[Balarama Krishna and Arunachalam, 2004] Balarama Krishna MV, Arunachalam J. *Ultrasound-assisted extraction procedure for the fast estimation of major, minor and trace elements in lichen and mussel samples by ICP-MS and ICP-AES.* Anal. Chim. Acta. 2004; 522, 179 – 187.

[Bald et al., 2005] Bald J, Borja A, Muxika I, Franco J, Valencia V. *Assessing reference conditions and physic-chemical status according to the European Framework directive: a case study from the Basque Country (North of Spain).* Mar. Pollut. Bull. 2005; 50, 1508 – 1522.

[Bartolome et al., 2010] Bartolome L, Navarro P, Raposo JC, Arana G, Zuloaga O, Etxebarria N, Soto M. *Occurrence and distribution of metals in mussels from the cantabrian Coast.* Arch. Environ. Contam. Toxicol. 2010; 59, 235 – 243.

[Belzunce et al., 2001] Belzunce MJ, Solaun O, Franco J, Valencia V, Borja A. *Accumulation of organic matter, heavy metals and organic compounds in surface sediments along the Nervion Estuary (northern Spain).* Mar. Pollut. Bull. 2001; 42, 1407–1411.

[Benoit et al., 1994] Benoit G, Oktay-Marshall SD, Cantu A, Hood EM, Cokeman CH, Corapcioglu MO, Santschi PH. *Partioning of Cu, Pb, Ag, Zn , Fe, Al and Mn between filter-retained particles, colloid and solution in six Texas estuaries.* Mar. Chem. 1994; 45, 249 – 264.

[Besser et al., 2003] Besser JM, Brumbaugh WG, May TW, Ingersoll CG. *Effects of organic amendments on the toxicity and bioavailability of cadmium and copper in spiked formulated sediments.* Environ. Toxicol. Chem. 2003; 22, 805 – 815.

[Bettinelli et al., 2000] Bettinelli M, Baffi C, Beone GM, Spezia S. *Soil and sediment analysis by spectroscopic techniques part I: determination of Cd, Co, Cr, Cu, Mn, Ni, Pb, and Zn.* Atom. Spectrosc. 2000; 21, 50 – 59.

[Björk, 1995] Björk M. *Bioavailability and uptake of hydrophobic organic contaminants in bivalve filter-feeders*. Ann. Zool. Fennici. 1995; 32, 237 – 245.

[Borja et al., 2004] Borja A, Valencia V, Franco J, Muxika I, Bald J, Belzunce MJ, Solaun O. *The water framework directive: water alone, or in association with sediment and biota, in determining quality standards?* Mar. Pollut. Bull. 2004; 49, 8 – 11.

[Borja et al., 2006] Borja A, Galparsoro I, Solaun O, Muxika I, Tello EM, Uriarte A, Valencia V. *The European water framework directive and the DPSIR, a methodological approach to assess the risk of failing to achieve good ecological status.* Estuar. Coast. Shelf. S. 2006; 66, 84 – 96.

[Borja et al., 2008] Borja A, Tueros I, Belzunce MAJ, Galparsoro I, Garmendia JM, Revilla M, Solaun O, Valencia V. *Investigative monitoring within the European water framework directive: a coastal blast furnace slag disposal, as an example.* J. Environ. Monitor. 2008; 10, 453 – 462.

[Borja et al., 2010] Borja A, Dauer DM, Elliot M, Simenstad CA. *Medium- and long-term recovery of estuarine and coastal ecosystems: patterns, rates and restoration effectiveness.* Estuar. Coast. 2010; 33, 1249 – 1260.

[Bourg et al., 1987] Bourg ACM. *Trace metal adsorption modeling and particle-water interactions in estuarine environments.* Cont. Shelf. Res. 1987; 7, 1319 – 1332.

[Bryan and Langston, 1992] Bryan GW, Langston WJ. *Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review.* Environ. Pollut. 1992; 76, 89 – 131.

[Bustamante et al., 2012] Bustamante J, Gredilla A, Fdez-Ortiz de Vallejuelo S, Rodríguez A, Arana G, de Diego A, Madariaga JM. *Distribution and posible sources of trace elements in estuarine sediments: comparative study among estuaries of the Basque Country and other parts of the worlds* in: De Leon Diego A, Aragón PR (Eds.). *Trace elements: Environmental sources, geochemistry and human health.* Nova Science Publishers, 2012.

[Burton et al., 2006] Burton ED, Phillips IR, Hawker DW. *Factors controlling the geochemical partitioning of trace metals in estuarine sediments.* Soil. Sediment. Contam. 2006; 15, 253 – 276.

[Clausen and Riisgård, 1996] Clausen I, Riisgård HU. *Growth, filtration and respiration in the mussel Mytilus Edulis: no evidence for psysiological regulation of the filter-pump to nutritional needs.* Mar. Ecol-Prog. Ser. 1996; 141, 37 – 45.

[Chon et al., 2010] Chon HS, Ohandaja DG, Voulvoulis N. *Implementation of EU Water Framework Directive: source assessment of metallic substances at catchment levels.* J. Environ. Monitor. 2010; 12, 36 – 47.

[Carrero et al., 2012] Carrero JA, Goienaga N, Olivares M, Martínez-Arkarazo I, Arana G, Madariaga JM. *Raman spectroscopy assisted with XRD and chemical simulation to assess the synergic impacts of guardrails and traffic pollutants on urban soils.* J. Raman. Spectrosc. 2012; 43, 1498 – 1503.

[Cearreta et al., 2004] Cearreta A, Irabien MJ, Pascual A. *Human activities along the Basque coast during the last two centuries: geological perspective of recent anthropogenic impact on the coast and its environmental consequences* in: Borja A, Collins M (Eds.). *Oceanography and Marine Environment of the Basque Country.* Elsevier, London, UK, 2004.

[Clarke et al., 2011] Clarke BO, Smith SR. *Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids.* Environ. Int. 2011; 37, 226 – 247.

[Couceiro et al., 2009] Couceiro F, Rauen W, Millward GE, Lin B, Turner A, Falconer R. *Transport and reactivity of nickel in estuarine sediments: results from a high capacity flume.* Mar. Chem. 2009; 117, 71 – 76.

[De Jonge et al., 2012] De Jonge M, Teuchies J, Miere P, Blust R, Bervoets L. *The impact of increased oxygen conditions on metal-contaminated sediments part I: effects on redox status, sediment geochemistry and metal bioavailability.* Water Res. 2012; 46, 3387 – 3397.

[de Souza Machado et al., 2016] de Souza Machado AA, Spencer K, Kloas W, Toffolon M, Zarfl C. *Metal fate and effects in estuaries: A review and conceptual model for better understanding of toxicity.* Sci. Total Environ. 2016; 541, 268 – 281.

[Dorfer et al., 2010] Dorfer T, Shumacher W, Tarcea N, Schmitt M, Poop J. *Quantitative mineral analysis using Raman spectroscopy and chemometric techniques.* J. Raman Spectrosc. 2010; 41, 684 – 689.

[Du Laing et al., 2009] Du Laing G, Rinklebe J, Vandecasteele B, Meers E, Tack FM. *Trace metal behaviour in estuarine and riverine floodplain soils and sediments: a review.* Sci. Total Environ. 2009; 407, 3972 – 3985.

[Dueri et al., 2008] Dueri S, Catro-Jimenez J, Comenges JMZ. *On the use of the partitioning approach to derive environmental quality standards (EQS) for persistent organic pollutants in sediments: a review of existing data.* Sci. Total Environ. 2008; 403, 23 – 33.

[Ellery et al., 2004] Ellery A, Wynn-Williams DD, Parnell J, Edwards HGM, Dickensheets D. *The role of Raman spectroscopy as an astrobiological tool in the exploration of Mars.* J. Raman Spectrosc. 2004; 35, 441 – 457.

[EPA Method 3051] *Microwave assisted acid digestion of sediments, sludges, soils and oils*, in: Test. Methods for Evaluating Solid Waste, 3rd Edition, 3rd Update, US Environmental Protection Agency, Washington DC, 1995.

[EPA Method 3052] *Microwave assisted acid digestion of siliceous and organically based matrices*, in: Test. Methods for Evaluating Solid Waste, 3rd Edition, 3rd Update, US Environmental Protection Agency, Washington DC, 1995.

[EU, Decision 2455/2001/EC] European Commission. *Decision 2455/2001/EC of the European Parliament and of the Council of 20 November 2001 establishing the list of priority substances in the field of water policy and amending the Directive 2000/60/EC*. Off. J. Eur. Comm. 2001; 331, 1.

[EU, Directive 2000/60/EC] European Commission. *Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy.* Off. J. Eur. Comm. 2000; 27, 1.

[EU, Directive 2008/105/EC] European Commission, *Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy.* Off. J. Eur. Union. 2008; 348, 84.

[EU, Directive 2009/90/EC] European Commission, *Directive 2009/90/EC of the European Parliament and of the Council of 31 July 2009 on technical specifications for chemical analysis and monitoring of water status.* Off. J. Eur. Union. 2009; 201, 36.

[EU, Directive 2013/39/EU] European Commission, *Directive 2013/39/EU of the European Parliament of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy.* Off. J. Eur. Union. 2013; 226, 1 – 17.

[EU, Regulation 2006/1881/EC] European Commission. *Regulation 2006/1881 of 19 December 2006 setting maximum levels for certain contaminants in foodstuff.* Off. J. Eur. Union. 2006; 364, 5.

[Fdez-Ortiz de Vallejuelo et al., 2009] Fdez-Ortiz de Vallejuelo S, Barrena A, Arana G, de Diego A, Madariaga JM. *Ultrasound energy focused in a glass probe: An approach to the simultaneous and fast extraction of trace elements from sediments.* Talanta. 2009; 80; 434 – 439.

[Fdez-Ortiz de Vallejuelo et al., 2010] Fdez-Ortiz de Vallejuelo S, Arana G, de Diego A, Madariaga JM. *Risk assessment of trace elements in sediments: the case study of the Nerbioi-Ibaizabal river (Basque Country).* J. Hazard. Mat. 2010; 181, 565 – 573.

[Fernandez et al., 2008] Fernandez S, Villanueva U, de Diego A, Arana G, Madariaga JM. *Monitoring trace elements (Al, As, Cr, Cu, Fe, Mn, Ni and Zn) in deep and surface waters of the estuary of the Nerbioi-Ibaizabal River (Bay of Biscay, Basque Country).* J. Mar. Syst. 2008; 72, 332 – 341.

[Fernandes et al., 2011] Fernandes L, Nayak GN, Ilangovan D, Borole DV. *Accumulation of sediment, organic matter and trace metals with space and time, in a creek along Mumbai coast, India.* Estuar. Coast. Shelf. S. 2011; 91, 388 – 399.

[Förstner and Wittmann, 1979] Förstner U, Wittmann GTW. *Metal pollution in the aquatic environment. New York : Springer-Verlag*, Berlin, 1979.

[Frontasyeva, 2011] Frontasyeva MV. *Neutron activation analysis in the life sciences.* Phys. Part. Nuclei. 2011; 42, 332 – 378.

[Fung et al., 2004] Fung CN, Lam JCW, Zheng GJ, Connell DW, Monirith I, Tanabe S, Richardson BJ, Lam PKS. *Mussel-based monitoring of trace metal and organic contaminants along the east coast of China using Perna viridis and Mytilus edulis.* Environ Pollut. 2004; 127, 203 – 216.

[Garret, 2000] Garret RG. *Natural sources of metals to the environment.* Hum. Ecol. Risk. Assess. 2000; 6, 945 – 963.

[Ge et al., 2005] Ge L, Lai W, Lin Y. *Influence of and correction for moisture in rocks, soils and sediments on in situ XRF analysis*. X-Ray Spectrom. 2005; 34, 28 – 34.

[Giddings, 1973] Giddings JC. *Chemistry, man and environmental change, an integrated approach.* CanField Press, San Francisco, USA, 1973.

[Gleyzes et al., 2002] Gleyzes C, Tellier S, Astruc M. *Fractionation studies of trace elements in contaminated soils and sediments: a review of sequential extraction procedures.* Trac. Trend Anal. Chem. 2002; 21, 451 – 467.

[Goienaga et al., 2011] Goienaga N, Arrieta N, Carrero JA, Olivares M, Sarmiento A, Martínez-Arkarazo I, Fernández LA, Madariaga JM. *Micro-Raman spectroscopic identification of natural mineral phases and their weathering products inside an abandoned zinc/lead mine.* Spectrochim. Acta. A 2011; 80, 66 – 74.

[Gredilla et al., 2013] Gredilla A, Fdez-Ortiz de Vallejuelo S, Arana G, de Diego A, Madariaga JM. *Long-term monitoring of metal pollution in sediments from the estuary of the Nerbioi-Ibaizabal River (2005-2010).* Estuar. Coast. Shelf S. 2013; 131, 129 – 139.

[Gredilla et al., 2014] Gredilla A, Fdez-Ortiz de Vallejuelo S, De Diego A, Arana G, Madariaga JM. *A new index to sort estuarine sediments according to their contaminant content.* Ecolog. Indic. 2014; 45, 364 – 370.

[Harmon et al., 2013] Harmon RS, Russo RE, Hark RR. *Applications of laser-induced breakdown spectroscopy for geochemical and environmental analysis: a comprehensive review.* Spectrochim. Act. B 2013; 87, 11 – 26.

[Hobbie, 2000] Hobbie JE. *Estuarine Science: a synthetic approach to research and practice.* Island Press, Washington DC, 2000.

[Hou et al., 2013] Hou D, He J, Lu C, Ren L, Fan Q, Wang J. *Distribution characteristics and potential ecological risk assessment of heavy metals (Cu, Pb, Zn, Cd) in water and sediments from Lake Dalinouer, China.* Ecotox. Environ. Safe. 2013; 13, 135 – 144.

[Kennish, 1997] Kennish MJ. *Practical handbook of estuarine and marine pollution.* CRC Press marine science series, New Jersey, 1997.

[Klüpfel et al., 2014] Klüpfel I, Keiluweit M, Kleber M, Sander M. *Redox properties of plant biomass derived black-carbon (biochar).* Environ. Sci. Technol. 2014; 48, 5601 – 5611.

[Kot et al., 2000] Kot A, Namiesnik J. *The role of speciation in analytical chemistry.* Trends Anal. Chem. 2000; 19, 69 – 79.

[Kundu et al., 2004] Kundu PK. Cohen IM. *Fluid Mechanics,* Elsevier, London, UK, 2004.

[Lu et al., 2014] Lu XQ, Zhang Y, Liu H, Xing M, Shao X, Zhao F, Li X, Liu Q, Yu D, Yuan X, Yuan M. *Influence of early diagenesis on the vertical distribution of metal forms in sediments of Bohai Bay, China.* Mar. Pollut. Bull. 2014; 88, 155 – 161.

[Manutsewee et al., 2007] Manutsewee N, Aeungmaitrepirom W, Varanusupakul P, Imyim A. *Determination of Cd, Cu, and Zn in fish and mussel by AAS after ultrasound-assisted acid leaching extraction.* Food Chem. 2007; 101, 817 – 824.

[Marigomez et al., 2013] Marigomez I, Zorita I, Izagirre U, Ortiz-Zarragoitia M, Navarro P, Etxebarria N, Orbea A, Soto M, Cajaraville MP. *Combined use of native and caged mussels to assess biological effects of pollution through the integrative biomarker approach.* Aquat. Toxicol. 2013; 136-137, 32 – 48.

[Marin et al., 2001] Marin A, Lopez-Gonzalvez A, Barbas C. *Development and validation of extraction methods for determination of zinc and arsenic speciation in soils using focused ultrasound application to heavy metal study in mud and soils*. Anal. Chim. Acta. 2001; 442, 305 – 318.

[Martin et al., 2003] Martin AJ, Jambor JL, Pedersen TF, Crusius J. *Post-depositional behavior of Cu in a metal-mining polishing pond (East Lake, Canada).* Environ. Sci. Technol. 2003; 37, 4925 – 4933.

[Moros et al., 2009] Moros J, Fdez-Ortiz de Vallejuelo S, Gredilla A, de Diego A, Madariaga JM, Garrigues S, de la Guardia M. *Use of Reflectance Infrared Spectroscopy for Monitoring the Metal Content of the Estuarine Sediments of the Nerbioi-Ibaizabal River (Metropolitan Bilbao, Bay of Biscay, Basque Country.* Environ. Sci. Technol. 2009; 43, 9314 – 9320.

[Mueller, 1981] Mueller G. *Heavy metals and other pollutants in the environment: a chronology based on the analysis of dated sediments* in Proc. Int. Conf. Heavy metals in the environment, WHO, Amsterdam, 1981.

[Ongeri et al., 2014] Ongeri DMK, Lalah JO, Michalke B, Schramm KW. *Speciation study of trace elements in surface sediment of Winam Gulf, Lake Victoria, by sequential extraction, aqua-regia acid digestion, and ICP-OES.* Toxicol. Environ. Chem. 2014; 96, 1489 – 1500.

[Panfili et al., 2005] Panfili F, Manceau A, Sarret G, Spadini L, Kirpichtchikova T, Bert V, Laboudigue A, Marcus MA, Ahamdach N, Libert MF. *The effect of phytostabilization on Zn speciation in a dredged contaminated sediment using scanning electron microscopy, X-ray fluorescence, EXAFS spectroscopy, and principal components analysis.* Geochim Cosmochim. Acta. 2005; 69, 2265 – 2284.

[Phillips et al., 1993] Phillips DJH, Rainbow PS. *Biomonitoring of Trace Aquatic Contaminants.* Elsevier Applied Science: New York, NY, 1993.

[Puy-Azurmendi et al., 2006] Puy-Azurmendi E, Ortiz-Zarragoitia M, Kuster M, Martínez E, Guillamón M, Dominguez C, Serrano T, Barbero MC, Alda ML, Bayona JM, Barceló D, Cajaraville MP. *An integrated study of endocrine disruptors in sediments and reproduction-related parameters in bivalve molluscs from the Biosphere's Reserve of Urdaibai.* Mar. Environ. Res. 2006; 69, S63 – S66.

[Rauret et at., 1999] Rauret G, Lopez-Sanchez JF, Sahuquillo A, Rubio R, Davidson C, Ure A, Quevauviller P. *Improvement of the BCR three step sequential extraction procedure prior to the certification of new sediment and soil reference materials.* J. Environ. Monit. 1999; 1, 57 – 61.

[Roose et al., 2005] Roose P, Brinkman UAT. *Monitoring organic microcontaminants in the marine environment: principles, programmes and progress.* Trends. Anal. Chem. 2005; 24, 897 – 926.

[Ruiz, 2009] Ruiz F. *Population of Spain website in Castilla - La Mancha University, http://alarcos.esi.uclm.es/per/fruiz/pobesp/map/fig/ccaa/r16-dens-2009p.pdf*.
Last access, October, 2015.

[Sagoff, 1994] Sagoff M. *Polulation and the Environment. In: Lakshman DG, Geoffrety WRP, Burns HW.* Int. Environ. Law and World Order 1994; 222.

[Saaedi et al., 2013] Saaedi M, Li LY, Karbassi AR, Zanjani AJ. *Sorbed metals fractionation and risk assessment of release in river sediment and particulate matter*. Environ. Monit. Assess. 2013; 185, 1737 – 1754.

[Sanz et al., 2004] Sanz J, de Diego A, Raposo JC, Madariaga JM. *Methylmercury determination in sediments and fish tissues from the Nerbioi-Ibaizabal estuary (Bilbao, Basque Country).* Anal. Chim. Acta. 2004; 508, 107 – 117.

[Spada et al., 2013] Spada L, Annicchiarico C, Cardellicchio N, Giandomenico S, di Leo A. *Heavy metals monitoring in the mussel Mytilus galloprovincialis from the Apulian coast (Southern Italy).* Medit. Mar. Sci. 2013; 14, 99 – 108.

[Sauve et al., 2000] Sauve S, Henderschot W, Allen HE. *Solid-solution partitioning of metals in contaminated soils: dependence on pH, total metal burden and organic matter*. Environ. Sci. Technol. 2000; 34, 1125 – 1131.

[Scancar et al., 2000] Scancar J, Milacic R, Strazar M and Burica O. *Total metal concentrations and partitioning of Cd, Cr, Cu, Fe, Ni and Zn in sewage sludge.* Sci. Tot. Environ. 2000; 250, 9 – 19.

[Spenser et al., 2002] Spenser KL, MacLeod CL. *Distribution and partitioning of heavy metals in esturiane sediment cores and implications for the use of sediment quality standards.* Hydrol. Earth Syst. Sci. 2002; 6, 989 – 998.

[Shuttleworth et al., 2014] Shuttleworth EL, Evans MG, Hutchinson SM, Rothwell JJ. *Assessmet of lead contamination in peatlands using field portable XRF.* Water Air Soil Pollut. 2014; 225, 1844 – 1857.

[Tessier et al., 1979] Tessier A, Campbell PGC, Bisson M. *Sequential Extraction procedure for the speciation of particulate trace metals.* Anal. Chem. 1979; 51, 844-851.

[Tueros et al., 2009] Tueros I, Borja A, Larreta J, Germán Rodríguez J, Valencia V, Millán E. *Integrating long-term water and sediment pollution data, in assessing chemical status within the European Water Framework Directive.* Mar. Pollut. Bull. 2009; 58, 1389 – 1400.

[Tsuji et al., 2012] Tsuji K, Kazuhiko N. *X-ray spectrometry.* Anal. Chem. 2012; 84, 636 – 668.

[UNESCO, 2009] Spain: the Autonomous Community of the Basque Country: The United Nations World Water Development, Report 3. Paris, 2009.

[USGS, 2015] *http://minerals.usgs.gov/minerals/pubs/commodity*. United States Geological Survey. Last access, October, 2015.

[Väisänen et al., 2002] Väisänen A, Suontamo R, Silvonen J, Rintala J. *Ultrasound-assisted extraction in the determination of arsenic, cadmium, copper, lead and silver in* *contaminated soil samples by inductively coupled plasma atomic emission spectrometry.* Anal. Bioanal. Chem. 2002; 373, 93 – 97.

[Vitousek et al., 1997] Vitousek PM, Mooney HA, Lubchenco J, Melilli JM. *Human domination of Earth's ecosystems.* Science. 1997; 277, 494 – 499.

[Wood et al., 2012] Wood CM, Farrell AP, Brauner CJ. *Homeostasis and Toxicology of Essential Metals.* Elsevier, San Diego, USA, 2012.

[Wu et al., 2005] Wu Y, Falconer R, Lin B. *Modelling trace metal concentration distributions in estuarine waters.* Estuar. Coast. Shelf S. 2005; 64, 699 – 709.

[Zhang et al., 2014] Zhang C, Yu Z, Zeng G, Jiang M, Yang Z, Cui F, Zhu M, Shen L, Hu L. *Effects of sediment geochemical properties on heavy metal bioavailability.* Environ. Int. 2014; 73, 270 – 281.

[Zwolsman et al., 1993] Zwolsman JJG, Berger GW, Van Eck GTM. *Sediment accumulation rates, historical input, postdepositional mobility and retention of major element and trace metals in salt marsh sediment of the Scheldt estuary.* Mar. Chem. 1993; 44, 73 – 94.

2. Objectives

2. Objectives

As discussed in the previous chapter, industry is one of the major pollution sources, but it is recognized that industry activity is necessary for mankind evolution. Regarding pollution caused by industry, sometimes, it is difficult to obtain analytical results over the polluted areas due to the difficult employed methods, expensive equipments or lack of economical resources. Thus, it is difficult to get correct answers or making decisions over the environment when there is neither a unified criteria on the methods nor on the samples to be used for assessing an environmental state situation. Besides, all the researchers and European directives focus on the necessity of a correct monitoring to obtain the overall state of the system and its evolution and not in a simple and punctual analysis.

In this framework, as it has been stated previously, quick, easy and cheap methodologies should be developed to help researchers, companies and consultancies to get a better understanding of the environmental issues. In this regard, not only legislation and governments can control the environment status, but also any company can investigate by their own the environmental impact produced by its industrial activity. With this continued information and with the whole background of knowledge about the environment, any required action over a particular polluted site can turn to a really fast and accurate operation.

In this sense, the main aim of the present PhD Thesis is centred on the **development of unified, fast and affordable methodologies for environmental analysis, focusing on the metal availability in different environmental bodies** and afterwards, their application to specific environmental scenarios. In order to accomplish this general and wide goal several specific objectives were established.

Firstly, for an adequate understanding of the mobilization process of minerals rich in toxic metals present in sediments, their identification must be performed. This geochemical characterization, which may inform about the presence of compounds of different sources (natural or anthropogenic), can be performed using Raman spectroscopy. However, the high amount of amount of organic matter present in sediment samples hinders the analysis performance. In this sense, the first specific objectives of this work will be:

- The development of a new methodology in order to reduce the fluorescence interference, easing the direct analysis of sediments by Raman spectroscopy.
- The application of the methodology on real sediment samples in order to identify and discern between natural and anthropogenic species of the solid phases in sediments.

The biohazard in sediments is often assessed by the determination of the acid-extractable metal concentration. However, clear misunderstandings can be outlined when only an environmental compartment is analyzed independently. In fact, the mobility of potential hazardous metals will be controlled by several factors such as pH, sediment type, concentrations of inorganic and organic ligands or the physicochemical characteristics of the water in contact with that sediment. To accomplish this framework, the second group of specific objectives of this work will be:

- The development of a new methodology to assess metal mobilization in the environmental water-sediment body using experimental conditions closer to the environmental ones. In this regard, two different scenarios will be evaluated: continental water (rivers)-sediment and estuarine water-sediment.
- The application of the previous methodologies to a particular case study: the arsenic behavior in both river and estuarine cases.

The knowledge of the speciation of metals and the role of organic matter in sediment-water interface is a key parameter to understand metal mobility and ecotoxicity. Humic substances are assumed to be the most reactive fractions that control the mobility of metals in sediment-water interface and in aquatic systems. However, there is little investigation dealing with the quantification of this fraction that acts as a bridge among environmental phases and as interference during conventional analyses. Its characterization and quantification is still being an analytical challenge. Within this scenario, the third group of specific objectives of this work are:

- The development of a new/fast methodology in order to quantitate the amount of organic matter (Humic substances) present in sediment samples and its application to real estuarine systems.
- Once all the sediment-water system is characterized, relationships among variables and seasonal trends will also be studied.

After these specific objectives focusing the metal movement in the estuarine ecosystem among water and sediment bodies and the relationship of the organic matter with the environment, the analysis of bioavailability of such elements must be carried out in biological samples. However, there is an important challenge when dealing with biological samples and metal analyses which is the matrix interference and reduced repeatability/precision. In this sense, the last objective of this work is:

- The development of an easy and inexpensive clean up step in order to reduce organic interferences in biota samples measurements and the application to real samples.

This work is just a proposed guideline of the total characterization study that an environmental research needs to include in order to obtain a real and global conclusion regarding metal pollution. The global study for an environmental system is a multidisciplinar work that includes various areas of study, not only chemistry but also biology, geology and mathematics in order to join all the data obtained from all the areas.

These proposed objectives will be developed in a series of chapters following this section of the work.

3. Raman spectroscopy speciation of mineral solid phases in sediments

3. Raman spectroscopy speciation of mineral solid phases in sediments

Sediments are considered to be a reservoir of pollutants that normally come from not well treated industrial wastewaters or from urban sewage treatment plants that are not prepared to eliminate modern micro-contaminants. As the sediment stores contaminants due to its contact with the polluted water, it is able to release them depending on changes in the natural conditions and/or physical variables of the surrounding water, modifying the bioavailability of the contaminants. Thus, salinity, pH, temperature, redox potential and most of the physical variables of the water become an important factor to control in order to perform the adequate modelling of the behavior of the different inorganic contaminants [Raposo et al., 2004].

Several methodologies are used to evaluate the bioavailability and mobility of such pollutants, but practically none of them provide the knowledge about the mineral phase of the given pollutants in the sediments [Förstner, 2004]. This information reaches the crucial level when it is necessary to differentiate anthropogenic species from natural ones. At this level, spectroscopic techniques that study surfaces are the most convenient ones [Olazabal et al., 2007]. Among them, Raman spectroscopy is a useful technique to differentiate several species of the same element or anion, especially if the spectral interpretation is assisted by thermodynamic modelling [Billon et al., 2003]. Moreover, Raman spectroscopy has the advantage to measure the sample practically without any pre-treatment and the chemical species can be identified as such in the solid state.

Raman spectroscopy has been used, alone or with other analytical techniques, by some authors to: (i) search for small quantities of pyrite and amorphous iron sulphide(s) [Boughriet et al., 1997] or metal sulphides in anoxic sediments of the Seine estuary (northern France) [Billon et al., 2001], (ii) analyze mineral particle reactions in soils [Birkefeld et al., 2005], (iii) look for different classes of compounds on igneous sediments from a perennially ice-covered lake (including sediments from surface and bottom oxic and anoxic zones, as analogues of Mars paleolake environments) [Edwards et al., 2004] and, (iv) characterize metallic contaminants through the analysis of heavy particles separated from the stream, canal and estuarine sediments [Moles et al., 2003].

Most of the samples analyzed in the previous works have low content of clay and the sample was pre-treated, when necessary, to minimize the interference of clays. However, clays and organic matter are two basic components of practically all sediments from highly populated areas. Both components are active in Raman spectroscopy, and they suppose a high interference when the analysis of sediments samples is performed directly by means of Raman spectroscopy.

This work has been performed in an attempt to develop a methodology to differentiate among natural and anthropogenic mineral phases in sediments, without altering the chemical speciation of solid inorganic compounds, by means of Raman spectroscopy. The analyzed samples were taken from the Bilbao estuary and from different continental rivers of the Basque Country (north of Spain). All the samples are rich in clays and organic matter, and belong to areas with different degrees of pollution.

3.1. Experimental

3.1.1. Material and reagents

Acetone, methanol and dichloromethane of HPLC quality (Lab-Scan, Dublin, Ireland) were used in order to extract and eliminate organic matter from the sediment samples. Cellulose filter papers (Whatman, Maidstone, England) were used to filter the sediments from the solvent extraction step applied as clean-up. Glassware (National Scientific Company, Rockwood, TN, USA) was used to store and treat the sediments.

3.1.2. Instrumentation

A Cryodos freeze dryer (Telstar, Terrasa, Spain), working at 10^{-1} bar and -52 °C, was used to dry the sediment samples. An oven (Heraeus, Haaksbergen, The Netherlands) was used to compare the percentage of organic material in sediments. An Octagon digital sieve (Endecotts, London, UK) was used to recover the 63 µm particle size sediment from the bulk sediment. An ultrasonic bath (J.P. Selecta, Barcelona, Spain) was used to assist the organic matter extraction from the sediment samples.

A fibre optic microprobe coupled to a Raman spectrometer, Renishaw RA-100 (Gloucestershire, UK), was used for the sediment characterization. The diode laser used has a power of 50mW at the source and an excitation wavelength of 785 nm. The spectra were recorded using a Peltier cooled charge-coupled device (CCD) detector and a mobile diffraction grating of 1200 lines·mm^{-1}, with integration times between 100 and 300 s. In order to avoid any thermal degradation of the samples, a 10% neutral density filter was used. The micro-probe is equipped with different objectives (4x, 20x and 50 x) which allows to focus the particles between 10 µm and 200 µm present in the sample. In order to help in the focus process, a colour micro-camera was mounted on the microprobe. The focusing of samples was performed by a joystick controlled X–Y plate mounted on a tripod. The Z axis was manually changed when required.

3.1.3. Sampling procedure

The samples were collected along the Bilbao estuary and some continental rivers of the Basque Country, in order to cover the two extreme organic matter compositions of the sediments.

The Bilbao estuary sampling points covered locations with different salinity due to its proximity to the coast. These points were Arriluze, Lamiako, Galindo and Ibaizabal. Continental rivers were chosen due to its anthropogenic interaction or industrial activity. The rivers were Estanda, Jundiz, Crispijana, Deba and Lasao.

Sediment samples were manually collected from the top sediments (5–10-cm depth), stored in sealed plastic bags under N_2 atmosphere, transported to the laboratory in cold boxes and frozen until freeze-drying. Most of the sediments were collected in oxidized areas (oxidant redox potential conditions at the surface sediments) trying not to take or minimize the sediment taken in anoxic areas (deeper sediment). The sediments were freeze-dried at -52 °C and 10^{-1} bar for 24 h. The lyophilized samples were sieved through a 63 µm sieve and stored in glass bottles at 4 °C until analysis.

3.1.4. Raman spectroscopy measurements

The entire laboratory ware and other equipments that come in contact with samples were cleaned with rigorous procedures in order to avoid cross-contamination of samples. Briefly, all glassware and plastic ware were washed with a common detergent and thoroughly rinsed with abundant pure water (Elix® quality, Millipore™, Bedford, MA, USA). After that, the entire laboratory ware was soaked in a clean diluted 10% HNO_3 bath for 24 h (Merck, Darmstadt, Germany). Afterwards, the material was rinsed with ultrapure water (Milli-Q® quality, Millipore™, Bedford, MA, USA), dried in an oven and stored (in sealed plastic bags used to collect samples in locations).

A small part of the gathered and dried sediments (0.5 g) was suspended with 10 ml of acetone in a glass vial and was submerged in an ultrasonic bath for at least half an hour. Thereafter, the mixture was filtered through cellulose filter paper and the solid was set to dry in air.

In order to perform the spectroscopic analysis, few milligrams of the treated sediments were set into a 5 mm depth solid holder, manually pressed and maintained in a perpendicular position to the Raman microprobe. Photobleaching was always applied to minimize the fluorescence of the sediments and, hence, to gather a well signal-to-noise ratio. Grain-to-grain characterization was possible, thanks to a precise focusing (approximately 5-200 µm) provided by the microscope lens (4x, 20x or 50x).

Raman acquisition conditions varied depending on the gathered signal-to-noise ratio and the observed fluorescence come from the clay matrix. Most of the Raman spectra were collected between 200 cm^{-1} to 2000 cm^{-1} with a spectral resolution of 1 cm^{-1}. The time of integration was between 1 s and 200 s and the accumulation was varied to get the best signal to noise ratio. Previous to the Raman acquisition, the sample was irradiated with the laser at least one hour (photobleaching) and afterwards, most of the analyses were conducted by taking 15 accumulations of 10 s each, and consequently, each complete measurement with more usual conditions last 7.5 min.

The measurement process was started by visually observing the mineral grains using the micro-camera, selecting an appropriate one to collect the Raman spectra and storing the image before an exposure to the laser beam. Then, the 1% filter was set for the laser and the spectra were collected. Another image was then recorded in order to be sure of the absence of sample burning. If the stored spectrum had a bad signal-to-noise ratio, the measurement process was repeated using the 10% filter for the laser or without filter if required. Very few spectra were collected at 100% power of the laser.

Spectral interpretation was assisted by home-made Raman databases [Perez Alonso et al., 2004; Castro et al., 2005] and other databases available in the literature [Burgio et al., 2001; Bouchard et al., 2003] or through official websites [Mineral and Inorganic Materials]. The software used to collect and treat spectra were WIRE (Renishaw, UK) and Omnic (Nicolet, Madison, WI, USA), respectively. When it was necessary, thermodynamic modelling was conducted in order to identify the most stable solid phase among several possible candidates, by means of the MEDUSA program [Puigdomenech, 2001].

3.2. Results and discussion

The Basque Country area (north of Spain, in front of the Gulf of Biscay) has been below the sea level until it emerged for thousand years. Consequently, its geology is conditioned by cycles of stratified deposits of the eroded near rocks and river-transported sediments. From the Palaeozoic ages to the Quaternary, the most common deposits of minerals and rocks have been calcareous minerals, quartz sand, micaceous silicates and oxides (the most common are aluminium and iron mixtures), and conglomerates (clay, gravel, mud, till, silt and any cementing material such as silica, calcium carbonate and some organic materials) [Arostegui et al., 1991].

Clay as well as organic matter are expected to be always present. However, if the organic matter fraction is effectively removed during a soft clean-up step of sediment samples, a cleaner Raman spectrum will be obtained, being the remaining clay the responsible of the background in the collected spectra. Clay is a mixture of very small particles of aluminium-hydrated silicates (i.e., illite, montmorillonite, smectite, kaolinite and sepiolite among others) together with other mineral compounds, depending on the geologic characteristics of the zone. Based on the previous knowledge about the eroded rocks composition, volcanic and metamorphic rocks like feldspars, pyroxenes, amphiboles and shale eroded into less than 4 µm particles, must be considered as natural compounds in the studied area. Besides, carbonated minerals must be considered as natural compounds

[Earth Science, 2008]. Apart from this natural distribution, it is necessary to emphasize the high iron concentration in the basin of Bilbao estuary. In fact, pyrite (FeS_2), cassiterite (SnO_2) and iron oxides are very common natural mineral phases occurring in the sediments of the studied area, as a consequence of natural transport processes in the basin. Therefore, these mineral phases are candidates to appear in the Raman measurements as natural inorganic compounds, but organic matter will always be present. The organic material is formed from the natural decay of flora, micro-organism activity and, in a smaller amount, animals.

3.2.1. Optimization of direct Raman analyses on sediments

The minimization of the effects (mainly fluorescence) yielded by the presence of organic compounds is highly recommended previous to Raman spectroscopy analyses. In order to achieve this goal, the sediments were treated with several organic solvents in order to remove the highest amount of the organic material without modifying the inorganic fraction. The selected organic solvents were chosen according to their chemical characteristics. Ideally, the solvents should show a high affinity for the organic fraction or for the fluorescence-causing compounds but inertness to the target compounds. In addition to the affinity, the selected solvents should have low boiling points in order to be eliminated fast afterwards. Thus, acetone, dichloromethane and methanol were tested as suitable organic solvents.

0.5 g of dried and sieved sediment (sampled from Ibaizabal) was treated with 10 ml of pure organic solvent for 30 min in an ultrasonic bath. Afterwards, the supernatant containing potential fluorescence causing elements was removed by filtration and sediments were air dried. All tests were performed with the same sediment by triplicate. The effectiveness of the different solvents to simplify the target matrix was evaluated by measuring the loss of organic matter content, which was estimated using the loss of ignition (LOI) method [Heiri et al., 2001]. Hence, the sediments were dried/burned in an furnace at 550°C for 4 h. Concurrently, the LOI of the same sediment samples without any kind of clean-up step (i.e., without any treatment of organic solvents) was determined, in order to compare the real effectiveness of the clean-up procedure.

The results obtained showed that only one organic solvent, acetone, was able to remove a considerable amount of organic fraction. An average of 39 ± 3% of the organic matter content was removed for the whole set of sediment samples (n=3 at 95% of confidence level). Treatment with dichloromethane removed only the 2.3 ± 0.6% of the organic matter whereas methanol showed the capacity to dissolve inorganic compounds altering the target compounds.

Consequently, further Raman analyses were performed using the following procedure: 0.5 g of pre-freeze dried and sieved sediment were mixed with 10 ml of acetone in a glass vial and submerged in an ultrasonic bath for 30 min (50–60 KHz). The organic supernatant was removed by filtration, and the sediment was re-extracted at least twice in order to quantitatively remove the extractable organic matter present in sediment samples. The sample treated in this way was finally air dried.

3.2.2. Natural compounds found in sediment samples

As it has been previously mentioned, clay is formed from the weathering of aluminium-silicate materials and it is found in every sedimentary matrix up to depths of 1200 m. Quartz and silicate-type micas, which are natural compounds of silicate oxides, often occur in metamorphic and sedimentary rocks. During the erosion of these rocks over thousand years, they suffered from aerial transport or water transport to the sea. Consequently, quartz and eroded micas are incorporated to the sediments, river and estuarine waters, becoming part of the natural compounds of the sediment.

Table 3.1 shows the natural compounds found in the different sample locations, while Table 3.2 shows the Raman signals used for their identification. These signals have been divided into three groups according to their relative intensity. Usually, the most intense signals and the secondary ones can be used to unambiguously identify the compounds.

Table 3.1. *Natural compounds found in sediments collected in the different sampling locations.*

		Natural compounds					
Sampling locations	***Tidal influence (estuarine)***	***Quartz***	***Mica***	***Aragonite***	***Calcite***	***Pyrite***	***Fe_2O_3***
Arriluze	Yes	X	X	X	X	X	X
Lamiako	Yes	--	X	X	X	X	X
Galindo	Yes	--	X	--	--	X	X
Estanda	No	X	--	X	X	--	--
Jundiz	No	X	X	--	X	--	--
Crispijana	No	X	X	--	--	--	X
Deba	No	--	X	X	--	--	X
Laso	No	X	--	X	--	--	--
Ibaizabal	No	--	X	X	X	--	X

Table 3.2. *Raman bands (cm^{-1}) used to identify the natural compounds in each sampling point.*

Mineral	***Chemical Formula***	***Primary bands (cm^{-1}, strong)***	***Secondary bands (cm^{-1}, medium)***	***Tertiary bands (cm^{-1}, weak)***
Quartz	SiO_2	463	--	--
Mica	--	464	708	--
Aragonite	$CaCO_3$	1084	206	705
Calcite	$CaCO_3$	1084	280	713
Hematite	Fe_2O_3	406, 291, 225	607, 494	--
Pyrite	FeS_2	342	462, 378	--

Figure 3.1 shows the Raman spectrum of clay, considered as the Raman background, and the Raman spectrum of a sample from the Estanda River showing the most intense signal of quartz (463 cm^{-1} – corresponding to the symmetric stretching band of SiO_2) on the clay Raman background. The same situation has been found in all the spectra, i.e. the signal of natural compounds over the signal of clay background. Similar situations have been observed in Crispijana (located before the wastewater treatment plant (WWTP)), where silicate-type mica is seen (464 cm^{-1} as the most intense Raman band and the secondary band at 708 cm^{-1}) together with the Raman background of clay.

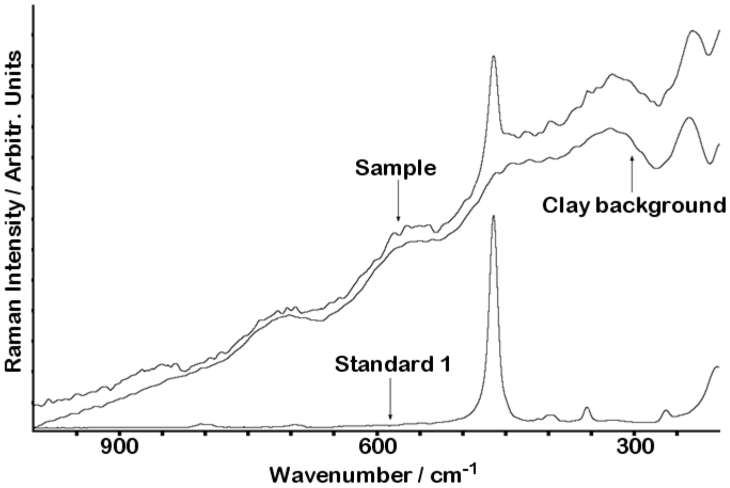


Figure 3.1. *Raman spectra of quartz clay in a sediment from the Estanda River. The standard 1 is the Raman spectrum of quartz from the database "e-VISARCH" [Perez-Alonso M. et al., 2004].*

Aragonite (1084 and 705 cm^{-1}; CO_2^{3-} stretching ν_1 and CO_2^{3-} bending ν_6 bands, respectively) is a natural phase of calcium carbonate. Although the sediments collected in this work do not have a high carbonate concentration, some compounds such as calcium carbonate occur in practically all the environmental sedimentary backgrounds. Aragonite is a stable metamorphic mineral with the same molecular formula as calcite (polymorph) but both minerals have different Raman features. The sampling location where the aragonite was found belongs to the Estanda river (a tributary of the Oria, Beasain), which is a large basin in Gipuzkoa (one of the counties belonging to the Basque Country, in the northeast of the sampling area). Industrial factories are found on both sides of the tributary, being the area of Beasain an important industrial zone in Gipuzkoa.

Calcium carbonate, probably coming from isolated carbonate-rich rock masses at the source of some rivers and transported across the whole river until their mouth, can be considered as a natural compound. This compound can be easily differentiated from the natural aragonite by means of Raman spectroscopy (characteristic Raman bands at 1084, 713 and 280 cm^{-1}). The spectrum was also obtained in samples from the Estanda River.

Hematite (α-Fe_2O_3, with characteristic Raman bands located at 607, 494, 406, 291 and 225 cm^{-1}) is considered a natural phase of iron (III) oxide. The basin of the Bilbao estuary is well known for its iron mines even from the Roman times. Thus, the iron containing minerals, mainly hematite and pyrite, are expected to be easily detected in the sampling locations on the Bilbao estuary. This compound was found in Lamiako, in the main channel of the Bilbao estuary. Pyrite (FeS_2, main Raman bands at 462, 378 and 342 cm^{-1}) was also found, not alone on a clay background but together with anthropogenic species of iron.

3.2.3. Anthropogenic compounds found in sediment samples

The compounds found in the spectra, not belonging to the natural mineral phases considered above were considered as coming from anthropogenic sources [Heiri et al., 2001]. Such anthropogenic mineral phases reached the sediments by either direct dump from spills, non-treated waters, solids not well retained in the treatment plants or in situ formed solid phases by chemical reaction among naturally dissolved compounds and dumped chemicals.

Table 3.3 summarizes the anthropogenic compounds found in some sampling locations and Table 3.4 shows the characteristic Raman bands used to identify them. As before, all the identified compounds have shown at least two characteristic bands of their Raman features on the clay background showing to their unambiguous identification.

Table 3.3. *Anthropogenic compounds found in sediments collected in the sampling locations.*

	Anthopogenic compounds								
Sampling locations	***TiO_2***	***Phthal blue***	***Phthal green***	***$CaSO_4$ $2H_2O$***	***$Ca_3(AsO_4)_2$***	***PbO***	***ZnO***	***Na_2S***	***FeO(OH)***
Arriluze	--	--	--	--	X	--	--	--	X
Lamiako	--	--	--	X	X	--	--	--	X
Galindo	--	--	--	--	X	--	--	--	X
Estanda	--	X	--	--	--	--	--	--	--
Jundiz	--	--	--	--	X	--	--	--	--
Crispijana	--	X	X	X	--	--	--	--	--
Deba	--	--	--	--	X	--	--	--	--
Laso	X	--	--	--	X	--	--	--	--
Ibaizabal	--	--	--	--	--	X	X	X	X

Table 3.4. *Raman bands (cm^{-1}) used to identify the anthropogenic compounds in sampling points.*

Compounds	***Primary bands (cm^{-1}, strong)***	***Secondary bands (cm^{-1}, medium)***	***Tertiary bands (cm^{-1}, weak)***
TiO_2	610, 447	238	--
Phthalocyanine blue	1527, 1340, 747, 680	140, 1143, 953, 483	1307, 1193, 1108, 831, 780, 594, 257
Phthalocyanine green	1527, 1340, 748, 680	1140	1451, 1305, 1183, 1083, 484
$CaSO_4 \cdot 2H_2O$	1008	1134, 494, 414	672, 620
$Ca_3(AsO_4)_2$	848	336	--
PbO	290	--	387
ZnO	438	382, 332	538
Na_2S	447, 407, 230	1533, 1341,990, 683, 325	813, 632, 523, 393
FeO(OH)	395, 301, 245	550, 479	--

Among the anthropogenic compounds detected in the analyzed sediments, titanium oxide was found (see Figure 3.2 – characteristic Raman bands located at 610 and 447 cm^{-1}). This compound was found in a sediment sample collected in a location settled close to an effluent of a WWTP in Lasao (Urola River). Titanium oxide was considered anthropogenic mineral, since it is often used as raw material in various industrial processes to produce industrial pigments. In fact, a pigment production industrial plant is located near to the Urola River.

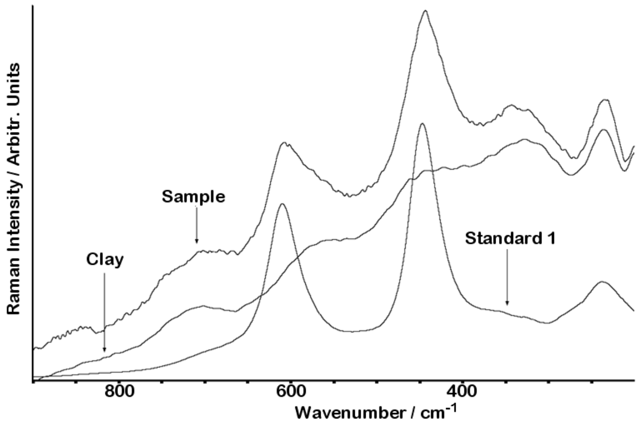


Figure 3.2. *Raman spectra of Titanium oxide in Lasao. The standard 1 is a Titanium oxide standard from the database ''e-VISART'' [Castro et al., 2005].*

Phthalocyanine blue (29H,31H-phthalocyaninate(2-)-N29,N30,N31,N32 copper; an aromatic macrocyclic compound) was another anthropogenic compound detected downstream of two WWTPs. The Raman spectrum shown in Figure 3.3 belongs to a sediment sample in the location of Crispijana, in Zadorra River, which is close to the effluent of the WWTP of Vitoria-Gasteiz, where both urban and industrial wastewaters are treated. Phthalocyanine green was also detected in the same location. Conventional WWTPs are not designed to settle such organic compounds, although most of them are removed through adsorption on particulate matter (especially iron hydroxioxide) or in sludge.

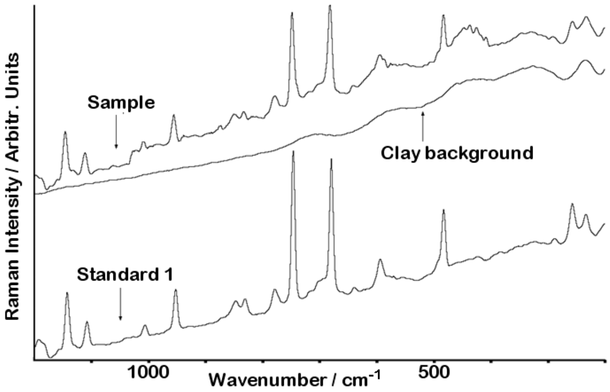


Figure 3.3. *Raman spectra of phthalocyanine blue in Crispijana. The standard 1 is a phthalocyanine blue standard from the database ''e-VISART'' [Castro et al., 2005].*

Also, the photography of the blue grain in which the spectra of phthalocyanine blue was taken from is shown in Figure 3.4.

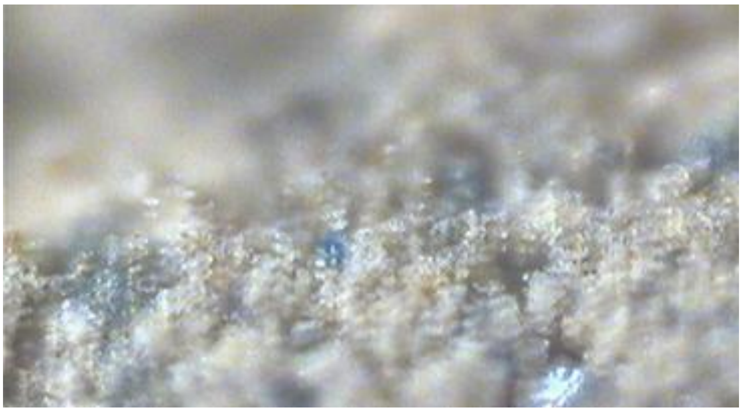


Figure 3.4. *Photography of the blue grain containing phthalocyanine blue in Crispijana.*

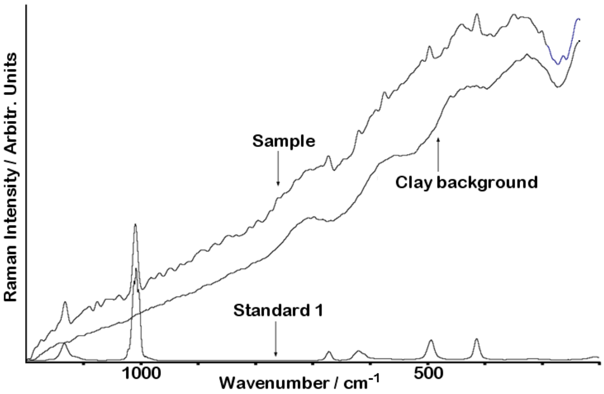


Figure 3.5. *Raman spectra of gypsum in Crispijana. The standard 1 is a gypsum standard from the database "e-VISART" [Castro et al., 2005].*

Gypsum ($CaSO_4 \cdot 2H_2O$) was also detected in Crispijana (see Figure 3.5), downstream the same central sewage treatment plant, where the urban and pre-treated industrial waste waters are processed by adding lime and sulphate-based flocculants. Gypsum was also found in Lamiako, a sampling location in front of the sewage treatment plant of the main iron production plant of the Basque Country, the ACB factory. The presence of this compound can be attributed to an *in situ* precipitation of the soluble sulphate fraction and calcium ionic species [Raposo et al., 1999; Puigdomenech, 2001].

Calcium arsenate ($Ca_3(AsO_4)_2$) was often found in sediments downstream of iron and steel production factories. In fact, this compound was considered an anthropogenic compound, was detected by Raman spectroscopy (see Figure 3.6) in Jundiz, Deba, Lasao and in other three sampling locations of Bilbao estuary. Arsenic is often associated with iron minerals and, hence, it is found in higher concentration near iron-producing factories and sulphuric production plants using pyrites [Van Elteren et al., 2006]. Arsenate compounds are not well retained in conventional WWTPs which favours their entering to the environment waters through WWTP effluents. The formation of compounds as calcium arsenate could be achieved when dissolved arsenate ions reaches a flow with high calcium concentration such as estuarine and river waters [Raposo et al., 1999; Puigdomenech, 2001].

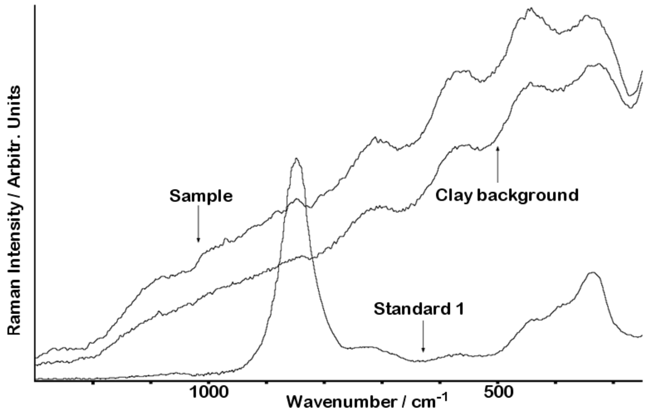


Figure 3.6. *Raman spectra of clay and calcium arsenate in Jundiz. The standard 1 is a calcium arsenate standard from the database "e-VISART" [Castro et al., 2005].*

Iron hydroxioxide (FeO(OH)) was found in several locations such as Galindo and other sampling locations in the estuary of Bilbao. The Raman spectrum shown in Figure 3.7 also includes Raman signals of natural pyrite. Iron hydroxioxide, usually associated with the presence of iron oxide, was also detected in sediments placed close to the main steel production plant in the Bilbao estuary. Hence, it is thought to have an anthropogenic origin associated with steel factory or by chemical reaction in the sea waters of the estuary where the pH of the river waters change from around 7.2 to around 8.3. Probably in both cases, the iron hydroxioxide is bounded to the surface of natural mineral particles, iron oxide or pyrite, suggesting the *in situ* formation of the FeO(OH) compound [Raposo et al., 1999; Puigdomenech, 2001].

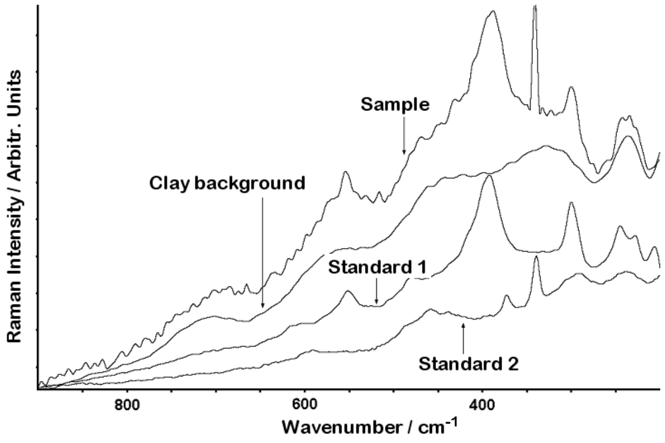


Figure 3.7. *Raman spectra of iron hydroxioxide and pyrite in Galindo. The standard 1 is an iron hydroxioxide and the standard 2 corresponds to a pyrite standard, both from the database "e-VISART" [Castro et al., 2005].*

3.3. Conclusions

Raman spectroscopy has demonstrated to be a useful technique to differentiate natural and anthropogenic compounds even if these compounds have the same molecular formula. This is the case of the calcium carbonate in the calcite and the aragonite polymorphs. The molecular speciation capability of this spectroscopic technique can be widely applied to multiple situations in environmental analysis.

Some variables must be considered before applying Raman spectroscopy to real environmental samples. The most important one is the fluorescence produced by the organic matter, an important component of the sediments. This interferes with Raman bands of compounds showing a medium to low Raman scattering. Moreover, the presence of such organic matter promotes the burning of most of the samples when a medium laser power is applied. Thus, to obtain adequate Raman spectra (i.e., high signal to noise ratio and low background), the minimization of the amount of fluorescence (organic matter) is often necessary, and hence, be able to get satisfactory Raman results, be able to apply higher laser powers or be able to use lower laser powers with more spectra accumulation without altering the samples.

In this sense, the method proposed in this work has demonstrated to be a very suitable procedure to reduce the fluorescence on sedimentary matrix and, consequently, to obtain good quality Raman spectra even on samples with high clay content. The soft clean-up of the sediment samples using organic solvents eased the Raman detection of contaminants present in highly complex matrix. In fact, although most of the pollutants detected in this work are often bounded to the surface of particulate clay, most of Raman spectra have been undoubtedly identified.

3.4. References

[Arostegui et al., 1991] Arostegui J, Zuluaga MC, Velasco F, Ortega-Huertas M, Nieto F. *Diagenesis of the central Basque-Cantabrian Basin (Iberian Peninsula) based on illite-smectite distribution.* Clay Miner. 1991; 4, 535 – 548.

[Billon et al., 2001] Billon G, Ouddane B, Laureyns J, Boughriet A. *Chemistry of metal sulfides in anoxic sediments.* Chem. Phys. 2001; 3, 3586 – 3592.

[Billon et al., 2003] Billon G, Ouddane B, Laureyns J, Boughriet A*. Analytical and thermodynamic approaches to the mineralogical and compositional studies on anoxic sediments.* J. Soils Sed. 2003; 3, 180 – 187.

[Birkefeld et al., 2005] Birkefeld A, Schulin R, Nowack B. *A New In Situ Method to Analyze Mineral Particle Reactions in Soils.* Envion. Sci. Technol. 2005; 39, 3302 – 3307.

[Boughriet et al., 1997] Boughriet A, Figueiredo RS, Laureyns J, Recourt P. *Identification of newly generated iron phases in recent anoxic sediments: ^{57}Fe Mössbauer and microRaman spectroscopic studies.* J. Chem. Soc., Faraday Trans. 1997; 93, 3209 – 3215.

[Bouchard et al., 2003] Bouchard M, Smith DC. *Catalogue of 45 reference Raman spectra of minerals concerning research in art history or archaeology, especially on corroded metals and coloured glass.* Spectrochim. Acta A 2003; 59, 2247 – 2266.

[Burgio et al., 2001] Burgio L, Clark RJH. *Library of FT-Raman spectra of pigments, minerals, pigment media and varnishes, and supplement to existing library of Raman spectra of pigments with visible excitation.* Spectrochim. Acta A 2001; 57, 1491 – 1521.

[Castro et al., 2005] Castro K, Perez-Alonso M, Rodriguez-Laso MD, Fernandez LA, Madariaga JM. *On-line FT-Raman and dispersive Raman spectra database of artists' materials (e-VISART database).* Anal. Bioanal. Chem. 2005; 382, 248 – 258.

[Edwards et al., 2004] Edwards HGM, Villar SEJ, Bishop JL, Bloomfield M. *Raman spectroscopy of sediments from the Antarctic Dry Valleys; an analogue study for exploration of potential paleolakes on Mars.* J. Raman Spectrosc. 2004; 35, 458 – 462.

[Earth Science, 2001] *Earth Science: Introduction to Clay Minerals and Soils*. Oakton Community College: Skokie, IL, USA, http://servercc.oakton.edu/.billtong/eas100/clays.htm, (last accessed 2015).

[Förstner, 2004] Förstner U. *Sediment dynamics and pollutant mobility in rivers: An interdisciplinary approach Lakes Reservoirs.* Res. Manage. 2004; 9, 25 – 40.

[Heiri et al., 2001] Heiri O, Lotter AF, Lemcke G. *Loss on ignition as a method for estimating organic and carbonate content in sediments: reproducibility and comparability of results.* J. Paleolimnol. 2001; 25, 101 – 110.

[Minerals and Inorganic Materials, 2008]. Minerals and Inorganic Materials. http://www.aist.go.jp/RIODB/rasmin/E_index.htm, Minerals. http://www.fis.unipr.it./bersani/raman/raman/spettri.htm, Minerals. http://www.sbg.ac.at/min/service/servicedaten.htm, Minerals. http://www .ens-lyon.fr/LST/Raman/index.php, Minerals. http://minerals.gps.caltech.edu/FILES/raman/Caltech data/index.htm, (last access, 2015).

[Moles et al., 2003] Moles NR, Betz SM, McCready AJ, Murphy PJ. *Replacement and authigenic mineralogy of metal contaminants in stream and estuarine sediments at Newtownards, Northern Ireland.* Mineral. Mag. 2003; 67, 305 – 324.

[Olazabal et al., 1997] Olazabal MA, Nikolaidis NP, Suib S, Madariaga JM. *Precipitation Equilibria of the Chromium(VI)/Iron(III) System and Spectrospcopic Characterization of the Precipitates.* Environ. Sci. Technol. 1997; 31, 2898 – 2902.

[Perez-Alonso et al., 2004] Perez-Alonso M, Castro K, Olazabal MA, Madariaga JM, *34th International Symposium on Archaeometry*, University of Zaragoza, 2004; 201.

[Puigdomenech et al., 2001] Puigdomenech I, MEDUSA (Make Equilibrium Diagrams Using Sophisticated Algorithms), version 15, Department of Inorganic Chemistry, The Royal Institute of Technology: Stockholm, Sweden, 2001.

[Raposo et al., 1999] Raposo JC, Sanz J, Borge G, Olazabal MA, Madariaga JM. *Development of a Modified Bromley's Methodology for the estimation of ionic media effects on solution equilibria: Part 3. Application to the construction of thermodynamic models.* Fluid Phase Equilibr. 1999; 155, 1 – 19.

[Raposo et al., 2002] Raposo JC, Sanz J, Zuloaga O, Madariaga JM. *The thermodynamic model of inorganic arsenic species in aqueous solutions. Potentiometric study of the hydrolitic equilibrium of arsenic acid.* Talanta 2002; 57, 849 – 857.

[Raposo et al., 2003] Raposo JC, Sanz J, Zuloaga O, Madariaga JM. *Thermodynamic Model of Inorganic Arsenic Species in Aqueous Solutions. Potentiometric Study of the Hydrolytic Equilibrium of Arsenious Acid.* J. Solution Chem. 2003; 32, 253 – 264.

[Raposo et al., 2004] Raposo JC, Olazabal MA, Madariaga JM. *Validation of chemical speciation model of inorganic arsenic in river waters with mobilisation processes.* Chem. Spec. Bioavailab. 2004; 16, 9 – 15.

[Van Elteren et al., 2006] Van Elteren JT, Slejkovec Z, Arcon I, Glass H. *An interdisciplinary physical-chemical approach for characterization of arsenic in a calciner residue dump in Cornwall (UK).* Environ. Pollut. 2006; 139, 477 – 488.

4. Mobility in river sediments, assessing new methodologies

4. Mobility in river sediments, assessing new methodologies

Among known pollutants, some trace elements are widely recognized as being potentially toxic to living organisms [Windom et al., 1999]. Toxic metals have been widely used in industrial and agricultural production and brought great harms to plant and animal through food chain [Dong et al., 2009]. In that sense, and because of the rapid development of urbanization and industrialization in recent years, metal contamination has become a topic of extensive study with many reports in the literature [Hu et al., 2006; Huang et al., 2006; Liu et al., 2006; Rodrigues et al., 2006; Shi et al., 2007; Chen et al., 2008].

It is generally considered that metals in nature come from two primary sources: natural inputs (e.g. parent material weathering) and anthropogenic inputs (e.g. metalliferous industries and mining, vehicle exhaust, agronomic practices, etc.), being adsorption processes the most important processes controlling their existence on solid phases, including sediments [Horváth et al., 2000; Veeresh et el., 2003].

River sediments often have very high amounts of metals due to the proximity to anthropogenic activities or to past depositions of industrial wastes. These pollutions bring along potential environmental risks being transferred into the natural system. In order to recognize and estimate both short and long term environmental impacts, the determination of the metal occurrence in river sediments as well as the identification of the factors that controls the metals distribution is often required. In fact, the accurate quantification of the metals in the river sediments is very useful to infer information about the spatial variability of the metal distribution between solid (river sediments) and aqueous phases (river water) [Atanassova, 1998].

The biohazard in soils is often assessed by the determination of the total metal concentration they contain. However, the total content of metals in the sediment is a poor indicator of environmental risk. Environmental risk assessment requires the determination not only of the total amount of pollutants in sediments but also of the metal presence in the available fraction. Mobility and bioavailability must be assessed to elucidate metals' behavior in sediments and prevent potential toxic hazards [Rauret, 1998; Daus et al., 1995; Einax et al., 1998; Windom et al., 2003; Caetano et al., 2003; Förstner, 2004; Bauer at al., 2003].

In general, metal mobility is controlled by several factors such as physicochemical variables in the environment, sediment type and the presence and concentration of inorganic and organic ligands or nutrients as well as biological activity. Moreover, biotic and abiotic redox reactions, for instance, are of great importance in controlling the oxidation state and thus, the mobility and toxicity of many elements. Metal adsorption and desorption process occurs simultaneously as an equilibrium and determines metal concentration in sediment solution [Navarro et al., 2006a].

In this framework, the speciation of metals in sediment solution is critical for assessing their bioavailability and potential threat to the environment [Kersten et al., 1995; Zoumis et al., 2001; Guevara et al., 2004; Sanz Landaluze et al., 2004]. Metals from anthropogenic sources are present in the oxic sediments mainly as oxides and/or hydroxides (with a very low solubility at the common sediment pH values among 6.8–8.4) a and as metal salts (sulfates, phosphates, arsenates, etc.) whereas metal sulphides are primarily found in the anoxic area of the sediments [Villanueva et al., 2008]. Therefore, their bioavailability for biota as well as their movement in the sediment-water interface and towards the water-flow depends on the possibility to form soluble complexes with inorganic and/or organic compounds [Sadiq et al., 1992]. Other natural processes including dilution, dispersion, sorption (absorption, adsorption and precipitation) and redox processes are factors that can be related to metal mobility.

In order to better quantify all of these processes in terms of metal availability, total and sequential extraction experiments are often carried out. In a first attempt, the treatment of sediments with a mixture of strong acids can provide a first estimation of the total extractable element concentration, which is related to the maximum potentially soluble metal concentration or related to the highest potential hazard that could occur in extreme environmental conditions. Regarding the mobility of metals in sediments, however, the possible risk is determined by their solid – solution partitioning rather than the total metal content itself [Sauve et al., 2000]. To this last purpose, selective leaching extraction methods have proven to be the most effective analytical assays to determine the availability of metals in sediments.

The use of sequential extractions provides information about the origin, mode of occurrence and bioavailability and mobility of metals in natural environments [Filgueiras et al., 2002]. The sequential extraction method proposed by European Community[1] [AFNOR, 1994], or any other derived sequential extraction procedure (like that of Baeyens [Baeyens et al., 2003]) from the original sequential extraction proposal [Tessier et al., 1997], is often employed in order to determine the metals geochemical distribution and to predict the mobility and their bioavailability.

---

[1] *The sequential extraction method proposed by the European Community consists on the subsequential addition of: (i) disodium ethylenediamintetraacetate (0.01mol·l*$^{-1}$*, EDTA) and ammonium acetate (1 mol·l*$^{-1}$*,* CH_3COONH_4*) at pH7; (ii) diethylene triamine pentaacetic acid (0.005 mol·l*$^{-1}$*), triethylamine (0.1 mol·l*$^{-1}$*, TEA) and calcium chloride (0.01 mol·l*$^{-1}$*,* $CaCl_2$*) at pH 7.3 [AFNOR, 1994].*

Although, the accuracy of the mobilization procedures can be evaluated using certified reference materials (e.g., by comparing the total concentration and the sum of the different phases of the sequential extraction) this multiple extraction procedure is quite tedious and it could lead to different quantification errors in comparison to the results obtained by a single step extraction procedure (total extractable element extraction methods). In this sense, the total extraction with 1.0 mol·l^{-1} ammonium nitrate solution (German standard DIN 19730) [Deutches Institut für Normung 19730, 1997] is one of the most requested extraction procedures for mobilization studies in sediments and soils. Anyhow, the lack of suitable reference materials for the extraction using ammonium nitrate solution makes difficult to establish the quality control/quality assurance of the performed methodology [Traub et al., 2001].

Many of the research works published in the literature use the mentioned *total extractable elements extraction* and *sequential extraction* procedures to assess the mobility of metals in sediment-water interface, but they do not fit with real field conditions. In fact, the reagents used for the extraction/mobilization of metals in current case studies in the lab, such as $Ca(NO_3)_2$, CH_3COONH_4, NH_4NO_3, assess poorly the real mobilization process because they are not naturally occurring in natural systems. For example, the possible formation of soluble ammoniacal complexes [Sillen et al., 1964] with metals like Cu(II), Co(II), Cd(II), Hg(II) and Ni(II) cannot be negligible when CH_3COONH_4 or NH_4NO_3 are used even in sediments with medium/low pH values. Moreover, these laboratory studies using pure solutions as extractants do not take into account the natural composition of the water in contact with the sediment, disregarding the possible influence of the real organic ligands present in the studied environment. In this sense, the use of the own river water as extracting agent could lead to obtain more realistic data and conclusions to be applied into the real systems. With this approach, the main inorganic species of the natural waters are considered as well as the effect of the organic compounds and the microbiological communities are taken into account in the laboratory mobilization studies.

In this section of the work, the concentration of the most relevant metals present in river sediments was determined. The selection of the elements (i.e., arsenic, cadmium, cobalt, chromium, cooper, iron, nickel and lead) was based on their occurrence in river sediments and on their different expectable mobility patterns. Although the proposed methodology was based on the DIN 19730 norm, the new procedure was performed using the own river water collected in contact with the sediments as extracting solvent. The aim of using river water is to fit the lab assays to a more realistic environmental conditions and, thus, reaching a more coherent metal availability data. Moreover, this methodology can allow defining the main physicochemical variables affecting the mobilization processes. Consequently, the proposed methodology takes into account the physicochemical characteristics of the river water and the sediments of the sampling points. The mobilization studies were completed taking into account the physicochemical parameters measured in water (i.e., conductivity, concentration of dissolved oxygen, pH, ORP and temperature) of each sampling location.

Once the chemical data of the performed mobilization studies is obtained, the interpretation of the final results is rather cumbersome due to the complex interactions of aquatic and particulate phases that cannot totally be described solely by univariate features [Rauret, 1998; Daus et al., 1995]. The use of multivariate analysis offers the possibility to obtain relationships among the different variables studied and to interpret correctly the potential effects on deposition and remobilization processes of metals in sediments [Raposo, 2001]. In this sense, principal component analysis (PCA) will be performed on the mobilization data obtained by analyzing different river sediments of the Basque Country (North of Spain) trying to focus on the importance of the water conditions in the metals mobilization.

Finally, Raman spectroscopy was employed to confirm some assumptions made as well as to understand some of the mobilization processes involved. Raman spectroscopy is an outstanding technique when speciation of compounds present in sediments is required, as it was demonstrated in other works [Raposo et al., 2006; Villanueva et al., 2008].

4.1. Experimental

4.1.1. Reagents and solutions

All reagents were of analytical-reagent grade and ultrapure water (Milli-Q® quality, Millipore™, Bedford, MA, USA) was used. The volumetric glassware was grade A and was calibrated at laboratory temperature. 1000 mg·l^{-1} Alfa Aesar (Specpure, Ward Hill, USA) standard solutions of As, Cd, Co, Cr, Cu, Fe, Ni and Pb were used. Hydrochloric acid (37%, HCl, Tracepur) and nitric acid (69%, HNO_3, Tracepur) were provided by Merck (Darmstadt, Germany). The 1.0 mol·l^{-1} NH_4NO_3 solution (Tracepur, Merck, Darmstadt, Germany) was freshly prepared for the mobilization studies.

4.1.2. Instrumentation

A Cryodos-50 (Telstar S.A., Terrasa, Spain) laboratory freeze-dryer was used to lyophilize sediment samples. An Octagon digital sieve (Endecotts, London, UK) was used to recover the 63 µm particle size sediment from the bulk sediment.

The microwave assisted digestion of sediment samples to determine total extractable elements concentration was carried out in a close microwave device 3000S (Anton Paar, Graz, Austria) equipped with 8 Teflon vessels and temperature and pressure controllers.

Inductively coupled plasma with mass spectrometry detector (ICP-MS, Perkin-Elmer, Elan 9000) was used for the analysis of metals in real system waters and mobilization extracts and using a Ryton cross-flow nebulizer, a Scott-type double pass spray chamber and standard nickel cones. For total concentration determination of metals in sediments, inductively coupled plasma - optical emission spectrophotometry (ICP-OES, Horiba Jobin Yvon, Activa) was employed using a quartz Meinhard concentric nebulizer, a Scott-type spray chamber and a standard quartz sheath connection between the spray chamber and the torch. Instrumental parameters for both ICP-MS and ICP-OES are listed in Table 4.1.

Table 4.1. *ICP-MS and ICP-OES operating and acquisition parameters used.*

	ICP-MS	***ICP-OES***
Instrumental conditions		
RF power (W)	1000	1200
Plasma gas flow (l·min^{-1})	14	12
Auxiliary gas flow (l·min^{-1})	1.0	0.0
Nebuliser gas flow (l·min^{-1})	0.91	0.95
Sample flow rate (ml·min^{-1})	1.0	1.0
Sheathing gas flow (ml·min^{-1})	---	0.3
Data acquisitions		
Dwell time (ms)	250	---
Integration time (ms)	---	2000
Sweeps for replicate	10	---
Replicates	4	3
Analytical detection		
Isotopes (ICP-MS) / Wavelength (nm, ICP-OES)	^{75}As, ^{114}Cd, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{56}Fe, ^{60}Ni, ^{208}Pb	As (193.695, 197.198, 200.334) Cd (214.438, 226.502, 228.802) Co (238.892, 240.767, 345.351) Cr (206.149, 267.716, 283.563) Cu (213.598, 324.754, 327.396) Fe (259.940, 273.955, 372.256) Ni (216.556, 341.476, 352.454) Pb (220.353, 283.306, 405.783)

For Raman analyses, a fibre optic microprobe coupled to a Raman spectrometer, Renishaw RA-100, was used in the sediment's characterization. The diode laser used had a power of 50 mW at the source and a wavelength of 785 nm. The spectra were recorded using a charge-coupled device (CCD) detector, with integration times between 100 and 300 s. In order to avoid any thermal degradation of the samples, a 10% neutral density filter was used. Objective lenses with a magnification of 20x allowed spectra to be collected from particles with a diameter down to 5 µm. The software used to collect and treat spectra were WIRE (Renishaw, UK) and Omnic (Nicolet, Madison, WI, USA), respectively.

4.1.3. Sampling points and procedure

4.1.3.1. Sediment samples

12 mixed samples were taken from the upper part of the sediments in each sampling point, located in 9 different rivers of the Basque Country: Kadagua, Nerbioi, Ibaizabal and Oka in Bizkaia; Deba-Elgoibar, Lasao (Urola River), Oria-Urnieta and Ormaiztegi (Estanda River) in Gipuzkoa; Crispijana and Zadorra in Araba. The sampling point names and their codes used are shown in Figure 4.1. Samples were gathered in three different seasonal time series, spring (March), summer (July) and autumn (November).

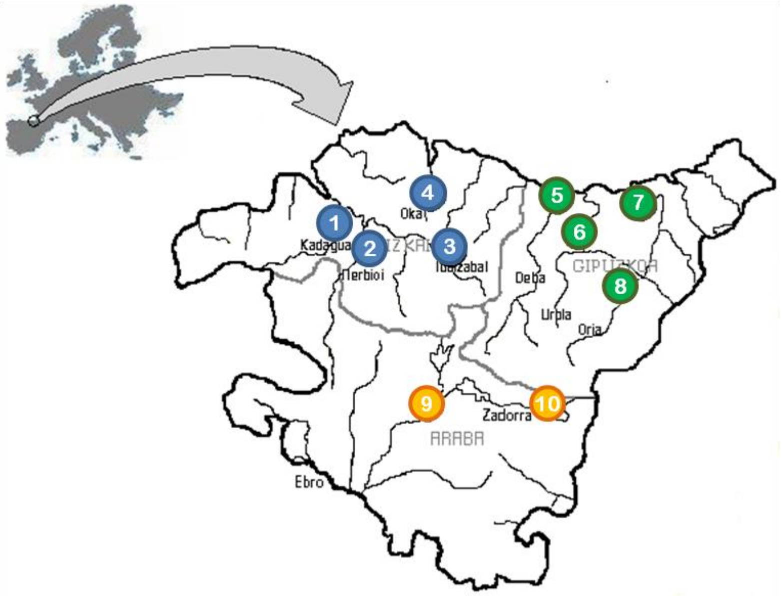


Figure 4.1. *Location of the sampling points: 1. Kadagua (KA-326), 2. Nerbioi (N-338), 3. Ibaizabal (I-160), 4. Oka (OK-114), 5. Deba-Elgoibar (DO-095), 6. Lasao (U-350 and U-410), 7 Oria-Urnieta (O-490), 8. Ormaiztegi (OE-160), 9. Crispijana (Z-516 and Z-576), and 10. Zadorra (Z-160).*

The sediment samples were scrapped from 5 cm of river-bed surface, introduced in plastic recipients, transported to the laboratory in cold boxes and frozen until freeze-drying. The sediments were freeze-dried at -52 °C and 10^{-1} bar for 24 h. The lyophilized samples were sieved through a 63 µm mesh and stored at −20 °C until analysis [Raposo et al., 2006].

The selection of the river sediments was based on geo-chemical properties: differences in the soil types, parent material and the exposition to different sources of pollution. In that sense, the presence of industrial sewage treatment plants in Crispijana and Lasao Rivers was taken into account and two different sampling sub-points were taken in both locations: before (Z-516 and U-350) and after (Z-576 and U-410) the treatment facilities, respectively.

4.1.3.2. Water samples

Water samples were collected at each point with a Van Dorm type all-plastic water sampler (KDDenmark, Research Equipment, Silkeborg, Denmark), specifically designed for metal sampling. River water samples were introduced in polystyrene bottles[2] and divided into two subsamples:

(i) a subsample for each sampling point was stored at 4 °C and used immediately for the mobilization studies.

(ii) the other subsample was filtered (0.45 µm PVDF, Whatman), acidified with HNO_3 (pH=2) and stored at 4 °C until total metal determination. Major elements were quantified by ICP-OES whereas metals were determined by ICP-MS [Fernández et al., 2008].

Immediately and during the sampling, physicochemical parameters of the river water were recorded: pH and pO_2 values, conductivity, oxidation-reduction potential (ORP) and temperature, by means of a pre-calibrated YSI 556 multiparametric probe (YSI Environmental, Yellow Springs, Ohio). The probe also provided an estimation of salinity

---

[2] *All plastic samples were first cleaned with a detergent, thoroughly rinsed with tap water, soaked in a 10% HNO_3 bath at least 24 h and finally rinsed again with ultrapure quality water. After drying the material in a laminar air flow hood, it was stored in clean plastic bags until being use for sampling or analysis purposes.*

and total dissolved solids (TDS) based on the temperature and electrical conductivity measurements.

The observed values were among 8.6 and 7.5 for pH, among 7.2 and 10.6 mg·l^{-1} for dissolved O_2, among 1510 and 404 µS·cm^{-1} for conductivity, among 294 and 148 mV for ORP, among 15.3 and 11.1°C for water temperature and among 20.0 and 11.1 °C for air temperature. It should be remarked that in I-160 sampling point, the conductivity (17 µS·cm^{-1}) and the ORP (41 mV) values obtained were out from the interval of values for the rest of the sampling points.

4.1.4. Sample pretreatment and analysis

4.1.4.1. Microwave assisted extraction for total extractable elements concentration determination in sediments

The freeze-dried sediments were subjected to acid digestions in a microwave oven in order to determine the acid soluble fraction of metals and following a previously optimized method [Navarro et al., 2006b]. Briefly, approximately 0.5 g of sediment samples were accurately weighted in PTFE vessels before adding 15 ml of an $HCl:HNO_3:H_2O$ (1:1:3 vol) mixture. During the extraction in the microwave, both temperature and pressure were controlled. After cooling, the extracts were filtered through syringe PTFE filters (0.45 µm, Waters, Milford, USA) and quantitatively transferred to polyethylene vials with a final volume of 50 ml with Milli-Q water. These extracts were kept in the dark in polyethylene vials at 4 °C until the analysis by ICP-OES (see section 4.1.2 for measurement parameters).

Together with the samples, reagents blanks submitted to the whole extraction procedure and certified reference materials (LGC 6187 and NIST 1640) were routinely analyzed in each sample batch to validate the extraction procedure used.

4.1.4.2. Metal mobilization assays: metal equilibrium leaching capacity

The potential mobility of metals from sediment samples in contact with river waters and under changing environmental conditions can be evaluated by several leaching extraction tests as stated previously (i.e., single batch extractions with selected reagents or sequential batch extractions). Generally, in leaching tests performed in the lab, sediment samples are put in contact with specific solution in order to simulate the real leaching conditions and consequently, the potential metal release can be worked out.

River waters from the same location of sediments were used as metal leaching reagent. For the equilibrium leaching tests, the leaching solution was mixed with the sediment, without its renewal, until equilibrium conditions were reached. This test was carried out following the specifications of the German DIN 19730 norm but using real river water instead of ammonium nitrate. Briefly, 20 g of sediment samples were mixed in a polyethylene vessel with 50 ml of both extractants (i.e., river water to simulate real conditions and ammonium nitrate to perform DIN 19730). The slurry was continuously mixed in a multiple rotating shaker (20 rpm, 2h and at room temperature). After the extraction step was over, all the extracts were filtered through PVDF filters (0.45 µm, Whatman) and acidified with concentrated HNO_3 (pH < 2) for stabilization and to adjust the acid percentage to 1% for their further analysis by ICP-MS (see section 4.1.2.).

4.1.5. Analytical fitness for purpose

The accuracy of the analytical methods was checked using two different certified materials, river sediment (LGC 6187, UKAS Reference Materials, UK) and natural freshwater (NIST 1640, National Institute of Standards and Technology, USA). The results obtained are shown in Table 4.2 and as it can be seen they were in good agreement with the certified values (at 95% confidence level). The precision of the analysis was evaluated using standard stock dilutions, 10 $\mu g \cdot l^{-1}$ and 10 $mg \cdot l^{-1}$ for ICP-MS and ICP-OES, respectively. The measurements were done at the same day (n=10) and in different days (n=10). The relative standard deviation (%RSD) was lower than 3% in all cases. The instrumental detection limits were calculated as three times the standard deviation of the intercept divided by the slope of the calibration curve. The detection limit values are also listed in Table 4.2 for all the metals considered.

Table 4.2. *Obtained results in natural freshwater (NIST 1640) and river sediment (LGC 6187) certified materials at 95% confidence level (n=10) and calculated limits of detection (LOD).*

	NIST 1640 ($\mu g\cdot kg^{-1}$)		**LGC 6187 ($mg\cdot kg^{-1}$)**		**ICP-MS**	**ICP-OES**
	Certified value	**Experimental value**	**Certified value**	**Experimental value**	**LOD ($\mu g\cdot kg^{-1}$)**	**LOD[a] ($mg\cdot kg^{-1}$)**
As	26.7 ± 0.4	28 ± 2	24.0 ± 3.2	18 ± 4	0.021	0.8 – 3.98
Cd	22.8 ± 1.0	22.3 ± 0.3	2.7 ± 0.3	2.4 ± 0.2	0.022	0.7 – 1.6
Co	20.3 ± 0.3	20.0 ± 0.1	---	---	0.020	1.0 – 2.3
Cr	38.6 ± 1.6	37.9 ± 0.4	84.0 ± 9.4	75 ± 5	0.026	0.3 – 2.4
Cu	85.2 ± 1.2	90 ± 2	83.6 ± 4.1	73 ± 7	0.028	0.9 – 2.3
Fe	34.3 ± 1.6	41 ± 3	23600 ± 1500	19480 ± 3000	0.470	0.1 – 1.6
Ni	27.4 ± 0.8	26.9 ± 0.9	34.7 ± 1.7	28 ± 5	0.024	1.5 – 3.1
Pb	27.9 ± 0.1	25.6 ± 0.9	77.2 ± 4.5	69 ± 4	0.110	0.3 – 3.1

[a] *Range defined by the less and most sensitive wavelengths, respectively.*

4.2. Results and discussion

4.2.1. Concentration of metals in sediment and water samples

Figures 4.2 and 4.3 sum up the concentration of metals found in sediment and river water, respectively. In brief, two different trends were observed based on the concentration levels of metals in sediment samples. On the one hand, almost all the sampling sites considered as impacted sites show, in general, high concentration levels of metals without any specific trend, regardless the sampling site and sampling period. This variability of metal occurrence could be a consequence of the important industrial activities and urban residues in the locations under study.

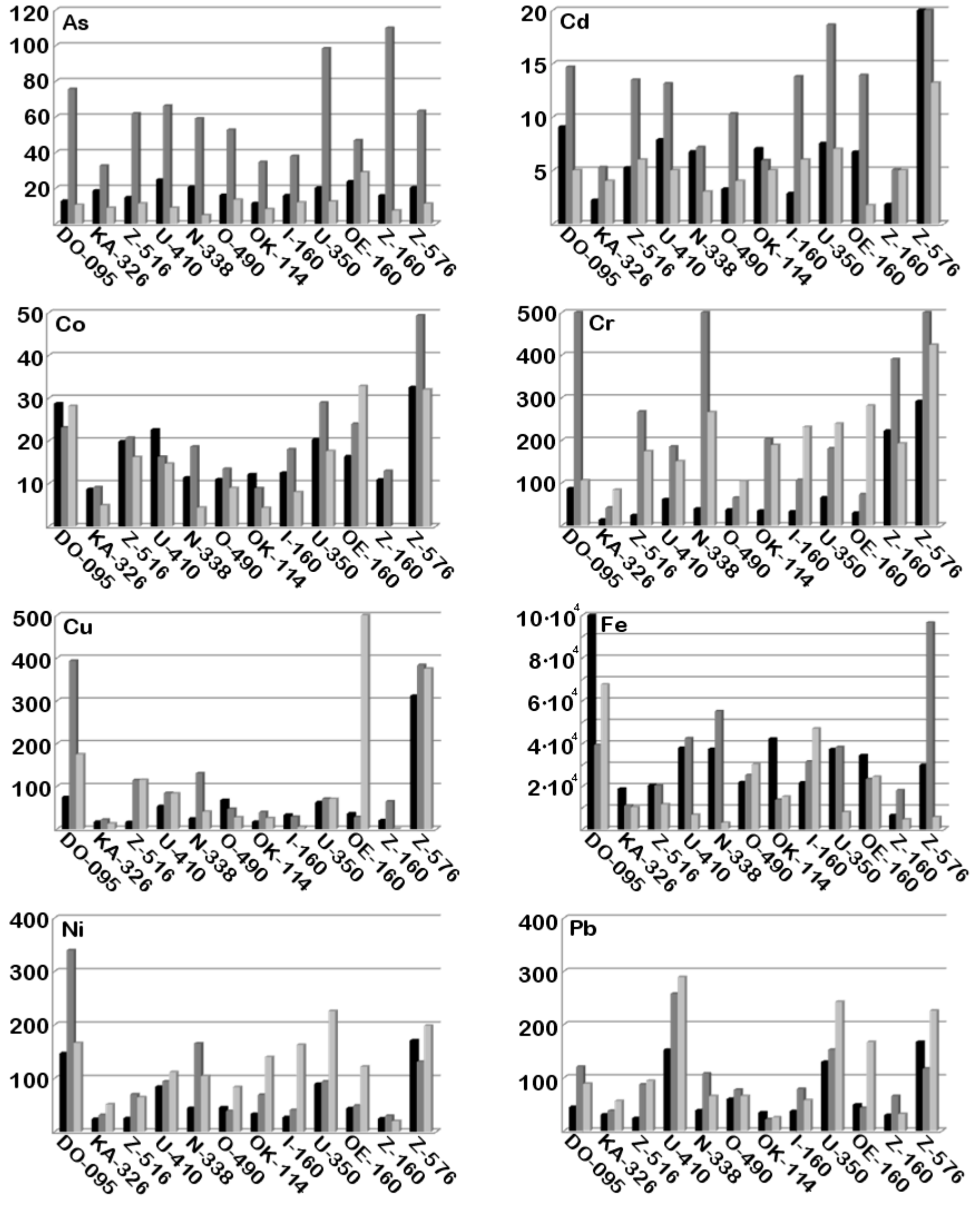


Figure 4.2. *Total concentration of metals (mg·kg^{-1}) in river sediment samples collected in three different sampling campaigns: March (in black), July (in dark grey) and November (in light grey).*

On the other hand, those sampling locations considered as less impacted, namely the upper parts of Kadagua (KA-326), Oria (O-490) and Zadorra (Z-160) Rivers, show similar concentration levels of metals (except for As in Z-160). In these sampling sites, maximum concentration levels were found in samples collected in summer whereas lower values were detected in autumn.

Going deeper into the results, the occurrence of arsenic in sediments deserves special consideration. The occurrence of As follows a natural profile, since it shows minimum and maximum concentration levels almost constant in all the sampling sites. The lowest values were found in March and November sampling campaigns whereas the maximum values were detected in July. This behavior could be explained by the occurrence of natural (geogenic) arsenic species (probably as oxyanions) in the systems under study. As an exception of this trend, higher concentrations than those expected for natural cycle of As (spikes) were found at three locations (DO-095, U-350 and Z-160) in July. Although it cannot be assured, the concentration increment could be attributed to an anthropogenic source. Other anomalous concentration increments (or spikes) were also detected in DO-095 and N-338 for Cr and in DO-095 and OE-160 for Cu (see Figure 4.2)

In the case of Cd, it shows similar behavior of that found for As in all the sampling points: higher concentration values were observed in July in comparison to the rest of sampling campaigns. This occurrence trend is broken in OK-114 and especially in Z-576, where the concentration of Cd is reduced along the seasons. This trend could be attributed to a natural regeneration of river systems to return to its natural equilibrium. Similar effect was also found for Co in U-410 and OK-114 sampling sites. Contrarily, a trend in which the concentration is increased along the seasons was found for Cr (in KA-326, O-490, I-160, U-350 and OE-160), Ni (in KA-326, U-410, OK-114, I-160, U-350 and OE-160) and for Pb (in KA-326, Z-516, U410 and U-350). This metal increasing tendency could be attributed to a continuous input of these elements to the river system.

A different occurrence trend was observed for Co in DO-095 and for Ni and Pb in Z-576, where the minimum concentration level of both metals was found in summer. This situation can be only explained by discontinued metal inputs to the river system.

Although high concentration levels were found for iron in all sampling sites it cannot be deduced any trend attributed to a contamination sources because it is a natural occurring element in the studied basins.

Sediment samples were also collected in different sampling points along the rivers, where it could be affected by wastewater treatment plant (WWTP) discharges. In this regard, two different trends were obtained according to the sampling locations. On the one hand, an increase of metals concentration, concretely Cd, Co, Cr, Cu, Ni and Pb, was found in sediment samples collected after the WWTP in Zadorra (Z-576) in comparison to the ones collected before the WWTP (Z-516). On the contrary, no relevant concentration difference was found for the sampling points before (U-350) and after (U-410) the WWTP of Urola.

Regarding the metals determined in river water samples (see Figure 4.3), all metals were found at low $ng \cdot ml^{-1}$ regardless the sampling site and period. Anyhow, some exceptions were found. On the one hand, taking into account the mean concentration values of each element in the different sampling points, some high concentration values were found for As (100 $ng \cdot ml^{-1}$) and Cr (60 $ng \cdot ml^{-1}$) in OK-114, for Co (12.5 $ng \cdot ml^{-1}$) in N-338 and for Cu (35 $ng \cdot ml^{-1}$) in Z-516. On the other hand, as it occurred for sediment samples, a continuous input of metals to river water bodies was observed. Particularly, the concentration of Ni increased along the sampling campaigns in DO-095, Z-516, U-410, N-338 and Z-576.

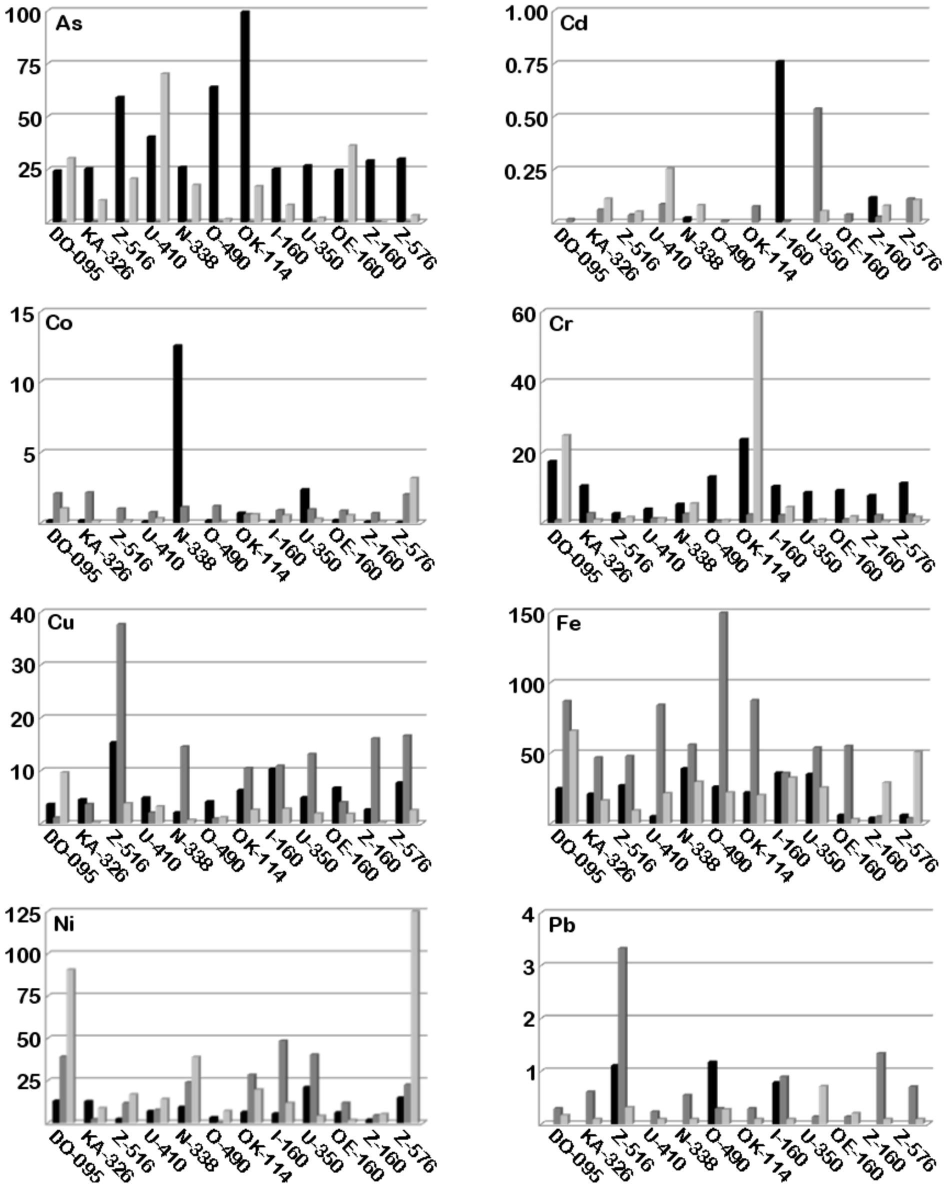


Figure 4.3. *Total concentration of metals (ng·ml^{-1}) in river water samples collected in three different sampling campaigns: March (in black), July (in dark grey) and November (in light grey).*

4.2.2 Mobilization methodologies

Environmental studies based on the concentration of metals found in water and sediment bodies using total extractable elements extraction methodologies (like those shown in Figures 4.2 and 4.3) must be considered to preliminary assessment of contamination impact. However, the potential risk associated to the re-dissolution of metals from sediments must be evaluated using other analytical strategies like the values given by the mobility studies for each metal. Two mobility methods were evaluated for all the river situations considered. The methods were based on the experimental method proposed in the DIN 19730 norm but using two different extracting agents: i) the 1.0 mol·l^{-1} NH_4NO_3 solution as the mobility extracting agent, and ii) the river water as the extracting agent.

Metal mobility in the different situations was measured as the ratio between the extracted concentrations against the total concentration in the sediment. Consequently, the mobilized portion (M_i, $^{0}/_{00}$) of the element, referred to dried sediment, could be calculated by means of the equation 4.1.

$$\mathrm{M_i} = \frac{2.5 * (\,C_1 - C_0)}{(1000 * C_{sed})}$$

Equation 4.1. *Calculation of the mobilized portion from each metal referred to the sediment.*

where: C_1 and C_0 are the concentrations (µg·l^{-1}) of mobilized element in the extractant after and before the extraction process, respectively. C_{sed} is the concentration (mg·kg^{-1}) of the considered element in the sediment.

The metal mobility values obtained in each sampling site using both natural river water and NH_4NO_3 solution as the extracting agents for spring season samples (March) are shown in Figures 4.4 and 4.5, respectively. Negative values indicated that the metal was highly retained or ad/absorbed in the sediment: the extracted concentration was lower than the metal concentration in water and hence, the metal was slightly mobilized or not mobilized at all.

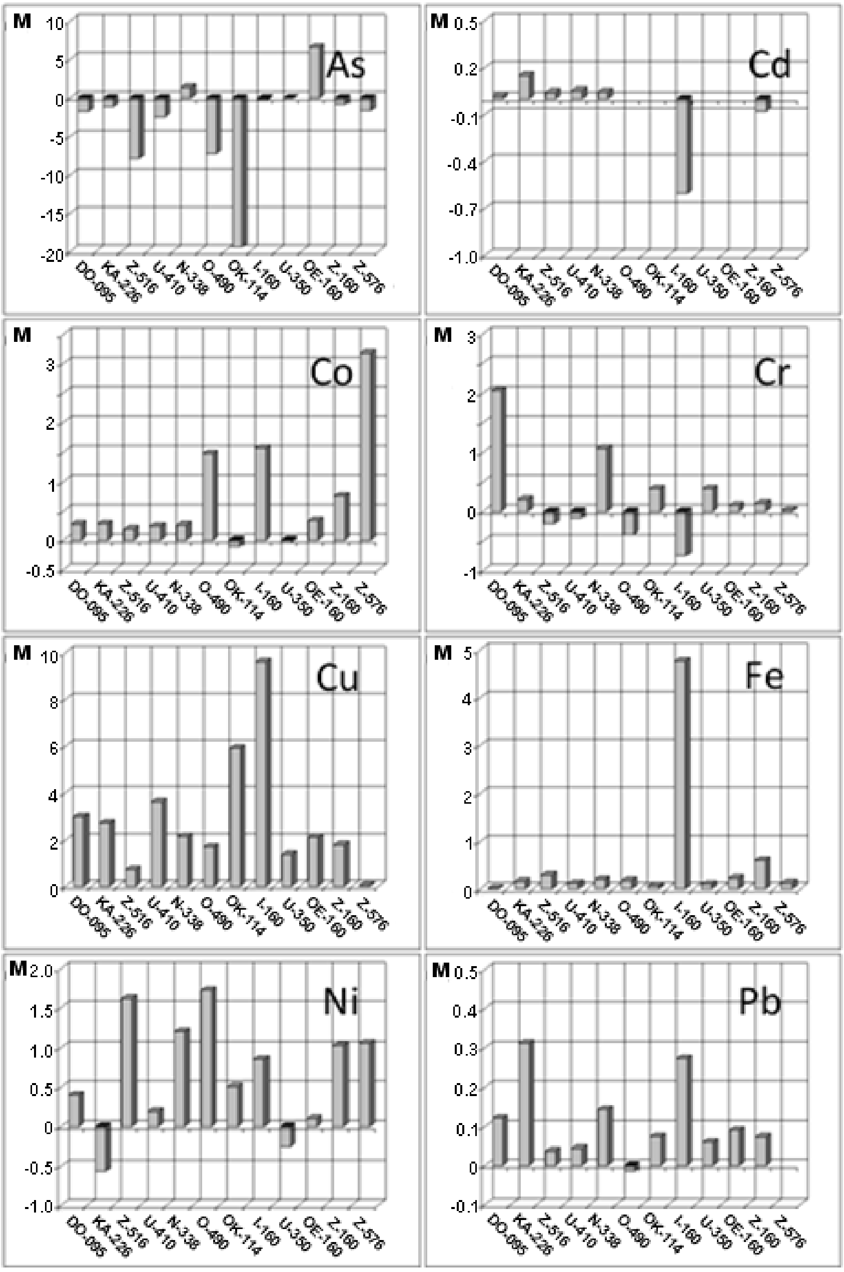


Figure 4.4. *Extractable fraction using river water as extracting agent in each sampling point collected in March.*

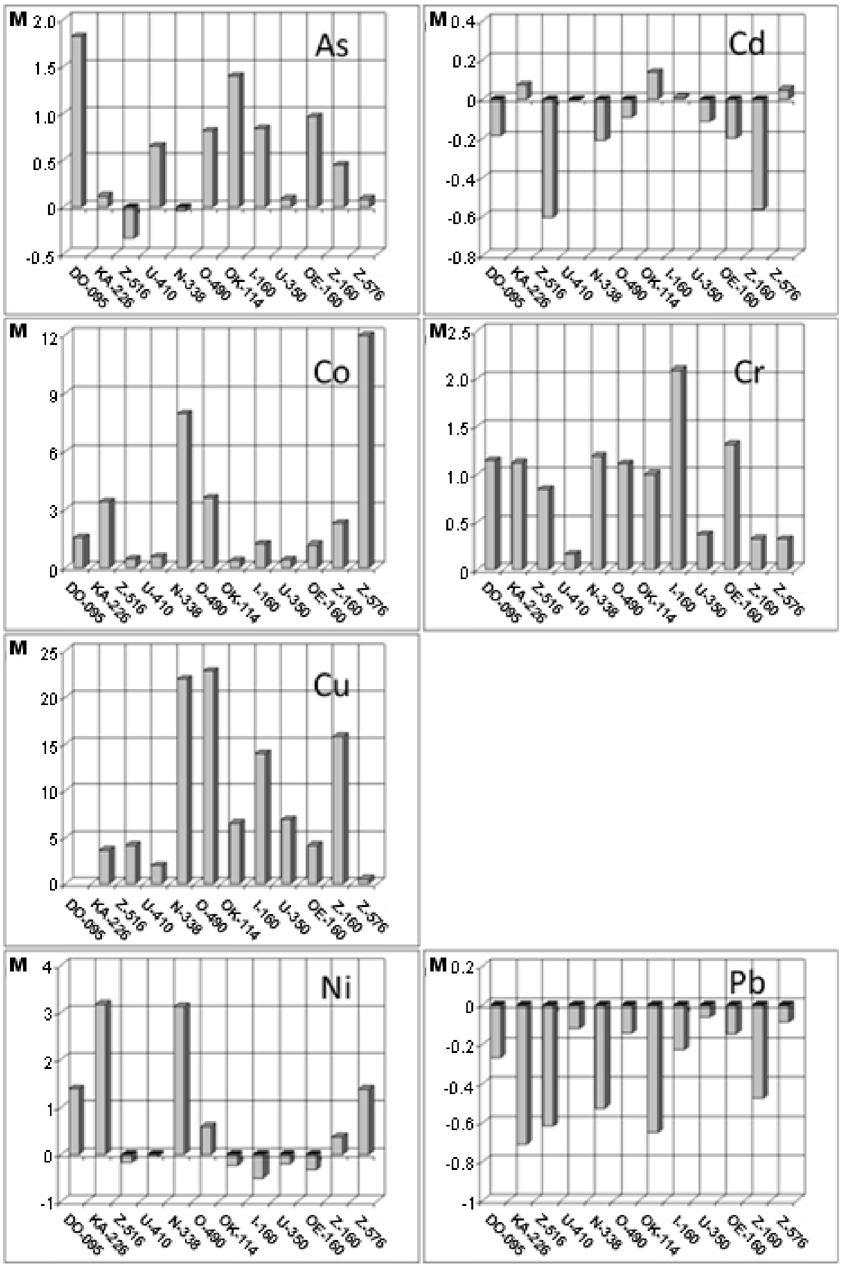


Figure 4.5. *Extractable fraction using NH_4NO_3 solution as extracting agent in each sampling point collected in March.*

During the mobilization studies, the pH of the slurry was monitored against time (Figure 4.6). The pH values at t=0 were the corresponding to the river waters (pH values among 7.5 – 8.6) and ammonium nitrate (pH=6.2) solutions. As it can be seen, the final pH values (pH=6.8 – 7.3) obtained for all the samples were nearly similar using both river water or ammonium nitrate except for the sample collected in Ibaizabal (I-160 sampling point).

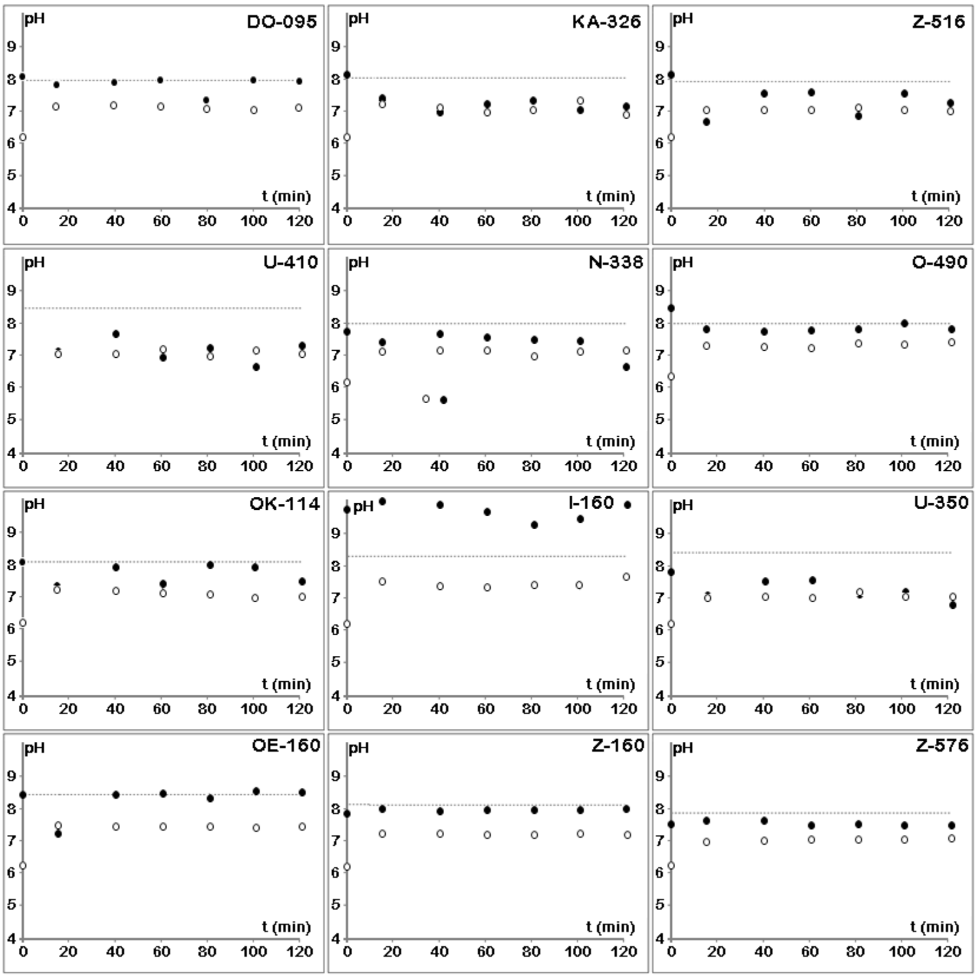


Figure 4.6. *pH values versus mobilization time for all the sampling points. (– – –) line is the pH value of the water river. Dark point is pH using water and light is pH using* NH_4NO_3.

The use of ammonium nitrate solution as the extracting agent could decrease the pH of the mixture during the mobilization assays due to its acid content. However, this fact was not observed in the monitored pH values, which supports the neutralization capacity of the studied sediments. This neutralization capacity can be attributed to the presence of basic anions which are mobilized together with the metals present in sediments.

A comparison of the paired mobility results (see Figures 4.4 and 4.5) shows how the NH_4NO_3 solution gave systematically higher values for the metals forming ammoniacal complexes (cobalt and cooper mainly). This fact can be explained by the excess of the NH_3 ligand concentration with regard to the $\mu g \cdot l^{-1}$ levels of mobilized metals (theoretically a $1.0\ mol \cdot l^{-1}$ NH_4NO_3 solution has, at the different pH values found in the mobility experiments, between 0.01 and 0.1 $mol \cdot l^{-1}$ of free NH_3). Arsenic and chromium were highly mobilized in ammonium nitrate in all the sampling points except arsenic in Ormaiztegi (OE-160) and Nerbioi (N-338) and chromium in Deba-Elgoibar (DO-095). The mobilities of cadmium and lead were lower in all sampling points except in I-160. For nickel, no clear systematical tendency was observed using any of the mobility tests.

4.2.3. Statistical analysis of the results

In order to improve the interpretation of the mobility results and concentration data, several chemometrical analysis were performed by means of the Unscrambler® software (v. 7.5, Camo, Norway) [Esbensen, 1994]. Among all statistical techniques used to identify the underlying features of any class of samples, and at the same time, allowing the class classification the use of principal components analysis (PCA)[3] should be highlighted [Tracela el al. 2007]. Hence, both correlation analysis (CA) and PCA were performed in the same run using the Unscrambler® software. Regarding the results and, in

---

[3] *Principal Component Analysis allows reducing the multivariate data by transforming the original variables into principal components (PC) which can explain the variance of the original data.*

order to distinguish each experimental situation studied, the statistical treatment was divided in two different subgroups (location site and variable dependence).

Correlation analysis was carried out using all available data. All the river samples were included as objects (rows) in a data matrix, while the measured physicochemical parameters and element mobilities were defined as variables (columns). The correlation coefficients are an association measure that allows the performance of a non-parametric contrast and it does not require the normality of the variables that is compulsory in regression models. These coefficients are grouped into the correlation matrix as results output and the correlation values with $r>0.6$ or $r<-0.6$, calculated in terms of the number of independent data, were considered as an important tendency. The correlation matrixes obtained for all variables measured using river water and NH_4NO_3 are collected in Table 4.3 and Table 4.4, respectively.

Table 4.3. *Correlation matrix obtained for all the variables measured using river water.*

	M_{As}	**M_{Cd}**	**M_{Co}**	**M_{Cr}**	**M_{Cu}**	**M_{Fe}**	**M_{Ni}**	**M_{Pb}**	**T_w**	**T_a**	**pH**	**ORP**	**C**	**O_2**
M_{As}	**1.00**	-0.29	0.03	0.10	0.02	0.15	-0.14	0.42	-0.16	-0.42	0.03	-0.21	0.12	0.36
M_{Cd}		**1.00**	**-0.96**	0.30	**-0.66**	**-0.97**	0.05	-0.42	-0.26	0.05	-0.54	**0.84**	0.41	0.13
M_{Co}			**1.00**	-0.13	-0.17	0.05	**0.88**	-0.02	-0.23	-0.14	**-0.64**	0.44	0.01	-0.10
M_{Cr}				**1.00**	0.17	-0.26	0.08	0.03	0.16	0.12	-0.22	-0.07	0.12	-0.38
M_{Cu}					**1.00**	**0.71**	-0.19	0.46	**0.60**	0.38	0.40	**-0.76**	-0.59	-0.13
M_{Fe}						**1.00**	-0.10	0.48	0.37	0.07	0.33	**-0.74**	-0.50	0.04
M_{Ni}							**1.00**	-0.52	-0.34	-0.01	**-0.80**	0.48	0.06	-0.26
M_{Pb}								**1.00**	**0.75**	0.10	0.55	-0.55	-0.35	0.19
T_w									**1.00**	0.40	0.41	-0.51	-0.46	-0.15
T_a										**1.00**	0.27	-0.07	**-0.69**	0.08
pH											**1.00**	**-0.63**	-0.44	0.27
ORP												**1.00**	0.19	0.20
C													**1.00**	-0.36
O_2														**1.00**

Bold data are found significant for $r>0.6$ or $r<-0.6$ as criteria from The Unscrambler program.

Table 4.4. *Correlation matrix obtained for all the variables measured using NH_4NO_3.*

	M_{As}	M_{Cd}	M_{Co}	M_{Cr}	M_{Cu}	M_{Ni}	M_{Pb}	T_w	T_a	**pH**	**ORP**	**C**	O_2
M_{As}	**1.00**	0.19	-0.28	0.24	0.11	-0.17	0.30	0.05	0.34	0.29	-0.38	-0.27	0.05
M_{Cd}		**1.00**	0.37	0.08	0.02	0.09	-0.28	0.32	0.28	0.13	-0.08	-0.14	-0.18
M_{Co}			**1.00**	0.53	-0.06	**0.73**	-0.08	-0.24	-0.09	**-0.63**	0.53	0.11	-0.14
M_{Cr}				**1.00**	0.14	**0.61**	0.28	-0.14	-0.16	-0.50	-0.13	0.15	-0.47
M_{Cu}					**1.00**	-0.01	0.44	-0.01	-0.03	-0.37	-0.22	-0.05	-0.38
M_{Ni}						**1.00**	-0.12	0.11	0.04	**-0.61**	0.33	0.09	-0.32
M_{Pb}							**1.00**	-0.21	-0.36	0.07	-0.22	-0.23	0.05
T_w								**1.00**	0.40	0.41	-0.51	-0.46	-0.15
T_a									**1.00**	0.27	-0.07	**-0.69**	0.08
pH										**1.00**	-0.43	-0.44	0.27
ORP											**1.00**	0.19	0.20
C												**1.00**	-0.36
O_2													**1.00**

Bold data are found significant for r>0.6 or r<–0.6 as criteria from The Unscrambler program.

From the correlation matrix obtained when using river water as the extractant (see Table 4.3), several assumptions were obtained:

i) Based on the previous results, it seems that the influence of dissolved oxygen, conductivity as well as ambient and water temperature is negligible on the mobilization of metals.

ii) pH is negatively correlated with ORP (r: -0.64), i.e. when pH increases the ORP decreases. This negative correlation can confirm the influence of the sulphide/bisulphide system in the water/sediment compartment because when its concentration increases, the water is more basic and the ORP is lowered. Moreover, this negative correlation between pH and ORP, or more concretely, the influence of the pair $S^{=}/HS^{-}$ in the system, diminish the influence of matrix effects and organic matter interactions. These conditions are often expected in spring times when microorganisms and plants are growing.

iii) The mobility of cobalt and nickel is highly correlated (r: 0.88). This correlation between both elements can be explained according to two factors: (a) the similar chemical behavior for both elements and (b) the same source (electroplating activities) of the spills introducing both metals in the estuary. It should be pointed out that the mobility of nickel is the only one that is inversely correlated with pH. This negative correlation can be attributed to a possible presence of nickel carbonate in the sediments. Unfortunately, this assumption cannot be corroborated because this mineral phase was not detected in the molecular analyses performed by means of Raman spectroscopy (see 4.2.3 section). The failure in detection can be attributed to: (a) low concentration of nickel carbonate in the analyzed sediments or (b) high concentration of calcium carbonate, since it present the main characteristic Raman band at 1086 cm^{-1} and nickel carbonate at 1084 cm^{-1}.

iv) Positive correlation value was also found for copper and iron mobilities (r: 0.71) whereas they present a negative correlation (r: -0.76 and r: -0.74 for Cu and Fe respectively) with ORP. This means that at oxidant conditions lower mobility of both Cu and Fe will be expected. This suggests the great influence of the redox properties of the water/sediment compartment for both metals, especially during the spring period.

v) The mobility of Cd in all sampling locations seems to have a completely different behavior in comparison to other elements. For instance, cadmium is the only one having a positive correlation with ORP value. This result can suggest the natural origin of the materials releasing cadmium to the river waters, like cadmium sulphide mineral (increasing of ORP means oxidation of sulphide and then release of cadmium), instead of anthropogenic sources.

Some of these statistical correlations obtained when mobilization of metals was studied using river waters (Table 4.3) were not so clear or even lost when the results obtained with metals mobilized with ammonium nitrate (Table 4.4) were studied. Only the positive correlation between the mobility for cobalt and nickel (M_{Co} and M_{Ni}), is maintained together with the negative correlation with aqueous pH. Besides, a slight correlation of Cr mobility with those of iron and nickel appears, showing similar mobilization tendency of these metals by forming ammonium complexes.

Once the correlation analyses were concluded, PCA of the whole datasets obtained after the analysis of mobilized fraction of metals with river water and ammonium nitrate was conducted. The aim of performing PCA in this specific case was to extract significant sources of variation in the dataset, expressing this information as a set of new orthogonal variables called principal components (PCs).

In this case, the data set consisted in one matrix (containing both metal mobilized data: mobilization with river water and with ammonium nitrate) with 12 rows (number of sediment samples) and 16 columns (number of elements for which concentration was measured – 8 with river water and 8 with ammonium nitrate). Concentrations bellow the detection limits were kept as missing values (m) in the calculations[4] instead of introducing a small quantity to avoid those values below the detection limits. The raw data were centered and scaled to assure an equal weight to all the variables and cross-validation method was used as internal validation method.

The projections of the scores and loadings on the bi-dimensional space defined by the first two principal components (PC1 and PC2) are shown in Figures 4.7 and 4.8 for the metal mobilities using river water and ammonium nitrate, respectively. As shown in both figures, the first two PCs explained up to 58% of the total variance for mobility data obtained using river water and up to 51% of the total variance when the metals were mobilized using ammonium nitrate.

---

[4] *Missing values in PCA. There are several strategies when the raw matrix data has missing values: (i) it can be maintained as missing value (m), (ii) it can be replaced by the limit of detection estimated for each trace metal, (iii) it can be replaced by the half of the limit of detection estimated for each trace metal, and (iv) it can be replaced by random values close to the limit of detection.*

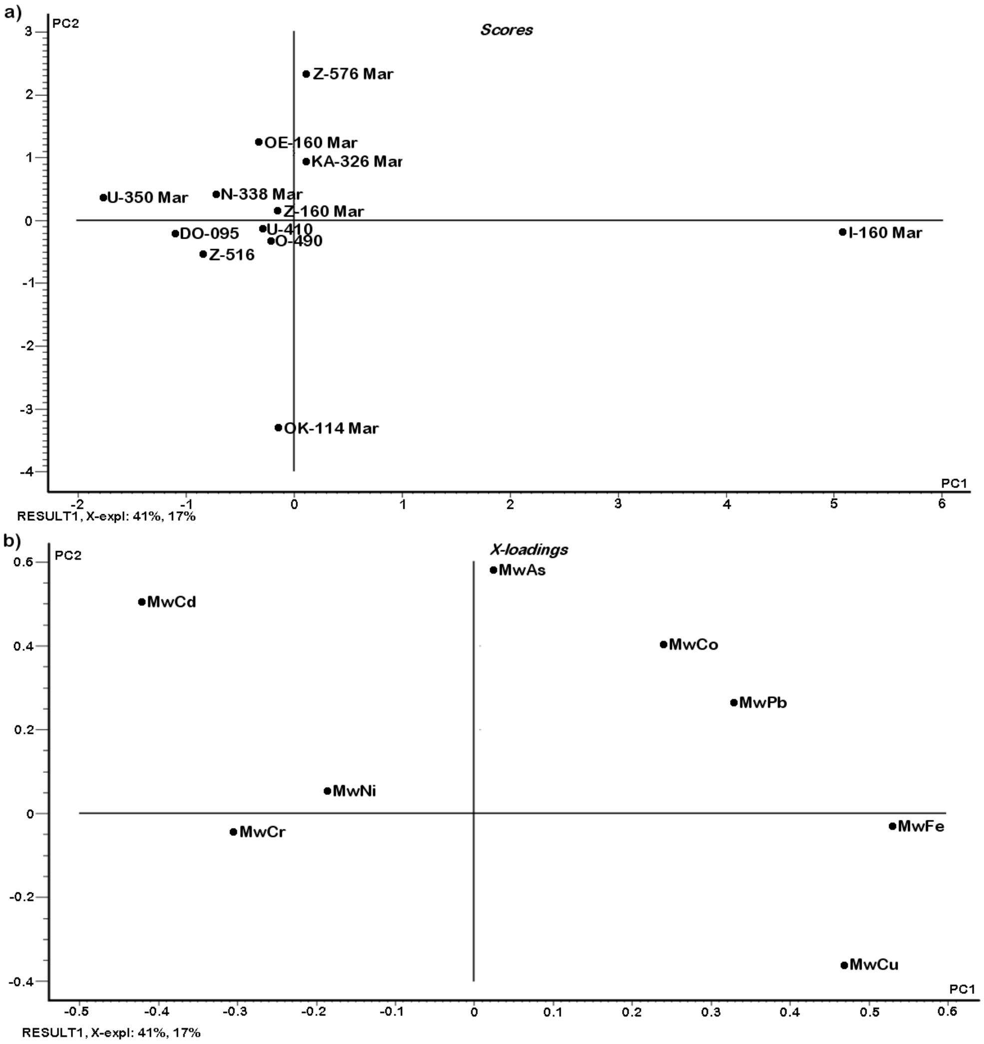


Figure 4.7. *Scores and loadings plots (PC1 vs PC2) of mobilized concentrations of metals using river water as extractant.*

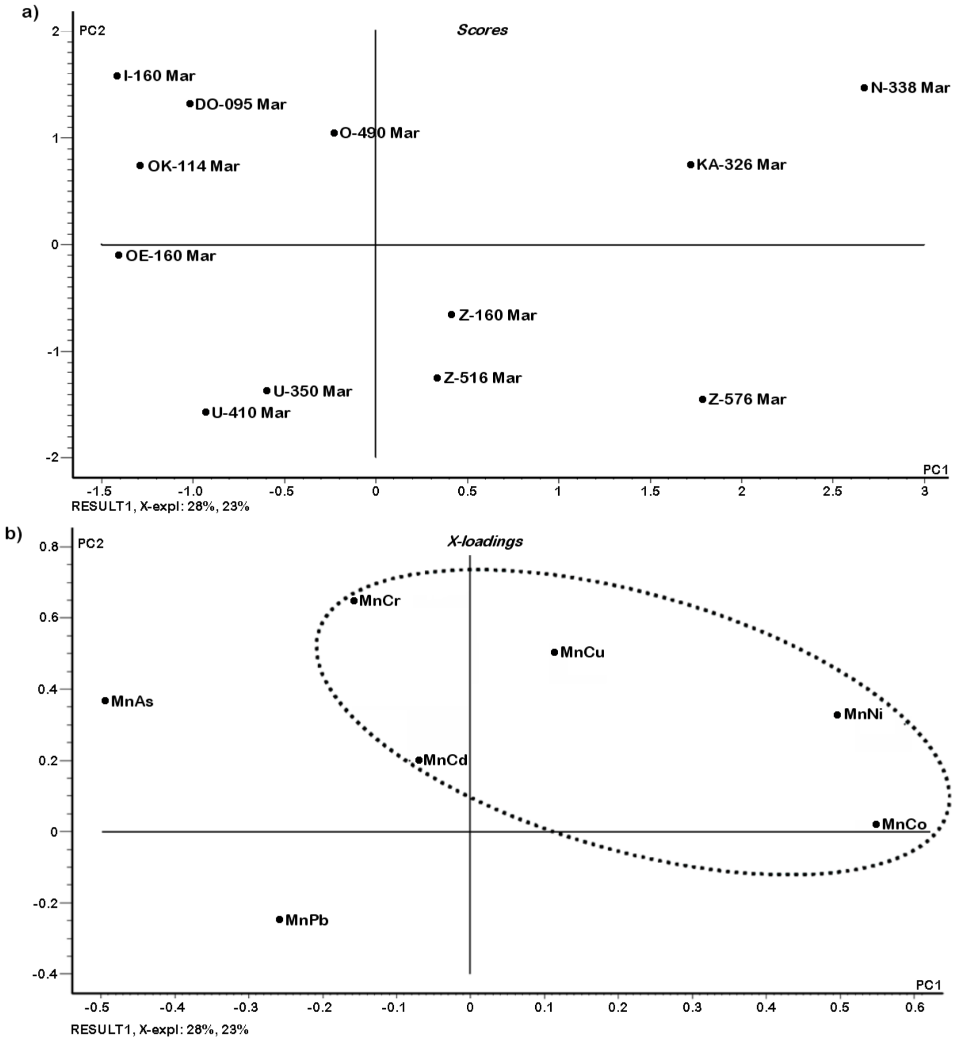


Figure 4.8. *Scores and loadings plots (PC1 vs PC2) of mobilized concentrations of metals using ammonium nitrate as extractant.*

In the representation of the scores obtained for metal mobilization with river water (Figure 4.7a), all the sampling points are grouped in a cluster except samples I-160, Z-576 and OK-114, which broke the general mobilization tendency. All the samples were collected during the same sampling day and hence, the different distributions observed are mainly related to the mobilized metal concentrations. PC1 is highly related to the mobilization of Fe (a natural element in sediments) whereas PC2 is more related to the mobilization of more hazardous elements such as As, Cd and Cu. These are related with the sampling point of Z-576, which is highly affected by industrial activities.

The distribution found for metal mobilization using ammonium nitrate didn't show any significant cluster and the sampling locations were randomly distributed along the PC1 and PC2. The variables influencing on the sampling behavior through the PCs were easily identified from the loadings and scores plots simultaneously. Since all the metals forming complexes with ammonium were shifted to the same side in the loadings plot, their effect on the samples distribution could be not negligible (see Figure 4.8) and it produced an important distortion to the sampling grouping leading to interpretation errors. This grouping of the metals forming ammonium complexes is not present in the river water case (loadings plot, Figure 4.7b).

Therefore, the cluster obtained for sampling sites using river water as extractant (scores plot, Figure 4.7a) could only be attributed to the general effect the natural system without any possible external influence from extraction reagents.

4.2.4. Spectroscopic evidences

Sediments samples were also analyzed by means of Raman spectroscopy in order to detect the metal elements that can be mobilized from the sediment matrix. Some of the detected compounds by Raman spectroscopy are shown in Figures 4.9, 4.10 and 4.11. The experimental Raman spectra were compared with the theoretical ones of different compounds contained in e-VISART database [Castro et al., 2005] taking into account the special behavior of the clay background for such Raman spectra of river sediments [Villanueva et al., 2008]. The Raman measurements were performed using the same experimental procedure used in the previous chapter (see Chapter 3).

In Figure 4.9 it can be seen the Raman spectrum of a sediment from Crispijana (Z-576) sampling point together with the most intense signal of a silicate (standard 1) type-mica (464 cm^{-1}) and its secondary band (708 cm^{-1}) as well as the Raman spectrum of clay. Other natural compounds appearing in all the river sediments considered in this work were aragonite and calcite, two polymorphs of calcium carbonate.

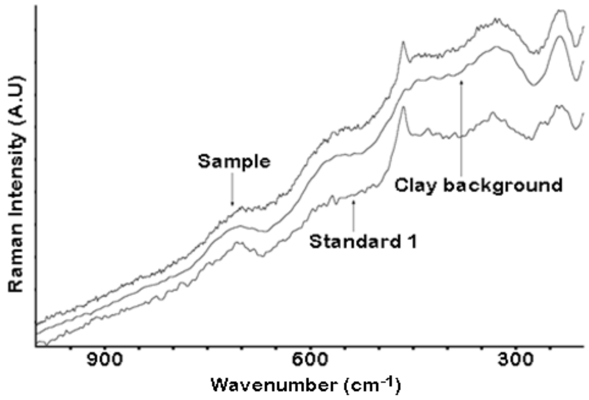


Figure 4.9. *Raman spectra of a silicate type mica+clay (standard 1) in a sediment from the Z-576 location.*

Figure 4.10 shows the Raman spectrum of a calcium carbonate compounds detected in sediment sample collected in OE-160. The first Raman spectra corresponds to the presence of aragonite (standard 1: Raman bands at 1084 cm^{-1} and 705 cm^{-1}) on the clay background. This calcium carbonate is probably coming from weathered carbonate-rich rock masses in the basin of the river, transported across the whole flow until their mouth. This compound can be easily differentiated from calcite due to the different wavenumbers of the Raman bands (at 1084, 713 and 280 cm^{-1}). The Raman spectra plotted in Figure 4.10b shows the characteristics Raman bands of aragonite over clay background spectra gathered in sediment sample from OE-160 sampling site. These carbonate species, detected in most of the river sediments, are probably the responsible to obtain similar pH profiles during the mobility assays of the sediments for all the sampling points (see Figure

4.6). However, the special behavior obtained in I-160 sampling point can be explained in terms of the compounds identified by Raman spectroscopy.

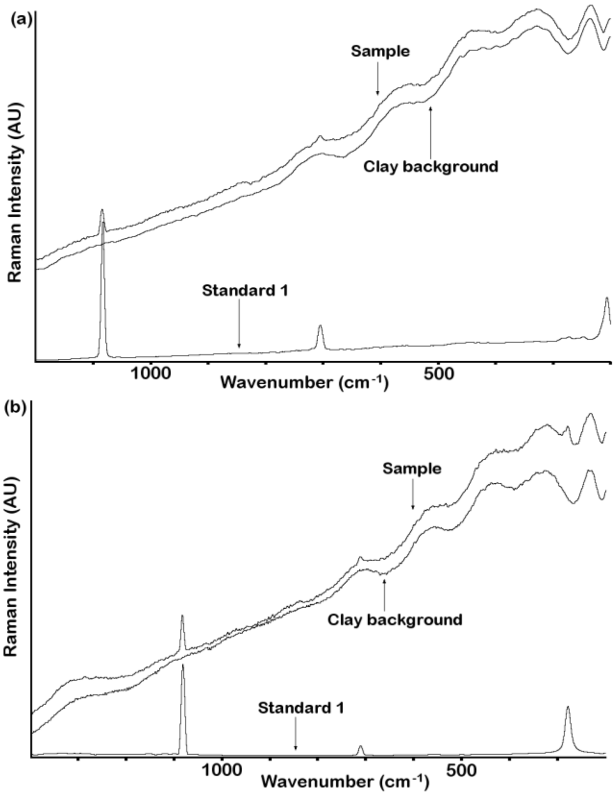


Figure 4.10. *Raman spectra of calcium carbonate compound detected in OE-160 sampling point:*
a) aragonite (standard 1) and clay in a sediment from the OE-160 sampling point.
b) calcite (standard 1) and clay in a sediment from the OE-160 sampling point.

The main species occurring in that sediment sample (I-160, see Figure 4.11), apart from the natural clay background, were sodium sulphide (standard 1) and carbon (standard 2). The presence of Na_2S is consistent with the high reducing conditions (ORP) found in the sediment sample collected in I-160 sampling site. Moreover, its presence can explain the pH increase shown in the slurry formed during the mobility experiments due to the solubility of Na_2S in water and forming, thereafter, OH^- and HS^- species.

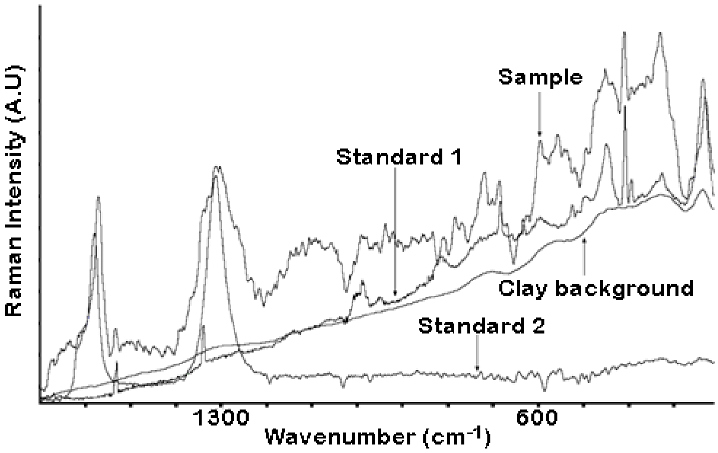


Figure 4.11. *Raman spectra Raman spectra of a sodium sulphide (standard 1) and Carbon (standard 2) together with the spectra taken on I-160 sediment.*

4.3. Conclusions

Until now, the mobilization studies of metals in the sediment/water interface has been usually performed in the laboratories using mainly sequential extraction procedures [Baeyens et al., 2003; Tessier et al., 1979; Gupta et al., 1975; Martin et al., 1987]. These procedures aimed to obtain a fast prediction of the processes that occur in the natural river/estuarine systems for years. The simple methodology assessed in this work has demonstrated to be an important tool in order to obtain results for environmental risks evaluation. This methodology is based on the DIN 19730 norm for soils but uses river water, in contact with the sediment during the sampling, as the extractant agent.

In this sense, the use of ammonium nitrate as the extractant agent proposed by the DIN 19730 norm must be disregarded in the mobilization studies because:

a) the solution of NH_4NO_3 modifies the final pH values in the mobilization experiments changing completely the metal concentration in the slurry,

b) the presence of NH_3 in high concentration, even 0.1 $mol \cdot l^{-1}$ at pH=8.4, promotes the formation of ammoniacal complexes resulting in an artificial increasing in the metal mobilization and

c) the statistical treatment showed the site influence on the mobilization processes performed using ammonium nitrate (high heterogeneity in the correlation matrix for sampling points).

The metals showed a bimodal cycle in sediments with important concentration levels in summer and lower in autumn and winter, probably, related to organic matter presence and its variation during the year.

Finally, and from the experimental results using the river water as extractant agent, it was concluded that the metal mobilization was directly depending on the total concentration in sediment.

4.4. References

[AFNOR, 1994] AFNOR (Association Française de Normalization), AFNOR, Paris, 1994. pp. 250.

[Atanassova, 1998] Atanassova I. *Competitive effect of copper, zinc, cadmium and nickel adsorption and desorption by soil clays.* Water Air Soil Pollut. 1998; 113, 115 – 125.

[Baeyens et al., 2003] Baeyens W, Monteny F, Leermakers M, Bouillon S. *Evaluation of sequential extractions on dry and wet sediments.* Anal. Bioanal. Chem. 2003; 376, 890 – 901.

[Bauer et al., 2006] Bauer M, Blodau Ch. *Mobilization of arsenic by dissolved organic matter from iron oxides, soils and sediments.* Sci. Total Environ. 2006; 354: 179–190.

[Caetano et al., 2003] Caetano M, Madureira MJ, Vale C. *Metal remobilisation during resuspension of anoxic contaminated sediment: short-term laboratory study*. Water Air Soil Pollut. 2003; 143, 23 – 40.

[Castro et al., 2005] Castro K, Pérez-Alonso M, Rodríguez-Laso MD, Madariaga JM. *On-line FT-Raman and dispersive Raman spectra database of artists' materials (e-VISART database).* Anal. Bioanal. Chem. 2005; 382, 248 – 258.

[Chen et al., 2008] Chen T, Liu XM, Zhu MZ, Zhao KL, Wu JJ, Xu JM, Huang PM. *Identification of trace element sources and associated risk assessment in vegetable soils of the urban–rural transitional area of Hangzhou, China.* Environ. Pollut. 2008; 151, 67 – 78.

[Daus et al., 1995] Daus B, Zwanziger HW*. Heavy metals in river sediments — a chemometrical evaluation.* Fresenius J. Anal. Chem. 1995; 352, 444 – 450.

[DIN 19730, 1997] DIN 19730 (Deutches Institut für Normung*). Bodenbeschaffenheit-Extraktion von Spurenelementen mit Ammonium Nitratlö sung*, Beuth, Berlin, 1997.

[Dong et al., 2009] Dong D, Zhao X, Hua X, Liu J, Gao M. *Investigation of the potential mobility of Pb, Cd and Cr(VI) from moderately contaminated farmland soil to groundwater in Northeast, China.* J. Hazard. Mater. 2009; 162, 1261 – 1268.

[Einax et al., 1998] Einax JW, Kampe O, Truckenbrodt D. *Assessing the deposition and remobilization behavior of metals between river water and river sediment using partial least squares regression.* Fresenius J. Anal. Chem. 1998; 361, 149 – 154.

[Esbensen, 1994] Esbensen KH. *Multivariate data analysis: an introduction to multivariate data analysis and experimental design*, Camo AB, Oslo, 1994.

[Fernández et al., 2008] Fernández S, Villanueva U, de Diego A, Arana G, Madariaga JM. *Monitoring trace elements (Al, As, Cr, Cu, Fe, Mn, Ni and Zn) in deep and surface waters of the estuary of the Nerbioi–Ibaizabal River (Bay of Biscay, Basque Country).* J. Mar. Syst. 2008; 72, 332 – 341.

[Filgueiras et al., 2002] Filgueiras AV, Lavilla I, Vendicho C. *Chemical sequential extraction for chemical partitioning in environmental soil samples.* J. Environ. Monitor. 2002; 4, 823 – 857.

[Förstner, 2004] Förstner U. *Sediment dynamics and pollutant mobility in rivers: an interdisciplinary approach, Lakes Reservoirs.* Res. Manage. 2004; 9, 25 – 40.

[Guevara-Riba et al., 2004] Guevara-Riba A, Sahuquillo A, Rubio R, Rauret G. *Assessment of metal mobility in dredged harbour sediments from Barcelona, Spain.* Sci. Total Environ. 2004; 321, 241 – 255.

[Gupta et al., 1975] Gupta SK, Chen KY. *Partitioning of trace metals in selective chemical fractions of nearshore sediments.* Environ. Lett. 1975; 10, 129 – 158.

[Horváth et al., 2000] Horváth T, Szilágyi V, Hartyáni Zs. *Characterization of trace element distributions in soils.* Microchem. J. 2000; 67, 53 – 56.

[Hu et al., 2006] Hu KL, Zhang FR, Li H, Huang F, Li BG. *Spatial patterns of soil heavy metals in urban–rural transition zone of Beijing.* Pedosphere*.* 2006; 16, 690 – 698.

[Huang et al., 2006] Huang B, Shi X, Yu D, Oborn I, Blomback K, Pagella TF, Wang H, Sun W, Sinclair FL. *Environmental assessment of small-scale vegetable farming systems in peri-urban areas of the Yangtze River Delta Region, China.* Agric. Ecosyst. Environ. 2006; 112, 391 – 402.

[Kersten et al., 1995] Kersten M, Förstner U. *Speciation of trace metals in sediments and combustion waste, in:* A.M. Ure, C.M. Davidson (Eds.) *Chemical Speciation in the Environment,* Balckie Academic, London, 1995, p. 250.

[Köhler et al., 2009] Köhler SJ, Buffam I, Seibert J, Bishop KH, Laudon H. *Dynamics of stream water TOC concentrations in a boreal headwater catchment: Controlling factors and implications for climate scenarios.* J. Hydrol. 2009; 373, 44 – 56.

[Liu et al., 2006] Liu XM, Wu JJ, Xu JM. *Characterizing the risk assessment of heavy metals and sampling uncertainty analysis in paddy field by geostatistics and GIS.* Environ. Pollut. 2006; 141, 257 – 264.

[Martin et al., 1987] Martin JM, Nirel P, Thomas AJ. *Sequential extraction techniques: promises and problems.* Mar. Chem. 1987; 22, 313–341.

[Müller, 1979] Müller G. *Schwermetalle in den Sedimenten des Rheins. Veränderungen seit.* Umschau, 1979.

[Navarro et al., 2006a] Navarro MC, Perez-Sirvent C, Martinez-Sanchez MJ, Vidal J, Marimon J. *Lead, cadmium and arsenic bioavailability in the abandoned mine site of Cabezo Rajao (Murcia, SE Spain).* Chemosphere 2006; 63, 484 – 489.

[Navarro et al., 2006b] Navarro P, Raposo JC, Arana G, Etxebarria N. *Optimisation of microwave assisted digestion of sediments and determination of Sn and Hg.* Anal. Chim. Acta 2006; 566, 37 – 44.

[Raposo, 2001] Raposo JC. *Contributions to the study of the inorganic arsenic interactions with the natural medium,* PhD Thesis, UPV-EHU, Leioa, Spain, 2001.

[Raposo et al., 2006] Raposo JC, Zuloaga O, Sanz J, Villanueva U, Crea P, Etxebarria N, Olazabal MA, Madariaga JM. *Analytical and thermodynamical approach to understand the mobility/retention of arsenic species from the river to the estuary. The Bilbao case study.* Mar. Chem. 2006; 99, 42 – 51.

[Rauret, 1998] Rauret G. *Extraction procedures for the determination of heavy metals in contaminated soil and sediment.* Talanta 1998; 46, 449 – 455.

[Rodrigues et al., 2006] Rodrigues S, Pereira ME, Sarabando L, Lopes L, Cachada A, Duarte A. *Spatial distribution of total Hg in urban soils from an Atlantic coastal city (Aveiro, Portugal).* Sci. Total Environ. 2006; 368, 40 – 46.

[Rodriguez et al., 2006] Rodriguez JG, Tueros I, Belzunce MJ, Franco J, Solaun O, Valencia V, Zuazo A. *Maximum likelihood mixture estimation to determine metal background values in estuarine and coastal sediments within the European Water Framework Directive.* Sci. Total Environ. 2006; 370, 278 – 293.

[Sadiq, 1992] Sadiq M. *Toxic Metal Chemistry in Marine Environments,* in: Marcel Dekker (Ed.) Prentice Hall, New Jersey, 1992.

[Saiz and Gonzalez, 2000] Saiz-Salinas JI, González-Oreja JA. *Stress in estuarine communities: lessons from the highly impacted Bilbao estuary (Spain).* J. Aquatic Eco. Stress Rec. 2000; 7, 43–55.

[Sanz Landaluze et al., 2004] Sanz Landaluze J, de Diego A, Raposo JC, Madariaga JM. *Methylmercury determination in sediments and fish tissues from the Nerbioi–Ibaizabal estuary (Basque Country, Spain).* Anal. Chim. Acta. 2004; 508, 107 – 117.

[Sauve et al., 2000] Sauve S, Henderschot W, Allen HE. *Solid-solution partitioning of metals in contaminated soils: dependence on pH, total metal burden and organic matter.* Envir. Sci. Tech. 2000; 34, 1125 – 1131.

[Shi et al., 2007] Shi JC, Wang HZ, Xu JM, Wu JJ, Liu XM, Zhu HP, Yu CL. *Spatial distribution of heavy metals in soils: a case study of Changxing, China.* Environ. Geol. 2007; 52, 1 – 10.

[Sillen et al., 1964] Sillen LG, Martell AE. *Stability Constants of Metal–Ion Complexes,* Metcalfe & Cooper Limited, London, 1964.

[Tessier et al., 1979] Tessier A, Campbell PCG, Bisson M. *Sequential extraction procedure for the speciation of particulate trace metals.* Anal. Chem. 1979; 51, 844 – 851.

[Tomlinson et al., 1980] Tomlinson JG, Wilson CR, Harris DW, Jeffrey DW. *Problems in the assessment of heavy-metal levels in estuaries and the formation of a pollution index.* Helgoland Mar. Res. 1980; 33, 566 – 575.

[Tracela et al., 2007] Tracela T, Strlic M, Kolar J, Lichtblau DA, Anders M, Mencigar DP, Pihlar B. *Nondestructive Analysis and Dating of Historical Paper Based on IR Spectroscopy and Chemometric Data Evaluation.* Anal. Chem. 2007; 79, 6319 – 6323.

[Traub et al., 2001] Traub H, Scharf H. *NH_4NO_3 extractable trace element contents of soil samples prepared for proficiency testing — a stability study.* Fresenius J. Anal. Chem. 2001; 370, 270 – 274.

[Veeresh et al., 2003] Veeresh H, Tripathy S, Chaudhuri D, Hart BR, Powell MA. *Sorption and distribution of adsorbed metals in three soils of India.* Appl. Geochem. 2003; 18, 1723 – 1731.

[Villanueva et al., 2008] Villanueva U, Raposo JC, Castro K, de Diego A, Arana G, Madariaga JM. *Raman spectroscopy speciation of natural and anthropogenic solid phases in river and estuarine sediments with appreciable amount of clay and organic matter*. J. Raman Spectrosc. 2008; 39, 1195 – 1203.

[Walinsky et al., 2009] Walinsky SE, Prahl FG, Mix AC, Finney BP, Jaeger JM, Rosen GP. *Distribution and composition of organic matter in surface sediments of coastal Southeast Alaska.* Cont. Shelf. Res. 2009; 29, 1565 – 1579.

[Windom et al., 1999] Windom HL, Niencheski LF, Smith Jr. RG. *Biogeochemistry of nutrients and trace metals in the estuarine region of the Patos lagoon (Brazil).* Estuar. Coast. Shelf S. 1999; 48, 113 – 123.

[Windom et al., 2003] Windom HL, Niencheski LF. *Biogeochemical processes in a freshwater–seawater mixing zone in permeable sediments along the coast of Southern Brazil.* Mar. Chem. 2003; 83, 121 – 130.

[Zoumis et al., 2001] Zoumis T, Schmidt A, Grigorova L, Calmano W. *Contaminants in sediments: remobilisation and demobilization.* Sci. Total Environ. 2001; 266, 195 – 202.

5. Application of the new mobility methodology to estuarine samples

5. Application of the new mobility methodology to estuarine samples

The monitoring of metals in rivers and estuaries has gained considerable relevance over the last decade. This increasing interest is based on the highly toxic potential of metals for living organisms in these real systems [Facchinelli et al., 2001, Mico et al., 2006]. These elements may enter and distribute themselves in the environment through superficial waters, contaminating soils and surrounding terrestrial ecosystems and producing a toxicity risk of more sensitive media (plants, animals, and human beings) [Holub et al., 1993]. Most of the research studies have been often limited to the determination of the total concentration of metals. Nevertheless, as it has been cited in the previous chapter, total concentration does not provide suitable information about the bioavailability of potentially toxic elements.

Fortunatelly, the determination of total metal concentrations in the solid phase (sediments, sludges or solid waste) and their potential mobility has been extended in environmental studies. The values obtained after these studies are very useful in order to obtain information about origin, potential mobility and transport of elements in natural environments [Chomchoei et al., 2002]. Metal metal mobilization is now recognized as key parameter to assess the chemical processes in environmental matrixes as well as the environmental impact [Tüzen, 2003; Beek and Sneddon, 2000; Tessier et al., 1979; Baeyens et al., 2003]. As stated previously, metals in natural sediments generally can be found in different oxidation states and are affected by many geochemical factors such as pH, redox potential or organic matter in both sediment or water column. A partial neutralization is produced by the primary minerals (mainly silicates) found on the exchangeable part of the soil and a stronger neutralization occurs if carbonate minerals are present [Cravotta, et al., 1999]. After these reactions, an increase in pH occurs, and the solubility of dissolved elements is reduced mainly by reactions of precipitation, co-precipitation, or adsorption in the exchangeable matrix of the soil [Xu et al., 1997].

The need to determine the available fraction of metals in different environmental monitoring programs has promoted the development of accurate analytical approaches. The new analytical method proposed in the previous chapter showed its suitability to assess metal mobilization factors in a more real approach. These conditions were ensured using the own natural water collected at the sampling site, since many of the variables affecting metal mobilization of sediments could be picked up. The preliminary assays showed that As, Cd, Co, Cr, Cu, Fe, Ni and Pb were the more mobilized metals in river sediments from Nerbioi-Ibaizabal.

Moreover, it is widely assumed that the physicochemical parameters of the estuary system could vary depending on the period of the year in which the analyses are performed. Hence, the present section is an extension of the work explained in the previous chapter as an application of the proposed mobility methodology on estuarine samples and taking into account the seasonal factor. In this sense, it is known that variables such as total dissolved organic carbon or total organic matter content in water and sediment, respectively, are directly dependent on the sampling time or season. For instance, different amount of dissolved organic matter is expected during the different seasons, mainly attributed to natural degradation processes of dead living beings [Walinsky et al., 2009; Köhler et al., 2009]. Besides the last, some other physicochemical parameters can be also variable depending on the seasonality such as temperature, redox potential, pH, specific conductivity or turbidity. Since all these parameters may affect metal mobilization, they must be considered when availability of metals is assessed.

Thus, the developed methodology in the previous chapter was applied to Nerbioi-Ibaizabal estuary samples (Basque Country, Spain). This emplacement, which was not studied in the previous chapter (only continental rivers were analyzed), is placed into an important industrial zone and suffers a very high degradation because of the burning industrial activity developed during the last decades [Departamento de ordenación del territorio, vivienda y medio ambiente, 1999]. This zone has a high tidal influence and hence, the ionic strength in the estuarine can be unsteady. Thus, three factors must be considered to have a closer interpretation of the mobilization results obtained in this estuary: metal concentration, mobility and tidal influence. Hence, an integrated study considering these three factors will be carried out.

The integration of large amount of data from different sampling points requires the use of statistical tools which allow the interpretation of the data showing features that remain hidden. Among many statistical techniques available for the identification of the principal features for defining “pattern recognition”, principal components analysis (PCA) is by far the most used chemometric tool of the unsupervised patter-recognition techniques [Einax et al., 1998; Gredilla et al., 2013]. PCA allows the reduction of the dimensionality of the data by transforming the set of original variables into another group of variables called principal components (PC), which are divided according to the explained variance. The use of PCA and correlation analysis will allow the identification of possible trends of metals found in estuarine sediments of Biscay basins.

5.1. Experimental

5.1.1. Reagents and solutions

All the laboratory material was carefully cleaned with abundant pure water (Elix® quality, Millipore™, Bedford, MA, USA) and without using detergent to avoid possible interferences produced by detergent residues. Afterwards, the material was maintained in a clean 10% nitric acid bath overnight and then, it was thoroughly rinsed with pure water and with ultrapure water (Milli-Q® quality, Millipore™, Bedford, MA, USA).

All chemical reagents were of analytical-reagent grade. Individual stock solutions of As, Cd, Co, Cr, Cu, Fe, Ni and Pb at a concentration of 1000 $mg \cdot l^{-1}$ were obtained from Aesar (Specpure stock solutions, Karlsruhe, Germany). Hydrochloric acid (37%, Tracepur) and nitric acid (69%, Tracepur) were provided by Merck (Darmstadt, Germany). Amonium nitrate was supplied by Merck ($NH_4NO_{3,}$ Tracepur, Darmstadt, Germany) and fresh solutions of 1.0 $mol \cdot l^{-1}$ were prepared daily according to the experimentation.

The acetone (HPLC grade) used for the treatment of sediment samples for Raman analyses was purchased from Labscan (Dublin, Ireland).

5.1.2. Instrumentation

A Cryodos-50 (Telstar S.A., Terrasa, Spain) laboratory freeze-dryer. An Octagon digital sieve (Endecotts, London, UK) was used to recover the 63 µm particle size sediment from the bulk sediment.

The microwave assisted digestion of the sediments was carried out in a close microwave device Multiwave 3000S (Anton Paar, Graz, Austria) equipped with 8 Teflon vessels and temperature and pressure sensors.

Inductively coupled plasma with mass detector (ICP-MS, 7700x, Agilent Technologies, Palo Alto, USA) was used for element determinations using a MicroMist micro-uptake glass concentric nebulizer (Glass Expansion, West Melbourne, Victoria, Australia). In order to reduce MO^+ formation in the plasma, the spray chamber was Peltier cooled at 2 °C. A standard quartz torch with 2.5 mm internal diameter injector was used. The instrument was equipped with an Agilent I-AS integrated autosampler, and the on-line internal standard addition kit was used for the on-line addition of the multi-element isotope spike solution or internal standards in external calibration mode. Finally, standard nickel cones (sample and skimmer) were used. The optimization of the ICP-MS conditions was achieved by adjusting the torch position and tuning for reduced oxide and doubly charged ion formation with a standard tuning solution containing 1.0 $\mu g \cdot l^{-1}$ of ^{7}Li, ^{24}Mg, ^{59}Co, ^{89}Y, ^{140}Ce and ^{205}Tl in 1.0% HNO_3. This equipment includes a collision cell (He gas, ORS3 system, Agilent Technologies$^{©}$) to discriminate spectral interferences with high performance for all the trace metals considered in here. All elements were measured using a single set of operating conditions without switching cell gas modes. Operating conditions are shown in Table 5.1 (acquisition masses and integration times). In addition, EPA 6020 [EPA, 2007] recommendations were followed for interference overcoming such as correction equations for Pb or Cd. Measurement of Cd by ICP-MS is known to suffer from spectral interference [Vassileva et al., 2003]. Finally, the formation of MO^+ oxides was overcome (< 1.0%) by means of the ORS3 He collision cell. As estimation, total analysis time per sample, including wash-in and wash-out, was 2.5 min.

Table 5.1. *ICP-MS operating and acquisition parameters used.*

Instrumental conditions		***Acquisition conditions***	
RF power (W)	1000	Dwell time (ms)	250
Plasma gas flow ($l \cdot min^{-1}$)	14	Integration time (ms)	---
Auxiliary gas flow ($l \cdot min^{-1}$)	1.0	Sweeps for replicate	10
Nebulizer gas flow ($l \cdot min^{-1}$)	0.91	Replicates	4
Sample flow rate ($ml \cdot min^{-1}$)	1.0		
Sheathing gas flow ($ml\ min^{-1}$)	----		
Analytical detection			
Isotopes	^{75}As, ^{111}Cd, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{56}Fe, ^{60}Ni, ^{208}Pb		

Micro-Raman measurements on dry sediment samples were performed using a Renishaw RA 100 Raman Spectrometer, equipped with a 785 nm excitation laser and a CCD detector (Peltier cooled). The micro-probe was joined to 4×, 20× or 50× long-range lenses which allowed us to focus the laser beam between 5 µm and 100 µm at the sample. Laser powers were set at low powers (not more than 1 mW at the sample) in order to avoid thermal decomposition. The data were obtained with WIRE 2.0 software (Renishaw, UK), and their analysis and treatment was performed with the Omnic software (Thermo Fisher-Nicolet, USA). Spectra were generally acquired between 200 and 3000 cm^{-1} and several scans were accumulated for each spectrum in order to improve the signal-to-noise ratio. Collected spectra were compared with standard spectra of different databases [Castro et al., 2005; Downs, 2006].

5.1.3. Study area

In this work, five different sampling points highly affected by tide in the Nerbioi-Ibaizabal estuarine were considered: Arriluze, Puente Colgate, Agra, Asua and San Inazio The location and sampling-codes of the sampling points are shown in Figure 5.1.

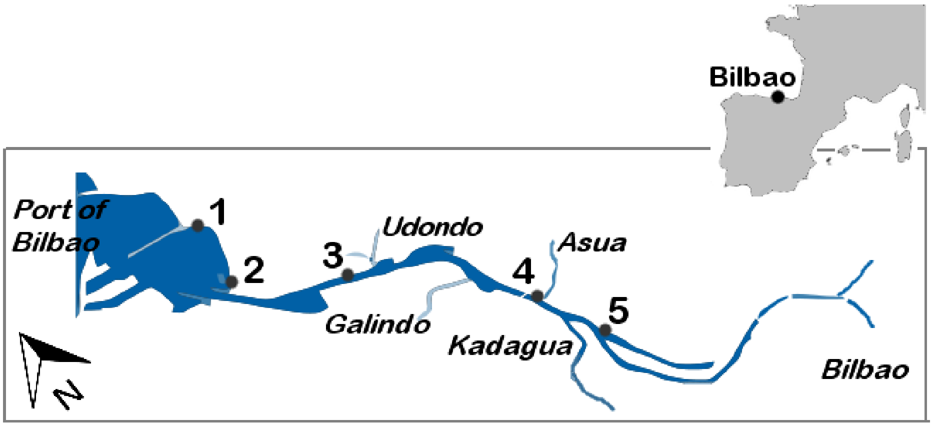


Figure 5.1. *Location of the sampling points in the Nerbioi-Ibaizabal River estuary: 1. Arriluze (Arr), 2. Puente Colgante (Pte), 3. Agra (Agr), 4. Asua (Asu), 5. San Inazio (Sin).*

The estuary of the Nerbioi-Ibaizabal is located in the continental shelf of the Cantabrian coastline in the northern coast of the Iberian Peninsula. This estuary is a macro-mesotidal temperate ecosystem located in the Basque Country ($43^{o}23'$ – $43^{o}14'$ N, $3^{o}07'$ – $2^{o}55'$ W) which drains into the southeast corner of the Bay of Biscay. It receives water from a drainage area of 1814 km^2 and its main rivers, the Nerbioi and the Ibaizabal merge, providing an average combined flow of 25 $m^3 \cdot s^{-1}$. Tides are semidiurnal with ranges between 1.2 and 4.6 m. The original morphology of the estuary has been strongly modified over the last 150 years by dyking up and dredging, to the extent that currently it lacks of intertidal areas in some zones of the estuary.

There are two different zones: the channel and the bay. The channel is short (16 km long), narrow (50–150 m) and shallow (2–9 m) and the water column is salt-stratified; the mid-enclosed coastal bay, also called El Abra, is 14–30 m deep, 3.8 km wide and with an area of 30 km^2 it contains the 95% of the total water volume of the estuary. Due to the industrial and urban development of the last 150 years, the estuary became highly polluted by wastes from many sources (mineral sluicing, industrial wastes and urban effluents) and its sediments accumulated high levels of organic matter and metals [Landajo et al., 2004].

The experimentation was performed in two different seasonal campaigns of the same year: July (summer) and November (autumn) to apply the proposed methodology taking into account the different physicochemical estuarine conditions in the different seasons.

5.1.4. Sampling

Water and estuarine sediments were collected in different seasonal campaigns (summer and autumn). All plastic and crystal material in contact with samples was first cleaned with a detergent, thoroughly rinsed with tap water, soaked in a 10% HNO_3 bath at least for 24 h and finally rinsed with ultrapure quality water. After drying the material in a laminar air flow hood, it was stored in clean plastic bags until use.

Deep water (in contact with the sediment, both at low and high tides) was collected in different sampling sites using a Van Dorm type all-plastic water sampler (KD Denmark, Research Equipment, Silkeborg, Denmark), specifically designed for trace element sampling. Physicochemical parameters of the estuarine water were in-situ recorded (pH, pO_2, conductivity, redox potential and temperature) by means of a previously calibrated YSI 556 multi-parametric probe (YSI Environmental, Yellow Springs, Ohio). The probe also provided an estimation of salinity and total dissolved solids (TDS) based on the temperature and electrical conductivity measurements. Total dissolved organic carbon was analyzed in the laboratory using a TOC-L analyzer (Shimadzu, Izasa, Bilbao, Spain).

Water samples were introduced in polystyrene bottles and divided into two sub-samples:
(i) one stored at 4°C and used immediately for the mobilization studies,
(ii) the other one filtered (0.45 µm), acidified (pH<2) and stored at 4°C until total trace element determination.

The superficial sediment samples were manually scrapped from 5 cm of river-bed surface, introduced in plastic recipients, freeze-dried, sieved (<63 µm mesh) and stored at -20°C until analysis.

5.1.5. Analysis for total concentrations

Filtered water samples (0.45 µm Cellulose, Whatman filters) were stabilized at pH<2.0 with HNO_3 and analyzed by ICP-MS to determine both major and minor elements.

Sediment samples were analyzed using the same procedure described in the previous work. Briefly, 0.5 g of freeze-dried sediments were subjected to acid digestion in a microwave system and using a mixture of 15 ml of HCl, HNO_3 and water (1:1:3, vol) [Navarro et al., 2006]. After cooling, the extracts were filtered through syringe PTFE filters (25 mm, 5 µm, Waters, Milford, USA) and diluted to 50 ml with ultrapure water. These extracts were kept in the dark in polyethylene vials at 4°C until the analysis by ICP-MS.

The accuracy of the analytical methods for metal quantification was checked using two different certified materials: estuarine sediment (NIST 1646, National Institute of Standards and Technology, USA) and natural freshwater (NIST 1643, National Institute of Standards and Technology, USA). The results obtained were always in good agreement with the certified values (95% confidence level).

5.1.6. Mobilization methodology

The determination of the mobilizable fraction of metals involves the mixing of the sediment with the leaching solution (without its renewal) until equilibrium conditions are reached. It was performed following the German DIN 19730 norm [Deutches Institut für Normung, 1997] but using own water from the estuary as extractant reagent (see chapter 4). In this work two different extractants were used: (i) estuarine water collected at high tide and (ii) estuarine water collected at low tide. According to this norm, the sediment/leaching solution ratio was fixed at 1:2.5 by mixing 20 g of sediment with a particle size bellow 63 µm with 50 ml of the corresponding leaching solvent in 50 ml polypropylene centrifuge tubes. The slurry was continuously shaken in a multiple rotating shaker at 20 rpm for 2 hours and at room temperature. Once the extraction period was over, the suspensions were filtered through 0.45 µm PVDF filters (Whatman). The sample extracts were then acidified with concentrated HNO_3 to adjust the acid percentage to 1% for their stabilization and ICP-MS analysis.

In order to study the repeatability of the method, assays were performed in triplicate for all samples collected in Arriluze sampling site (Arr). Adequate repeatability values, in terms of relative standard deviation (RSD %), were obtained being in all the cases lower than 10%.

As it was conducted in the previous work, metals mobility (i.e., metal mobilized fraction referred to sediment concentration M_i, $^0/_{00}$) was calculated by the equation 5.1.

$$\mathrm{M_i} = \frac{2.5 * (\,C_1 - C_0)}{(1000 * C_{sed})}$$

Equation 5.1. *Calculation of the mobilized portion from each metal referred to the sediment.*

where: C_1 and C_0 are the concentrations ($\mu g \cdot l^{-1}$) of mobilized element in the extractant after and before the extraction process, respectively. C_{sed} is the concentration ($mg \cdot kg^{-1}$) of the considered element in the dried sediment.

5.1.7. Chemometric analysis

Chemometric analysis is widely used in order to define any trend in the measured variables or contamination sources in the sampling points. The results were analyzed by means of PCA using The Unscrambler® software (v.7.5, Camo, Trondheim, Norway) [Esbensen, 1994]. PCA looks for dimensionality reduction of the data, and at the same time gives associations between the objects under study that previously were difficult to detect, for example, after the correlation analysis [Einax et al., 1998a; Einax et al., 1998b, Daus and Zwanziger, 1995]. This chemometric tool is able to transform the original variables in new ones known as Principal Components (PCs), which are lineal combination of the original ones and orthogonal among them.

The PCA models were systematically performed. On the one hand, those values that were below limit of detection were considered as missing values during the treatment. On the other hand, all the variables were centered and scaled so that they maintain the sample variance. Finally, for PCA model validation purposes, the standard internal validation (i.e., cross-validation) was always used in a first attempt. Once the number of PCs needed to explain the largest variance was defined as well as outlier samples were kept out, the cross validation algorithm was used. Although this last algorithm involves longer calculation events in comparison to leverage correction, it supplies models with better prediction ability.

5.2. Results and Discussion

Each sampling point considered was influenced by specific parameters leading to a different effect in the real system from a similar cause or incident. Linked to that, the different physicochemical parameters (redox potential, pH, pO_2, turbidity and conductivity) were measured in situ and listed in Table 5.2. It should be highlighted that two different values were determined as a function of the tide and thus, low and high tide were differentiated in the estuary case.

Table 5.2. *In-situ measured physicochemical parameters of estuarine waters at high and low tide in different sampling campaigns: pH value, redox potential (pE: mV),* pO_2*, conductivity (C:* $\mu S \cdot cm^{-1}$*), turbidity (T: NTU) and total dissolved organic carbon (TOC:* $mg \cdot l^{-1}$*).*

Sampling period	Sampling site	Tide	pH	pE	pO_2	C	T	TOC
Nov (autumn)	**Pte**	**low**	7.6	6.5	6.3	33800	10	5.95
		high	7.9	76	8	37200	10	5.83
	Arr	**low**	8.0	103	7.7	36500	9.8	4.85
		high	8.0	79.5	7.8	37600	10	4.28
	Asu	**low**	7.9	85.5	6.4	19200	10	7.75
		high	7.6	68.5	5.6	28000	10	7.68
	Sin	**low**	7.8	90	7.3	14100	10	8.21
		high	7.7	85.5	6.5	19400	10	6.56
	Agr	**low**	7.7	72.5	4.9	24200	10	8.37
		high	7.8	78	6	32700	10	5.68
July (summer)	**Pte**	**low**	7.7	4	6.87	32900	58	3.7
		high	8.2	64	8.1	30000	18	6.26
	Arr	**low**	8.0	23	7	33800	56	7.06
		high	8.3	69	10.1	28800	15	3.48
	Asu	**low**	8.1	-1	7.54	12400	14	10.7
		high	8.0	56	7.1	18400	45	8.9
	Sin	**low**	8.0	-28	7.5	11900	>100	6.72
		high	8.3	37	9	15300	22	12.25
	Agr	**low**	8.1	45	4.9	10500	45	10.1
		high	8.0	43	6	11000	43	11.0

Regarding the information summarized in Table 5.2, two values should be highlighted: the high redox potential and conductivity values measured in water samples collected in autumn. On the one hand, the high redox potential could be the consequence of the increase of dissolved oxygen percentage observed in this season sampling campaign [Nelson, 2002]. On the other hand, the increase of conductivity of water samples collected in autumn could be usual for estuarine areas due to higher water levels easing the seawater entrance. Dissolved solids are more concentrated during summer [Fernández et al., 2008]. The amount of dissolved organic matter in autumn is completely different than in summer due to natural degradations [Walinsky et al., 2009; Köhler et al., 2009]. This last variable must be also taken into account in the interpretations of the mobilization results.

5.2.1. Sediment characterization

In order to confirm the molecular composition of the sediments, dry sediment samples were analyzed by Raman spectroscopy employing a new fluorescence reducing methodology. Briefly, Raman analyses were performed directly on sediment samples which were previously sonicated for 30 minutes with acetone (10 ml x 3 times) and dried at 35°C (see chapter 3 for more details). Different compounds were identified and classified as natural and anthropogenic ones.

Clay was found everywhere in the Basque Country up to depths of 1200m. Quartz and silicate-type micas, which are natural compounds of silicate oxides, are compounds spread in metamorphic and sedimentary rocks like those present in the basins considered in this work [Fernández et al., 2014]. Table 5.3 summarizes the natural compounds found in the different sampling sites. Specifically, different carbonate compounds were found, mainly calcite and aragonite ($CaCO_3$), and siderite ($FeCO_3$, Raman bands at 290 and 1089 cm^{-1}). Hematite is considered as a natural phase of iron (III) oxide. The basin of the Nerbioi-Ibaizabal River estuary is well known for its iron mines from the Roman times. Thus, the amount of iron minerals, mainly hematite (Figure 5.2b) and pyrite (FeS_2), occurring in the sampling sites was expected to be high. This compound was found in Puente Colgante, in the main channel of the estuary. FeS_2 was also found together with other species of iron.

Table 5.3. *Natural and anthropogenic compounds found in each sampling location. Raman bands shown in bold are the primary bands.*

Source	**Compound**	**Raman bands (cm^{-1})**[1]	**Arr**	**Pte**	**Agr**	**Asu**	**Sin**
Natural	Quartz (α-SiO_2)	**463**	✓	---	✓	✓	---
	Mica	**464**, 708	✓	✓	✓	✓	✓
	Aragonite ($CaCO_3$)	**1084**, 206	✓	✓	✓	✓	✓
	Calcite ($CaCO_3$)	**1084**, 280	✓	✓	✓	✓	✓
	Pyrite (FeS_2)	**342**, 462, 378	✓	✓	---	---	---
	Siderite (Fe_2CO_3)	**1089,** 290	✓	✓	---	✓	✓
	Hematite (α-Fe_2O_3)	**406**, **291**, **225**, 607, 494	✓	✓	---	✓	✓
Anthropogenic	Gypsum ($CaSO_4 \cdot H_2O$)	**1009**, 1134, 494, 414	✓	✓	---	---	---
	$Ca_3(AsO_4)_2$	**848**, 336	✓	✓	---	---	---
	PbO	**290**	---	---	---	---	✓
	ZnO	**438**, 382, 332	---	---	---	---	✓
	Na_2S	**447**, **407**, **230**, 1533, 1341, 990, 683, 325	---	---	---	---	✓
	FeO(OH)	**395**, **301**, **245**, 550, 479	✓	---	✓	✓	✓

On the other hand, compounds that could have an anthropogenic origin were identified in the sediment samples (see Table 5.3). The Raman spectra of gypsum ($CaSO_4 \cdot 2H_2O$) was obtained in sediment samples from Puente Colgante together with aragonite (a natural phase of $CaCO_3$). This location is placed in front of the sewage treatment plant of the main iron production plant of the Basque Country, the ACB factory (Figure 5.2a). The presence of this compound is mainly attributed to the precipitation of calcium species (expected to

---

[1] *The Raman bands have been obtained from the database e-VISARCH [Castro et al., 2005].*

be found at high concentration levels in this sampling site) by reacting with the soluble sulphate [Puigdomenech, 2001]. Also, various iron species were found nearby (see chapter 3, figure 3.7 for pyrite and iron hydroxioxide in Galindo).

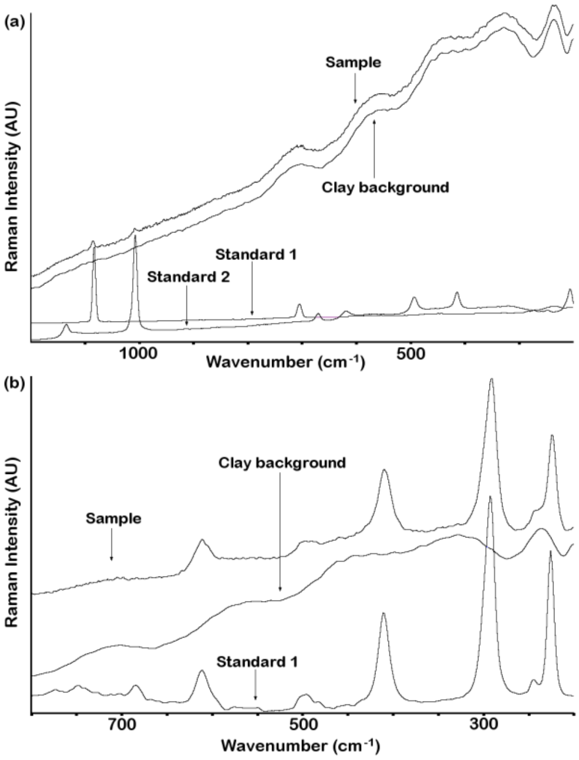


Figure 5.2. *Raman spectra of compounds of anthropogenic/natural origin detected in sediments: (a) Gyspsum (Standard 2) and Aragonite (Standard 1) in sediment from Puente Colgante. (b)* Fe_2O_3 *(Standard 1) detected also in sediment from Puente Colgante. All the standards were obtained from the database ''e-VISARCH'' [Castro et al., 2005].*

Calcium arsenate was also found in sediments collected downstream of iron and steel production factories. Arsenic is often associated with iron minerals and it is found in higher concentrations near iron-producing factories and sulfuric production plants using pyrites. Finally, iron hydroxioxide (FeO(OH)) was found in several locations in the Nerbioi-Ibaizabal River estuary. Probably in both cases, the iron FeO(OH) is bounded to the surface of natural mineral particles, iron oxide or pyrite, suggesting the in situ formation of the FeO(OH) compound.

5.2.2. Trace element concentration in water and sediment samples

The total concentrations of metals found in estuarine sediment samples and water collected in the different sampling sites and periods are plotted in Figures 5.3 and 5.4, respectively.

In general terms, high concentration levels of metals were found in both water samples (from 1 $\mu g \cdot ml^{-1}$ to 800 $\mu g \cdot ml^{-1}$) and sediment samples (from 5 $\mu g \cdot g^{-1}$ to 30 $mg \cdot g^{-1}$).

In the case of sediment samples (see Figure 5.3) high concentration levels of metals were found in all the studied sites as a consequence of the important industrial activities and urban residues in the zones under study. According to the spatial distribution of sampling sites, higher concentrations of metals were found in the innermost sites than in sites closer to sea.

Going deeper into the results, some elements in specific locations reached concentration levels that may be considered harmful for the environment bodies. Exemplarily, concentrations up to 45 $mg \cdot kg^{-1}$ of Cu, 380 $mg \cdot kg^{-1}$ of Fe, 120 $mg \cdot kg^{-1}$ of Ni and 12 $mg \cdot kg^{-1}$ of Pb were found in sediments collected in Puente Colgante. Asua (As: 32 $mg \cdot kg^{-1}$, Fe: 425 $mg \cdot kg^{-1}$ and Ni: 88 $mg \cdot kg^{-1}$) and Agra (Fe 250 $mg \cdot kg^{-1}$, Ni: 120 $mg \cdot kg^{-1}$ and Pb: 13 $mg \cdot kg^{-1}$) can be considered as impacted sites with higher concentration levels for all of the metals considered.

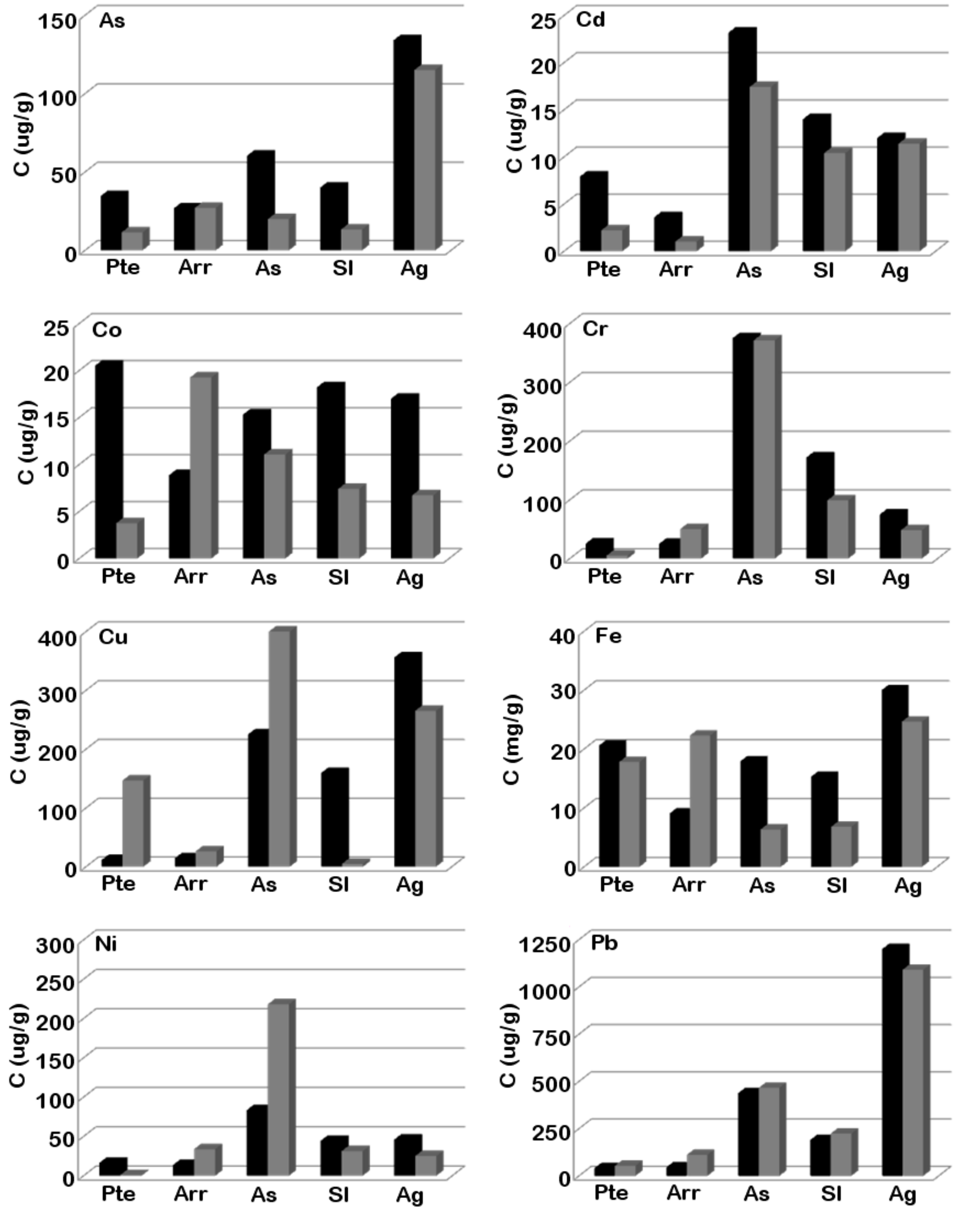


Figure 5.3. *Acid extractable concentration (µg·g^{-1}) obtained in estuarine sediment for the two seasons considered: summer in black and autumn in grey.*

The evaluation of the results in view of sampling campaigns can provide information about the possible seasonal tendency of metals in estuarine sediments. Although we have analyzed only two sampling campaigns to assure this seasonal influence, higher concentrations levels of metals were observed in the dry season (summer) regardless their location. This trend corresponds to a natural cycle of absorption-desorption of metals in sediments, with minimum accumulation values during autumn-winter seasons [Fernandez, 2008].

Concerning estuarine waters, a natural dilution effect attributed to the water input of the tide was generally observed notwithstanding the sampling site, except for the specific cases of Arriluze and Puente Colgante. It should be taken into account that these two sampling points are located in the external zone of the estuarine (see Figure 5.1) and the water flow decreases to these sites, especially in Arriluze. In addition, these sampling sites are the most directly impacted by industrial (shipyard), seaport and recreational activities producing a distortion effect on the natural profile. Finally, higher concentration levels were obtained for As, Cr, Cu, Fe, Ni and Pb while lower concentrations were found for Co and Cd.

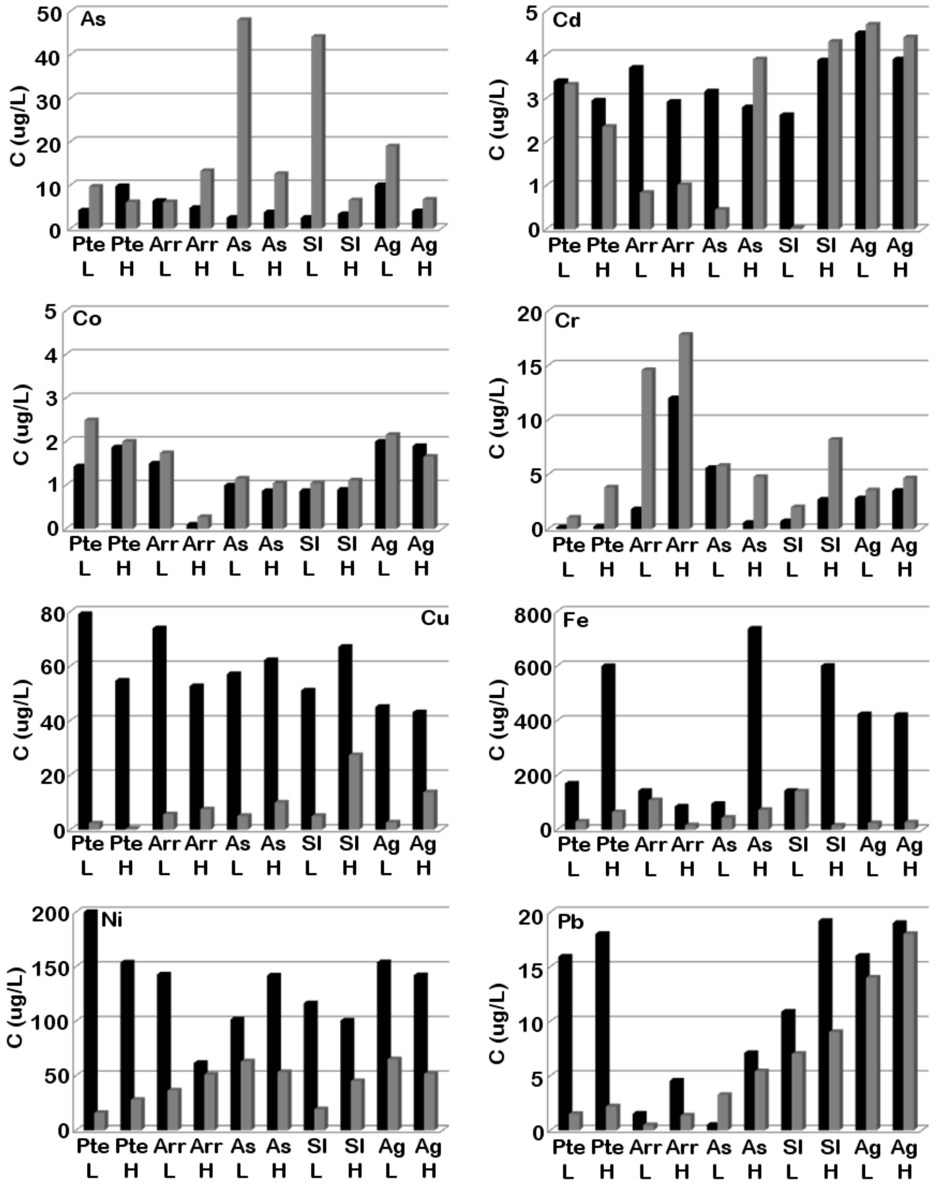


Figure 5.4. *Total concentration (µg·l^{-1}) obtained in estuarine water for the two seasons considered: summer in black and autumn in grey. (L) means water collected at low tide and (H) corresponds to water collected at high tide.*

5.2.3. Mobility tests

The mobility results obtained were expressed as element mobility (M_i, $^0/_{00}$) following the equation 5.1 described previously. A negative M_i value means that the concentration of the element is lower in the extract in comparison to the concentration of that element in the extractant solution (that means, a retention of the metal). This fact would indicate that the element is not mobilized and the sediment is enriched with the target element. On the contrary, positive M_i value indicates that a part of the element has been incorporated to the extractant solution, that is, the leaching conditions would be favorable for the re-dissolution of the element accumulated in sediment.

The estuarine sampling sites are highly dependent of the tidal influence; therefore, composition and ionic strength could be critical factors in the application of the mobilization methodology in estuarine environments. In this study, estuarine waters from both low and high tide were used for the mobility assays, since their properties and/or composition are rather different. These data were compared with those obtained using ammonium nitrate as extracting reagent, as it is defined in DIN 19730.

From the mobility tests performed from the DIN 19730 norm, the mobilization values of metals were always positive ($M_i > 0$) regardless the sampling site and period (see Figure 5.5). These values indicate that this reagent has a high capacity, even higher than natural waters, to mobilize metals from sediments to water bodies. The obtained element mobility value can be directly related to the thermodynamic complexation constant between element and ammonium (Co > Cu > Ni > Cd), being the main species involved $Co(NH_3)_6^{3+}$, $Ni(NH_3)_6^{2+}$, $Cd(NH_3)_6^{2+}$, $Cu(NH_3)_4^{2+}$ [Sillen and Martell, 1964]. The mobilization of arsenic can be explained by the sub-sequent mobilization of metals linked to As in the arsenate salts, such as $XAsO_4$ (X= Fe or Cr) and $Y_3(AsO_4)_2$ (Y= Cd, Co, Cu, Ni or Pb).

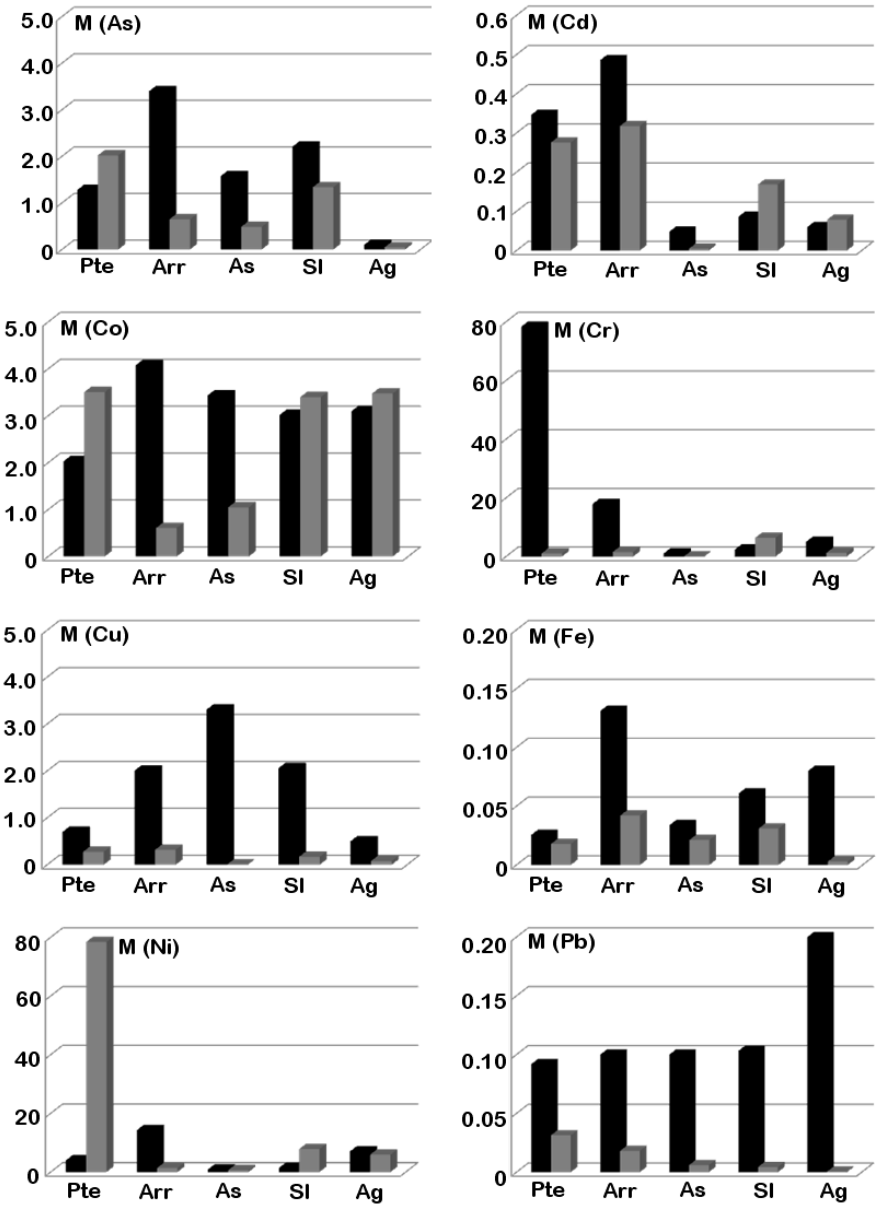


Figure 5.5. *Mobility of metals in each sampling site using ammonium nitrate (M) as extracting reagent. Two seasons were considered: summer in black and autumn in grey.*

As an example, the possible occurrence of copper species in presence of ammonium nitrate was computed by means of the MEDUSA program [Puigdomenech, 2001]. Figure 5.6 shows the theoretical distribution of the Cu species in the mobilization conditions using NH_4NO_3 1.0 mol·l^{-1}. Ammoniacal complexes are clearly predominant avoiding the processes of re-adsorption or precipitation onto the sediment and stabilizing the metal in solution (mobilization). The same thermodynamic modeling could be extrapolated to the other studied elements.

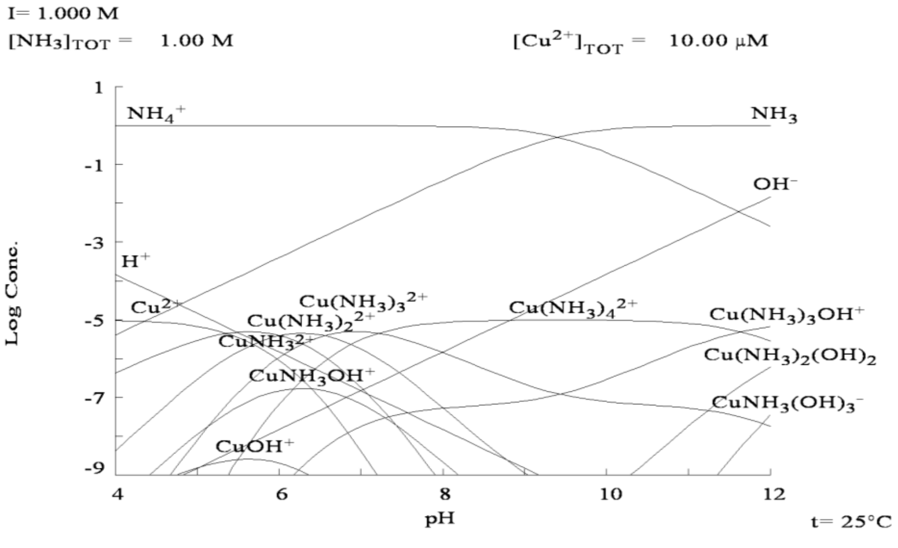


Figure 5.6. *Thermodynamic modeling of Cu using 1.0 mol·l^{-1}* NH_4NO_3 *for mobilization purposes.*

Concerning mobilization using estuarine water, random mobility values (positive, $M_i > 0$: mobilization and negative, $M_i < 0$: re-adsorption) were obtained regardless the type of water (collected at low and high tide) and sampling site (see Figure 5.7). The random pattern ($M_i > 0$ and $M_i < 0$) was followed by all the elements except for Co with ($M_i > 0$). However, the signal and magnitude of mobilization value was directly dependant on the tide and season considered, because of the different physicochemical parameters of water in each situation.

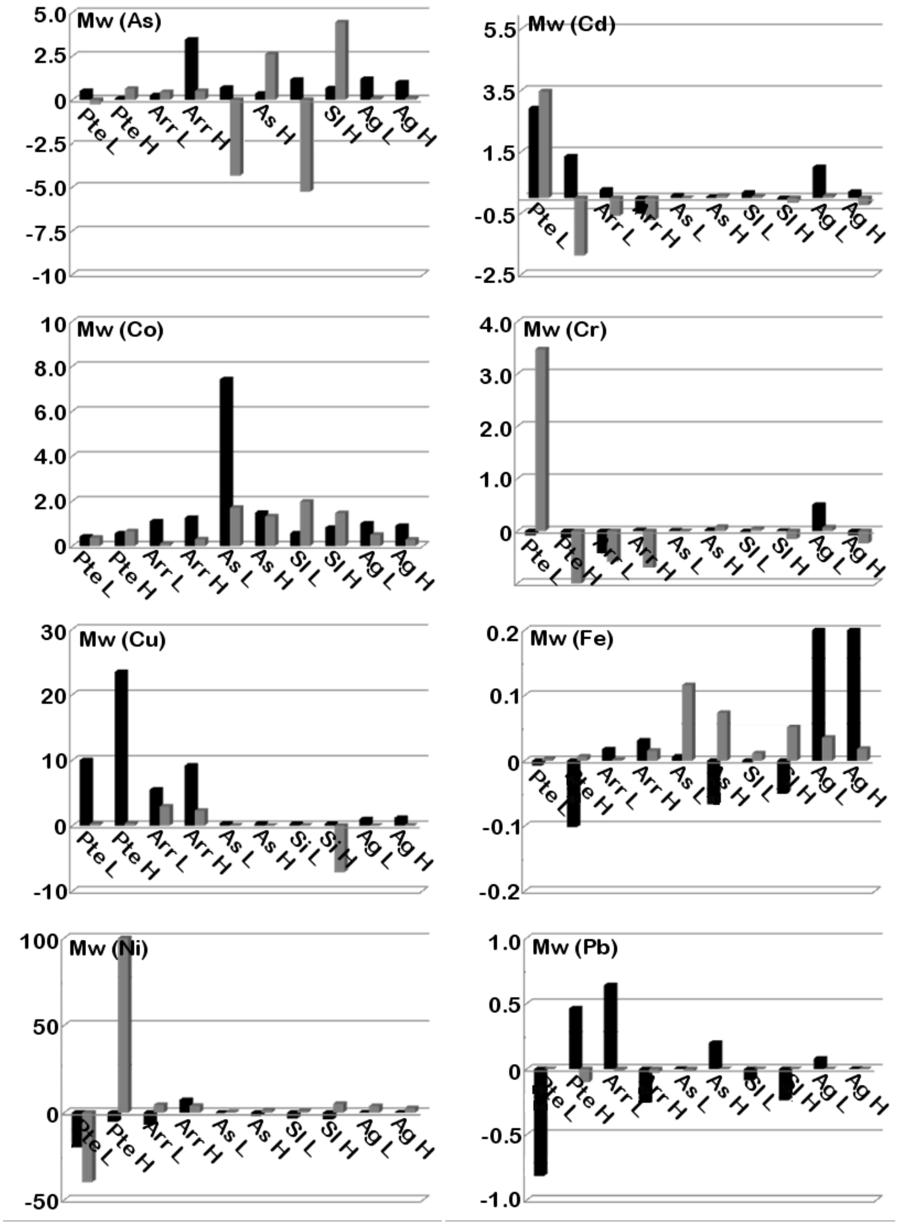


Figure 5.7. *Mobility using estuarine water (Mw) as extracting reagent in each sampling point and for the two seasons considered: summer in black and autumn in grey.*

Thermodynamic modeling was done in order to predict the mobilization process of metals for each case. As an example, the theoretical distribution of the Cu species was simulated by means of MEDUSA program and using different mobilization conditions depending on the sampling site in the estuary: Arriluze (Arr, Figure 5.8a) and San Inazio (Sin, Figure 5.8b), respectively.

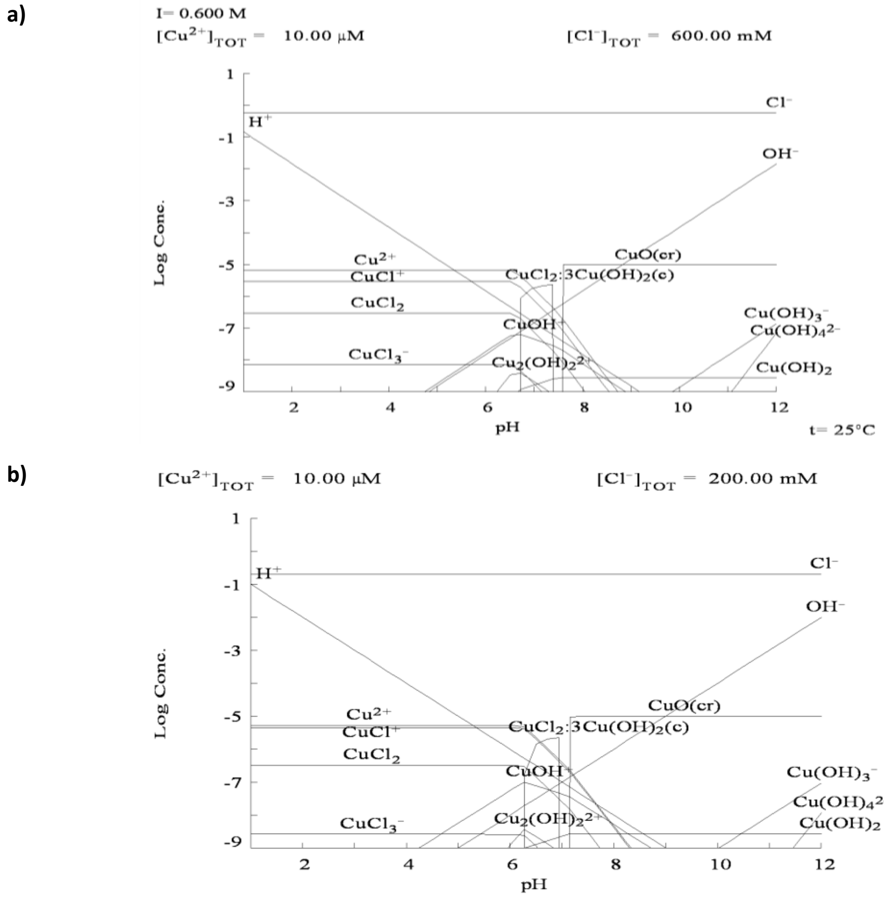


Figure 5.8. *Thermodynamic modeling of Cu using the corresponding physicochemical parameters of estuarine water. (a) Simulation of Cu mobilization using the conditions of Arriluze site. (b) Simulation of Cu mobilization using the conditions of San Inazio site.*

The thermodynamic modeling was performed by establishing the ionic strength and pH conditions of the two sampling sites (the introduction of more variables could contribute to a very complex interpretation):

(i) NaCl was defined as the main contribution to the ionic strength in both sites, the ionic strength was fixed at 0.2 mol·l^{-1} for the inner emplacement (San Inazio) whereas a higher ionic strength was established for the site closer to sea (Arriluze = 0.6 mol·l^{-1}).

(ii) The pH was fixed at pH =8, taking into account the experimental values measured in-situ and detailed in Table 5.2.

At these established conditions, the presence of CuO(s) and/or $CuCl_2$:3$Cu(OH)_2$(s) [Sillen and Martell, 1964] could cause the re-adsorption of the mobilized cooper. This fact was more marked in San Inazio (Sin) than in Arriluze (Arr). According to the simulation shown in Figure 5.8b the predominance of CuO(s) was found to be higher at conditions fixed for San Inazio than for Arriluze. As other particular case, the thermodynamic modeling was also useful to predict the mobilization results obtained for Cobalt. In the estuary conditions, the presence of $Co(OH)_2$ (s) (see Figure 5.9) could alter the mobilization process but it was negligible at estuarine pH values. Consequently, $M_i > 0$ was experimentally obtained for all sampling sites (see Figure 5.7).

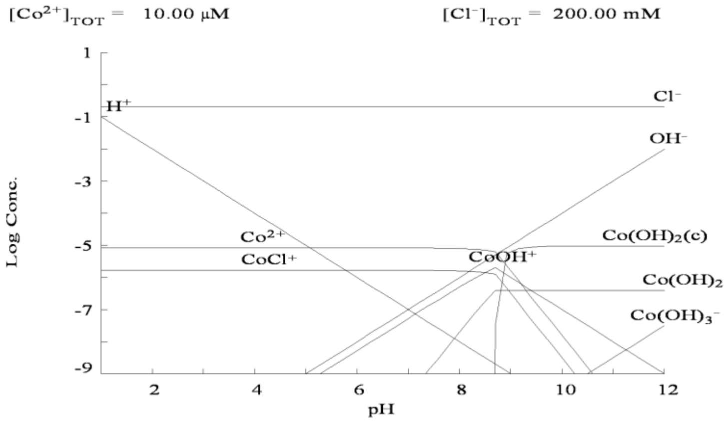


Figure 5.9. *Thermodynamical simulation of Co species using the conditions of San Inazio site.*

As it was performed in the previous chapter, and in order to find any hidden trend in the experimental mobilization results, the whole data was statistically re-processed by PCA using the Unscrambler® program. The data set consisted in one matrix containing both metal mobilized data: mobilization with estuarine water (low tide) and with ammonium nitrate in the different sampling sites (see section 5.1.7 for the methodology used in the PCA).

Figure 5.10 shows the loading plot of the first two principal components (PCs) for the metal mobilities using ammonium nitrate (Figure 5.10a) and using estuarine water collected at low tide (Figure 5.10b). The total metal concentration found in sediment samples in both cases was also taken into account for both cases. PC1 and PC2 explain the 69% and 72% of the total variance in the first and second scenarios, respectively.

As it can be seen in both loading projections (mobility of metals after treating with ammonium nitrate and with estuarine water, respectively) different variable distribution was obtained. In the first case (see Figure 5.10a), the first PC try to separate the concentrations found in sediments vs the mobilized metal concentration. All the mobilized metals are negatively correlated with the corresponding sediment samples, indicating a mobilization of metals from sediment to the extractant, probably in form of ammonium complexes. A clear exception is observed for Pb, since the mobilized fraction is positively correlated with the Pb concentration found in sediment. This fact indicates that Pb is the less mobilizable metal among the assessed elements in that extraction conditions.

However, the x-loadings using the mobilized metal fraction with estuarine water (Figure 5.10b) showed random grouping along to the PC1 and PC2, with different statistical sense and no clear influence of the capacity of forming ammonium complexes.

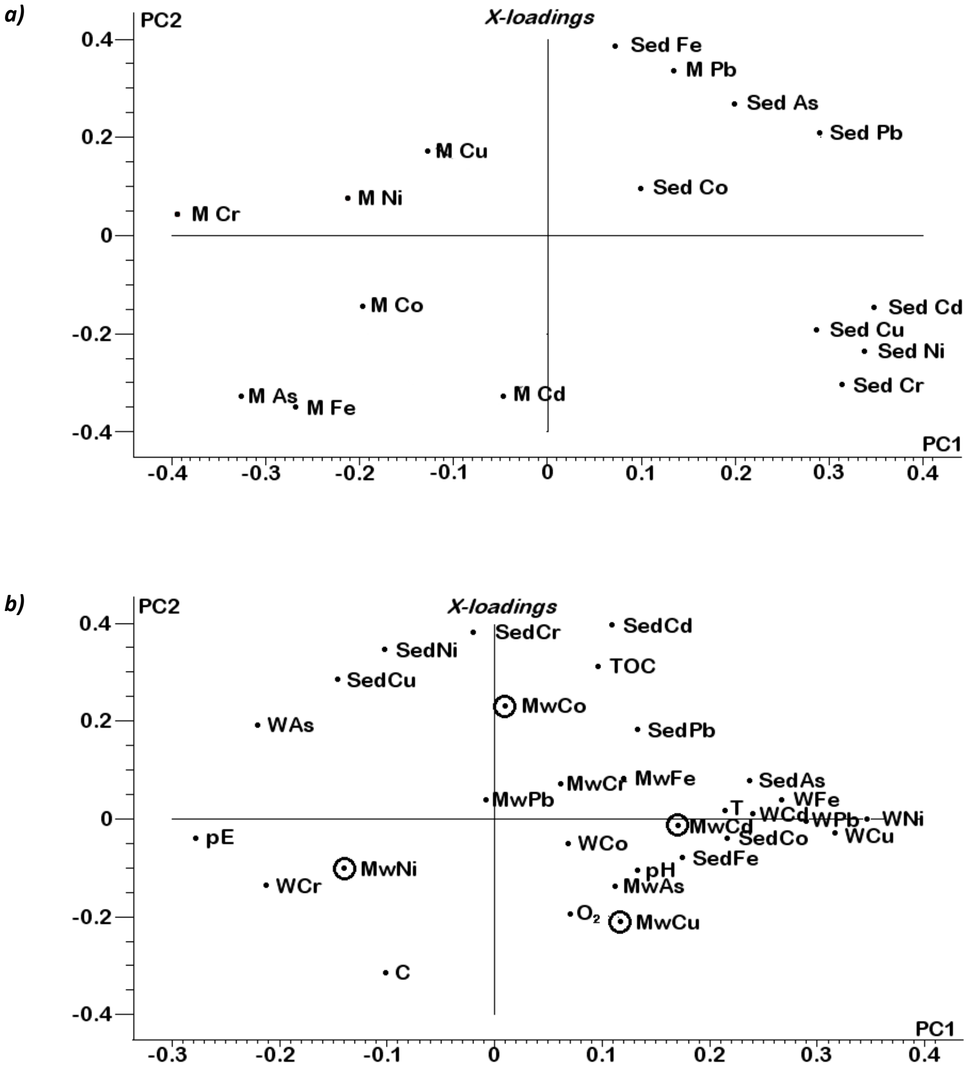


Figure 5.10. *X-loadings plot (PC1 vs PC2) obtained for: (a) mobilization process considering ammonium nitrate as extracting reagent.* M_i *is the mobility for each metal using ammonium nitrate as extractant and* Sed_i *the total concentration in sediment. (b) mobilization process considering estuarine water collected at low tide as extracting reagent.* Mw_i *is the mobility for each metal using water as extractant and* Sed_i *the total concentration in sediment.*

This random grouping of mobility of metals was directly correlated with the metal concentration in the estuarine sediments and waters along PC1. These correlations could define the saturation cycle of metals in natural systems. Sediments operate as a drain, being able to remove metals from sediment to the estuarine water to obtain saturation conditions or in contrast, to precipitate them into the estuarine sediments in water super saturation conditions. The significant correlation among turbidity and the total content of iron, nickel, cadmium, copper and lead in water shows that there could be an adsorption of those elements in suspended particles increasing turbidity value. Taking into account that nickel, cadmium and lead are not natural elements, the presence of these elements indicates the influence of the industrial activities in the estuarine system.

Finally, TOC had also an important role in the clustering depending on the sampling time, mainly for cadmium, lead and chromium. Though the role of organic matter in natural systems is complex, the variation of metal concentrations seems to be closely related to the variation of organic matter [Saiz and Gonzalez, 2000].

5.3. Conclusions

Raman spectroscopy has shown a high potential to identify molecular species, helping in the identification of certain substances and distinguishing natural species from anthropogenic ones. Also, the methodology for partial removing organic matter (soft cleaning) without alteration of inorganic species or fluorescence reducing cleaning, has demonstrated promising results to provide well defined Raman spectra.

Regarding metals concentration found in sediments, high concentration levels of metals (mainly As, Cr, Cu, Fe, Ni and Pb) were found in all the studied sites as a consequence of the important industrial activities and urban residues in the zones under study.

About the new mobilization methodology, as it was also demonstrated in the case of river sediment samples, the use of estuarine water to assess metal mobility has shown to be a powerful alternative to the DIN 19730 standard method. In fact, this method has shown great ability to mimic the real mobilization produced in estuarine samples giving realistic values about metal availability. The comparison performed against DIN 19730 (using ammonium nitrate as metal mobilization reagent) indicates that the use of additional chemical not present in the environment can lead to non-accurate results for metal availability.

5.4. References

[Baeyens et al., 2003] Baeyens W, Monteny F, Leermakers M, Bouillon S. *Evaluation of sequential extractions on dry and wet sediments.* Anal. Bioanal. Chem. 2003; 376, 890 – 901.

[Beek and Sneddon, 2000] Beek JN, Sneddon J. *Metal Concentrations in Soils and Sediments in Southwest Louisiana.* Anal. Lett. 2000*;* 33, 1913 – 1959.

[Castro et al., 2005] Castro K, Pérez-Alonso M, Rodríguez-Laso MD, Madariaga JM. *On-line FT-Raman and dispersive Raman spectra database of artists' materials (e-VISART database).* Anal. Bioanal. Chem. 2005; 382, 248 – 258.

[Chomchoei et al., 2002] Chomchoei R, Shiowatana J, Pongsakul P. *Continuous-flow system for reduction of metal readsorption during sequential extraction of soil.* Anal. Chim. Acta 2002; 472, 147 – 159.

[Cravotta et al., 1999] Cravotta CA, Trahan MK. *Limestone drains to increase pH and remove dissolved metals from acidic mine drainage.* Appl. Geochem. 1999; 14, 581 – 606.

[Departamento de ordenación del territorio, vivienda y medio ambiente, 1999]. Departamento de Ordenación del Territorio, Vivienda y Medio Ambiente. *Vigilance of the Basque Country River Waters Quality*, Servicio Central de Publicaciones del Gobierno Vasco, Vitoria-Gasteiz, 1999.

[Deutches Institut fur Normung, 1997] DIN 19730 (Deutches Institut für Normung*). Bodenbeschaffenheit-Extraktion von Spurenelementen mit Ammonium Nitratlösung*, Beuth, Berlin, 1997.

[Downs, 2006] Downs RT. *The RRUFF Project: an integrated study of the chemistry, crystallography, Raman and infrared spectroscopy of minerals. Program and Abstracts of the 19th General Meeting of the International Mineralogical Association in Kobe*. Japan, 2006.

[Einax et al., 1998] Einax JW, Kampe O, Truckenbrodt D. *Assessing the deposition and remobilisation behaviour of metals between river water and river sediment using partial least squares regression,* Fresenius J. Anal. Chem. 1998; 361, 149 – 154.

[EPA, 2007] EPA 6020A, *Inductively coupled plasma mass spectrometry*. Environmental protection agency, USA, 2007.

[Esbensen, 1994] Esbensen KH. *Multivariate data analysis: an introduction to multivariate data analysis and experimental design*, Camo AB, Oslo, 1994.

[Facchinelli et al., 2001] Facchinelli A, Sacchi E, Mallen L. *Multivariate statistical and GIS-based approach to identify heavy metal sources in soils.* Environ. Pollut. 2001; 114, 313 – 324.

[Fernández, 2008a] Fernández S, *Diagnóstico, evolución y predicción de la concentración metálica en el estuario del rio Nerbioi-Ibaizabal*. PhD Thesis, UPV-EHU, Leioa, Spain, 2008.

[Fernández et al., 2008b] Fernández S, Villanueva U, de Diego A, Arana G, Madariaga JM. *Monitoring trace elements (Al, As, Cr, Cu, Fe, Mn, Ni and Zn) in deep and surface waters of the estuary of the Nerbioi–Ibaizabal River (Bay of Biscay, Basque Country).* J. Mar. Syst. 2008; 72, 332 – 341.

[Fernández et al., 2014] Fernández S, Gredilla A, de Diego A, Arana G, Madariaga JM. *Methodology to assess the mobility of trace elements between water and contaminated estuarine sediments as a function of the site physicochemical characteristics.* Sci. Total Environ. 2014; 473, 359 – 371.

[Gredilla et al., 2013] Gredilla A, Fdez-Ortiz de Vallejuelo S, Amigo JM, de Diego A, Madariaga JM, Uns*upervised pattern-recognition techniques to investigate metal pollution in estuaries.* TRAC Trends Anal. Chem. 2013; 46, 59 – 69.

[Holub et al., 1993] Holub Z, Simonovicova A, Banasova V. *The influence of acidification on some chemical and microbiological properties of soil, determining plant viability.* Biologia 1993; 48, 671 – 675.

[Köhler et al., 2009] Köhler SJ, Buffam I, Seibert J, Bishop KH, LaudonH. *Dynamics of stream water TOC concentrations in a boreal headwater catchment: controlling factors and implications for climate scenarios*. J. Hydrol. 2009; 373, 44 – 56.

[Landajo et al., 2004] Landajo A, Arana G, de Diego A, Etxebarria N, Zuloaga O, Amouroux D. *Analysis of heavy metal distribution in superficial estuarine sediments (estuary of Bilbao, Basque Country) by open-focused microwave-assisted extraction and ICP-OES, Chemosphere.* 2004; 56, 1033 – 1041.

[Mico et al., 2006] Mico C, Recatala L, Peris M, Sanchez J. *Assessing heavy metal sources in agricultural soils of an European Mediterranean area by multivariate analysis.* Chemosphere 2006; 65, 863 – 872.

[Navarro et al., 2006] Navarro P, Raposo JC, Arana G, Etxebarria N. *Optimisation of microwave assisted digestion of sediments and determination of Sn and Hg.* Anal. Chim. Acta. 2006; 566, 37 – 44.

[Nelson, 2002] Nelson D. *Natural variations in the composition of groundwater.* Springfield, region: Oregon, Department of Human Services; 2002.

[Puigdomenech, 2001] Puigdomenech I. *MEDUSA (Make Equilibrium Diagrams Using Sophisticated Algorithms), version 15*, Department of Inorganic Chemistry, The Royal Institute of Technology: Stockholm, Sweden, 2001.

[Saiz and Gonzalez, 2000] Saiz-Salinas JI, González-Oreja JA. *Stress in estuarine communities: lessons from the highly impacted Bilbao estuary (Spain).* J. Aquatic Eco. Stress Rec. 2000; 7, 43 – 55.

[Sillen and Martell, 1964] Sillen LG, Martell AE. *Stability Constants of Metal–Ion Complexes, Metcalfe & Cooper Limited*, London, UK. 1964.

[Tessier et al., 1979] Tessier A, Campbell PGC, Bisson M. *Sequential extraction procedure for the speciation of particulate trace metals.* Anal. Chem. 1979; 51, 844 – 851.

[Tüzen, 2003] M. Tüzen. *Determination of trace metals in the River Yeşilırmak sediments in Tokat, Turkey using sequential extraction procedure.* Microchem. J. 2003; 74, 105 – 110.

[Vassileva et al., 2003] Vassileva E, Quétel CR, Petrov I. *Certification of the Cu and Cd amount contents in artificial food digest using isotope dilution inductively coupled plasma mass spectrometry for Pilot Study 13 of the Comité Consultatif pour la Quantité de Matière.* Spectrochim. Acta B 2003; 58, 1553 – 1565.

[Walinsky et al., 2009] Walinsky SE, Prahl FG, Mix AC, Finney BP, Jaeger JM, Rosen GP. *Distribution and composition of organic matter in surface sediments of coastal southeast Alaska*. Cont. Shelf Res. 2009; 29, 1565 – 1579.

[Xu et al., 1997] Xu Y, Schwartz FW, Traina S J. *Treatment of acidic-mine water with calcite and quartz.* Environ. Eng. Sci. 1997; 14, 141 – 152.

6. Mobility and Raman methodologies: application on an arsenic species thermodynamical study

6. Mobility and Raman methodologies: application on an arsenic species thermodynamical study

Toxicity, bioavailability and/or biodisponibility of a given element is highly dependent on the oxidation state and/or the molecular species it occurs. Thus, speciation analysis is highly recommended when metal mobilization assessments are required. Speciation analysis can be performed using: (i) hyphenated analytical techniques (analytical speciation), where the total concentration of the different inorganic and organometallic species of the same oxidation state are measured, and (ii) using theoretical computations where chemical reactions are modeled taking into account all the known equilibrium reactions of the element in a particular system (thermodynamical speciation).

Although they are consistent procedures, both speciation approaches are often used separately, but they have some limitations. On the one hand, atomic analytical techniques cannot distinguish among the different inorganic species belonging to the same oxidation state. On the other hand, it is not always possible to perform the thermodynamical speciation due to the absence of both correct thermodynamic models and consistent and accurate thermochemical data for some important chemical species of the element. In order to have a better picture of the species distribution, both speciation studies should be performed.

An example of this approach was published on the speciation of inorganic arsenic species, As(III) and As(V), in river waters [Raposo et al., 2004a]. The thermodynamical speciation of each oxidation state (H_3AsO_4, $H_2AsO_4^-$, $HAsO_4^{2-}$, AsO_4^{3-}, $FeAsO_4$, H_3AsO_3, $H_2AsO_3^-$...) was performed using a basic chemical model and the predicted total concentration of the As(III) species was confirmed by analytical speciation procedures.

The basic thermodynamic model of inorganic arsenic was defined through the acid-base, redox, complexation and precipitation equilibria [Raposo et al., 2004c; Raposo et al., 2003a, Raposo et al., 2003b; Raposo et al., 2003 c; Raposo et al., 2002]. From these results, the corresponding thermodynamic constants for those equilibria were defined. Besides, the interaction parameters for inorganic arsenic species involved in the equilibria considered were also calculated using the Modified Bromley Methodology [Raposo et al., 2004a].

Mobilization studies are necessary to understand, for example, the arsenic behavior in natural systems where solid phases are involved because the sediments are important sinks of inorganic arsenic in natural systems [Irabien and Velasco, 1999]. Inorganic arsenic is not permanently accumulated in sediments since pH, temperature or redox potential changes and the presence of organic matter or ionic exchange processes can cause its mobilization [Luoma and Bryan, 1981]. As stated before, the mobilization studies were normally based on extraction procedures [Kennedy et al., 1997; Gupta and Chen, 1975; McLaren and Crawford, 1973; Oughton et al., 1992; Tessier et al., 1979; Martin et al., 1987] in order to predict the processes taking place in the natural systems. A new methodology to evaluate the metal mobilization/retention potential of a sediment has been proposed in this PhD work based on the application of the German Norm DIN 19730 using the river water as the extractant of metals from riverine sediments as well as for estuarine water with estuarine sediments (see chapter 4 & chapter 5). In this chapter, the thermodynamic speciation of inorganic arsenic and the mobilization methodology is applied, to understand the arsenic behavior in estuarine conditions (Bilbao estuary, north of Spain).

The Bilbao estuary spreads 16 Km along the Nerbioi river (see Figure 6.1). Several sampling sites were selected to cover the three different zones in the estuary: Arriluze, Las Arenas, Lamiako, Asua, San Inazio, Nerbioi, Kadagua and Ibaizabal.

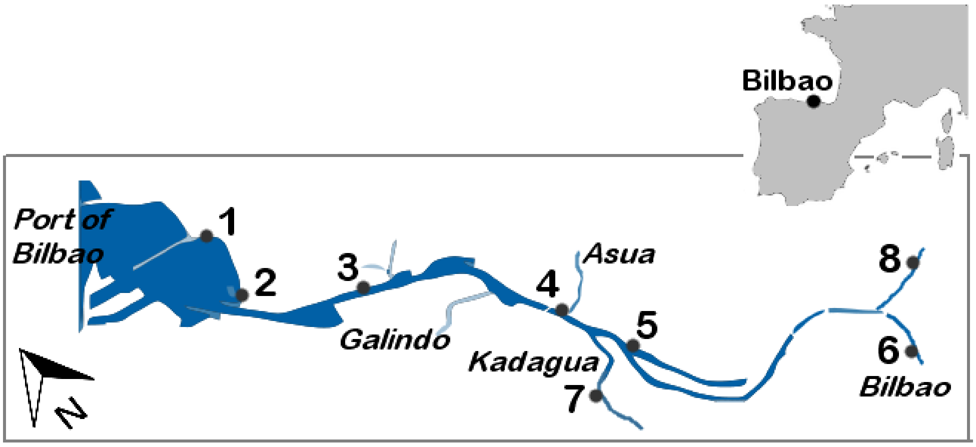


Figure 6.1. *Sampling points in the Bilbao estuary: 1. Arriluze, 2. Las Arenas, 3. Lamiako, 4. Asua, 5. San Inazio, 6. Nerbioi, 7 Kadagua and 8. Ibaizabal.*

In each sampling site sediment and water samples were collected and several physicochemical variables were measured: pH, pE, medium composition, ionic strength, Ca(II) and Mg(II) [Sadiq, 1992] and Fe(III) [vanderHoek and Comans, 1996]. These parameters were selected as the main variables conditioning the arsenic distribution between the aqueous and solid (sediment) phases. Finally, the presence of solid arsenates in the estuary sediments was confirmed by Raman spectroscopy employing a new methodology to reduce fluorescence interference (see chapter 3).

6.1. Experimental

6.1.1. Sampling Procedure

Surface water samples were collected along different sites of Bilbao estuary system (see Figure 6.1.). Five of the sampling points were collected along the estuary system with tidal influence, and so, water samples were collected both at low (L) and high (H) tides. These sampling points corresponded to Arriluze located in a corner beach of the Abra Bay (N 43° 20' 15.6", W 03° 00' 38.4"), Las Arenas (N 43° 19' 36.6", W 03° 01' 03.6"), Lamiako (N 43° 19' 00.5", W 02° 59' 55.7"), Asua (N 43° 17' 36.0", W02° 58' 11.0") and San Inazio (N 43° 17' 00.5", W 02° 05' 48.1"). The rest sampling sites were located in rivers without tidal influence: Kadagua (N 43° 16' 25.6", W 02° 58' 39.2"), Nerbioi (N 43° 14' 02.5", W 02° 53' 01.4") and Ibaizabal rivers (N 43° 13' 57.7", W 02° 52' 03.4").

Natural water samples were manually collected from the top surface on the banks of the river in pre-cleaned polyethylene bottles and stored at 4°C until analysis. As each sample was collected, temperature, pH and electrical conductivity were in-situ measured by means of a precalibrated YSI 556 multiparametric probe (YSI Environmental, Yellow Springs, Ohio).

The sediment samples were collected in the same sampling sites where water samples were taken. Sediment samples were scrapped from 5 cm of river-bed surface, introduced in plastic recipients, transported to the laboratory in cold boxes and frozen until freeze-drying. The sediments were freeze-dried at -52 °C and 10^{-1} bar for 24 h. The lyophilized samples were sieved through a 63 µm mesh and stored at −20 °C until analysis [Raposo et al., 2006].

6.1.2. Reagents and solutions

1000 mg·l^{1} Merck (p.a.) stock solutions of As(V), Fe(III), Ca(II) and Mg(II) were used. HCl (p.a.), HNO_3 (69%, Tracepure), NaOH (p.a.) and $NaBH_4$ (p.a.) were also purchased from Merck (Darmstadt, Germany). All reagents were of analytical-reagent grade and ultrapure water (Milli-Q® quality, Millipore™, Bedford, MA, USA) was used. Regarding the acetone (HPLC grade) used for the treatment of sediment samples for Raman analyses, it was purchased from Labscan (Dublin, Ireland).

6.1.3. Instrumentation

A Cryodos-50 (Telstar S.A., Terrasa, Spain) laboratory freeze-dryer, an Octagon digital sieve (Endecotts, London, UK) was used to recover the 63 µm particle size sediment from the bulk sediment. A Perkin-Elmer model 4110 ZL atomic absorption spectrophotometer equipped with a FIAS 400 and graphite furnace with Zeeman background correction (GF-AAS) was employed in the As and Fe determinations for water samples. Inductively coupled plasma – optical emission spectrometry (ICP-OES) (ARL Fisons 3410) was used for Ca and Mg determinations in aqueous samples and for As, Fe, Ca and Mg measurements in sediments.

A fiber optic microprobe coupled to a Raman spectrometer, Renishaw RA-100, was used in the sediment characterization. The diode laser used had a power of 50 mW at the source and a wavelength of 785 nm. The spectra were recorded using a charge-coupled device (CCD) detector, with integration times between 100 and 300 s. In order to avoid any thermal degradation of the samples, a 10% neutral density filter was used. An objective lens with a magnification of 20x allowed spectra to be collected from particles with a diameter down to 5 µm. Spectra were generally acquired between 200 and 3000 cm^{-1} and several scans were accumulated for each spectrum in order to improve the signal-to-noise ratio. The data were obtained with WIRE 2.0 software (Renishaw, UK), and their analysis and treatment was realized with the Omnic software (Thermo Fisher-Nicolet, USA).

6.1.4. Analytical procedure

Rigorous cleaning procedures of all the laboratory ware and other equipment that comes into contact with samples must be employed in order to avoid contamination of samples. All glassware and plastic ware were washed with a common detergent and thoroughly rinsed with abundant pure water (Elix® quality, Millipore™, Bedford, MA, USA). After that, all the laboratory ware was soaked into a clean dilute HNO_3 (15%) bath for 24 h. Thereafter, the material was rinsed with ultrapure water (Milli-Q® quality, Millipore™, Bedford, MA, USA).

The water samples (500 ml) were filtered through cellulose filters (0.45 µm, Whatman) in a pre-cleaned standard borosilicate vacuum system. All the filtered solutions were acidified with concentrated HNO_3 (pH<2), stored at 4°C and protected against light until analysis of Ca, Mg, Fe and As by means of GF-AAS and ICP-OES.

The pretreated sediments (freeze-drying and sieving), were acid digested (0.5 g) with 15 ml of an $HCl:HNO_3:H_2O$ (1:1:3 vol) mixture in a microwave system (Prolabo, Microgest 3.6). The digestion program was optimized in a previous work [Navarro et al., 2006]. All the acid extracts were measured by ICP-OES. The samples were analyzed in triplicate.

For metal mobilization experiments, the extraction procedure described in chapter 4, which is based on German Standard DIN 19730 with modifications, was used. Briefly, 20 g of each sediment sample was constantly shaken for 2 hours using 50 ml of the own natural water as the extracting agent. Hence, the estuarine sediments were subjected to two different extractions: (a) using waters collected in high tide (H) and (b) using low tidal (L) waters. Once the extraction time was over, all the samples were filtered through PVDF filters (0.45 µm, Whatman) and the Ca, Mg, Fe and As total concentrations of each solution were measured afterwards by means of GF-AAS and ICP-OES.

Regarding the Raman analyses of sediment samples, the measurements were performed directly on samples which were previously sonicated for 30 minutes with acetone (10 ml x 3 times) and dried at 35°C (see chapter 3 for more details).

6.2. Results

The physicochemical parameters (i.e., pH, redox potential and electrical conductivity) of water samples at high (H) and low (L) tides measured in situ are summarized in Table 6.1. Calculated ionic strength is also shown in Table 6.1.

Table 6.1. *Ionic strength (I: mol·dm^{-3}), pH, redox potential (pE: mV), and conductivity (C: µS·cm^{-1}) in water samples.*

Sampling point*	Tide	I	pH	pE	C
Arriluze (H)	High	0.74 ± 0.01	8.0 ± 0.1	82 ± 1	37600 ± 150
Las Arenas (H)	High	0.63 ± 0.01	7.9 ± 0.1	79 ± 1	37200 ± 150
Lamiako (H)	High	0.60 ± 0.01	7.8 ± 0.1	81 ± 1	32700 ± 130
Asua (H)	High	0.41 ± 0.01	7.6 ± 0.1	72 ± 1	28000 ± 110
San Inazio (H)	High	0.31 ± 0.01	7.7 ± 0.1	88 ± 1	19400 ± 70
Arriluze (L)	Low	0.71 ± 0.01	8.0 ± 0.1	106 ± 1	36500 ± 140
Las Arenas (L)	Low	0.59 ± 0.01	7.6 ± 0.1	95 ± 1	33800 ± 140
Lamiako (L)	Low	0.38 ± 0.01	7.7 ± 0.1	76 ± 1	24200 ± 100
Asua (L)	Low	0.32 ± 0.01	7.9 ± 0.1	88 ± 1	19200 ± 70
San Inazio (L)	Low	0.22 ± 0.01	7.8 ± 0.1	93 ± 1	14100 ± 60
Nerbioi	--	0.013 ± 0.005	7.8 ± 0.1	152 ± 1	763 ± 3
Kadagua	--	0.011 ± 0.005	8.2 ± 0.1	182 ± 1	603 ± 2
Ibaizabal	--	0.007 ± 0.005	8.5 ± 0.1	28 ± 1	300 ± 1

** See Locations in Figure 6.1. H: high tide, L: low tide.*

The total metal concentrations found for As (mg·kg^{-1}) and Fe (g·kg^{-1}) in sediments collected at each sampling site are shown in Table 6.2.

Table 6.2. *Concentration of As (mg·kg^{-1}) and Fe (g·kg^{-1}) in sediments (95% confidence level, n=3).*

Sampling point	As	Fe
Arriluze	27.0 ± 0.7	22.3 ± 0.7
Las Arenas	11.4 ± 0.5	17.8 ± 0.5
Lamiako	115 ± 3	24.6 ± 0.7
Asua	20.0 ± 0.5	6.3 ± 0.2
San Inazio	13.3 ± 0.3	6.8 ± 0.2
Nerbioi	21.0 ± 0.5	37 ± 1
Kadagua	19.0 ± 0.5	18.9 ± 0.6
Ibaizabal	16.0 ± 0.4	21.8 ± 0.7

Table 6.3. shows the total concentration of metals in natural waters for As, Fe, Ca and Mg at high and low tides. The samples were analyzed in triplicate. Calculated limit of detection for As was 0.50 µg·l^{-1}, for Fe was 1.00 µg·l^{-1}, for Ca was 0.80 mg·l^{-1} and for Mg was 0.60 mg·l^{-1}. Calculated limits of quantification were established in 0.65 µg·l^{-1} for As, 1.20 µg·l^{-1} for Fe, 0.90 mg·l^{-1} for Ca and 0.71 mg·l^{-1} for Mg.

Table 6.3. *Total concentration of As (µg·l^{-1}), Fe (µg·l^{-1}), Ca (mg·l^{-1}) and Mg (mg·l^{-1}) in natural waters and mobility values ($^{0}/_{00}$) for As and Fe. (H) Corresponds to High tide values and (L) to low tide values. (n=3, 95% of confidence level).*

Sampling point	As	Fe	Ca	Mg	M_{As}	M_{Fe}
Arriluze (H)	13.2 ± 0.4	15.8 ± 0.5	406 ± 1	1148 ± 1	0.51	0.02
Las Arenas (H)	6.1 ± 0.3	15.8 ± 0.5	365 ± 1	1141 ± 1	0.64	0.01
Lamiako (H)	6.7 ± 0.3	26.3 ± 0.6	354 ± 1	980 ± 1	0.10	0.02
Asua (H)	12.6 ± 0.4	71.1 ± 0.9	321 ± 1	876 ± 1	2.62	0.07
San Inazio (H)	6.5 ± 0.3	14.8 ± 0.5	312 ± 1	575 ± 1	4.45	0.05
Arriluze (L)	6.0 ± 0.3	108 ± 1	412 ± 1	1112 ± 1	0.46	0.01
Las Arenas (L)	9.6 ± 0.4	29.3 ± 0.6	393 ± 1	891 ± 1	-0.25	0.01
Lamiako (L)	18.9 ± 0.5	23.6 ± 0.6	291 ± 1	690 ± 1	0.09	0.04
Asua (L)	48.0 ± 0.9	43.8 ± 0.8	231 ± 1	567 ± 1	-4.34	0.12
San Inazio (L)	44.4 ± 0.9	140 ± 1	178 ± 1	178 ± 1	-5.25	0.01
Nerbioi	26.6 ± 0.8	12.4 ± 0.5	80.0 ± 0.7	8.0 ± 0.4	1.29	-0.04
Kadagua	25.3 ± 0.8	4.6 ± 0.1	91.0 ± 0.7	8.0 ± 0.4	-1.21	0.12
Ibaizabal	25.2 ± 0.8	18.0 ± 0.5	63.0 ± 0.6	1.0 ± 0.2	-0.19	0.84

The mobilized fractions of As and Fe using the natural waters are also summarized in Table 6.3. The mobilized portion (M_i, $^{0}/_{00}$) of a given element, referred to dried sediment, can be calculated by means of the equation 6.1:

$$\mathrm{M_i} = \frac{2.5 * (\,C_1 - C_0)}{(1000 * C_{sed})}$$

Equation 6.1. *Calculation of the mobilized portion from each trace metal referred to the sediment.*

where: C_1 and C_0 are the concentrations ($\mu g \cdot l^{-1}$) of mobilized element in natural waters after and before the extraction process, respectively. C_{sed} is the concentration ($mg \cdot kg^{-1}$) of the mobilized element in the dried sediment.

In order to found similarities in the behavior of the studied sampling sites and the measured physicochemical parameters, a statistical analysis of the whole data was carried out. The correlation analysis to determinate the Person coefficient was performed using The Unscrambler® (v. 7.5) program [Camo, 1998]. In all the correlation analysis conducted, a threshold value of 0.7 ($r > 0.7$ or $r < -0.7$ were considered as indicators of a positive or negative correlation respectively) was set in order to indicate a strong correlation between the studied variables.

In a first attempt, the correlation values (see Table 6.4) between different sampling points were calculated taking into account all the studied variables (i.e., physicochemical parameters and metal concentrations of sediment samples for each sampling point).

Table 6.4. *Correlation matrix obtained for all sampling points. Significant parameters marked in bold.*

	Arriluze	Las Arenas	Lamiako	Asua	San Inazio	Nerbioi	Kadagua	Ibaizabal
Arriluze	1.00							
Las Arenas	**1.00**	1.00						
Lamiako	**0.96**	**0.94**	1.00					
Asua	**0.97**	**0.98**	**0.87**	1.00				
San Inazio	**0.99**	**1.00**	**0.93**	**0.99**	1.00			
Nerbioi	0.48	0.42	**0.70**	0.26	0.39	1.00		
Kadagua	0.49	0.43	0.69	0.27	0.40	**1.00**	1.00	
Ibaizabal	0.47	0.40	0.68	0.24	0.37	**1.00**	**1.00**	1.00

Secondly, the correlation between the chemical parameters was also assessed taking into account the two different environmental systems studied. On the one hand, the parameters measured in the three sites of the river system were considered (Kadagua, Nerbioi and Ibaizabal) and the results are summarized in Table 6.5. On the other hand, the parameters of the five sampling points corresponding to the estuary system were considered (Arriluze, Las Arenas, Lamiako, Asua and San Inazio) both high (see Table 6.6) and low (Table 6.7) tidal conditions.

Table 6.5. *Correlation matrix obtained for different physicochemical parameters of river water samples. (Significant parameters are marked in bold).*

	pE	**pH**	**As(w)**	**As(s)**	**Fe(w)**	**Ca(w)**	**Mg(w)**	**M_{As}**	**M_{Fe}**	**Fe(s)**	**C**
pE	1.00										
pH	**-0.70**	1.00									
As(w)	0.39	**-0.93**	1.00								
As(s)	**0.83**	**-0.98**	**0.84**	1.00							
Fe(w)	0.19	0.34	0.03	-0.52	1.00						
Ca(w)	**0.98**	-0.53	0.19	0.69	**-0.98**	1.00					
Mg(w)	**0.98**	**-0.82**	0.55	**0.92**	**-0.82**	**0.92**	1.00				
M_{As}	-0.01	-0.65	**0.89**	0.49	0.49	-0.29	0.10	1.00			
M_{Fe}	**-0.94**	**0.91**	-0.69	**-0.97**	**0.70**	**-0.84**	**-0.98**	-0.27	1.00		
Fe(s)	0.19	**-0.83**	**0.98**	**0.71**	0.24	0.02	0.37	**0.96**	-0.52	1.00	
C	**0.93**	**-0.92**	**0.71**	**0.98**	-0.68	**0.82**	**0.98**	0.30	**-0.99**	0.55	1.00

Table 6.6. *Correlation matrix obtained for different physicochemical parameters of estuarine water samples, at high tide. (Significant parameters are marked in bold).*

	I	**pH**	**pE**	**C**	**Fe(s)**	**As(w)**	**As(s)**	**Fe(w)**	**Ca(w)**	**Mg(w)**	**M_{As}**	**M_{Fe}**
pE	1.00											
pH	**0.89**	1.00										
pE	-0.11	0.30	1.00									
C	**0.96**	**0.77**	-0.34	1.00								
Fe(s)	0.65	**0.79**	0.12	**0.78**	1.00							
As(w)	0.22	0.03	-0.48	0.22	-0.08	1.00						
As(s)	0.26	0.04	0.03	0.18	0.63	-0.24	1.00					
Fe (w)	-0.37	**-0.72**	**-0.84**	-0.18	-0.49	0.51	-0.03	1.00				
Ca (w)	**0.97**	**0.94**	0.03	**0.88**	**0.81**	0.33	0.12	-0.45	1.00			
Mg (w)	**0.94**	**0.74**	-0.40	**0.99**	**0.75**	0.29	0.15	-0.12	**0.87**	1.00		
M_{As}	**-0.92**	-0.69	0.28	**-0.93**	**-0.91**	-0.07	-0.52	0.19	**-0.80**	**-0.92**	1.00	
M_{Fe}	**-0.81**	**-0.88**	-0.31	**-0.74**	**-0.87**	0.37	-0.30	**0.75**	**-0.76**	-0.69	**0.77**	1.00

Table 6.7. *Correlation matrix obtained for different physicochemical parameters of estuarine water samples, at low tide. (Significant parameters are marked in bold).*

	I	**pH**	**pE**	**C**	**Fe(s)**	**As(w)**	**As(s)**	**Fe(w)**	**Ca(w)**	**Mg(w)**	**M_{As}**	**M_{Fe}**
pE	1.00											
pH	0.14	1.00										
pE	0.62	0.49	1.00									
C	**0.99**	0.06	**0.91**	1.00								
Fe(s)	0.67	-0.17	-0.04	**0.73**	1.00							
As(w)	**-0.89**	0.18	-0.34	**-0.93**	**-0.89**	1.00						
As(s)	-0.11	-0.23	-0.64	-0.03	0.63	-0.22	1.00					
Fe (w)	-0.13	0.54	0.58	-0.25	-0.35	0.23	-0.46	1.00				
Ca (w)	**0.98**	-0.03	0.49	**0.99**	**0.74**	**-0.94**	-0.08	-0.28	1.00			
Mg (w)	**0.96**	0.14	0.42	**0.97**	**0.74**	**-0.86**	0.08	-0.34	**0.96**	1.00		
M_{As}	**0.82**	-0.18	0.11	**0.88**	**0.96**	**-0.96**	0.44	-0.43	**0.89**	**0.88**	1.00	
M_{Fe}	-0.41	0.27	-0.42	-0.41	-0.46	0.62	0.05	-0.42	-0.42	-0.20	-0.42	1.00

6.3. Discussion

All the sampling points considered showed an apparent homogeneity in the parameter data measured (see Tables 6.1, 6.2 and 6.3). The sampling points near to open sea (Arriluze and Las Arenas) showed As concentration levels in natural waters lower than the inside ones (Lamiako, Asua and San Inazio) in low tide. As shown in Table 6.3, this decrease of dissolved As seems to be related to an increase of the Ca^{2+} and Mg^{2+} concentrations in water samples. This fact could lead to the possible solid arsenate formation in the estuarine sediments.

Considering the mobility results, a dynamic chemical cycle (low tide – high tide) could be concluded for the inorganic arsenic (see Table 6.3). On the one hand, using high tide water as extractant for mobilization experiments, a positive mobilization potential was obtained. In fact, within a few hours, the arsenic content of the waters increased in the samples collected in the next tidal cycle. On the other hand, using low tide water with higher arsenic content for the mobilization experiments, a negative mobilization potential of As (retention) was obtained. This retention potential would produce a decrease in the arsenic contents in the natural waters; higher arsenic concentration is detected thereafter in high tides. Therefore, a mobilization/retention cycle took place depending on the tidal situation.

A transport of inorganic arsenic from inside zones (San Inazio) to open sea (Arriluze) should be expected. This was supported by the increase in the arsenic concentration in sediments from San Inazio to Arriluze locations (see Table 6.2). The high content of both arsenic and iron in Lamiako sampling site was probably due to the sampling site is close to the effluent of the wastewater treatment plant of the high furnace factory. The values of arsenic and iron found in the three river sediments (Nerbioi, Kadagua and Ibaizabal) can probably come from natural sources (i.e., the natural composition of geological materials in the basin) rather than industrial ones.

6.3.1. Correlation analysis of the chemical parameters

In order to assess the homogeneity of the whole set of chemical data, all the chemical parameters of the sampling sites (see results in Tables 6.1, 6.2 and 6.3) were statistically evaluated.

From the correlation coefficients summarized in Table 6.4, the high correlation among the three sampling sites (Ibaizabal, Kadagua and Nerbioi) of river waters without tidal influence could be highlighted. Besides, the five sampling sites with estuarine waters were highly correlated, being Lamiako location the site less correlated with the other four ones; the anomalous arsenic and iron concentration in the sediments from Lamiako could explain this lower correlation.

When comparing the sites with and without tidal influence, the absence of correlation was clearly shown. Thus, the correlation analysis among the chemical variables should be performed separately because the absence of homogeneity between the two groups of sampling sites (estuarine and river systems) would promote unreliable correlation indexes.

In fact, the three sampling sites without tidal influence show similar behavior to that previously described in the literature [Raposo et al., 2004b] for five river waters. This is summarized in Table 6.5 where the correlation indexes among all the chemical parameters for the three sampling sites without tidal influence are shown.

The arsenic mobility was only, but highly correlated, with the iron in the sediment (0.96) and with the dissolved arsenic (0.89). The dissolved arsenic was highly correlated with the iron content in the sediment (0.98). Moreover, the arsenic in the sediment was negatively correlated with the iron mobility (-0.97) and positively (0.71) with the iron in the sediment. All this was in agreement with previous results found for river waters [Raposo et al., 2004b], suggesting the influence of iron on the arsenic behavior in such waters, probably through the formation of iron arsenate.

The correlations found in river waters disappeared in the case of estuary system. Table 6.6 summarizes the correlation values among the chemical parameters in high tide conditions and Table 6.7 shows the correlation values obtained using the data in low tidal conditions for the five estuarine sampling sites.

As it can be seen, in high tidal conditions, the concentration of arsenic found in sediments as well as the dissolved arsenic were not correlated with any of the other studied parameters. Regarding the arsenic, its mobility was the only parameter that showed significant correlation with the rest of parameters. It was negatively correlated with the dissolved magnesium (-0.92), the dissolved calcium (-0.80), the ionic strength (-0.92) and the conductivity (-0.93). It was also negatively correlated with the iron content in sediments (-0.91) and positively with the iron mobility (0.77), just in an opposite trend to that shown for the river system.

With regard to the low tidal conditions, as for the previous conditions, the content of arsenic in sediment was not correlated with any other parameter. Similarly to the previous described correlation factors, the arsenic mobility was correlated with dissolved magnesium (0.88), the dissolved calcium (0.89), the ionic strength (0.82) and the conductivity (0.88). However, the sign of the correlation values was just the opposite of what has been found in high tide water (positive instead of negative or vice versa). Actually, the dissolved arsenic was correlated with the arsenic mobility (-0.96) but in the opposite way to the correlation shown in river waters.

Thus, the arsenic behavior in the estuarine system was not controlled by iron, as it has seen in rivers, but by parameters related to the sea influence such as the concentration of magnesium and calcium and the salinity (which is reflected in the ionic strength and conductivity values). The negative correlation between arsenic mobility and these parameters in high tidal conditions confirmed the higher mobilization potential of the sediments with the less saline waters. On the other hand, in low tidal conditions, the inner locations showing lower salinity, were the less mobile. In fact, San Inazio and Asua sampling sites show high negative mobilities that meant retention potential for dissolved arsenic (see Table 6.3).

6.3.2. Thermodynamical speciation and spectroscopic confirmation

In an attempt to explain such different behavior of arsenic from river to estuarine waters and the tidal influence in the estuarine system, some theoretical calculations were performed by using the thermodynamic model of inorganic arsenic.

This model covers all the possible equilibria of arsenic (i.e., acid-base, redox, complexation with iron, precipitation with iron, calcium and magnesium), defined by the corresponding thermodynamic equilibrium constants, together with the interaction parameters of the different arsenic species and the ions present in the waters considered in this work.

As the theoretical calculations must be performed in molar scale, thus, the stoichiometric equilibrium constants must be first calculated from the thermodynamic ones by computing the activity coefficient of each species involved in a given equilibrium. For example as per equation 6.2.

$$K_{H_3AsO_3} = {}^{o}K_{H_3AsO_3} \frac{\gamma_{H^+} \cdot \gamma_{H_2AsO_3^-}}{\gamma_{H_3AsO_3}}$$

Equation 6.2. *Calculation of the stoichiometric equilibrium constant from the thermodynamic constant for the specie H_3AsO_3. K is the stoichiometric constant, ${}^{0}K$ is the thermodynamical constant and γ is the activity coefficient for each specie involved in the equilibrium.*

The activity coefficient values depend on the composition and ionic strength [Borge et al., 1996a], which is defined considering only the majority ions in solution as per equation 6.3.

$$I = 0.5 \sum C_i Z_i^2$$

Equation 6.3. *Calculation of the Ionic strenght as per sum of the product of each specie concentration by the square of its charge.*

being Z_i the charge of each ion. As the composition and ionic strength change for each sampling point, the set of stoichiometric equilibrium constants will change from one site to another.

Different models have been developed to determine the activity coefficients as a function of the ionic strength. The Modified Bromley´s Methodology (MBM) has been proved as a consistent tool for these calculations in different equilibria and for both single and multicomponent systems [Borge et al., 1996a; Borge et al., 1996b].

The activity coefficients were calculated using the equations 6.2 and 6.3 and considering all the thermodynamic data of inorganic arsenic calculated as well as the chemical parameters of all the sampling sites. The arsenic distribution was computed by means of the MEDUSA program taking into account the set of stoichiometric constants previously calculated [Puigdomenech, 1999]. These computations require the knowledge of the different stoichiometric equilibrium constants. To this purpose, the concentration of ions can be used to estimate the activity coefficient value (γ) of the inorganic arsenic species. Then, the corresponding γ values are used to compute the stoichiometric equilibrium constant from the corresponding thermodynamic ones: inorganic arsenic, iron [Belaustegui et al, 1999] and calcium and magnesium species [Borge et al., 1996a; Borge et al., 1996b]. These sets of stoichiometric equilibrium constants allow solving the mass balance equations of inorganic arsenic in order to calculate the free concentrations (taking into account the possible side reactions with iron, calcium and magnesium). From the thermodynamic model and based on the experimental redox potential of samples it could be concluded that the As(III) are always minor species not more than 1.2% molar.

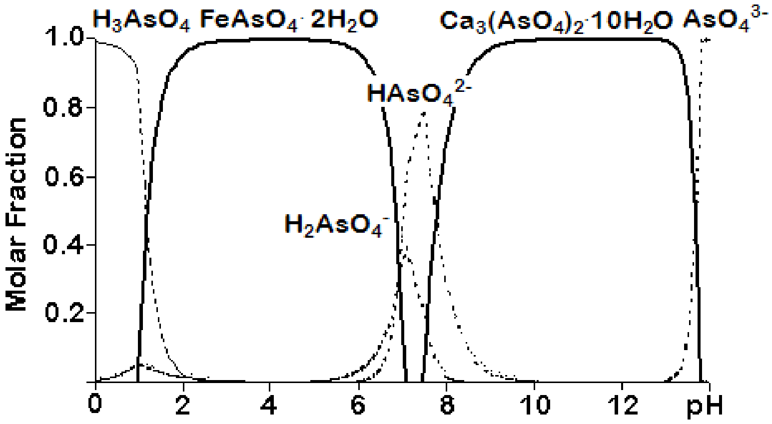


***Figure 6.2**. Theoretical distribution of arsenate species in river waters for the Ibaizabal experimental conditions.*

The computations performed with the particular conditions of the three sampling sites of river waters show Ca(II) and Fe(III) as the main factors to control the arsenate distribution through the formation of the solid Ca3(AsO4)2(s) and FeAsO4(s) species. As an example, Figure 6.2 shows the distribution of arsenic (V) species as a function of pH for the Ibaizabal sampling point (pH: 8.5). When the dissolved iron concentration is higher than dissolved arsenic the formation of $FeAsO_4$(s) is favored at pH values of river waters, against calcium arsenate. This is in agreement with previous works where the iron arsenate precipitation was defined as the variable that controls the distribution of arsenate species between solid and aqueous phases [Raposo et al., 2004a; Raposo et al., 2004b].

On the contrary, in estuary conditions, the inorganic arsenic distribution can be affected by the presence of salinity, Ca(II) and mainly Mg(II), which can be different depending on the tidal situation. In high tidal conditions, where a high ionic strength and low dissolved arsenic was observed for the five sampling sites, precipitation of any solid phases is not favored at the standard pH values of the estuarine waters. As an example, Figures 6.3 and 6.4 show the arsenic(V) species distribution for the particular conditions of the Asua sampling site at high and low tides respectively.

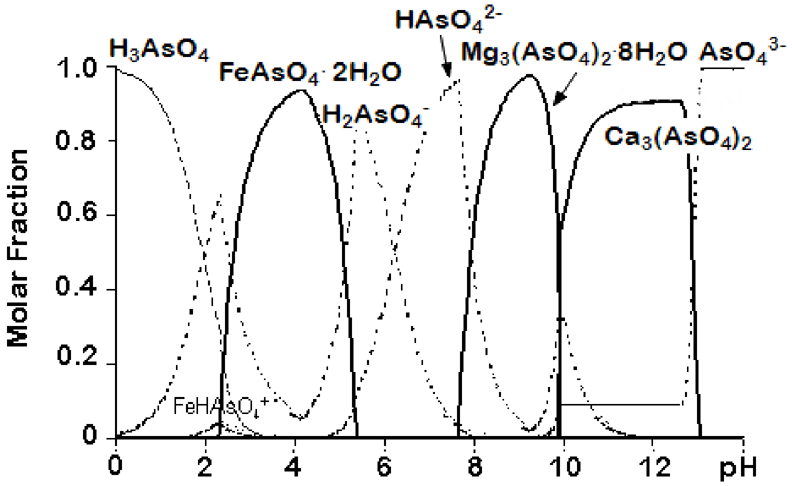


Figure 6.3. *Theoretical distribution of arsenate species in estuarine waters for the Asua location concentrations in high tide.*

However, in low tides, the formation of $Mg_3(AsO_4)_2$(s) is favored in practically all the estuary locations (high dissolved arsenic), being $Ca_3(AsO_4)_2$(s) slightly favored in the inside part of the estuary when dissolved magnesium concentration decreases sensibly. Figure 6.4 shows the arsenic (V) species distribution for the particular conditions in the Asua sampling site in low tide. This arsenic precipitation with magnesium and calcium will explain the high retention potential for inorganic arsenic observed in the mobilization experiments using the waters collected in low tide conditions.

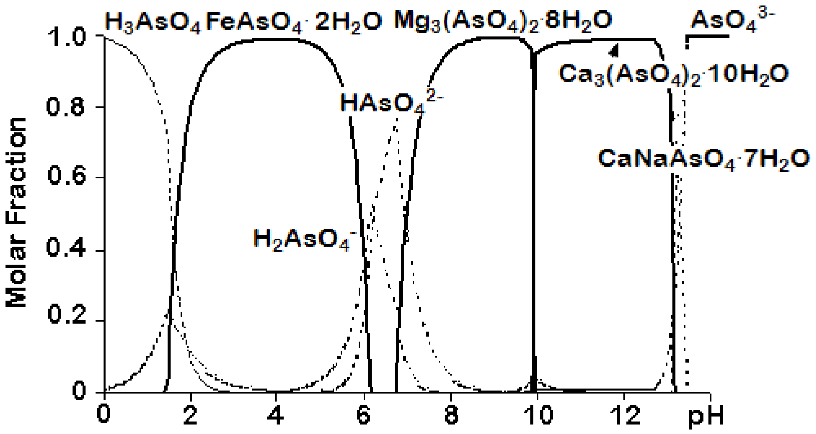


Figure 6.4. *Theoretical distribution of arsenate species in estuarine waters for the Asua location concentrations in low tide.*

In order to confirm the possible presence of these species in sediment samples experimentally, sediment samples were analyzed by Raman spectroscopy as detailed in experimental section. $FeAsO_4$(s) was detected in the sediment samples from the three river sampling sites. Together with these species, the presence of $Ca_3(AsO_4)_2$(s) was also detected. In the case of sediments from estuarine sites both $Mg_3(AsO_4)_2$(s) and $Ca_3(AsO_4)_2$(s) were detected in different relative proportions.

As an example, Figure 6.5 shows one of the Raman spectrum collected from the sediment of the Arriluze sampling site, together with the spectra of standard $Mg_3(AsO_4)_2$(s) and $Ca_3(AsO_4)_2$(s) obtained in our laboratory [Raposo et al., 2004c]. According to this last study, the characteristic Raman bands of $Mg_3(AsO_4)_2$ are 877 cm^{-1}, 810 cm^{-1}, whereas 848 cm^{-1} is the characteristic Raman band for $Ca_3(AsO_4)_2$. As can be seen, the presence of both arsenates (calcium and magnesium) could be confirmed.

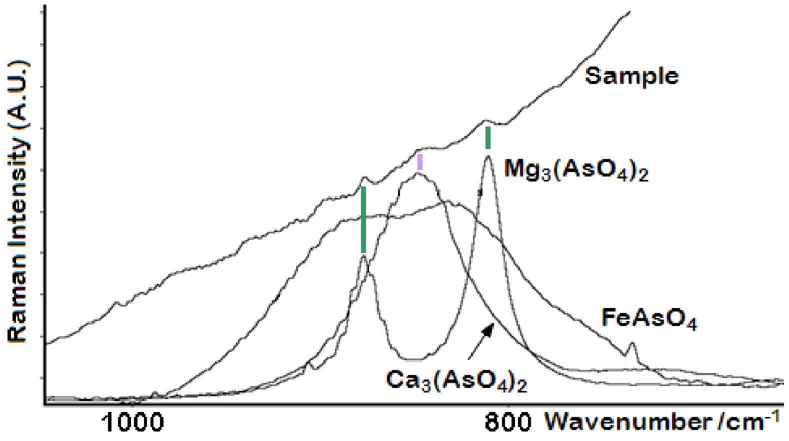


Figure 6.5. *Raman spectrum obtained from the sediment collected in Arriluze sampling point together with the Raman spectra of standard calcium, magnesium and iron arsenates.*

6.4. Conclusions

A basic chemical model for inorganic arsenic has been successfully applied to define the different chemical behavior of arsenic in estuarine systems.

On the one hand, modified mobility methodology (Chapter 4) has been used to evaluate the behavior of the arsenic estuarine system. The more abundant metals in the estuarine system (Ca, Mg, Fe) were studied in order to predict arsenic conduct and relationships. Stoichiometric constants were calculated having into account the ionic strength and concentrations and the system model was described using a computer program in order to plot a logarithmic diagram of the majority species versus the system pH. This plot helped to describe which arsenic species were forming and what were the main factors affecting the arsenic distribution.

Different situations can be expected for estuarine and river systems. In river situations, tidal influence can be disregarded due to there is almost no change in the conductivity values. In this case, arsenic distribution is mainly controlled by Ca and Fe concentration. Usually iron is more abundant being the $FeAsO_4$ the main arsenic solid specie precipitated.

In estuarine situations when the tidal influence is high (high conductivity in high tide but lower conductivity in low tide, i.e., great contribution of the sea when high tide appears, but river water replacement at low tide), the main factors controlling the arsenic system are the concentration of Ca and Mg. Whilst in high tide there is almost no arsenic solid specie formation, in low tide $Mg_3(AsO_4)_2$ precipitation is preferred or $Ca_3(AsO_4)_2$ in case the concentration ratio between Mg and Ca changes in favor of Ca.

On the other hand, Raman spectroscopy data allowed the confirmation of the predictions performed from the basic chemical model as well as the assumptions made from the mobilization studies carried out [Raposo et al., 2006]. The use of the proposed methodology (Chapter 3) to diminish fluorescence effect from the direct Raman measurement on sediments, allowed finding some spectra where arsenic species could be elucidated.

6.5. References

[Belaustegui et al., 1999] Belaustegui Y, Olazabal MA, Madariaga JM. *Development of a Modified Bromley's Methodology for the estimation of ionic media effects on solution equilibria Part 4. The chemical model of Fe(III) with the halide ligands in aqueous solution at 25 degrees C.* Fluid Phase Equilibr. 1999; 155, 21 – 31.

[Borge et al., 1996a] Borge G, Castaño R, Carril MP, Corbillon MS, Madariaga JM. *Development of a modified Bromley´s methodology (MBM) for the estimation of the ionic media effects. 1. Calculation of the interaction parameters in the molar and molal scales at 25ºC.* Fluid Phase Equilibr. 1996; 121, 85 – 98.

[Borge et al., 1996b] Borge G, Etxebarria N, Fernandez LA, Olazabal MA, Madariaga JM. *Development of a modified Bromley´s methodology (MBM) for the estimation of the ionic media effects. 2. Correlation of the molar and molal interaction parameters with the charge and crystal radii of the ions.* Fluid Phase Equilibr. 1996; 121, 99 – 109.

[CAMO, 1998] CAMO. *Unscrambler User Manual*, Oslo, Norway. 1998.

[Gupta and Chen, 1975] Gupta SK, Chen KY. *Partitioning of trace metals in selective chemical fractions of nearshore sediments.* Environ. Lett. 1975; 10, 129 – 158.

[Irabien and Velasco, 1999] Irabien MJ, Velasco F. *Heavy metals in Oka river sediments (Unrdaibai National Biosphere Reserve, northern Spain): lithogenic and anthropogenic effects.* Environ. Geol. 1999; 37, 54 – 63.

[Kennedy et al., 1997] Kennedy VH, Sanchez AL, Oughton DH, Rowland AP. *Use of single and sequential chemical extractants to assess radionuclide and heavy metal availability from sediments for root uptake.* Analyst. 1997; 122, 89-100.

[Navarro et al., 2006] Navarro P, Raposo JC, Arana G, Etxebarria N. *Optimisation of microwave assisted digestion of sediments and determination of Sn and Hg.* Anal. Chim. Acta 2006; 566, 37 – 44.

[Luoma and Bryan, 1981] Luoma SN, Bryan GW. *A statistical assessment of the form of trace metals in oxidized estuarine sediments employing chemical extractants.* Sci. Total Environ. 1981; 17, 165 – 196.

[Martin et al., 1987] Martin JM, Nirel P, Thomas AJ. *Sequential extraction techniques: promises and problems.* Mar. Chem. 1987; 22, 313 – 341.

[McLaren and Crawford, 1973] McLaren RG, Crawford DV. *Soil copper I. Fractionation of copper in soils.* J. Soil Sci. 1973; 24, 172 – 181.

[Oughton et al., 1992] Oughton DH, Salbu B, Riise G, Lien H, Ostby G, Noren A. *Radionuclide mobility and bioavailability in Norwegian and soviet solids.* Analyst. 1992; 117, 481 – 486.

[Puigdomenech, 1999] Puigdomenech I. *Medusa: Make Equilibrium Diagrams using Sophisticated Algorithms. Department of Inorganic Chemistry*, The Royal Institute of technology, Stokholm, Sweden, 1999.

[Raposo et al., 2002] Raposo JC, Sanz J, Zuloaga O, Olazabal MA, Madariaga JM. *Thermodynamic model of inorganic arsenic species in aqueous solutions. Potentiometric study of the hydrolytic equilibrium of arsenic acid.* Talanta. 2002; 57, 849 – 857.

[Raposo et al., 2003a] Raposo JC, Zuloaga O, Olazabal MA, Madariaga JM. *Development of a modified Bromley´s methodology (MBM) for the estimation of the ionic media effects. 6. The chemical model of phosphoric acid in aqueous solution at 25°C and comparison with arsenic acid.* Fluid Phase Equilibr. 2003; 207, 69 – 80.

[Raposo et al., 2003b] Raposo JC, Zuloaga O, Olazabal MA, Madariaga JM. *Development of a modified Bromley´s methodology (MBM) for the estimation of the ionic media effects. 6. The chemical model of boric acid in aqueous solution at 25°C and comparison with arsenious acid.* Fluid Phase Equilibr. 2003; 207, 81 – 95.

[Raposo et al., 2003c] Raposo JC, Sanz J, Zuloaga O, Olazabal MA, Madariaga JM. *Thermodynamic model of inorganic arsenic species in aqueous solutions. Potentiometric study of the hydrolytic equilibrium of arsenious acid.* J. Sol. Chem. 2003; 32, 253 – 264.

[Raposo et al., 2004a] Raposo JC, Sanz J, Zuloaga O, Olazabal MA, Madariaga JM. *Validation of the thermodynamic model of inorganic arsenic in non polluted river waters of the Basque Country (Spain).* Talanta. 2004; 63, 683 – 690.

[Raposo et al., 2004b] Raposo JC, Olazabal MA, Madariaga JM. *Validation of chemical speciation model of inorganic arsenic in river waters with mobilization processes.* Chem. Spec. Bioavailab. 2004; 16, 9 – 15.

[Raposo et al., 2004c] Raposo JC, Zuloaga O, Olazabal MA, Madariaga JM. *Study of the precipitation of arsenate anion with calcium and magnesium in sodium perchlorate at 25°C.* App Geochem. 2004; 19, 855 – 862.

[Raposo et al., 2006] Raposo JC, Zuloaga O, Sanz J, Villanueva U, Crea P, Etxebarria N, Olazabal MA, Madariaga JM. *Analytical and thermodynamical approach to understand the mobility/retention of arsenic species from the river to the estuary. The Bilbao case study.* Mar. Chem. 2006; 99, 42 – 51.

[Sadiq, 1992] Sadiq M. *Toxic Metal Chemistry in Marine Environments.* Marcel Dekker (Ed.) Prentice Hall, New Jersey, 1992.

[Tessier et al., 1979] Tessier A, Campbell PCG, Bisson M. *Sequential extraction procedure for the speciation of particulate trace metals.* Anal. Chem. 1979; 51, 844 – 851.

[vanderHoek and Comans, 1996] vanderHoek EE, Comans RNJ. *Modeling arsenic and selenium leaching from acidic fly ash by sorption on iron hydroxide in the fly ash matrix.* Environ. Sci. Technol. 1996; 30, 517 – 523.

7. New methodological approach to determinate humic substances in sediments by focussed ultrasound energy (FUSLE)

7. New methodological approach to determinate humic substances in sediments by focussed ultrasound energy (FUSLE)

Organic substances are formed in the environment by residues from living beings and the transformations of these substances by chemical reactions or biological activity. A substantial proportion of carbon-containing substances in the environment can be referred to humic substances (HS) – it is estimated that humic substances account for around 50-90% of dissolved organic matter in freshwater systems [Hayes et al., 1990].

HS can be generally described as naturally occurring, highly decomposed, oxidized and polymerized organic substances with very complex structures [Stevenson and Schnitzer, 1982; Schulten and Schnitzer, 1993; Buffle et al., 1977]. They are derived from plant and animal residues (any kind of organic molecule including carbohydrates, lipids, terpenoids, tannins, pigments, aminoacids, etc) and are usually dark in colour as well as coal, leonardite and peat. HS can be defined as biomass in the Van Krevelen diagram (see Figure 7.1) [Kim et al., 2003]. The final compound of all of these C/H non-biological molecules after their deposition and sedimentation for years is kerogen [Vandenbroucke, 1980; Huc, 1980]. The basic characteristics, physicochemical properties and reactivity of HS are widely described in many classical monographs and review works [Aiken et al., 1985; Hayes et al., 1990; Stevenson et al., 1994; Davies et al., 1998].

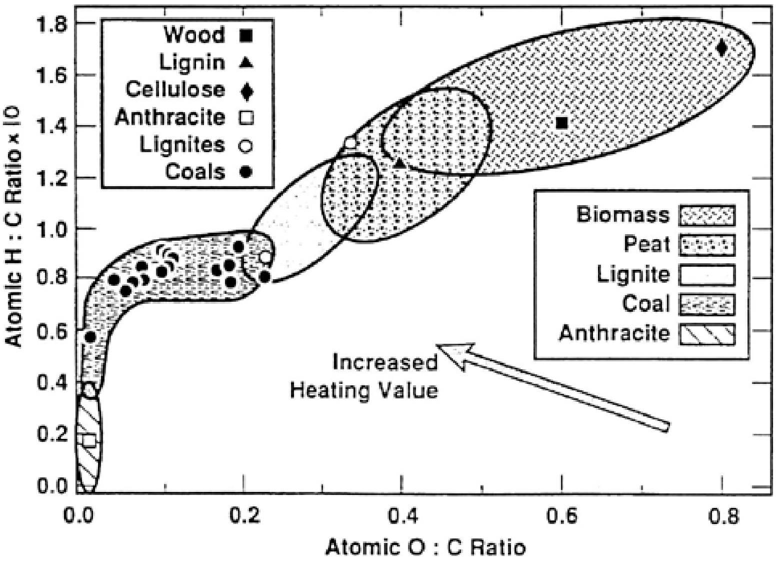


***Figure 7.1**. Van Krevelen diagram. In the Y axis, the value of the 10·H/C ratio is displayed. In the X axis, the O/C ratio. The area within the ratios clasiffies the organic matter within a group [Van Loo and Koppejan, 2007.*

HS are classified depending on their solubility [Aiken et al., 1985] and are divided into three groups trying to classify them or sorting them somehow despite their heterogeneity.

Humic acids (HAs) are defined as the fraction of humic substances that are not soluble under acid conditions (bellow pH 2) but are soluble at higher pH values. They are often refered to the highest molecular weight fraction (weight ranges of 1500-5000 Da and 50000-500000 Da in streams and sediments, respectively). In addition, they have a higher polymerization degree than the rest of the fractions. In figure 7.2, it can be seen the behaviour of the humic acid depending on the pH in solution.

Fulvic acids (FAs) are corresponding to the fraction of humic substances that are soluble in water at any pH conditions. This fraction is composed with substances with lower molecular weight (ranging from 600 to 1000 Da in streams and a little bit higher in soils (1000 to 5000 Da)) and contains more acidic functional groups.

Humin (HN) is composed by humic substances that are not soluble in water at any pH. Their molecular weights are considerably higher than the rest, and due to their deposition tendencies and ground accumulation and sedimentation, these molecules became the main kerogen precursor [Durand, 1980].

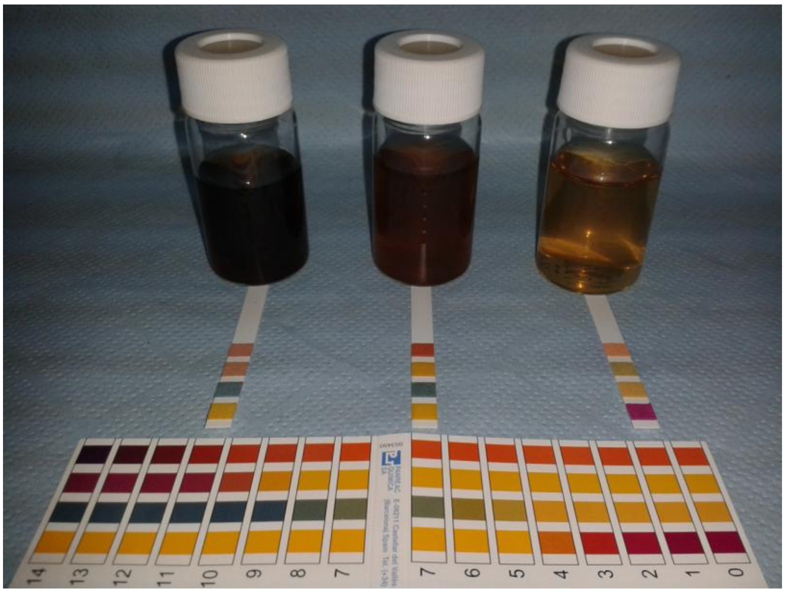


Figure 7.2. *Behaviour of the humic acid in solution. From left to right it can be seen a precipitation due to pH decrease.*

Even though it is not possible to accurately detail the structure of a typical HS, it is widely accepted that HAs and FAs show a great complexing ability for metals. This property is attributed to the different oxygen-containing functional groups they have, such as carboxylic, phenolyc and carbonyl groups. More concretely, FAs can solubilize metal ions from sediments and affect their mobilization due to their complexation habilities [Buffle et al., 1977], while HAs can act as a sink of multicharged cations. As a matter of fact, metal-HS interactions have a great influence on the bioavailability of metal ions in aquatic ecosystems [Lund et al., 1990]. The sediment works as a store of substances, including potentially hazardous ones, and HS can interact with those dangerous substances, changing their bioavailability to living beings and controlling their supply from water to sediment and vice-versa, dragging other substances with them. Knowing, not only the amount of hazardous substances but also the amount of HS that can make them available, allow us assessing with better accuracy the real danger of contaminated places.

7.1. Isolation and analysis of humic substances

Several methods have been already used to isolate and characterize HS and have been reviewed in some outstanding works [Janoš, 2003; McDonald et al., 2004]. Due to the heterogeinity of HS, there are many research works trying to improve the selectivity and sensitivity of the developed methods in an attempt to unravel the complex properties of these substances. Until now, there is no standarized analytical approach or combination of methods that provide accurate data about the structure of HS, and so, many efforts are being conducted to this end.

Determination of elemental composition, acidic functional group content and UV-Vis absorbance or average molecular weight are some of the most bulk parameters measured to characterize HS [McDonald et al., 2004]. Currently, there are more proposals that combines spectroscopic, chromatographic and electrophoretic hyphenated techniques [Janoš, 2003; McDonald et al., 2004], and even computational analytical approaches [Schulten, 1995], but the structure is still being unclear [Peña-Mendez et al., 2005]. Nuclear Magnetic Resonance (NMR) [Hertkorn et al., 2005], infrared spectroscopy [Tanaka et al., 2001], Surface Enhanced Raman Spectroscopy (SERS) [Francioso et al., 2008], Fluorescence spectroscopy [Senesi et al., 1991], Pyrolisis-Gas Chromatography coupled to mass spectrometry (Py-GC-MS) [Sainz-Jimenez, 1994] and infrared detectors (Py-GC-IR)

[Kuckuk et al., 1994] and mass spectrometry techniques [Mugo et al., 2004] have been widely used for the characterization and quantification of HS. Figures 7.3a, 7.3b and 7.3c show various structural proposals for these substances, suggested by different research works published in the literature [Stevenson and Schnitzer, 1982; Schulten and Schnitzer, 1993; Buffle et al., 1977].

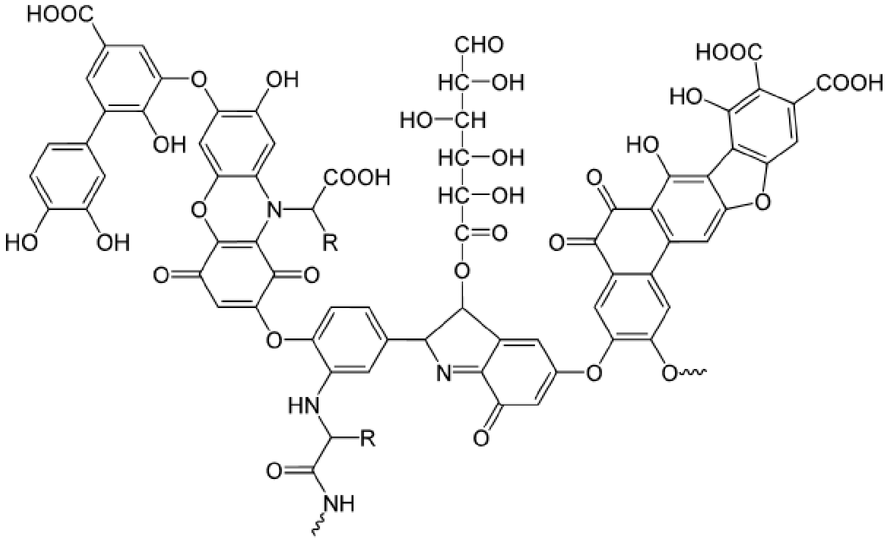


***Figure 7.3a**. Proposed structure of a humic acid molecule [Stevenson and Schnitzer, 1982].*

Prior to the detection of HS, the isolation of HAs and FAs from sediments must be conducted. The published methods often require various and tedious steps with lots of solutions and with a final column (XAD-8, cation exchange phase) purification.

Most of the extraction methods use aqueous sodium/potasium hidroxyde and sodium pyrophosphate as extracting solutions, but other saline solutions, chelating agents and organic solvents have been also used [Janoš, 2003]. Among them, those methods using alkaline solutions, especially sodium hydroxide solutions, yielded greater extractable HS concentrations due to: (i) it is hypothesized that the rest of reagents may change the nature of HS [Tonelli et al., 1997] and (ii) they are more soluble in aqueous solutions at high pH and ionic strength [Belzile et al., 1997]. Anđelković et al. [Anđelković et al., 2001], showed that the use of $MnSO_4$ decreases the degradation of HS during the extraction step, but there are no more references where it is employed. Sodium hydroxide solution has been recommended by the International Humic Substances Society (IHSS) for the HS extraction from organic sediments and soils [IHSS, 2006].

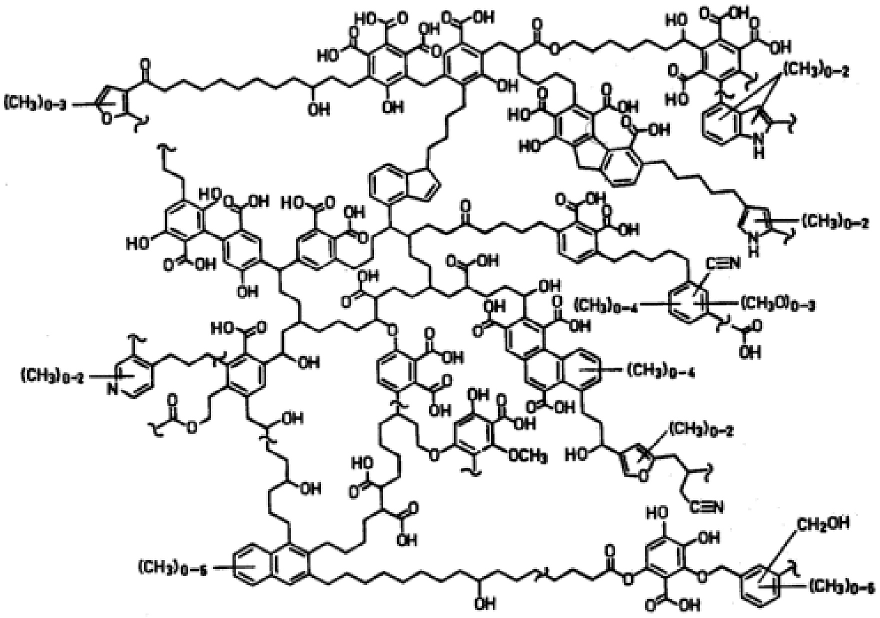


Figure 7.3b. *Proposed structure of a humic acid molecule [Schulten and Schnitzer, 1993].*

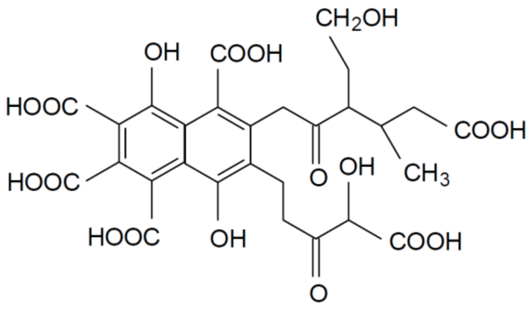


Figure 7.3c. *Proposed structure of a fulvic acid molecule [Buffle et al., 1977].*

Regarding the extraction mechanism, mechanical stirring and shaking devices are the most common ones, regardless the long extraction periods (often 24 hours) and poor reproducibilities [Aiken et al., 1985; Thurman et al., 1981; Watanabe et al., 1991; Swift, 1996; Kuwatsuka et al., 1992]. Hence, the development of new and fast HS extraction methods would be desirable. In this regard, Romaris-Hortas et al. [Romaris-Hortas et al., 2007] developed an extraction method based on microwave energy in order to accelerate the extraction process, which allows the simultaneous extraction of multiple samples. However, the stability of the HS during the extraction step could not be assured.

Ultrasound based solid-liquid pretreatments drive effective extraction processes in shorter time, with less analyte degradation during the sample pretreatment and using a safe, low-cost and eco-friendly methodology [Bendicho et al., 2012]. Currently, among the different ultrasonic processors, ultrasound bath (USB) is one of the most common instruments to accelerate the extraction of both organic and inorganic compounds from several matrices [Bendicho et al., 2012]. The first work dealing with the use of USB to accelerate extractable HS extraction from marine sediments was published by Mecozzi et al. [Mecozzi et al., 2002] and improved by Moreida-Piñeiro et al. later on [Moreida-Piñeiro et al., 2004]. However, in many applications, USB is replaced by focussed ultrasound solid-liquid extraction (FUSLE) systems because of they higher sonication power (100 times higher than USB), higher reproducibility and efficiency. This latter set-up consists on a titanium probe (in which the ultrasound is focussed in) and it is immersed directly in the solution of the sample. Although FUSLE has been satisfactorily used for the extraction of organic compounds from sediments samples [Errekatxo et al., 2008], it has not still been applied to the extraction of HS.

In this framework, the main objective of this work was to develop a fast, cost-effective, eco-friendly and sensitive analytical method based on FUSLE, which allow the isolation and separation of HAs and FAs from sediment samples to be afterwards quantified by means of UV-Vis. The developed method is based on the proposed method by the IHSS

[IHSS, 2006] and taking into account various published methods to extract HS via different techniques [Moreda-Piñeiro et al., 2004; Romarís-Hortas et al., 2007]. As far as we know, most of the published works deal with the isolation of pure HS in order to elucidate their chemical structure. The present work, however, aims to extract HS quantitatively joining the better steps from the previous works and deleting unnecessary steps for quantification in which HS can be lost [Thurman et al., 1981].

7.2. Experimental

7.2.1. Sampling procedure

The method development was carried out using real estuarine sediments collected in the Nerbioi-Ibaizabal estuary (Metropolitan Bilbao, Bay of Biscay, Basque Country). The sediment sample used for optimization purposes was collected in Gobela river (Metropolitan Bilbao, Bay of Biscay, Basque Country, N 43°19'4.5''; W2°59'35.1'').

Sediment sample was collected manually from the top sediments with 2 cm of depth assuring that only fresh sediment was gathered. The sample was stored in sealed plastic containers, transported to the laboratory in cold boxes (4 ^{o}C) and frozen until freeze-drying. The sediments were freeze-dried at −52 ^{o}C and 10^{-1} bar during 24 h using a Cryodos 50 freeze-drier. The lyophilized samples were sieved through a 63 µm sieve, homogenized and then stored in glass bottles at -20 ^{o}C until analysis.

7.2.2. Reagents

All the laboratory material was carefully cleaned with abundant pure water (Elix® quality, Millipore™, Bedford, MA, USA) and without using detergent to avoid possible interferences produced by detergent residues. Afterwards, the material was maintained in a clean 10% nitric acid (Merck, Darmstadt, Germany) bath overnight and then, it was thoroughly rinsed with pure water and with ultrapure water (Milli-Q® quality, Millipore™, Bedford, MA, USA).

HAs (technical grade) were obtained from Fluka (Barcelona, Spain) and FAs were purchased from the International Humic Substances Society (IHSS, Saint Paul, Minnesota, USA). Individual stock solutions from each standard were dissolved to prepare a ≈1000 $\mu g \cdot g^{-1}$ stock solutions in water at pH 10.0 for HA and pH 1 for FA, which were stored at 4 ^{o}C. Mixed fresh solutions at lower concentrations were daily prepared according to the experimentation.

Hydrochloric acid (37%, tracepur), nitric acid (69%, tracepur) and manganese sulfate ($MnSO_4 \cdot H_2O$, puriss. p.a.) were supplied by Merck (Darmstadt, Germany). Sodium hydroxide (NaOH pellets, 98% puriss) and ammonia (NH_3 solution, 28%) were purchased from Fluka (Barcelona, Spain).

7.2.3. Apparatus and Instruments

The FUSLE extractions were carried out using an HD 2070 Sonoplus Ultrasonic Homogenizer (Bandelin, Germany) equipped with a GM 2070 generator (70 W, 20 kHz), an UW 2070 ultrasonic converter and either a SH 70G horn and MS 73 titanium probe (3 mm). A variable power setting (0 – 100 %) allows controlling amplitude of the delivered ultrasound. A Labquake Tube Shaker Rotator from Thermo Fisher (Massachusetts, USA) was used for the extraction of HA and FAs when conventional shaking extraction was assessed. Also, after FUSLE extraction, the same Labquake Tube Rotator was used for HA precipitation and separation from FA. The separation of liquid and solid extracts was performed using a Centromix centrifuge from Selecta (Barcelona, Spain).

The analysis of the HA and FA extracts was performed using a Jasco v-670 duble-beam UV-Vis-NIR spectrophotometer (Jasco, Tokio, Japan) equipped with 10 mm quartz cells with a single monochromator design covering a wavelength range from 190 to 2700 nm. The monochromator features dual gratings (automatically exchanged): 1200 grooves/mm for the UV-Vis region and 300 grooves/mm for the NIR region. A PMT detector is provided for the UV-Vis region and a Peltier-cooled PbS detector is used for the NIR region.

The solid residues of the extracts were also characterized by means of infrared spectroscopy using a Jasco-6300 spectrometer (Jasco, Tokio, Japan). Unlike measures performed in transmittance mode, the analyses carried out in reflectance mode allow the direct analysis of the solid samples without any pretreatment. Hence, the infrared spectra were acquired in reflectance mode by attaching an attenuated total reflectance (ATR-IR) module (Single reflection ATR, Miracle, Pike, USA). The measurements were performed using a single-reflection sampling plate with a 1.8 mm round crystal (diamond/ZnSe) surface allowing reliable analysis of small samples. The module is provided with a high-pressure rotating clamp which is calibrated to deliver over 10,000 psi. All the measurements were carried out in the same pressure conditions which were controlled by the specifications of the equipment.

7.2.4. Humic substances extraction method

The IHSS method to isolate extractable humic substances (i.e., HA and FA) from soil samples [IHSS, 2006] was adapted to estuarine sediments. Briefly, this method consists on shaking 1 g of sample with HCl 0.1 mol·l^{-1} for 1h at room temperature. Both phases are then separated by centrifugation and the supernantant, containing FAs, is reserved. The solid residue, which is firstly neutralized with 1 ml of NaOH 1 mol·l^{-1}, is treated with 9 ml of NaOH 0.1 mol·l^{-1} under Ar atmosphere for 4 hours at room temperature and kept overnight. The supernatant is acidified (HCl, pH:1) and after 12-16 hours is centrifuged to separate HA (solid fraction) from FA (liquid fraction). This second liquid fraction is combined with the FA fraction obtained after the HCl treatment, and both extracts are purified using a non-ionic macroporous Amberlite XAD-8 resin[1].

---

[1] ***XAD-8 resin**. It is a crosslinked aliphatic polymer (acrylic ester) with more hydrophilic structure than other resins used for the same purpose such as XAD-4. Although this resin is widely used for the purification of humic substances and also for the isolation of dissolved organic matter from environmental water bodies, an insufficient elution time may result in the incomplete desorption of humic substances [Kożuch et al., 1992; Peuravuori, 2001; McDonald et al., 2004].*

Although this is the best accepted methodology to isolate humic substances with the aim of characterize the humic substances, it is time consuming and the recoveries of the extractable fraction could be diminished after the purification step. Hence, in order to develop a cost-effective, eco-friendly and quantitative extraction method based in FUSLE, the procedure was fully optimized and its results compared to those obtained with the conventional method based on mechanical stirring.

For the isolation of humic substances by means of FUSLE, all the parameters affecting the extraction step were thoroughly optimized by means of an experimental desingn approach. Figure 7.4 shows the flow chart that summarizes the analytical procedure.

Moreover, the extractions were performed at controlled temperature using a water bath in order to avoid compounds degradation. The titanium probe was placed inside the suspension and after each run the probe was rinsed several times with ultrapure water to avoid cross-contamination. The effect of extractant volume and sonication time, amplitude and cycles was evaluated using an experimental design approach to obtain the optimum extraction conditions for humic and fulvic acids. Under optimal conditions, 15 ml of NaOH 1 $mol \cdot l^{-1}$ were required to extract the target compounds using focussed ultrasound at 95% of amplitude and 10 cycles for 30 minutes.

Under optimum conditions the experimental procedure was as follows: ca. 0.1 g of dry sediment were accurately placed in a polyethylene vial and extracted with deoxygenated NaOH 1 $mol \cdot l^{-1}$. The deoxigenation of NaOH under N_2 atmosphere and $MnSO_4$ was performed just before the extraction step in order to minimize the oxidation of HS [Anđelković et al., 2001].

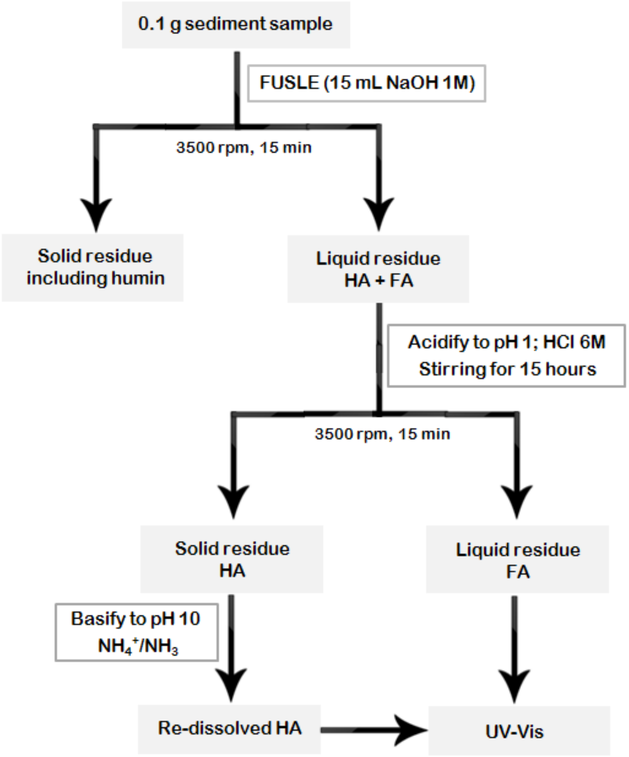


Figure 7.4. *Flow chart outlining procedures for the extraction of humic substances from sediments.*

Once the sonication was over, the liquid fraction containing HA and FA (both soluble at pH >10 [Aiken et al., 1985]) was separated from the residual solid (known as humin fraction) by centrifugation at 3500 rpm during 15 min. The liquid supernatant containing HA and FA was quantitatively recovered. Afterwards, the leachate was acidified with 5 ml of HCl 6 mol·l^{-1} and stirred for 15 hours. In this acidic media HA precipitate while FA remain in solution. The separation of FA (supernatant) and HA (precipitate) fractions was achieved by centrifugation (3500 rpm, 15 min). The HAs were afterwards re-dissolved with 5 ml of 1.0 mol·l^{-1} NH_4^+/NH_3 (pH=10) to be analyzed by means of UV-Vis absorption spectroscopy.

7.2.5. UV-Vis spectrophotometric analysis

UV-Vis measurements of HA and FA leachates were conducted by recording UV-Vis spectra within 200 nm to 750 nm range against spectrometric blanks consisting of 6.0 mol·l^{-1} of HCl for FA determination or 1.0 mol·l^{-1} NH_4^+/NH_3 (pH=10) for HA determination, respectively. These pH values were previously optimized in order to maximize the absorbance. Moreover, since the maximum absorbance of the spectra can vary for different estuarine sediments [Mecozzi et al., 2002] the UV-Vis spectra of several samples were recorded in order to guarantee the absence of variations on the maximum wavelenght. Thus, the repeatability of the spectra was assured and the maximum wavelengths were set at 271 nm and 273 nm for FA and HA, respectively.

7.3. Results and discussion

7.3.1. Selection of UV-Vis acquisition parameters

Due to the high complexity of the molecules to be determined, the UV-Vis spectra can vary depending on the conditions they are acquired. Moreover, the target compounds can also be oxidized with large period of extraction times or if the extraction is performed under severe conditions [Mecozzi et al., 2002]. Consequently, the identification of adequate acquisition conditions and maximum wavelength identification for HA and FA was carried out. In this work, the oxidation processes were minimized by the addition of $MnSO_4$ during the extraction procedure. Moreover, taking into account the whole analytical procedure (detailed in section 7.2.4.) the pH of the final extract is influentless for the HS extraction but affects the UV-Vis response, so it was optimized. In order to evaluate the best pH conditions for UV-Vis measurements, 10 µg·ml^{-1} stock solutions of HA and FA were prepared at different pH values. The assessed pH values ranged from 4 to 14 for the HA (HA not soluble at pH values below to 4) and from 1 to 14 for the FA. The UV-Vis spectra within 200 nm to 750 nm range were recorded against Milli-Q water prepared at the corresponding pH in order to obtain the maximum absorbance wavelength as well as to select the best working pH values.

According to the obtained spectra (see Figure 7.5), the maximum absortion wavelengths were set at 273 nm and at 271 nm for HA and FA, respectively. These correspond to the overlapped bands of different functional groups such as quinonoid, semiquinonoid and aromatic groups [Mecozzi et al., 2002].

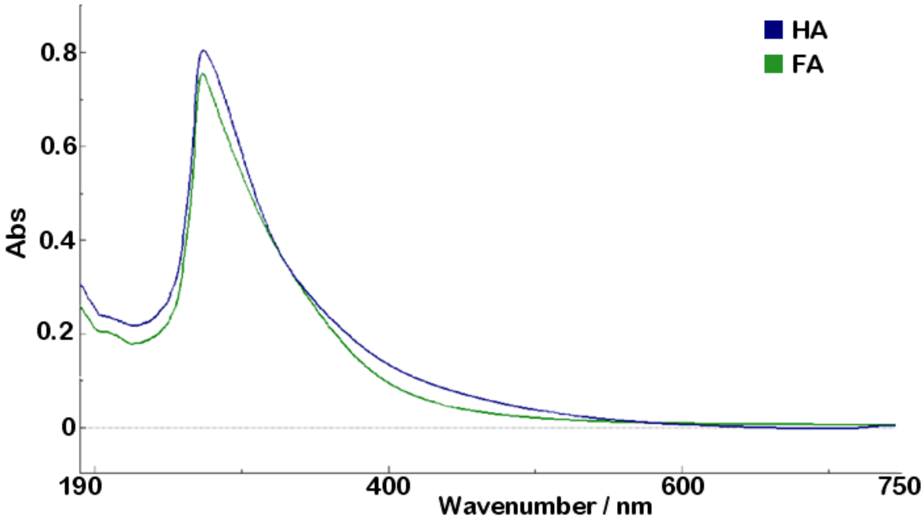


Figure 7.5. *UV-Vis spectra recorded for: HA (blue) and FA (green) standard solutions.*

Figure 7.6 shows the absorbance values for HA (273 nm) and FA (271 nm) at the different pH values. The highest absorbance value for HA was obtained at pH 10, and hence, the redisolution of HA was performed with a buffer solution of NH_4^+/NH_3 later on. Regarding the FA, there was not found a significant absorption variation as a function of pH, that's why, for the measurement of FA, the pH was fixed to pH=0, which is the pH of the liquid extract after HCl addition required for the HA precipitation.

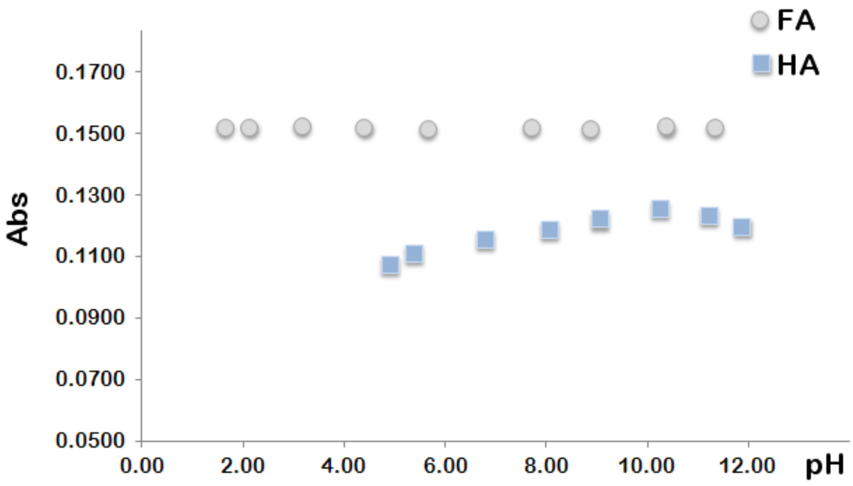


Figure 7.6. *Absorption of 5 µg·ml^{-1} HA and 8 µg·ml^{-1} FA prepared at different pH values. The wavelengths were fixed at 271 nm and 273 nm for HA and FA, respectively.*

7.3.2. Physicochemical conditions for the separation of HA and FA

Regardless the extraction mechanism used for the isolation of HS, the last step of the procedure is the quantitative separation of HA and FA. It is assumed that at pH<2, HA precipitates quantitatively while FA remains dissolved. However, both pH and time for the precipitation equilibria can affect the quantitativity of the process. To the best of our knowledge, there are no studies about the importance of this step since most of the works aim the characterization of HS and not the quantification itself.

The kinetics of the HA precipitation in acidic media can take from minutes to hours depending on the experimental conditions. Thus, the time course of the precipitation process (assisted by stirring) was evaluated using a 50 µg·ml^{-1} HA stock solution at pH<0 (6 mol·l^{-1} HCl). Theses acidic conditions promote the charge neutralization of the carboxilate (–COO^{-}) groups in the HA, losing their stability in solution and making them precipitating. The HCl buffer only changes its pH value with large dilution volumes; using the volume selected on the extraction optimization section, the pH value has always a pH<1 value, maintaining HA precipitated. Assays were performed in triplicate at 14 different stirring times comprized between 1h and 30 h.

Once the stirring time was over, the extracts were centrifuged at 3500 rpm (maximum rpm without vial glass breakage) for 15 minutes and the clear liquid fraction was removed. The remaining solid fraction was redissolved with NH_3/NH_4^+ (pH=10) to be measured by UV-Vis. This fraction containing HA was quantitated and compared with known concentration dissolutions of the HA standard. Recoveries for HA corresponding to different stirring times are shown in Figure 7.7.

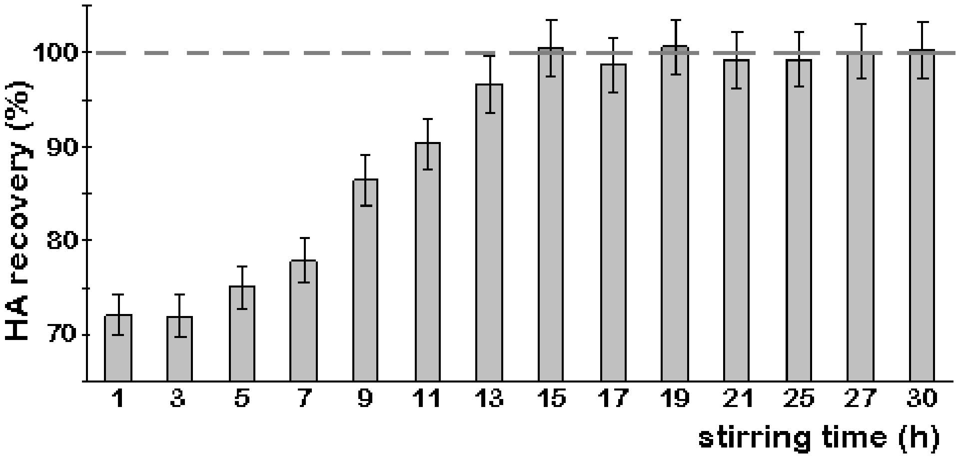


Figure 7.7. *HA recoveries after different stirring times. Assays were performed in triplicate (n=3, 95% confidence level).*

According to the time-profile shown in Figure 7.7, the quantitative precipitation of HA required 15 h at least to reach the equilibrium, which was considered as the optimal time. Although this stirring time is relatively long, but still in the range of the whole procedure proposed by IHSS (24 - 48 h), several samples can be stirred simultaneously overnight, and thus, the method throughput is still assured.

7.3.3. Optimization of focussed ultrasound assisted alkaline extraction of humic substances

The extraction of HS from sediment samples can be affected by both process variables (i.e., ultrasound power, cycles and extraction time) and alkaline extractant characteristics (i.e, NaOH concentration and volume) [Moreda-Piñeiro et al., 2004; Baglieri et al., 2007].

In order to achieve the optimum working conditions that improve the efficiency of the extraction step, an experimental design approach was performed using the Unscrambler® program (v. 7.1., Camo) [Esbensen, 1994]. The influence of ultrasound power (FUS_p, %), ultrasound cycles (FUS_c, s^{-1})[2], extraction time (FUS_t, s), NaOH concentration (C_{NaOH}, $mol\cdot l^{-1}$) and NaOH volume (V_{NaOH}, ml) as well as their interactions was simultaneously evaluated by means of a full factorial (FF) design extended to a central composite design (CCD). Although the extracted mass amount could be significant, some research works concluded that the FA extraction efficiency decreased when increasing the mass of sample [Moreida-Piñeiro et al., 2004]. Based on this fact, the experiments were conducted using 0.1 g of homogeneized sediment sample from Gobela (this real sample was gathered from the estuary of the Nerbioi-Ibaizabal). The design factor space was limited as follows: FUS_p (30 – 100%), FUS_c (1 – 10 s^{-1}), FUS_t (786 – 2214 s), C_{NaOH} (0.05 – 1.95 $mol\cdot l^{-1}$) and V_{NaOH} (7.86 – 22.14 ml). The studied ranges were chosen based on manufacturer's recommendations. The design matrix, involving 48 experiments and the responses of HA and FA (expressed as concentration in sediment sample, $\mu g\cdot g^{-1}$) are summarized in Table 7.1. The precision of the measurements was estimated from the six replicates of the

---

[2] *The cycles for the ultrasound represent the percent of time that the ultrasound actuates over the dissolution to extract taking into account that 10 cycles represent to a continuous extraction and 1 cycle represents that for each second, the ultrasound is working during 1/10 period of a second.*

central point getting relative standard deviations (RSD %) of 10% and 4% for HA and FA, respectively.

Table 7.1. *Central composite design matrix and the responses obtained for HA and FA (expressed as µg·g^{-1}). A: FUS$_t$ (s); B: C$_{NaOH}$ (mol·l^{-1}); C: V$_{NaOH}$ (ml); D: FUS$_p$ (%); E: FUS$_c$ (s^{-1}). The replicates of the central point are marked with an *.*

Experiment	Optimized variables					Responses	
#	A	B	C	D	E	HA	FA
1	786	1.00	15.00	65.0	6	2502	7593
2	2214	1.00	15.00	65.0	6	4871	11060
3	1500	0.05	15.00	65.0	6	5256	4816
4	1500	1.95	15.00	65.0	6	4217	8208
5	1500	1.00	7.86	65.0	6	3416	9256
6	1500	1.00	22.14	65.0	6	3435	9196
7	1500	1.00	15.00	29.3	6	4685	6600
8	1500	1.00	15.00	100.0	6	5253	9074
9	1500	1.00	15.00	65.0	1	1598	4890
10	1500	1.00	15.00	65.0	10	6053	11203
11	1200	0.60	12.00	50.0	4	3380	7428
12	1800	0.60	12.00	50.0	4	3776	8065
13	1200	1.40	12.00	50.0	4	2150	6568
14	1800	1.40	12.00	50.0	4	3047	7001
15	1200	0.60	18.00	50.0	4	3352	7254
16	1800	0.60	18.00	50.0	4	3857	8217
17	1200	1.40	18.00	50.0	4	4152	7113
18	1800	1.40	18.00	50.0	4	2596	7536
19	1200	0.60	12.00	80.0	4	3607	6997
20	1800	0.60	12.00	80.0	4	4640	8548
21	1200	1.40	12.00	80.0	4	2861	8380
22	1800	1.40	12.00	80.0	4	4701	8510
23	1200	0.60	18.00	80.0	4	3505	7334

Table 7.1. Continuation. *Central composite design matrix and the responses obtained for HA and FA (expressed as µg·g^{-1}). A: FUS$_t$ (s); B: C$_{NaOH}$ (mol·l^{-1}); C: V$_{NaOH}$ (ml); D: FUS$_p$ (%); E: FUS$_c$ (s^{-1}). The replicates of the central point are marked with an *.*

Experiment	**Optimized variables**					**Responses**	
	A	**B**	**C**	**D**	**E**	**HA**	**FA**
24	1800	0.60	18.00	80.0	4	4370	8133
25	1200	1.40	18.00	80.0	4	2781	7774
26	1800	1.40	18.00	80.0	4	5187	8755
27	1200	0.60	12.00	50.0	8	5429	7959
28	1800	0.60	12.00	50.0	8	6280	8392
29	1200	1.40	12.00	50.0	8	4981	7515
30	1800	1.40	12.00	50.0	8	4834	10205
31	1200	0.60	18.00	50.0	8	5702	8755
32	1800	0.60	18.00	50.0	8	4984	9200
33	1200	1.40	18.00	50.0	8	6694	10578
34	1800	1.40	18.00	50.0	8	4691	10883
35	1200	0.60	12.00	80.0	8	7066	9710
36	1800	0.60	12.00	80.0	8	7083	9889
37	1200	1.40	12.00	80.0	8	5257	10790
38	1800	1.40	12.00	80.0	8	6017	11108
39	1200	0.60	18.00	80.0	8	6233	9877
40	1800	0.60	18.00	80.0	8	6394	10067
41	1200	1.40	18.00	80.0	8	5574	11288
42	1800	1.40	18.00	80.0	8	7191	11824
43*	1500	1.00	15.00	65.0	6	4881	10586
44*	1500	1.00	15.00	65.0	6	4457	10071
45*	1500	1.00	15.00	65.0	6	5256	10289
46*	1500	1.00	15.00	65.0	6	5915	9916
47*	1500	1.00	15.00	65.0	6	5241	9767
48*	1500	1.00	15.00	65.0	6	4655	9588

The assessment of the effect of each variable on the yields of the HA and FA alkaline extraction was made by using the analysis of variance, as a significant testing method, available in the Unscrambler® program. Table 7.2. summarizes the p-values of the main variables and their interactions for HA and FA. Those variables with p-values < 0.05 were considered statistically significant[3].

Table 7.2. *p-values obtained for the studied variables and variable interactions in the FUSLE experiments after multilinear regression. p-values < 0.05 were considered as significant (values marked in bold).*

Variable	**HA**	**FA**	**β (HA)**	**β (FA)**
FUS_t (A)	**0.0063**	**0**	0.326	1482
C_{NaOH} (B)	**0.0350**	**0.0001**	-543.429	1.04E+03
V_{NaOH} (C)	0.6083	0.0739	16.925	56.799
FUS_P (D)	**0.0029**	**0**	21.414	34.169
FUS_C (E)	**0**	**0**	543.059	570.737
AB	0.8483	0.8571	20.275	17.865
AC	0.2410	0.6171	-125.852	-49.706
AD	**0.0078**	0.6333	301.641	-47.415
AE	0.1203	0.8119	-168.417	-23.608
BC	**0.0396**	0.2724	226.994	110.094
BD	0.9380	0.1038	8.237	165.423
BE	0.8027	**0.0042**	-26.495	307.547
CD	0.5606	0.1332	-61.86	-152.15
CE	0.7606	0.0764	-32.312	181.055
DE	0.6106	0.0996	54.085	167.559
AA	**0.0475**	0.3391	-172.184	-75.545
BB	0.9893	**0**	-1.127	-534.063
CC	0.0153	0.2468	-214.704	-91.894
DD	0.6612	**0.0003**	36.750	-318.151
EE	0.0825	**0.0011**	-149.539	-284.002

---

[3] *The effect of a variable is considering significant when the corresponding source of variation explains more of the variations of the response variable than could be expected from random phenomena.*

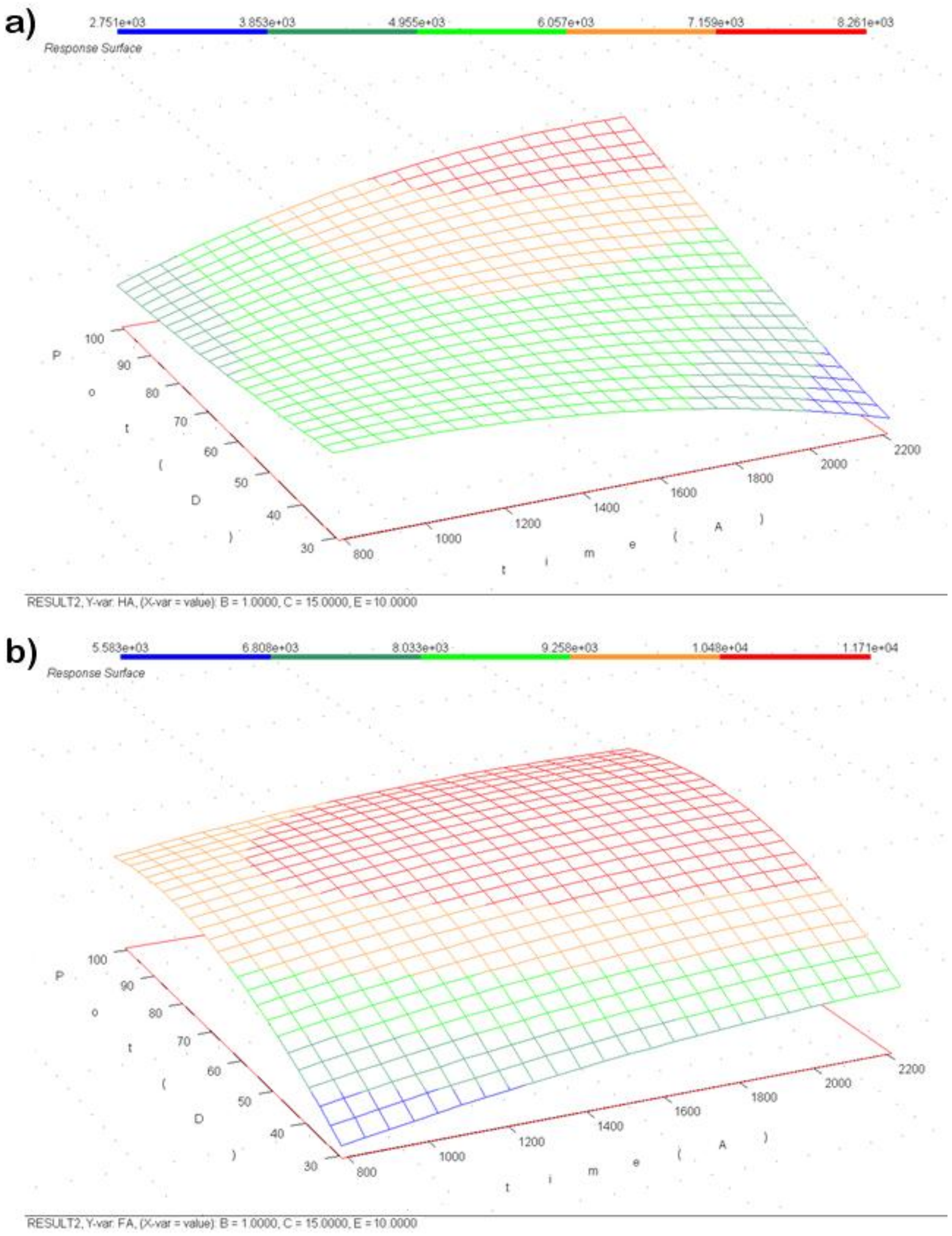


Figure 7.8. *Response surfaces built against extraction time (s) and sonication power (%) obtained for: (a) HA and (b) FA. The rest of the variables were fixed in the optimum values at C_{NaOH}: 1 mol·l^{-1}, V_{NaOH}: 15 ml and ultrasound cycles: 10 s^{-1}.*

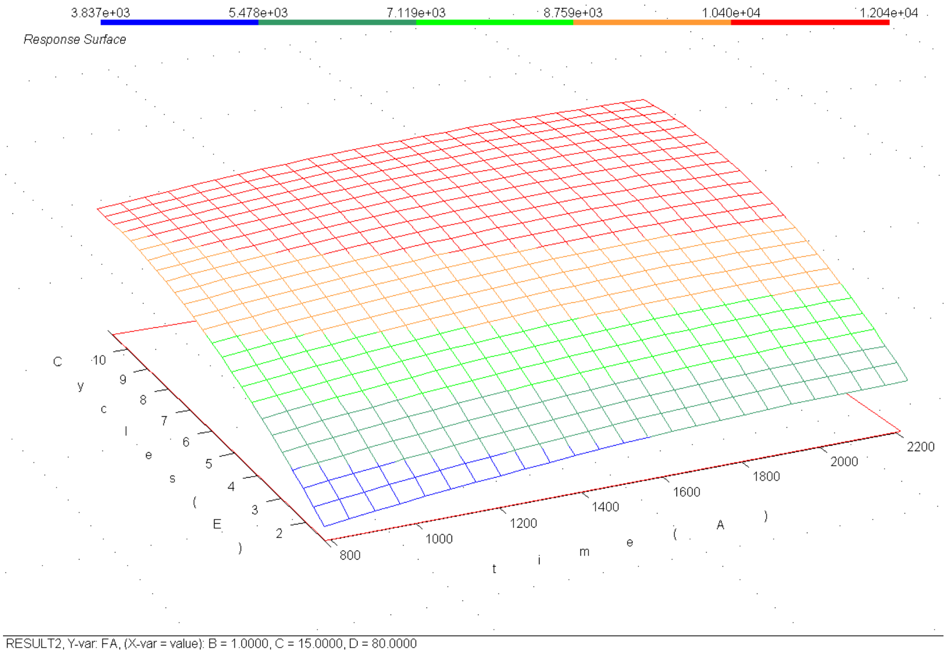


Figure 7.9. *Response surface built varying extraction time (s) and cycles (s^{-1}) obtained for HA (for FA, it was obtained a similar response surface). The rest of the variables values were fixed at: C_{NaOH}: 1 mol·l^{-1}, V_{NaOH}: 15 ml and ultrasound power: 95%.*

All the main variables involved in the experimental design were significant (see Table 7.2) except the volume of NaOH used for the extraction of HA and FA, being fixed at 15 ml in order to ease posterior steps. The selection of the optimum value for the rest of the variables was performed using the response surface models built with multiple linear regression (MLR).

Regarding the effect of the process variables, the effect of ultrasound power and extraction time as well as their interactions were significant for HA and FA. As can be seen in the response surfaces plotted in Figure 7.8, the extraction yield of HS is higher when longer extraction times (i.e., 1740 s or 29 min) and higher ultrasound power are used. Although higher extraction times could be then checked, higher sonication times can promote the oxidation of HS [Mecozzi et al., 2002].

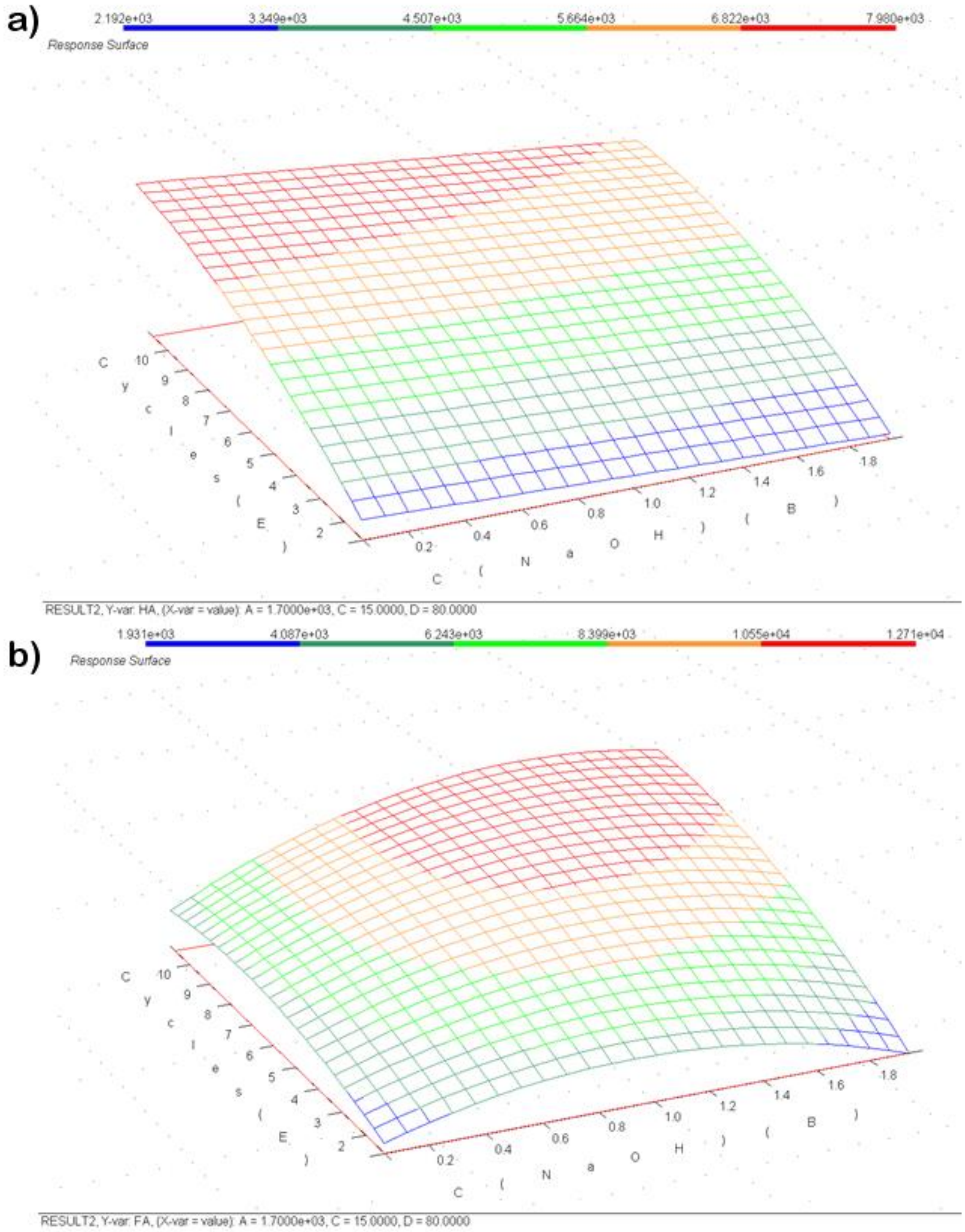


Figure 7.10. *Response surfaces built varying concentration of NaOH (mol·l^{-1}) and cycles (s^{-1}) obtained for (a) HA and (b) FA. The rest of the variables were fixed at: V$_{NaOH}$: 15 ml, ultrasound power: 95% and extraction time 29 min.*

The evaluation of the number of ultrasound cycles was afterwards performed by fixing the ultrasound power at 95%. The application of ultrasounds continiously (i.e, maximum number of cycles allowed: 10) rendered the best responses for HA and FA (see Figure 7.9).

Concerning the extractant concentration, two slightly different trends were observed for the optimal values of NaOH concentration. Taking into account the response surfaces plot in Figure 7.10, higher concentration of NaOH (i.e., between 1-1.8 mol·l^{-1}) provided the highest extraction yields for FA, whereas the opposite phenomena occurred for HA (i.e., 0.2-1.2 mol·l^{-1}). Hence, it was decided to fix the concentration of NaOH at a consensus value of 1 mol·l^{-1}.

As a result, optimum alkaline extraction conditions were set as follows: continuous application of ultrasounds with a power of 95 % for 29 min and using 15 ml of NaOH 1 mol·l^{-1} as the extracting solution.

7.3.4. Quality assurance / quality control (QA/QC)

In order to assure the quality of the developed method (FUSLE-UV-Vis), the figures of merit involving the extraction and analysis step were determined for extractable HS. Regarding the figures of merit for UV-Vis measurements, external calibration curves showed good linearities over a wide concentration range (i.e., 0.3 - 45 µg·ml^{-1} and 0.8 – 60 µg·ml^{-1} for HA and FA respectively) with coefficients of determination (r^2) higher than 0.998 and 0.999 for HA and FA, correspondingly.

Working range of the method was estimated between LOQ and the corresponding amount to obtain 1 absorption units (1 AU). Higher values of absorption were discarded in order to avoid dispersion interferences due to the big size of the HA and FA molecules.

Sensitibity is a parameter directly related to the analyte response of the analyzed concentration. The sensitibity of the measurements was estimated considering the value of the slopes average recorded from the calibration curves built during 5 consecutive days. The obtained average value was 24 ± 1 mAUs / µg·g^{-1} for HA and 16.3 ± 0.6 mAUs / µg·g^{-1}

for the FA. Different calibration curves were performed in different days in order to evaluate sensitibity changes for the measurements during the days. The reproducibility of the slope change was estimated in terms of relative standard deviation (RSD %) for six consecutive days. It was established at 4.0% for both HA and FA, being the absolute intercept variation during the six days of 0.02 AU. According to these results, no tendency on the slopes or intercepts was observed.

Instrumental limits of detection (LODs) were estimated and defined as the average response (n=3) of the lowest concentration level of the calibration curve for each compound plus three times the standard deviation. The obtained LODs were bellow 0.12 $\mu g \cdot g^{-1}$ and 0.30 $\mu g \cdot g^{-1}$ for HA and FA, respectively.

In order to calculate the procedural limits of detection (LOD_{proc}) and limits of quantitation (LOQ_{proc}), procedural blanks (i.e., the whole optimized extraction procedure performed without sediment sample but using 15 ml of NaOH as well as all the reactives used during all the steps) were analyzed (n=3). The LOD was calculated as the average signal of the blank plus three times the standard deviation of the blank and the LOQ was calculated as the average signal of the blank plus ten times the standard deviation of the blank. The use of other approaches to determine method detection limits that include the presence of matrix were not applicable in this work, due to: (i) HS are expected at high concentration levels in real matrices and; (ii) the impossibility to find sedimentary samples without HS. For HA, the LOD_{proc} and LOQ_{proc} were 0.1 $\mu g \cdot g^{-1}$ and 0.3 $\mu g \cdot g^{-1}$ respectively. In the case of FA, the LOD_{proc} was 0.2 $\mu g \cdot g^{-1}$ whereas the LOQ_{proc} was 0.8 $\mu g \cdot g^{-1}$.

The repeatability of the FUSLE-UV-Vis involving all the steps was checked using five replicate extractions of a real sediment sample analyzed in the same day. The RSD% value for HA was 2.9% and 3.3% for FA. The reproducibility was calculated by the five replicates analyzed within three consecutive days being the RSD% values 3.3% and 3.7% for HA and FA, respectively.

Finally, due to the abscence of certified reference material for HS in sediment samples, two different procedures were used to assess the accuracy of the proposed FUSLE-UV-Vis method. On the one hand, consecutive extractions of the same sample were performed in order to assess the maximum extractable concentration of HS. On the other hand, the same sediment sample was analyzed by the classical extraction method proposed by the IHSS.

Four consecutive extractions of a sediment sample were performed under selected extraction conditions described above and adding fresh extractant (i.e., NaOH 1 mol·l^{-1}). The results obtained for six replicates performed in the same conditions are shown in Figure 7.11. The responses are expressed as the recovery of HS obtained in each extraction step, which have been calculated as the extracted concentration on each step for each acid divided by the sum of all the concentrations extracted from the sediment in all of the extractions performed.

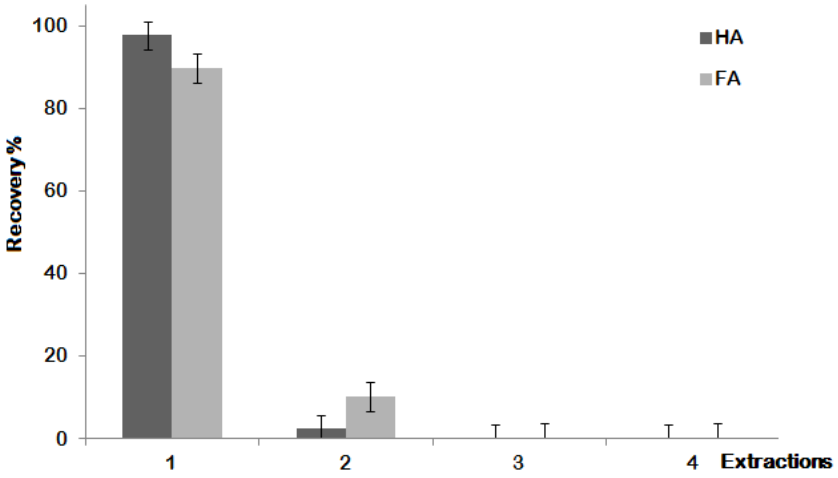


Figure 7.11. *Four consecutive extractions of sediment sample to quantify HS (n=6, 95% of confidence level) performed by FUSLE and using NaOH 1 mol·l^{-1}.*

According to these results, the average recovery in the first extraction was 97 ± 3% for HA and 89 ± 4% for FA (at 95% of confidence level). The recoveries in the second extraction step were negligible for both compounds (i.e., 2 ± 10% and 10 ± 2% for HA and FA, respectively) and the amount of HS recovered in the rest of extractions was bellow the limit of quantification. Hence, it can be concluded that quantitative extraction of HS was achieved in the first extraction. Up to three successive extractions were required in the method developed by Moreida-Piñeiro et al. [Moreida-Piñeiro et al., 2004], but they used 10 minutes of sonication time in an ultrasound-bath. Therefore, the developed method meets the same goal but in one extraction step.

Besides, in order to compare the effectiveness of the developed method with those provided by traditional methods (i.e., the method proposed by IHSS), the extraction of the same amount of sediment sample was performed by classical mechanical stirring and using NaOH 1 mol·l^{-1}. Since the IHSS method proposes large period of extraction times, the stirring process was monitored for HA and FA for 16 different times ranging from 5 hours to 80 hours.

Figure 7.12 shows the recovery of HA of FA for the different assessed stirring times. The response (expressed as recovery %) was calculated by dividing the concentration obtained after mechanical stirring of the sample with the concentration obtained after focussed ultrasound extraction of the same sample, and then multiplying by 100. According to these results, mechanical stirring procedure yielded lower concentration (values bellow 100% in Figure 7.13) of HS no matter the extraction time. This fact prove the efficiency of FUSLE for HA and FA extraction. Moreover, although using the deoxygenated extractant, the concentration values diminish with the extraction time, showing the possible HS degradation that is widely reported in the literature [Anđelković et al., 2001]. Finally, the repeatability obtained with the classical methodology was determined as worse than using the focalized ultrasound extraction and evaluated in 15% for HA and 9% for FA.

Taking these problems into account, mechanical stirring cannot be considered as the first choice for the quantitative analysis of HS in sediments.

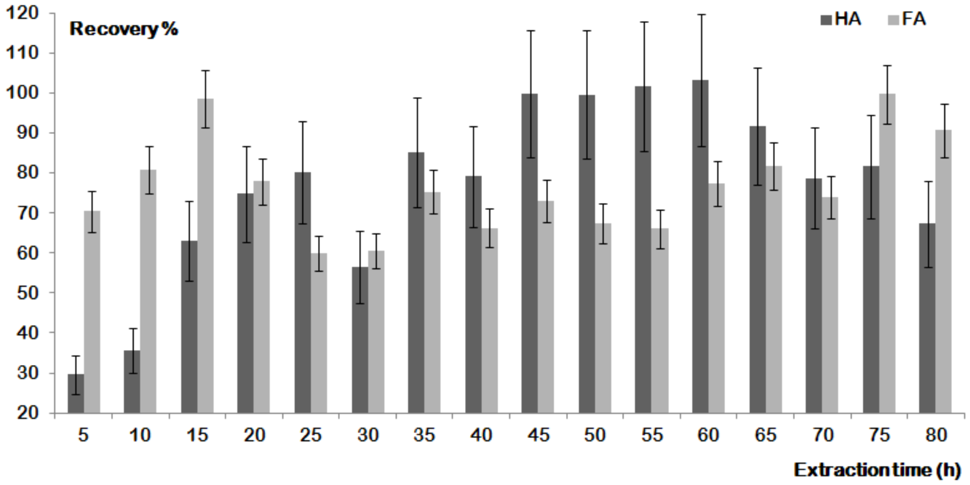


Figure 7.12. *The response obtained for HA and FA (expressed as recovery % and calculated by comparing the concentration obtained after mechanical stirring of the sample with the concentration obtained after focussed ultrasound extraction of the same sediment sample) at different extraction times. (n=3, 95% of confidence level).*

7.3.5. Characterization of extracted Humic acid

Once the extraction procedure is completed, the solid residue obtained after the whole isolation procedure shown in Figure 7.4 can be checked by means of infrared spectroscopy in the ATR mode. To this aim, a real sediment sample was treated following the common extraction procedure proposed by IHSS, but using our focussed ultrasound extraction step with the parameters optimized in chapter 7.3.3. Briefly, approximately 0.1 g of sediment were treated with NaOH 1 mol·l^{-1}, the liquid fraction was quantitatively recovered and treated afterwards with HCl. The solid residue was isolated, washed, air-dried and analyzed by means of ATR directly.

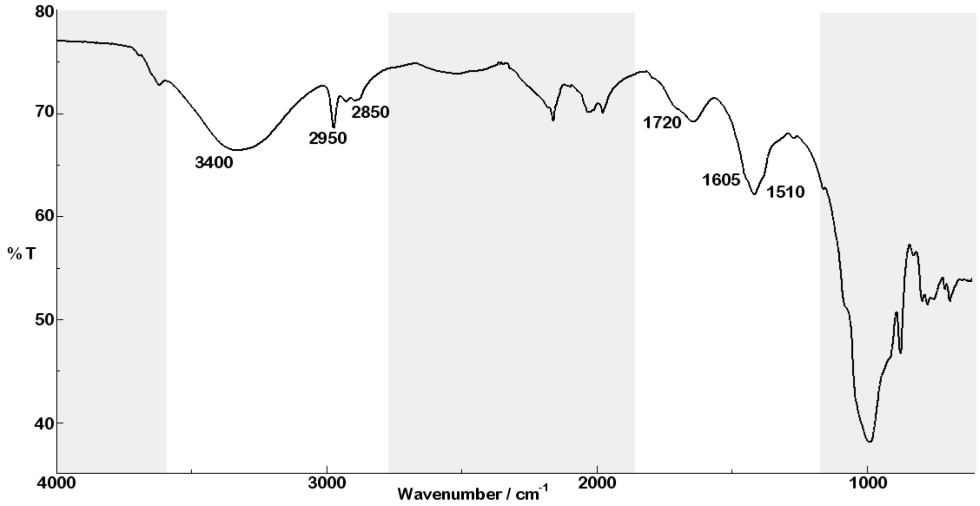


Figure 7.13. *ATR spectra of HA isolated from a estuarine sediment sample (Arriluze location) by means of focussed ultrasound extraction.*

The ATR spectra obtained for the isolated HA from sediment sample is shown in Figure 7.13, and the bands match with the infrared spectra published in the literature [Romaris-Hortas et al., 2007]. The isolated fraction includes many typical bands of extractable humic substances in the range of 1200 – 4000 cm^{-1}:

a) the wide band centered at 3400 cm^{-1} corresponds to the –OH stretching vibration of acid, protein and polysaccharide groups,
b) the bands located at 2950 and 2850 cm^{-1} are associated with the stretching vibration of $-CH_3$ and $-CH_2$ in saturated aliphatic chains
c) an intense band at 1720 cm^{-1} is related to the –C=O stretching vibration of acid group
d) two bands located at 1605 and 1510 cm^{-1} (corresponding to the ring mode vibration of aromatic structures).

Hence, focussed ultrasound extraction was thoroughly optimized in order to isolate extractable HS quantitatively.

7.4 Conclusions

The analysis of HS in sediments samples is an analytical challenge from both characterization and quantitation point of view. Most of the works published in the literature often use conventional extraction techniques, which require high amount of extraction solvent and/or long extraction times. Ultrasound focussed solid-liquid extraction followed by separation of HS in acidic media and determination by means of UV-Vis turned out to be a very suitable analytical procedure to quantitate HS. In fact the developed method showed to be a fast procedure (in comparison to the classical methods) that allowed the detection of HA and FA with high precision (RSD < 4%) and accuracy (> 89%). The addition of Mn (II) has proven to be a good alternative to prevent the degradation of HS, thanks to which reproducible analyses were obtained.

Taking into account that in this work there is not any purification step (as the intention of the proposed method is quantification and not pure solid isolation), the time saved until final concentration is achieved, is much larger than in any other method proposed until this moment. Moreover, in this work it has been optimized the precipitation time of HA once the extraction from the sediment is completed. To the best of our knowledge, there is no evidence studies about this step in the literature.

Hence, the innovative application of the developed method provide a promising new approach for the monitoring of the HS in sediment samples, thanks to which the mobility of metals through environmental compartments can be better understand. That is the reason why, the developed method will be applied to the quantification of these substances in sediment samples collected in the Nerbioi-Ibaizabal estuarine system in subsequent works, and will be explained in the next chapter of the present work.

7.5. References

[Aiken et al., 1985] Aiken GR, McKnight DM, Wershaw RL, MacCarthy P. *Humic Substances in Soil, Sediment and Water.* Wiley, New York, 1985.

[Anđelković et al., 2001] Anđelković T, Anđelković D, Perović J, Purenović M, Polić P. *Decrease of oxygen interference on Humic Acid structure alteration during isolation*. FU Phys. Chem. Tech. 2001; 2, 163 – 171.

[Baglieri et al., 2007] Baglieri A, Ioppolo A, Ne`gre M, Gennari M. *A method for isolating soil organic matter after the extraction of humic and fulvic acids. Org. Geochem.* 2007; 38, 140 – 150.

[Belzile et al., 1997] Belzile N, Joly HA, Li H. *Characterization of humic substances extracted from Canadian lake sediments.* Can. J. Chem. 1997; 75, 14 – 27.

[Bendicho et al., 2012] Bendicho C, De la Calle I, Pena F, Costas M, Cabaleiro N, Lavilla I. *Ultrasound-assisted pretreatment of solid samples in the context of green analytical chemistry,* Trends Anal. Chem. 2012; 31, 50 – 60.

[Buffle et al., 1977] Buffle J, Greter FL, Haerdi W. *Measurement of complexation properties of humic and fulvic acids in natural waters with lead and copper ion-selective electrodes. Anal. Chem.* 1977; 49, 216 – 222.

[Davies et al., 1998] Davies G, Ghabour EA. *Humic Substances – Structures, properties and use. Royal Society of Chemistry*, Cambrigde, 1998.

[Durand, 1980] Durand B. *Definition and quantitative importance of kerogen* in *insoluble organic matter from sedimentary rocks*, Editions Technip, Paris, 1980.

[Errekatxo et al., 2008] Errekatxo A, Prieto A, Zuloaga O, Usobiaga N, Etxebarria N, Fernández LA. *Simultaneous extraction of several persistent organic pollutants in sediments using focussed ultrasound solid-liquid extraction.* Anal. Bioanal. Chem. 2008; 392, 1471 – 1478.

[Esbensen, 1994] Esbensen KH. *Multivariate data analysis: an introduction to multivariate data analysis and experimental design, Camo AB*, Oslo, 1994.

[Francioso et al., 2001] Francioso O, Sànchez-Cortés S, Tugnoli V, Marzadori C, Ciavatta C. *Spectroscopic study (DRIFT, SERS and ^{1}H NMR) of peat, leonardite and lignite humic substances.* J. Mol. Struc. 2001; 565, 481 – 485.

[IHSS, 2006] International Humic Substances Society ©2014, *http://www.humicsubstances.org/index.html*, last access 2015/08/04.

[Hayes et al., 1990] Hayes MHB, Swift RS. *Genesis, Isolation, Composition and Structures of Soil Humic Substances* in Soil Colloids and Their Associations in Aggregates, De Boodt MF, Hayes MHB, Herbillon A, De Strooper EBA, Tuck JJ. NATO ASI Series 1990; 214.

[Hertkorn et al., 2005] Hertkorn N, Kettrup A. *Molecular Level Structural Analysis of Natural Organic Matter and of Humic Substances by Multinuclear and Higher Dimensional NMR Spectroscopy* in Use of Humic Substances to Remediate Polluted Environments: From Theory to Practice. Perminova IV, Hatfield K, Hertkorn N.. NATO Science Series 2005; 52.

[Huc, 1980] Huc AY. Origin *and formation of organic matter in recent sediments and its relation to kerogen in* Kerogen. Insoluble organic matter from sedimentary rocks, Editions Technip, Paris, 1980.

[Janoš, 2003] Janoš P. *Separation methods in the chemistry of humic substances*. J. Chromatogr. A. 2003; 983, 1 – 18.

[Kim et al., 2003] Kim S, Kramer RW, Hatcher PG. *Graphical Method for Analysis of Ultrahigh-Resolution Broadband Mass Spectra of Natural Organic Matter, the Van Krevelen Diagram.* Anal. Chem. 2003; 75, 5336 – 5344.

[Kożuch et al., 1992] Kożuch J, Pempkowiak J, Grzybowski W. *Complexing properties of humic substances towards cadmium and their influence on accumulation of cadmium by Mytilus edulis*. Bull. Sea Fish. Inst., Gdynia, 1992; 1, 15 – 18.

[Kuckuk et al., 1994] Kuckuk R, Hill W, Burba P, Davies AN. *Pyrolysis-GC-FTIR for structural elucidation of aquatic humic substances. Fresenius J. Anal. Chem.* 1994; 350, 528 – 532.

[Kuwatsuka et al., 1992] Kuwatsuka S, Watanabe A, Itoh K, Arai S. *Comparison of Two Methods of Preparation of Humic and Fulvic Acids, IHSS Method and NAGOYA Method.* Soil Sci. Plant Nutr. 1992; 38, 23 – 30.

[Lund et al., 1990] Lund W, Helbak IA, Seip HM. *Studies of the complexation properties of aquatic humic material by differential pulse polarography.* Sci. Tot. Environ. 1990; 92; 269 – 281.

[McDonald et al., 2004] McDonald S, Bishop AG, Prenzler PD, Robards K. *Analytical chemistry of freshwater humic substances.* Anal. Chim. Acta. 2004; 527, 105 – 124.

[Mecozzi et al., 2002] Mecozzi M, Amici M, Pietrantonio E, Romanellli G. *An ultrasound assisted extraction of available humic substance from marine sediments.* Ultrason. Sonochem. 2002; 9, 11 – 18.

[Moreda-Piñeiro et al., 2004] Moreda-Piñeiro A, Bermejo-Barrera A, Bermejo-Barrera P. *New trends involving the use of ultrasound energy for the extraction of humic substances from marine sediments.* Anal. Chim. Acta. 2004; 524, 97 – 107.

[Mugo et al., 2004] Mugo SM, Bottaro CS. *Characterization of humic substances by matrix assisted laser desorption/ionization time-of-flight mass spectrometry.* Rapid Commun. Mass. Spectrom. 2004; 18, 2375 – 2382.

[Peña-Mendez et al., 2005] Peña-Mendez EM, Havel J, Patočka J. *Humic substances compounds of still unknown structure: applications in agriculture, industry, environment, and biomedicine.* J. Appl. Biomed. 2005; 3, 13 – 24.

[Peuravuori , 2001] Peuravuori J. *Binding of pyrene on lake aquatic humic matter: the role of structural descriptors.* Anal. Chim. Acta. 2001; 429, 75 – 89.

[Romarís-Hortas et al., 2007] Romarís-Hortas V, Moreda-Piñeiro A, Bermejo-Barrera P. Application of microwave energy to speed up the alkaline extraction of humic and fulvic acids from marine sediments. Anal. Chim. Acta. 2007; 602, 202 – 210.

[Sainz-Jimenez, 1994] Sainz-Jimenez C. *Analytical pirolisis of humic substances: pitfalls, limitations and possible solutions.* Environ. Sci. Technol. 1994; 28, 1773 – 1780.

[Schulten et al., 1991] Schulten HR, Plage B, Schnitzer M. *A chemical structure for humic substances.* Naturwissenschaften. 1991; 78, 311 – 312.

[Schulten and Schnitzer, 1993] Schulten HR, Schnitzer M. *A state of the art structural concept for humic substances.* Naturwissenschaften. 1993; 80, 29 – 30.

[Schulten, 1995] Schulten HR. *The three dimensional structure of humic substances and soil organic matter studied by computational analytical chemistry.* Fresenius J. Anal. Chem. 1995; 351, 62 – 73.

[Senesi et al., 1991] Senesi N, Miano T, Provenzano MR, Brunetti G. *Characterization, differenciation and clasiffication of humic substances by fluorescence spectroscopy.* Soil Sci. 1991; 152, 259 – 271.

[Stevenson and Schnitzer, 1982] Stevenson FJ, Schnitzer M. *Transmission electron microscopy of extracted fulvic and humic acids.* Soil Sci. 1982; 133, 334 – 345

[Stevenson et al., 1994] Stevenson FJ. *Humus Chemistry. Genesis. Composition. Reactions.* Wiley. Chichester, 1994.

[Swift, 1996] Swift RS. *Organic matter characterization (chap 35), 1018 – 1020* in Methods of soil analysis. Part 3. Chemical methods. Sparks DL, Page AL, Helmke PA, Loeppert RH, Soltanpour PN, Tabatabai MA, Johnston CT, Sumner ME. Soil Sci. Soc. Am (eds). Book Series: 5. Soil Sci. Soc. Am. Madison, WI, 1996

[Tanaka et al., 2001] Tanaka T, Nagao S, Ogawa H *Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy of functional groups of humic acid dissolving in aqueous solution.* Anal. Sci.2001; 17, 1081 – 1084.

[Tonelli et al., 1997] Tonelli D, Seeber R, Ciavatta C, Gessa C. *Extraction of humic acids from a natural matrix by alkaline pyrophosphate. Evaluation of the molecular weigth of fractions obtained by ultrafiltration.* Fresenius J. Anal. Chem. 1997; 359, 555 – 560.

[Thurman et al., 1981] Thurman EM, Malcom RL. *Preparative isolation of aquatic humic substances.* Environ. Sci. Technol. 1981; 15, 463 – 466.

[Van Loo and Koppejan, 2007] Van Loo S, Koppejan J. *The handbook of Biomass Combustion & Co-firing*, Earthscan publishing house, London. 2007

[Vandenbroucke, 1980] Vandenbroucke M. *Structure of kerogens as seen by investigations on soluble extracts in* Kerogen. Insoluble organic matter from sedimentary rocks, Èditions Technip, Paris, 1980.

[Watanabe et al., 1991] Watanabe A, Kuwatsuka S. *Fractionation of soil fulvic acids using polyvinyl-pyrrolidone and their ionization difference spectra.* Soil Sci. Plant Nutr. 1991; 37, 611 – 617.

8. Distribution of humic substances in sediments: seasonal and mobility trends

8. Distribution of humic substances in sediments: seasonal and mobility trends

As it has been stated in previous chapters, sediments work as storage of various organic and inorganic components, absorbing new materials and maintaining cohesion among constituents. Also, the sediment is an unbeatable substrate where chemical reactions and microbiological interactions occur, all of them regulated by equilibrium reactions. Water, oxygen, inorganic salts, organic materials, organic wastes, sunlight and temperature/humidity cycles (in exposed estuarine sediments during low tide) as well as microbial activity can shift any of the equilibrium processes increasing or decreasing the presence of toxic or essential substances into the ecosystem and even reaching the food chain.

Organic matter is an important component of marine and estuarine sediments and it is considered as one of the main natural reservoirs where many chemical reactions are catalyzed. In this sense, organic matter is involved in many biogeochemical processes occurring in the environment such as photochemical reactions, microbial growth, nutrient or contaminant mobilization and metal complexation [Davis, 1984; Fletcher and Beckett, 1987; Kögel-Knabner et al., 1998; Weng et al., 2002; Iskrenova-Tchoukova et al., 2010].

Consequently, the determination of its composition, quantity and reactivity may be essential for a better understanding of environmental processes. Besides, the input of dissolved organic matter through estuaries may have effects on the biogeochemical cycles in the coastal ocean [Canuel et al., 2012]. Despite its importance, the composition and fate of riverine and estuarine dissolved organic matter have been poorly studied and remain being still one of the major concerns in current biogeochemistry [Bianchi et al., 2011; Marie et al., 2015].

Organic matter shows a great complexation capacity favoring the transport and/or immobilization of metal ions and hence, it can act as a potential mechanism to distribute metals in the different environmental compartments [Lund, 1990]. As an example, some research studies have concluded that nearly 99% of dissolved iron is associated with dissolved organic matter forming complicated and unknown complexes that are ubiquitous in aquatic environments and play a key role in iron speciation [Fang et al., 2015]. In this framework, some authors assessed the behavior of metal species in the environment in the presence of organic matter through the determination of the humic substances – metal conditional stability constants [Pantano et al., 2014].

The main components of dissolved organic matter are the humic substances (HS), which are mainly fractionated into humic acid (HA) and fulvic acid (FA), according to their solubility [Aiken et al., 1985]. Although their complete characterization and quantification is still an analytical challenge, several efforts have been performed in the literature in order to monitorize them in different environmental scenarios [Fabiano et al., 1995; Grémare et al., 1997; Tockner et al., 1999; Halbedel et al., 2013]. Besides, environmental factors (e.g., light and temperature cycles, water pH and ionic strength, nutrients, microbiological activity, etc.) as well as hydrodynamic factors (e.g., discharge events or the water input from tributaries to the estuary) are known to be main drivers of possible temporal variability of HS, an so, the effects they produce [Halbedel et al., 2013].

Within this context, the aim of the present study was to assess the concentration and variations of HS (mainly HA and FA) by examining the sediment and water samples collected from seven sampling locations along the Nerbioi-Ibaizabal estuary using a previously developed method for HS determination in such samples (see chapter 7). This estuary has been extensively studied by our research group finding the evolution of metals along the estuary over several years as well as their mobility [Gredilla et al., 2013; Fdez-Ortiz de Vallejuelo et al., 2014]. Although its regeneration is fairly assumed, it has not recovered its original state. Thus, this work aims to evaluate if the seasonal evaluation of HS can be a complementary tool to detect pollution sources and environmental impacts.

8.1 Experimental

8.1.1 Sampling sites

Sediment and water samples, both at high and low tide, were gathered along the estuary of the Nerbioi-Ibaizabal river (Metropolitan Bilbao, Bay of Biscay, Basque Country, N 43°19'4.5''; W2°59'35.1''), involving the main stream, the tributaries and the mouth of the estuary, including one basin. Seven sampling points distributed along the Nerbioi-Ibaizabal estuary (shown in Figure 8.1) were taken into account, specifically:

- *Alde Zaharra* (AZ, in the main stream of the Nerbio-Ibaizabal river, highly affected by human daily activities, N43.263785, W2.924692).
- *Galindo* (GA, tributary to the main flow with an industrial sewage treatment plant along its bed, N43.304086, W2.983395).
- *Gobela* (GO, small tributary without much industrial activity and low conductivity water in low tide, N43.318091, W2.993019).
- *Kadagua* (KA, tributary with a lot of industrial activity in the past but in recovery process, N43.277808, W2.977435).
- *Udondo* (UD, basin by a chemical company, near different industrial activities such as chemical and shipyard activities, N43.315294, W2.992077).
- *Zorroza* (ZO, in the main stream of the river, N43.275722, W2.969464).

- *Arriluze* (AR, in the mouth of the Nerbioi-Ibaizabal river with high sea influence, N43.337801, W3.010881).

The samples were gathered every three months from January to December at all locations except for AZ, GA and GO which were collected monthly during a year. The samples were collected along the Bilbao estuary in order to cover the whole variability of organic matter composition presented in the estuarine sediments as a function of the location and season, involving the main stream, the tributaries and the mouth of the estuary, including one basin.

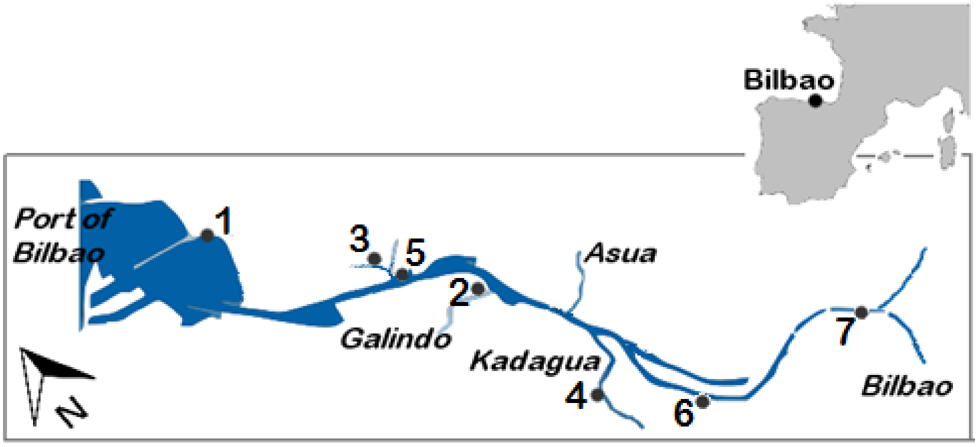


Figure 8.1. *Sampling points in the Nerbioi-Ibaizabal estuary: 1. Arriluze (AR), 2.Galindo (GA), 3. Gobela (GO), 4.Kadagua (KA), 5.Udondo (UD), 6. Zorroza (ZO), 7 Alde Zaharra (AZ).*

8.1.2 Sampling procedure

Sampling was carried out on the highest level of the tide for high tide water samples and in the lowest level of the tide for low tide water samples and sediment. Samples were taken on January, February, March, May, June, July, September, November and December in AZ, GA and GO sampling sites while samples from the rest of sampling points were collected on January, May, September and December.

Sediment samples were manually gathered from the top sediments with 2 cm of maximum depth assuring that only fresh sediment was gathered. Samples were stored in sealed plastic containers, transported to the laboratory in cold boxes and afterwards frozen until. The sediments were freeze-dried at −52 °C and 10^{-1} bar during 24 h. The lyophilized samples were sieved through a 63 µm sieve and stored in glass bottles at -20 °C until analysis. Regarding water samples, they were not filtered in order to avoid losses of HS adsorbed to suspended particulate material being maintained at 4 °C in darkness until their analysis which was performed within 48 hours. Physicochemical parameters of the waters were measured in situ during the water sampling.

8.1.3 Reagents and standards

All the laboratory material was carefully cleaned with abundant pure water (Elix® quality, Millipore™, Bedford, MA, USA) and without using detergent to avoid possible interferences produced by detergent residues. Afterwards, the material was maintained in a clean 10% nitric acid (Merck, Darmstadt, Germany) bath overnight and then, it was thoroughly rinsed with pure water and with ultrapure water (Milli-Q® quality, Millipore™, Bedford, MA, USA).

Hydrochloric acid (37%, Tracepur), nitric acid (69%, Tracepur), manganese sulfate ($MnSO_4·4H_2O$), silver nitrate ($AgNO_3$), potassium dicromate ($K_2Cr_2O_7$, p.a.), iron (II) ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$, p.a.), sulphuric acid (98%, p.a.), phosphoric acid (85%, p.a.) were provided by Merck (Darmstadt, Germany). Sodium hydroxide (NaOH pellets, 98% puriss) and ammonia (NH_3 solution, 28%) were purchased from Fluka (Barcelona, Spain).

HA (technical grade) were obtained from Fluka (Barcelona, Spain) and FA were purchased from International Humic Substances Society (IHSS, Saint Paul, Minnesota, USA).

8.1.4 Apparatus and Instruments

A Cryodos 50 freeze-drier was used for sediments freeze-drying. The focused ultrasound extractions (FUSLE) were carried out using an HD 2070 Sonoplus Ultrasonic Homogeniser (Bandelin, Germany) equipped with a GM 2070 generator (70 W, 20 kHz), an UW 2070 ultrasonic converter and either a SH 70G horn and MS 73 titanium probe (3 mm). A variable power setting (0 – 100 %) allows controlling amplitude of the delivered ultrasound.

A Labquake Tube Shaker Rotator from Thermo Fisher (Massachusetts, USA) was used for the separation of HA from FAs after extraction from sediment. The separation of liquid and solid extracts was performed using a Centromix centrifuge from Selecta (Barcelona, Spain). The analysis of the HA and FA extracts was performed using a Jasco v-670 double-beam UV-Vis-NIR spectrophotometer (Jasco, Tokio, Japan) equipped with 10 mm quartz cells.

Physicochemical parameters of the estuarine water were in-situ recorded (pH, pO_2 (DO), conductivity (C), redox potential (ORP)) by means of a previously calibrated YSI 556 multi-parametric probe (YSI Environmental, Yellow Springs, Ohio). The probe also provided an estimation of salinity and total dissolved solids (TDS) based on the temperature and electrical conductivity measurements. Total dissolved organic carbon in water was analyzed in the laboratory by a TOC-L analyzer (Shimadzu, Izasa, Bilbao, Spain). Elemental analysis was performed by a LECO CHNS-932 (Michigan, USA).

8.1.5 Analytical procedure

The TOC contained in the dried sediment was determined by redox titration following the procedure proposed by Page et al [Page et al., 1982]. Briefly, 0.5 g of sediment was weighed into an erlenmeyer flask. The organic matter present in the sediment was oxidized adding 10 ml of $K_2Cr_2O_7$ 0.045 $mol \cdot l^{-1}$ with a bit amount of $AgNO_3$ to avoid chlorides interferences and side reactions and afterwards 10 ml of concentrated

H_2SO_4. The solution stand 30 minutes to ease oxidation and 10 ml of H_3PO_4 were added to ensure acid media. The solution was carried to 100 ml with distillated water and back-titrated with 0.1 $mol \cdot l^{-1}$ of $Fe(NH_4)_2(SO_4)_2$ to calculate the $K_2Cr_2O_7$ consumed by the organic matter. This method has been widely used to calculate TOC in sediments [Bartolomé et al., 2003].

The determination of HS in sediment samples was performed using a previously optimized method (see chapter 7). Briefly, 0.1 g of dried and sieved sediment were accurately weighed in a polyethylene vial and extracted with 15 ml of deoxygenated NaOH 1 $mol \cdot l^{-1}$ during 29 minutes using focused ultrasound at 95% of amplitude and 10 cycles. Once the sonication was over, the liquid fraction containing HA and FA (both soluble at pH >10 [Aiken et al., 1985]) was separated from the residual solid (known as humin fraction) by centrifugation at 3500 rpm during 15 min. The liquid supernatant containing HA and FA was quantitatively recovered. Afterwards, the leachate was acidified with 5 ml of HCl 6 $mol \cdot l^{-1}$ and stirred for 15 hours. In acidic media, HA precipitates while FA remains in solution. The separation of FA (supernatant) and HA (precipitate) fractions was achieved by centrifugation (3500 rpm, 15 min). The HAs were afterwards re-dissolved with 5 ml of 1.0 $mol \cdot l^{-1}$ NH_4^+/NH_3 (pH=10) in order to be analyzed by means of UV-Vis absorption spectroscopy. FA was directly measured in the previous step supernatant (see chapter 7 for more details).

The same procedure was used for water samples but without the need of FUSLE step (e.g., 35 ml of water samples were acidified with 5 ml of HCl 6 $mol \cdot l^{-1}$, stirred for 15 hours and submitted to the rest of steps as indicated previously). The sample amount for water was fixed in around 35 ml due to physical restrictions of the plastic vial used in the stirring.

Instrumental limits of detection were 0.12 $\mu g \cdot g^{-1}$ and 0.30 $\mu g \cdot g^{-1}$ for HA and FA, respectively. Regarding the procedural limits of detection (LOD_{proc}) were 0.1 $\mu g \cdot g^{-1}$ and 0.2 $\mu g \cdot g^{-1}$ for HA and FA respectively for sediment samples whereas the LOD_{proc} for water samples were 0.3 $ng \cdot ml^{-1}$ and 0.5 $ng \cdot ml^{-1}$ for HA and FA, respectively.

8.2 Results and discussion

8.2.1 Physicochemical parameters

Before the analysis of humic substances in sediment and water samples, the physicochemical parameters of both matrixes were acquired. The measured parameters are presented in Figure 8.2 for water samples (e.g., inorganic carbon (IC), total organic carbon (TOC), dissolved oxygen (DO), turbidity (TDS), oxidation-reduction potential (ORP), conductivity (C) and pH) and in Figure 8.3 for sediment samples (e.g., TOC%, C, H and N), respectively.

Regarding the waters samples, high tide water collected samples present lower conductivity values than expected in January, February, March, November and December but it can be explained by the rain water abundance in those months, according to the climatological data recovered from Euskalmet during the sampled seasons.

The turbidity of water samples (expressed as TDS) collected in summer showed higher values in comparison to the rest of the seasons, which can be explained by the larger amount of tributaries freshwater entering into the estuary. It must be also remarked the high correspondence among TDS and conductivity trends during all the seasons, both showing a tidal influence as well as estuarine system dependence.

In the case of DO, slight variations were observed in water during low tide cycles with mean concentration values of 6 $mg \cdot l^{-1}$. A bit larger differences were observed at high tides. The higher values were found in January, and then decreasing DO values were measured through the seasons. ORP values show a similar tendency through the seasons. Overall, values in high tide were around 10 mV higher than in low tide. pH value of water samples remained almost constant at slightly alkaline conditions (mean value 8.5) at both low and high tide conditions except in January and March, where values decreased to 7.

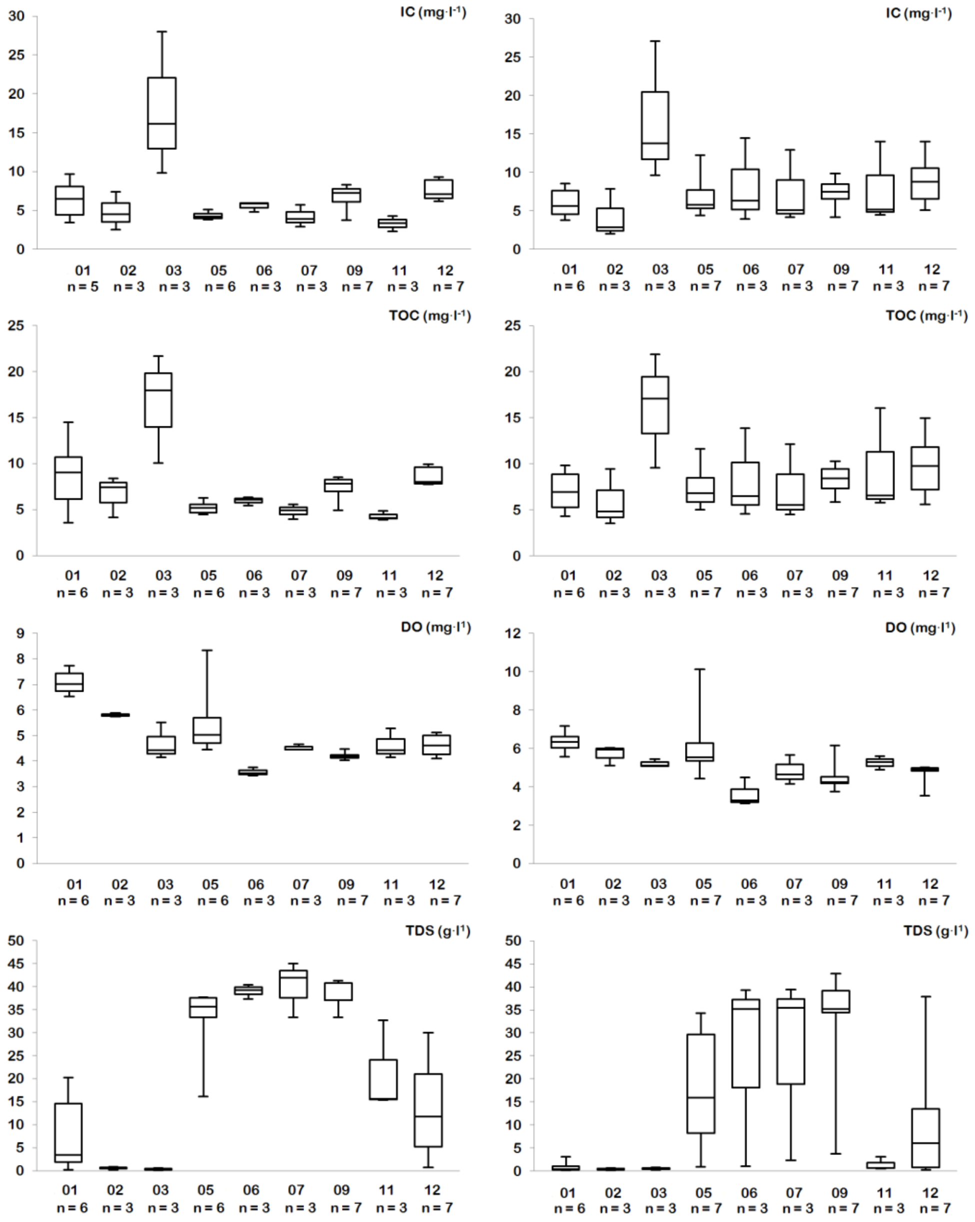


Figure 8.2. *Physicochemical parameters measured in gathered waters in high tide (left) and in low tide water (right) for all sampling points and all sampling seasons. n value refers to the number of sampling points processed.*

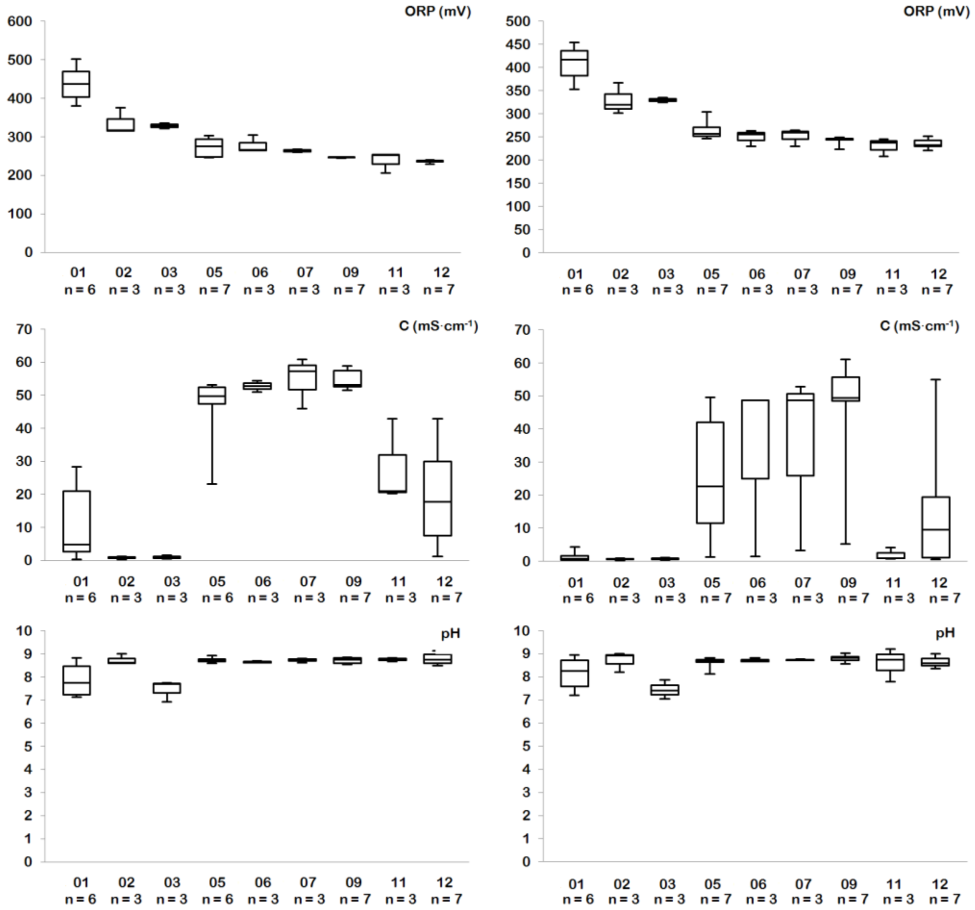


Figure 8.2. Continuation. *Physicochemical parameters measured in gathered waters in high tide (left) and in low tide water (right) for all sampling points and all sampling seasons. n value refers to the number of sampling points assessed.*

With regard to sediment parameters, total organic carbon percentage values are illustrated in Figure 8.3 together with the elemental composition (e.g., content of C, H and N). As an overall trend, lower concentration of TOC% was observed during summer months whereas the values were increased in winter. As will be later discussed, the same distribution pattern was observed for HS in sediment.

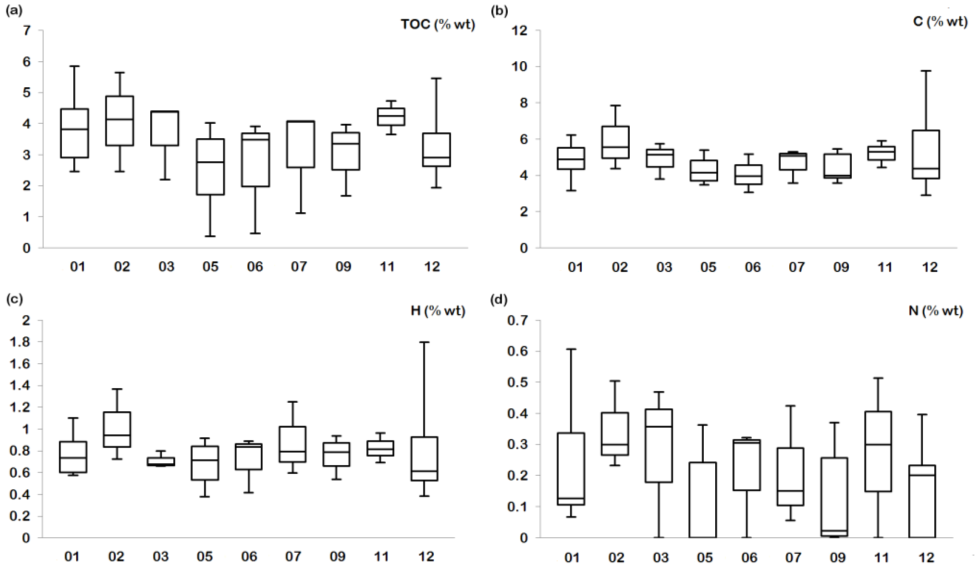


Figure 8.3. *TOC % measured in sediment samples (a) together with elemental composition C% (b), H% (c) and N% (d) measured in sediments for all sampling points and all sampling seasons.*

8.2.2 HS in sediments and water samples

HA and FA concentrations in sediment samples collected during the year are shown in Figure 8.4. The analyses were performed in triplicate, being the intermediate precision of the measures (expressed in terms of relative standard deviation, RSD %) lower than 3.3 % and 3.6 % for HA and FA, respectively.

As it can be seen in the Figure 8.4, the concentrations of HA and FA in the estuarine sediments were in the range of 0.3 – 6.8 $g \cdot kg^{-1}$ and 1.9 – 16 $g \cdot kg^{-1}$, respectively. The mean concentration of FA was about 4-5 times higher than that of HA. According to the literature, the predominance of FA fraction over HA came from different solids with sorption capability in natural water [Yamada et al., 2000; Ma et al., 2001]. Water pH was slightly alkaline (mean pH value 8.5), conditions that can favor the formation of FA instead of HA [Hou et al., 2013]. The relation of FA/HA has been used in the literature as an indicator of humus quality [Naidja et al., 2002]. According to that work, high FA/HA values found in sediments can be indicative of a relatively good humus quality. In this work, the ratios of FA/HA ranged from 2.1 to 21.4 with a mean of 6.6, so the quality of humus gathered in Nerbioi-Ibaizabal sediments cannot be considered poor.

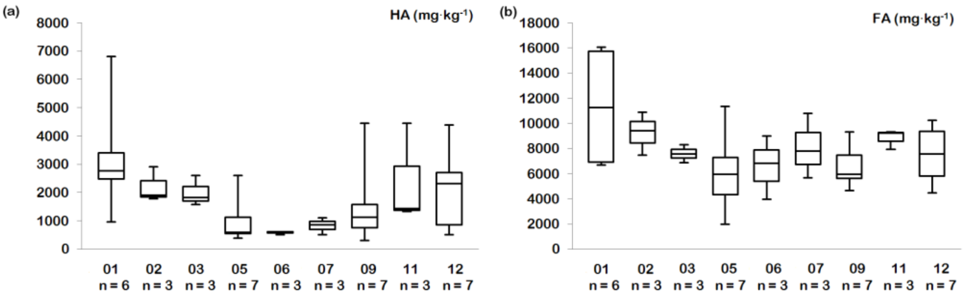


Figure 8.4. *HA (a) and FA (b) concentrations found on sediments for each campaign. n value refers to the number of sampling points assessed.*

On the other hand, larger seasonal variability was found for HAs in comparison to that found for FAs. This could be attributed to the smaller size and higher solubility of FAs in comparison to HAs, which make its distribution in the environmental compartments more homogeneous [Buffle et al., 1977; Aiken et al., 1982]. In this sense, lower concentrations of HAs were overall found in summer time, whereas no specific trend was observed for FAs within its random variability.

Regarding the concentration of HS in estuarine water samples, the concentrations analyzed in water samples collected at low and high tides in all sampling points for all seasons are summarized in Figure 8.5. As occurred for sediment samples, FA concentration in both high and low tide water was more than 10 times higher than HA concentration. The higher presence of FA can be explained by: (i) higher mobilization of FA in comparison of HA taking into account the physicochemical characteristics of water, and

(ii) the characteristics of some locations, where the conditions of flow ease the HA deposition, making some locations richer in HA than others.

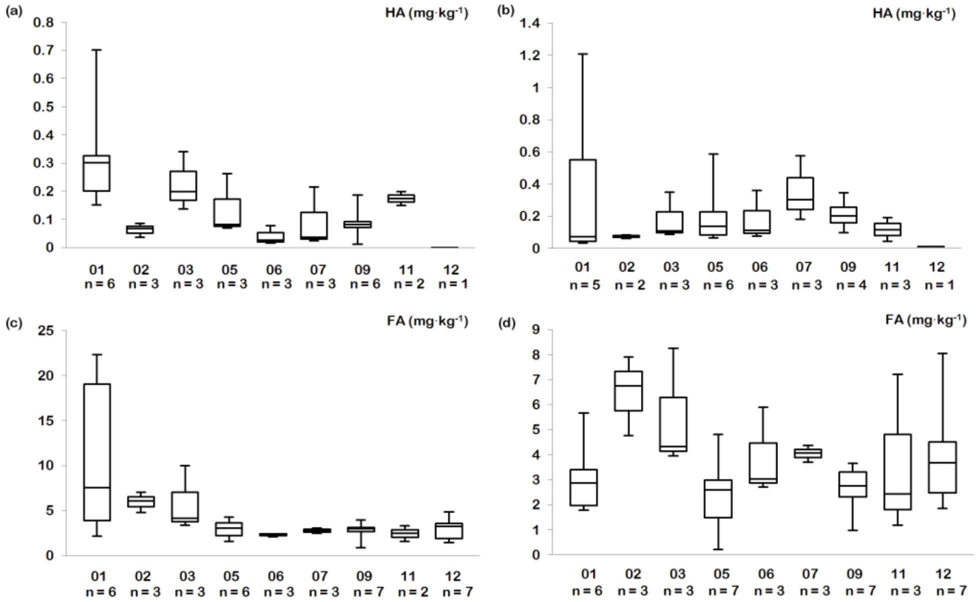


Figure 8.5. *HA (a, low tide; b, high tide) and FA (c, low tide; d, high tide) concentrations (in mg·l^{-1}) found on waters for each sampling campaign. n value refers to the number of sampling points assessed.*

The HA concentration is in the range of 0.1 – 0.6 mg·l^{-1} regardless tide and sampling site. Mean concentration values were slightly higher in low water tides. Larger variability was found for FA distribution. Concentrations up to 20 mg·l^{-1} were detected in low water tides whereas in high water tides the maximum concentration of 8.3 mg·l^{-1} was reached in some sampling sites. This issue can be explained because of the higher homogeneity of FA due to its increased solubility compared to HA [Aiken et al., 1985].

Going deeper into the results, the different patterns of seasonal distribution for HS in water (expressed as the sum of HA and FA in low and high tides) and sediment samples (expressed as HA and FA) in each sampling site are shown in Figure 8.6.

Regarding water samples, and specifically for high tide water samples, maximum values for HS were observed during winter season (January-February) regardless the sampling site. On the contrary, no clear trend was found in low tide water samples, where the concentration for HS remains almost constant in all the sampling points with unsystematic variability.

In the case of sediments, different distribution patterns of HS were observed. On the one hand, the distribution of HS showed an apparent seasonal dependence in AZ, GA and KA sampling sites. This seasonality trend is specially marked for FAs, showing a maximum concentration in winter and minimum values in summer time. The rest of the sampling sites didn't show any special trend regarding the seasonality. With the exception of some isolated larger concentrations of HS in some sampling points (ZO in May and GO in January) the concentration of HS remained almost constant (mean concentration values of FAs between 6000 – 8000 mg·kg^{-1}) during the year.

Finally it should be highlighted the different global behavior (waters plus sediment) in the most oceanic sampling site (AR) with regard to the sites (KA, UD, ZO, AZ, GA, GO) with a higher influence of the continental river waters.

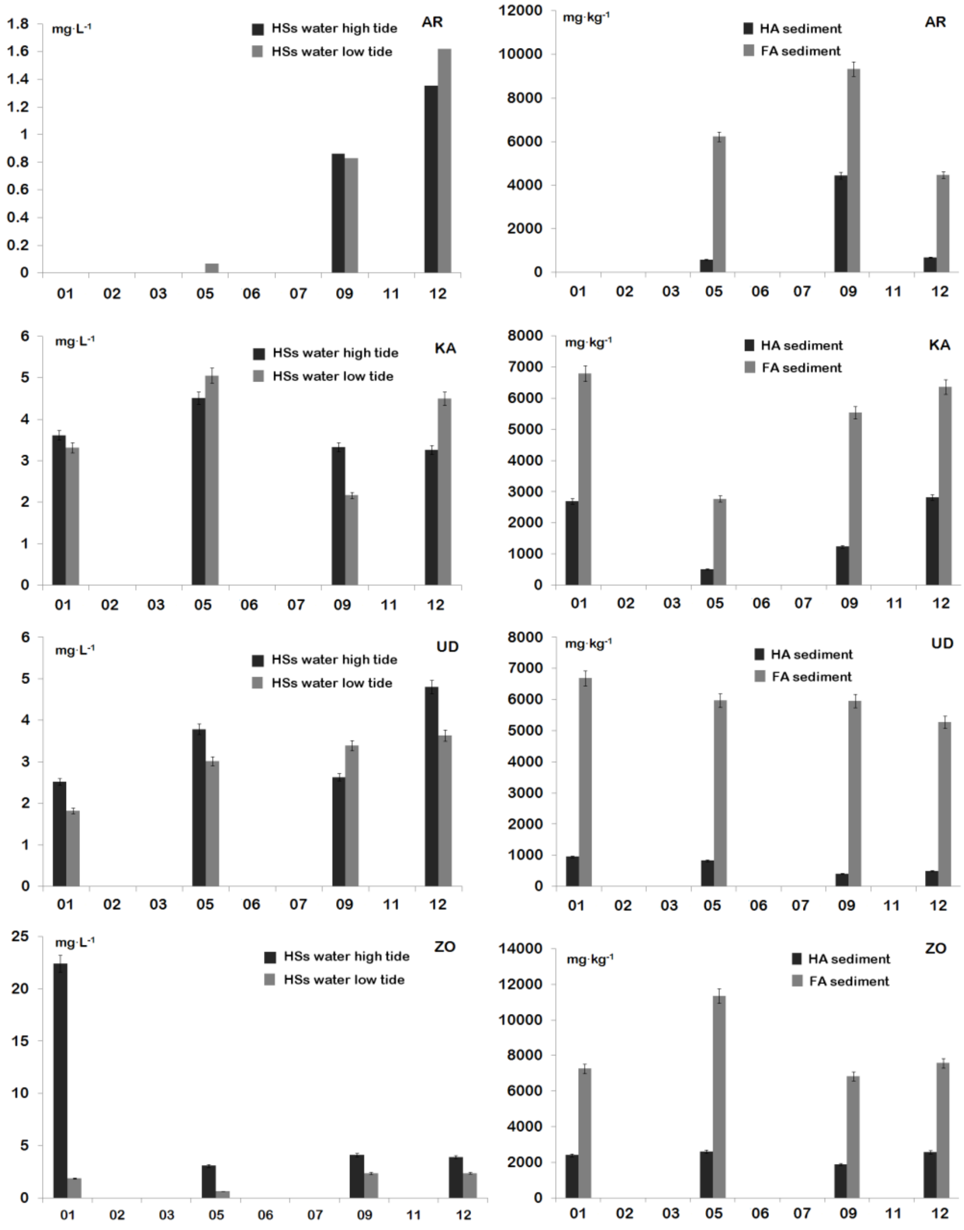


Figure 8.6. *Tendency for HS in Arriluze (AR), Kadagua (KA), Udondo (UD) and Zorroza (ZO) in water (left) and sediments (right) during all the sampled seasons.*

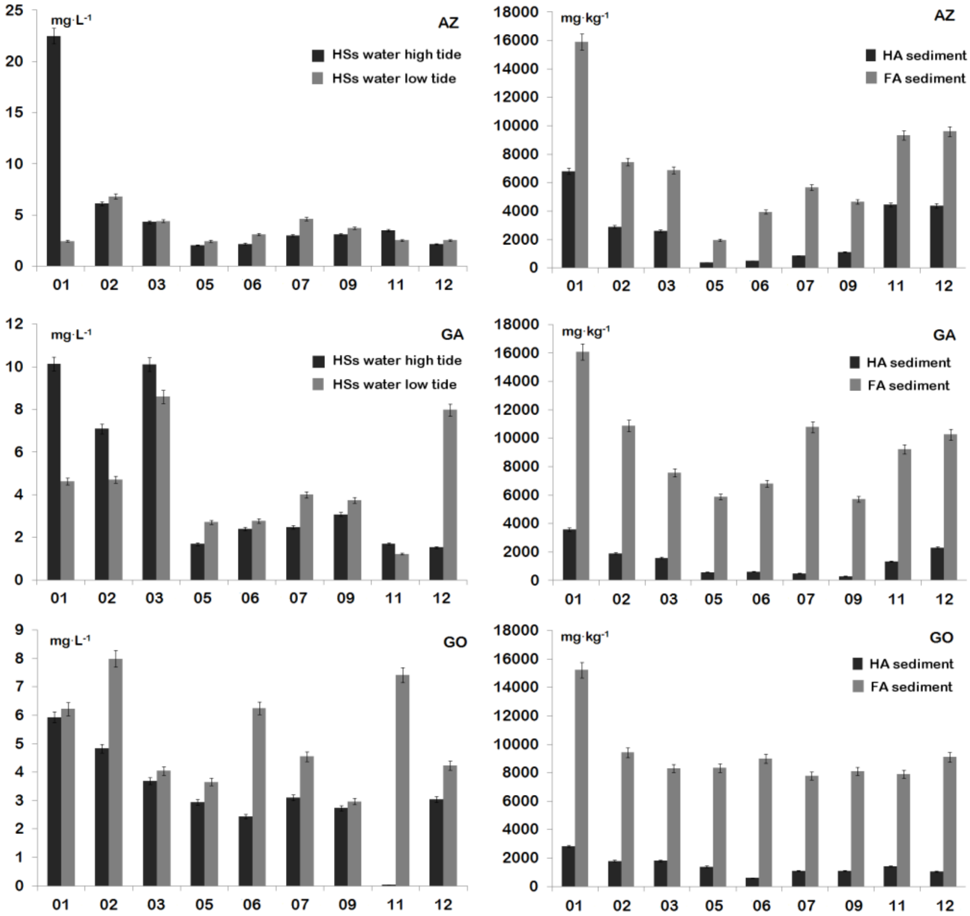


Figure 8.6 Continuation. *Tendency of HS in Alde Zaharra (AZ), Galindo (GA) and Gobela (GO) in water (left) and sediments (right) during all the sampled seasons.*

8.2.3 Correlation and PCA data analysis

In order to relate the concentrations of HA and FA in sediment and water and the physicochemical parameters measured in water and sediment, the correlation analysis of the data was performed by means of the Unscrambler® program (v. 7.1., Camo)

[Esbensen, 1994]. According to the number of data included in the correlation, the critical r value was set to 0.7 in order to consider a correlation among values.

As it has been previously discussed, high correlation was found for IC and TOC in water samples (r: 0.938) as well as for TDS and conductivity (r: 0.998). ORP was found to be negatively correlated with pH (r: -0.743), which can be explained by the influence of the $S^{=}/HS^{-}$ pair in the media as it was largely discussed in other sections of the work (see Chapter 4, section 4.3.3). The concentration of HAs in sediment samples is positively correlated with the concentration of FAs (r: 0.757), which is highly correlated with TOC% in sediment (r: 0.729). TOC% in sediment is also positively correlated with nitrogen content in sediments revealing that the nitrogen content in the sediment may be derived from the decomposition of organic matter. Another positive correlation found is the carbon content with the hydrogen (r: 0.853). This correlation can be explained due to the similar saturation ratio among organic compound constituting the organic matter.

Trying to eliminate any disturbance because of the particular sampling point characteristics or season weather condition, the performance of correlation analysis of the different sampling sites was independently considered. High positive correlations (r >0.70) among the following variables were found in all sampling locations: (i) TOC and IC in waters showing that the carbon present in the estuarine system could come not only from the organic matter, but also from naturally eroded carbonates; (ii) TDS and conductivity; (iii) HA and FA in sediment and both concentrations with TOC% in sediment, which ensures that one of the main component of organic matter are HS and (iv) TOC% and C% of sediment and C% and H% of sediment, evidencing the similar saturation ratio commented before [Laborda et al., 2008].

Other variables were negatively (r > - 0.70) or positively correlated (r > 0.70), but only in some sampling points showing the influence of site characteristics. For example, negative correlation values were observed between the pH and ORP water samples collected in AZ, KA and ZO, whereas a positive correlation was found in AR sampling site. This may indicate that the presence of sulfides or their participation in the system is practically negligible in AR. Besides, a positive correlation was found between TDS of water and FA in sediment in AR. This correlation can be derived from the specific location of AR in the estuary. It is located in the mouth of the estuary, where the water movement is more turbulent in comparison to the rest of sites, which can favor the input of FAs from sediment samples to water body as dissolved organic particles, and hence, increase the turbidity.

The correlation factors found in some sampling sites between HS and physicochemical parameters of water and sediment samples show the dependence of their distribution with sampling site characteristics. In this sense, some different scenarios were found:

- High positive correlation ($r > 0.7$) was found between water ORP (and also DO) with concentration of HS in sediment samples collected in AR, GO and UD. The higher ORP values measured in these sampling sites in comparison to the rest of sites favors the formation of HS.
- The correlation factors found between HS concentration and ORP values in UD allow the identification of HS formation/deposition processes in this specific sampling site. On the one hand, as in the previous case, positive correlation (r: 0.86) was observed between ORP and HS in sediment, which justifies the formation of these compounds. On the other hand, negative correlation factor were obtained for FAs in water and ORP (r: -0.72) as well as for FAs in water and FAs in sediments (r: -0.87). These last results indicate that the deposition of HS and so their accumulation is favored. This last assumption is also supported by the negative correlation found among FAs concentration in water and TOC% in sediments.

In order to establish a possible grouping among all assessed variables and sampling points, Principal Component Analysis (PCA) analysis was also performed using The Unscrambler® software. First of all, all the sampling points (n=7) and all the measured variables (e.g., 11 variables regarding water samples and 7 variables regarding sediment samples) were considered. The raw data were centered and scaled to assure an equal weight to all the variables and cross-validation method was used as internal validation method. The first results indicated that the 65% of the total variance can be explained using the first three principal components (PC1, PC2 and PC3 explained the 35%, 17% and 13% of the total variance, respectively). The scores and loadings plot for the first two PCs are shown in Figure 8.7.

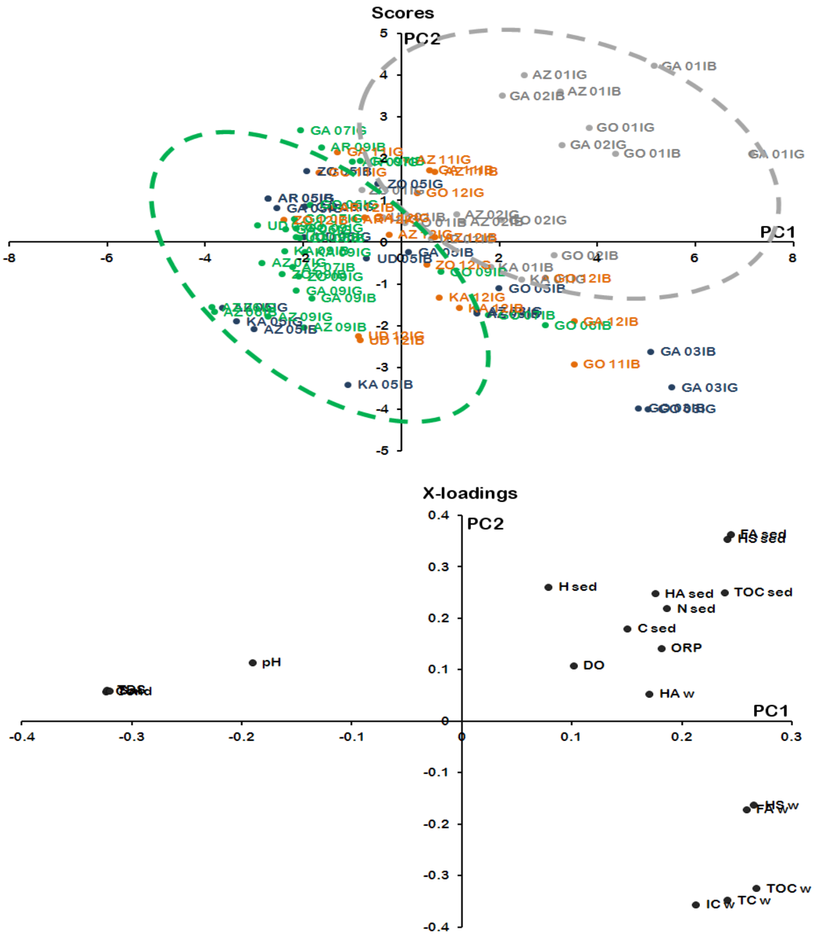


Figure 8.7. *Scores and loadings plot (PC1 vs PC2) of physicochemical parameters and HS concentration of water and sediment samples in all sampling locations and seasons.*

According to the scores plot, samples seem to be distributed along the space according to the seasonality (grey corresponds to winter, green to summer, the rest of seasons are distributed across the middle). It seems that there is a distribution of groups through the season of the year based on the influence of some variables (see loadings plot in Figure 8.8). Hence, samples collected in winter (grey group) are more influenced by the parameters measured in sediments. Along the seasons, water related variables (TDS, pH and conductivity) gain strength especially in summer. Intermediate seasons among winter and summer (spring colored in dark blue and autumn colored in orange), show an intermediate influence between water and sediments related variables.

Going deeper into the results, the trend along the year can be explained according to the high influence observed in winter by DO, ORP and HS in sediment and in summer by TDS and conductivity. That is, on the one hand, in autumn and winter, organic matter accumulated during the year in sediments is oxidized and the concentration of HS in sediments is increased; alternatively, new HS formed during the decomposition of the terrestrial flora can enter in the waters from the run-off of the soils near the river after rainfall events in autumn-winter time. On the other hand, in spring, increased water flow can reduce HS concentrations in water by pulling or dilution; alternatively, the growing of microorganisms mass in spring time reduce the HS in solution due to their metabolic processes (they “eat” cutting the C-C and C-O bonds of the complex HS substances).

In order to assess the HS behavior in water and sediment samples, separate PCA analyses of data were considered. When water samples were only considered, the previous described displacement along the seasons was also observed. These results are shown in the Figure 8.8 where the scores and loadings plot of PC1 (explaining the 47% of the total variance) and PC2 (explaining the 19% of the total variance) are plotted. According to these results, it can be noticed that the distribution of water samples through the seasons is mainly dominated by oxidation parameters (e.g., DO and ORP) and the water related parameters (e.g. conductivity, TDS and pH).

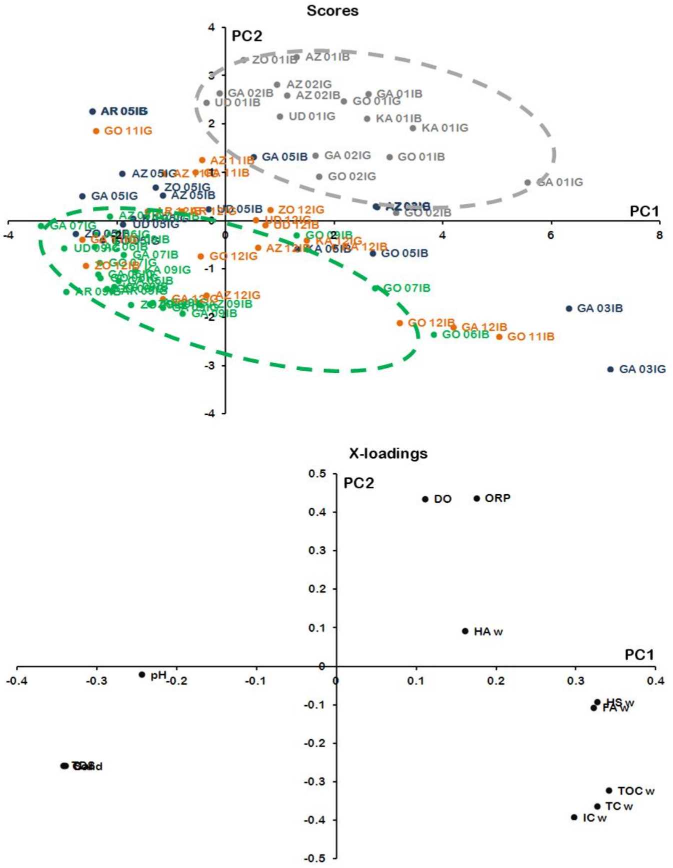


Figure 8.8. *Scores and loadings plot (PC1 vs PC2) of physicochemical parameters and HS concentration in water samples for all sampling locations and seasons.*

Although the PC3 only explains the 10% of the variance, the representation of PC1 vs PC3 loadings plot (see Figure 8.9) allowed us to identify the relation between the HA concentration in water with oxidation/oxygen parameters, as HA content in water can be found separated from the rest of variables related with organic matter or carbon content in water (e.g., FA, TOC_w and IC_w).

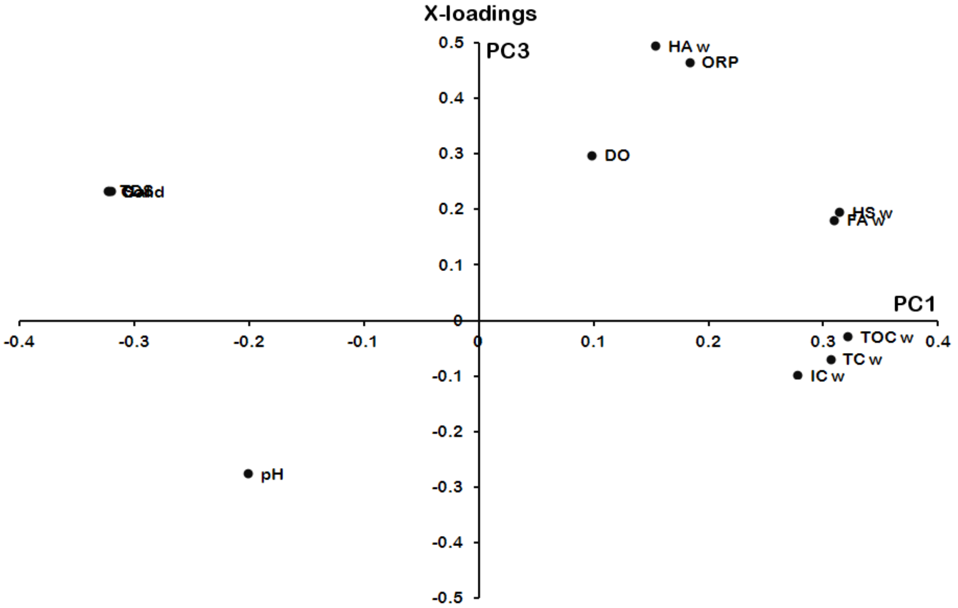


Figure 8.9. *Loadings plot (PC1 vs PC3) of physicochemical parameters and HS concentration for all sampling locations and seasons taking into account only water values.*

The same PCA procedure was also performed taking into account only sediment samples and sediment related variables. The obtained PCA scores and loading plots for PC1 and PC2 are illustrated in Figure 8.10. In this case, the first PC explains up to 48% of the total variance and the second PC explains the 28% of the variance.

According to the scores plot, the samples are randomly distributed along the bidimensional plot regardless the sampling location, indicating that there is not a clear site dependence. However, when FA vs HA ratio was calculated two different groupings can be observed: those samples with a FA/HA $\geq$ 4.5, which are related to a higher content of FA (e.g., GA, UD, GO) and those samples with a FA/HA < 4.5, which are related to a lower content of FA (e.g. AZ, ZO, AR, KA).

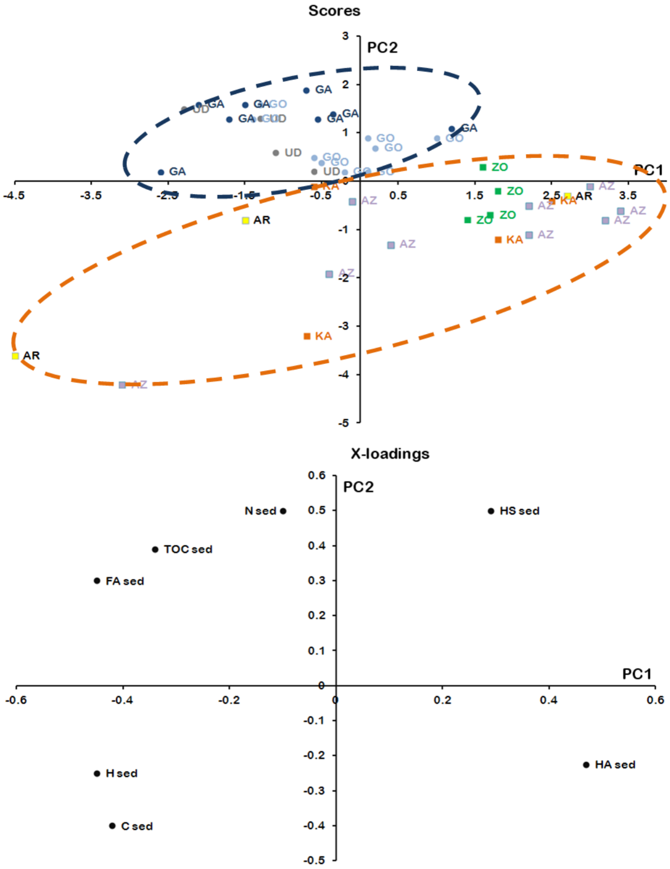


Figure 8.10. *Scores and loadings plot (PC1 vs PC2) of physicochemical parameters and HS concentration in sediments for all sampling locations and seasons. Blue color ellipse indicated samples with FA/HA > 4.5 and orange one, samples with FA/HA < 4.5.*

Besides, it should be taken in mind the specific location of sampling points: AZ, ZO, UD and AR are placed in the main course of the estuary, whereas GA, KA and GO correspond to tributaries emplacement. According to this, it seems that there is a distribution of the samples based on their specific location and FA/HA ratio in sediment: those placed in the main course (FA/HA < 4.5) and those related with tributaries (FA/HA > 4.5). This trend is quite clear except for the UD emplacement, which is grouped together with GA and GO instead of with those sampling points located in the main course of the estuary. This exception can be explained by the human-made obstacles to the main course situated in here (it is a dock), protecting the sampling point from the direct flow of the main course (see Figure 8.12). This can force UD to get inside the group of the tributaries and not in the main course group showing bigger accumulation of HS. The barriers and dykes in the estuary demonstrate the capacity of altering a location from its natural trend. On the contrary, KA (a tributary) is grouped with main course locations except in summer when water level and water flow is minimum, conditions that favor the accumulation of HS.

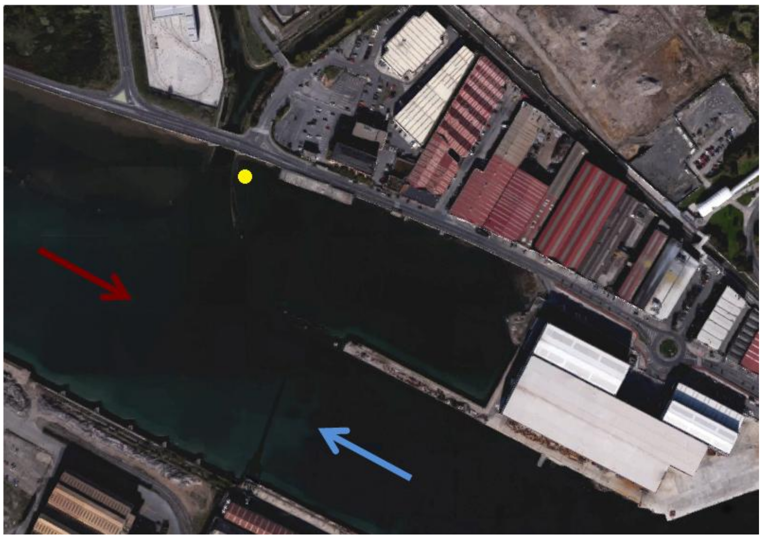


Figure 8.12. *Detailed sampling location maps for UD. The sampling point is marked with a yellow point. Blue arrow shows the continental water course direction whereas red arrow shows the seawater direction in high tide.*

8.2.4 Mobility trends

In order to obtain the possible availability of FA and HA from sediment to water and vice versa, mobility experiments were conducted with sediments sampled on September and December and using the previously proposed conditions (for more details see chapter 4). The mobilized portion (M_i, $^0/_{00}$) of the substance, referred to dried sediment, was calculated by means of the equation 8.1.

$$\mathrm{M_i} = \frac{2.5 * (\,C_1 - C_0)}{(1000 * C_{sed})}$$

Equation 8.1. *Expression used to calculate HS mobilities in the sediments.*

where: C_1 and C_0 were the concentrations (μg·l^{-1}) of mobilized substance in the extract before and after the extraction process, respectively. C_{sed} was the concentration (mg·kg^{-1}) of the mobilized substance in the dried sediment. To these assays, water gathered in high and low tide was used and the mobilized concentrations of HS are shown in Table 8.1.

Table 8.1. *Mobility of HS using both high tide and low tide estuarine water.*

		September		December	
Sample	**Tide**	**HA M_i**	**FA M_i**	**HA M_i**	**FA M_i**
AZ	High	0.20	0.20	-0.65	-0.53
GA	High	-3.46	0.33	0.11	0.38
GO	High	0.70	0.38	-0.38	0.43
KA	High	-0.57	1.02	-1.37	-0.81
UD	High	-0.64	0.39	0.07	0.31
ZO	High	0.83	0.37	-0.95	-0.61
AZ	Low	0.73	0.91	1.35	1.96
GA	Low	-3.20	-1.04	-0.32	-0.38
GO	Low	-1.43	-0.06	-4.04	-0.57
KA	Low	-1.05	-1.84	0.88	0.62
UD	Low	-1.00	-1.23	-2.02	-2.27
ZO	Low	0.99	-0.55	1.77	1.04

Overall, the mobilization of FA is favored in comparison to HA regardless the sampling site, period or even tide, since most of the points showed higher mobilization values for FA (except ZO). This can be explained by the higher FA solubility compared to HA. Taking into account this previous assumption, different mobilization behaviors were detected for FA and HA under specific conditions (e.g. tide and sampling location) as it will be following discussed:

- Overall, higher mobilities or lower deposition indexes are found on FA compared to HA.
- In the September sampling campaign, regardless the sampling location, the physicochemical characteristics of low tide water samples do not promote the mobilization of HS, being all the mobilization values larger at high tide conditions in comparison to low tide conditions. Hence, higher accumulation/deposition of HS are expected in environmental scenarios where larger input of freshwater occur, except for AZ and ZO.
- In December, the main tendency is to deposit HS in low tide conditions (e.g., GA, GO, UD), whereas some locations tend to behave contrarily (e.g., AZ, KA, ZO).
- Overall, those sampling points behaving as tributaries (e.g., GA, KA and UD) showed lower mobilization values for HA (among -4.04 and -0.32) in comparison to the sampling points located in the main course (e.g. AZ and ZO with mobilization values of HA among -0.65 and 1.77). This is in correspondence with the previous results obtained by means of PCA, where samples can be classified based on their FA/HA ratio, emplacement and according to the mobilization trends.

8.3. Conclusions

The proposed methodology for the determination of HS in water and sediments has proven to be very useful in the quantification of HS in estuarine environmental matrices. Moreover, the monitoring of these compounds in estuarine sampling locations, affected by different anthropogenic and natural stressors, may enable having a more comprehensive picture of the quality of the estuarine system.

In the specific scenario of Nerbioi-Ibaizabal estuary, higher concentration of FA were found in sediment samples (among 1957 $mg \cdot kg^{-1}$ - 16082 $mg \cdot kg^{-1}$) in comparison to HA (among 293 mg·kg^{-1}- 6812 mg·kg^{-1}), which can indicate a good quality of the humus. In fact, the FA/HA ratio can be a good indicator to assess the behavior of estuarine sampling sites, this is, sites where the deposition of HS is favored versus those where larger mobilization of HS can occur. Besides, the behavior of the HS concentration showed dependence with the physicochemical parameters and tidal/weather events. In general terms, higher mobilization of HS was found at high tide or when lower freshwater input occurs. This statement is also related with water/sediment physicochemical parameters. In fact, the correlation obtained between pH and ORP has been found to be a clue about the sulphides influence on the system.

TOC percentage in sediments has proven to be a basic estimation of the HS, for example, higher values of TOC were found on GA, GO and UD locations (proven deposition areas) and ORP and DO have proven to be important parameters in order to determine the deposition or releasing of HS in the estuarine system.

Besides, the assessment of HS methodology using real water samples has proven to be a powerful tool to mimic the natural trend of HS regarding deposition/solubilization trends. In fact, this methodology allowed to assess the effect of tide events in the HS distribution in different environmental compartments.

This work has revealed the importance of the HS analysis at the time of analyzing contaminants as well as physicochemical parameters in estuarine water and sediments. Moreover, the monitoring processes must take this into account and make different evaluation of the compounds along the year and not in short periods of time because the presence of humic substances is seasonally dependent.

8.4 References

[Aiken et al., 1985] Aiken GR, McKnight DM, Wershaw RL, MacCarthy P. *Humic Substances in Soil, Sediment and Water.* Wiley, New York, 1985.

[Bartolomé, 2003] Bartolomé L J. *Monitorización de diversos contaminantes organicos en sedimentos de los estuarios de Urdaibai y Nerbioi-Ibaizabal.* Bachelor thesis, 2003.

[Bianchi 2011] Bianchi TS. *The role of terrestrially derived organic carbon in the coastal ocean: a changing paradigm and the priming effect.* Proc. Natl. Acad. Sci. U.S.A. 2011; 108, 19473 – 19481.

[Buffle et al., 1977] Buffle J, Greter FL, Haerdi W. *Measurement of complexation properties of humic and fulvic acids in natural waters with lead and copper ion-selective electrodes. Anal. Chem.* 1977; 49, 216 – 222.

[Canuel et al., 2012] Canuel EA, Cammer SS, McIntosh HA, Pondell CR. *Climate change impacts on the organic carbon cycle at the land–ocean interface.* Annu. Rev. Earth Planet. Sci. 2012; 40, 685 – 711.

[Davis, 1984] Davis JA. *Complexation of trace metals by adsorbed natural organic matter. Geochim. Cosmochic. Ac.* 1984; 48, 679 – 691.

[Esbensen, 1994] Esbensen KH. *Multivariate data analysis: an introduction to multivariate data analysis and experimental design, Camo AB*, Oslo, 1994.

[Fabiano et al., 1995] Fabiano M, Danovaro R, Fraschetti S. *A three-year time series of elemental and biochemical composition of organic matter in subtidal sandy sediments of the Ligurian Sea (northwestern Mediterranean). Cont. Shelf Res*. 1995; 15, 1453 – 1469.

[Fang et al., 2015] Fang K, Dongxing Y, Zhang L, Feng L, Chen Y, Wang Y. *Effect of environmental factors on the complexation of iron and humic acid*. J. Environm. Sci. 2015; 27, 188 – 196.

[Fdez-Ortiz de Vallejuelo et al., 2014] Fdez-Ortiz de Vallejuelo S, Gredilla A, de Diego A, Arana A, Madariaga JM. *Methodology to assess the mobility of trace elements between water and contaminated estuarine sediments as a function of the site physicochemical characteristics.* Sci. Tot. Environ. 2014; 473 – 474, 359 – 371.

[Fletcher and Beckett, 1987] Fletcher P, Beckett PHT. *The chemistry of heavy metals in digested sewage sludge—II. Heavy metal complexation with soluble organic matter. Water Res*. 1987; 21, 1163 – 1172.

[Gredilla et al., 2013] Gredilla A, Fdez-Ortiz de Vallejuelo S, Arana G, de Diego A, Madariaga JM. *Long-term monitoring of metal pollution in sediments from the estuary of the Nerbioi-Ibaizabal River 2005 – 2010.* Estuar. Coast. Shelf S. 2013; 131, 129 – 139.

[**Grémare et al., 1997]** Gremare A, Amouroux JM, Charles F, Dinet A, Riaux-Gobin C, Baudart J, Medernach L, Bodiou JY, Vetion G, Colomines JC, Albert P. *Temporal changes in the biochemical composition and nutritional value of the particulate organic matter available to surface deposit-feeders: a two year study. Mar. Ecol. Prog. Ser*. 1997; 150, 195 – 206.

[Halbedel et al., 2013] Halbedel S, Büttner O, Weitere M. *Linkage between the temporal and spatial variability of disolved organic matter and whole-stream metabolism.* Biogesciences. 2013; 10, 5555 – 5569.

[Hiraide, 1992] Hiraide M. *Heavy Metals Complexed with Humic Substances in Fresh Water. Anal. Sci.* 1992; 8, 453 – 459.

[Hou et al., 2013] Hou DK, He J, Lu CW, Fan QY, Wang JH, Xie ZL. *Distribution characteristics and potential ecological risk assessment of heavy metals (Cu, Pb, Zn, Cd) in water and sediments form Lake Dalinquer*. Ecotoxicol. Environ. Saf. 2013; 93, 135 – 144.

[Iskrenova-Tchoukova et al., 2010] Iskrenova-Tchoukova E, Kalinichev AG, Kirkpatrick RJ. *Metal Cation Complexation with Natural Organic Matter in Aqueous Solutions: Molecular Dynamics Simulations and Potentials of Mean Force. Langmuir*. 2010; 26, 15909 – 15919.

[Kögel-Knabner et al., 1998] Kögel-Knabner I, Totsche KU. *Influence of dissolved and colloidal phase humic substances on the transport of hydrophobic organic contaminants in soils. Phys. Chem. Earth.* 1998; 23, 179 – 185.

[Laborda et al., 2008] Laborda F, Bolea E, Górriz MP, Martín-Ruiz MP, Ruiz-Beguería S, Castillo JR. *A speciation methodology to study the contributions of humic-like and fulvic-like acids to the mobilization of metals from compost using size exclusion chromatography-ultraviolet absorption-inductively coupled plasma mass spectrometry and deconvolution analysis. Anal Chim Acta.* 2008; 606, 1 – 8.

[Lund, 1990] Lund W. *The complexation of metal ions by humic substances in natural waters*. In: Broekaert JAC, Gücer S, Adams F (Eds.) *Included in metals speciation in the environment*, pp. 43 – 56, Springer-Verlag, Berlin, Germany. 1990.

[Ma et al., 2001] Ma H, Allen HE, Yin Y. *Characterization of isolated fractions of dissolved organic matter from natural waters and a wastewater effluent.* Wat. Res. 2001; 35, 985 – 996.

[Marie et al., 2015] Marie L, Pernet-Coudrier B, Waeles M, Gabon M, Riso R. *Dynamics and sources of reduced sulfur, humic substances and dissolved organic carbon in a temperate river system affected by agricultural practices.* Sci. Tot. Environm. 2015; 537, 23 – 32.

[Naidja et al., 2002] Naidja A, Huang PM, Anderson DW, van Kessel C. *Fourier transform infrared, UV-visible and X-ray diffraction analyses of organic matter in humin, humic acid and fulvic acid fractions in soil exposed to elevated CO2 and N fertilization.* Appl. Spectrosc. 2002; 56, 318 – 324.

[Page et al., 1982] Page AL, Miller RH, Keeney DR. *Methods of Soil Analysis: Part 2, Chemical and Microbiological Properties. Agronomy Series No 9 part 2, American Society of Agronomy*, Madison, WI (USA), 1982.

[Pantano et al., 2014] Pantano G, Amanda MR, Bisinoti MC, Benedito Moreira A. *Seasonal variability of a conditional stability constant and characterization of sedimentary humic substances form typocal agricultural and urban areas.* J. Soil Sed. 2014; 14, 385 – 393.

[Tockner et al., 1999] Tockner K, Pennetzdorfer D, Reiner N, Schiemer F, Ward LV, *Hydrological connectivity, and the exchange of organic matter and nutrients in a dynamic river–floodplain system (Danube, Austria)* Freshwater Biol. 1999; 3, 521 – 535.

[Weng et al., 2002] Weng L, Temminghoff EJM, Lofts S, Tippping E, Van Riemsdijk WH. *Complexation with Dissolved Organic Matter and Solubility Control of Heavy Metals in a Sandy Soil.* Environ. Sci. Technol. 2002; 36, 4804 – 4810.

[Yamada et al., 2000] Yamada E, Doi K, Okano K, Fuse Y. *Simultaneous determinations of the concentration and molecular weigth of humic substances in environmental water by gel chromatography with a fluorescence detector.* Anal. Sci. 2000; 16, 125 – 129.

9. New methodology for reducing matrix effect in biologic samples

9. New methodology for reducing matrix effect in biologic samples

Bivalve mollusks, mainly mussels (Mytilus galloprovincialis), result very suitable for aquatic environment biomonitoring due to their outstanding characteristics as their abundance, their suitable size, sedentary state, ease of collection, transplantation, maintenance in the laboratory, their wide distribution and their filter feeding activity that favors the bioaccumulation of contaminants [Khadim, 1990]. For that reason, they have been widely used as sentinel organisms for the evaluation of coastal contamination [do Amaral et al., 2005; Viarengo et al., 2000; Franco et al., 2002].

As a consequence of the increasing environmental and toxicological requirements, several metal profiling studies in mussel tissues have been exhaustively performed [Poperechna and Heumann, 2005; Yoshinaga et al., 1999; Yang et al., 2004] using inductively coupled plasma mass spectrometry (ICP-MS) as sample analysis method. This instrumental technique has several advantages, such as the ability to determine several metal ions simultaneously, very low detection limits and high repeatability [Hirata et al., 2001]. However, ICP-MS technique is liable to show spectral interferences, e.g. isobaric, doubly charged, and polyatomic interferences in addition to chemical interferences from the matrix [Hattendorf and Günther, 2001; May and Wiedmeyer, 1998; Evans and Giglio, 1993]. In this sense, the presence of interfering compounds as fat content and its organic residues should be minimized.

To this end, both the digestion procedure conditions and the clean-up step of the extracts are key steps to get reliable results by means of ICP-MS. The requirements to develop a suitable method for metal determination in mussel tissue are: (i) the use of a clean-up step that eliminates quantitatively the elimination of the interfering fats without losing target analytes and, (ii) the use a digestion procedure that involve total recoverable processes. The use of a hard oxidative process for the digestion step can favor the minimization or even the reduction of the interferences.

Microwave-assisted extraction (MAE) systems are widely used as digestion systems for several environmental matrixes including mussel tissues [Jayaraman et al., 2001; Soto-Ferreiro et al., 1991; Vaidya and Rantala, 1996]. However, before the routine use of this acid digestion procedure, the optimization of the chemical and instrumental variables must be undertaken preferably by experimental design [Davies, 1993] in order to assure the fitness of the digestion procedure.

When ICP-MS is used, it should be rather avoided hydrochloric acid due to the interference of the classic argide $^{40}Ar^{35}Cl$ with the monoisotopic ^{75}As [Montaser, 1998]. In this sense, and for the extraction process, chemical (concentration of HNO_3) and instrumental variables (radio frequency power and digestion time) were fully optimized. The goodness of the analytical method was evaluated using different certified reference materials

However, using high nitric acid concentration and under high pressure and high temperature conditions (such as microwave systems), it was observed an increase in the oxidant power of the reaction mixtures, oxidizing a greater amount of compounds and generating a wider variety of organic residues [Gonzalez et al., 2009]. Several published works have reported fat and protein by-products formation after microwave acid digestion like benzoic, oxalic, picric, terephthalic, and m- and p- nitrobenzoic acids [Gonzalez et al., 2009; Pratt et al., 1988]. These products are sources of carbon which may give space charge effects or salt build up on the orifice of the interface sampler cone. Another isobaric interference comes from the formation of $^{40}Ar^{12}C$ which difficults the analysis of ^{52}Cr [Montaser, 1998].

Besides, as the metal standards are usually prepared in 1.0% vol HNO_3, the use of higher concentration of HNO_3 or the presence of a random amount of organic residuals, can introduce a bias in the metal analysis. In fact, unexpected higher relative standard deviations were pointed out critically by some authors [Bartolome et al., 2010a]. Consequently, the use of a previous clean-up step was considered in order to get rid of the fats and to improve the quality of the analytical results.

The suitability of the methodology was checked using mainly spectroscopic techniques, such as Fourier Transform infrared spectroscopy (FTIR) and Energy Dispersive X-ray spectroscopy (ED-XRF), allowing the characterization of isolated fraction that will be discarded. Moreover, based on the studied best solvent, the extraction of the fat content in a rotary shaker, and the recovery of metals in the MAE was optimized by means of full factorial and central composite designs [Esbensen, 1994].

Finally, the effectiveness of the analytical method was proven in order to test the accuracy and precision by re-analyzing exactly the same real individuals collected at 10 locations of Bay of Biscay, northern Spain [Bartolome et al., 2010a]. The mean concentration and standard deviation were compared for both without clean step method and with the clean up step method developed in this chapter.

9.1. Materials and methods

Real samples collected and pre-treated (freeze-dried mussel tissues) by Bartolome [Bartolome et al., 2010a] were used in the analytical method development and optimizations performed in this chapter. The stabilization of these real samples is an important step to preserve material characteristics, avoiding any physicochemical degradation. Proper storage conditions are essential to guarantee that the material remains unaltered for a long time. Thus, as soon as the monitoring study was finished [Bartolome et al., 2010a], the samples were stored in containers at −42 °C [Navarro et al., 2010].

9.1.1. Reagents

All reagents were of analytical-reagent grade and ultrapure water (Milli-Q® quality, Millipore™, Bedford, MA, USA) was employed. The volumetric glassware was grade A and was calibrated at laboratory temperature. 1000 $mg \cdot l^{-1}$ Alfa Aesar (Karlsruhe, Germany) Specpure Plasma Standard stock solutions of As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, Sn, V and Zn were used. Internal standard solutions (Be, Sc, In and Bi) were purchased from Alfa Aesar (Specpure, Karlsruhe, German). Nitric acid (69%, Tracepur) was provided by Merck (Darmstadt, German). Dichloromethane was used as extracting agent and provided by Labscan (HPLC grade, Barcelona, Spain). For the evaluation of the efficiency of the analytical method, the standard reference material SRMs 2977 (mussel tissue) purchased from NIST (Gaithersburg, USA) was used.

9.1.2. Instrumentation

A Planetary Mono Mill Pulverisette 6 (Fritsch GmbH, Oberstein, Germany) agate ball mill and a Laborette 27 rotary cone sample divider (Fritsch GmbH, Oberstein, Germany) were used for the preparation and homogenization of the mussel samples after stabilizations at room temperature.

The qualitative analyses were performed using spectroscopic techniques. According to infrared analysis, all FTIR spectra were collected in the middle infrared region (400–4000 cm^{-1}) recording 40 scans per spectrum at a spectral resolution of 4 cm^{-1}. A Jasco 6300 FTIR spectrometer was used for this purpose.

The elemental measurements were carried out using a portable Röntec (at the moment, Bruker AG) ArTAX µ-EDXRF with an X-ray tube with molybdenum anode working at a maximum voltage of 50 KV and a maximum current of 0.6 mA. The X-ray fluorescence is detected by means of a thermoelectrically cooled Si-drift (XFlash) detector which has an active area of 5 mm^2 and 8 µm beryllium window. The operating conditions during the µ-EDXRF measurements were fixed at 1800 s, at a voltage of 50 KV and a current of 0.6 mA.

In order to perform the quantitative analysis, microwave assisted digestion was carried out in a close microwave device Multiwave 3000 (Anton Paar, Graz, Austria) equipped with 8 Teflon vessels and temperature controllers.

Inductively coupled plasma with mass detector (ICP-MS 7700x, Agilent Technologies, Palo Alto, USA) was used for metal determinations using a MicroMist micro-uptake glass concentric nebulizer (Glass Expansion, West Melbourne, Victoria, Australia) in a class 100 clean room. In order to reduce MO^+ formation in the plasma, the spray chamber was Peltier cooled at 2 °C. Finally, standard nickel cones (sample and skimmer) were generally used. Operating conditions are shown in Table 9.1. The acquisition masses and integration times (Table 9.1) provided more than sufficient sensitivity to meet all certified values. The optimization of the ICP-MS conditions was achieved by adjusting the torch position and tuning for reduced oxide and doubly charged ion formation with a standard tuning solution containing 1.0 $\mu g \cdot l^{-1}$ of ^{7}Li, ^{24}Mg, ^{59}Co, ^{89}Y, ^{140}Ce and ^{205}Tl in 1.0% HNO_3. This equipment includes a collision cell (He gas, ORS^3 system, Agilent Technologies©) for discriminate spectral interferences with high performance for all the metals considered in here. In addition, EPA 6020 [EPA, 2007] recommendations were followed for interference overcoming such as correction equations for arsenic, lead or cadmium.

Table 9.1. *ICP-MS operating and acquisition parameters.*

RF power (W)	1550	**Plasma gas flow ($l\cdot min^{-1}$)**	15
Cell output (V)	-30	**Carrier gas flow ($l\cdot min^{-1}$)**	0,85-0,90
Octopole RF (V)	150	**Sample flow rate ($ml\cdot min^{-1}$)**	0,1
Octopole bias	-6	**He flow rate ($ml\cdot min^{-1}$)**	4,3
QP bias	-3	**Data acquisition**	(Dwell time, 300 ms)
Extraction lens 1 (V)	2	**Sweeps per replicate**	8
Extraction lens 2 (V)	-140	**Replicates**	3
Omega bias (V)	-30	**Detection mode**	Peak hopping
Omega lens (V)	1		
Cell input (V)	-34	**Isotopes**	^{75}As, ^{114}Cd, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{202}Hg, ^{55}Mn, ^{56}Ni, ^{208}Pb, ^{207}Pb, ^{206}Pb, ^{78}Se , ^{82}Se, ^{118}Sn, ^{123}Sb, ^{51}V, ^{66}Zn.
QP focus	2		

9.1.3. General analytical procedures

Rigorous cleaning procedures of all the laboratory ware and other equipment that comes into contact with the samples must be employed in order to avoid contamination. All glassware and plastic ware were washed with a common detergent and thoroughly rinsed with pure water Elix® quality, Millipore™, Bedford, MA, USA). After that, all the laboratory ware was soaked into a clean dilute HNO_3 (10%) bath for 24 h. The material was rinsed with ultrapure water (Milli-Q® quality, Millipore™, Bedford, MA, USA).

For the clean-up step, 1.0 g of each sample was weighed (after determination of percent moisture) in the extraction tube and 5 ml of pure dichloromethane was added. After that, the extraction was carried out at 1.0 rpm for 5.0 min in a rotary shaker. This methodology is operationally based on the procedure defined by the NOAA [Wade et al., 1993].

Once the agitation period was over, the mixture was centrifuged and the supernatant was weighed after evaporation at 65 °C (% dry residue or fat content). On the other hand, the solid residue (mussel tissue) was dried at 65 °C until constant weight and stored at room temperature in a desiccator until acid treatment for metals extraction.

Briefly, 0.2 g of this solid residue was exactly weighed and digested using 15 ml of HNO_3 (7.0% vol) by microwave assisted digestion (980W for 18 minutes). The digestion program was run according to the experimental designs and optimal extraction conditions. The extracts were filtered through 0.45 µm filters (PVDF, Whatmann) and brought to a final volume of 100 ml using Milli-Q water with a final HNO_3 percentage of 1.0% in order to be analyzed by ICP-MS.

9.2. Results and discussion

9.2.1. Qualitative spectroscopic analysis

FT-IR or µ-ED-XRF methods are well suited for molecular and elemental analysis studies on biological systems. The possible aims of such analysis include structural proteins, lipids, enzymes, domains thereof or nucleic acid derivatives [Szoboszlai et al., 2009]. In this work both spectroscopic methods were used for qualitative purposes.

Different extracting agents were tested in this work to isolate the fat content of mussel tissues such as dichloromethane, hexane and different alcohols. A standard extraction procedure defined by NOAA[1] was followed [Wade et al., 1993]. Briefly, 1.0 g of mussel tissue was extracted using 15 ml of each solvent for 1 h. The organic liquid fraction was poured drop by drop into a pressed KBr powder pellet (applying a total pressure of 10 tons with a resulting diameter of 10 mm and 1 mm width). The pellet was allowed to stand for the evaporation of solvent in a flux cabin. Thereafter, XRF analysis was performed to qualitatively elucidate the presence of metal in the final extracts and FT-IR for the qualitative characterization of the final extracts.

---

[1] *National Oceanic and Atmospheric Administration (USA)*

Figure 9.1 shows examples of the FT-IR spectra obtained for the final extracts: (a) in dichloromethane and (b) in methanol. Figure 9.1a shows the comparison between the sample (A) and a triglyceride standard (B) extracted in dichloromethane. The spectrum of triglyceride is characterized by a C=O band in 1740–1750 cm^{-1}, the methylene stretching band occurred at 2850–2960 cm^{-1} with a C=C–H band at 3020 cm^{-1}. Other bands such as C–H at 1464, 1379 and 725 cm^{-1} and C–O at 1240, 1165 and 1103 cm^{-1} also appeared; this fact produced a specific profile being the intermediate C=O band the most intense one.

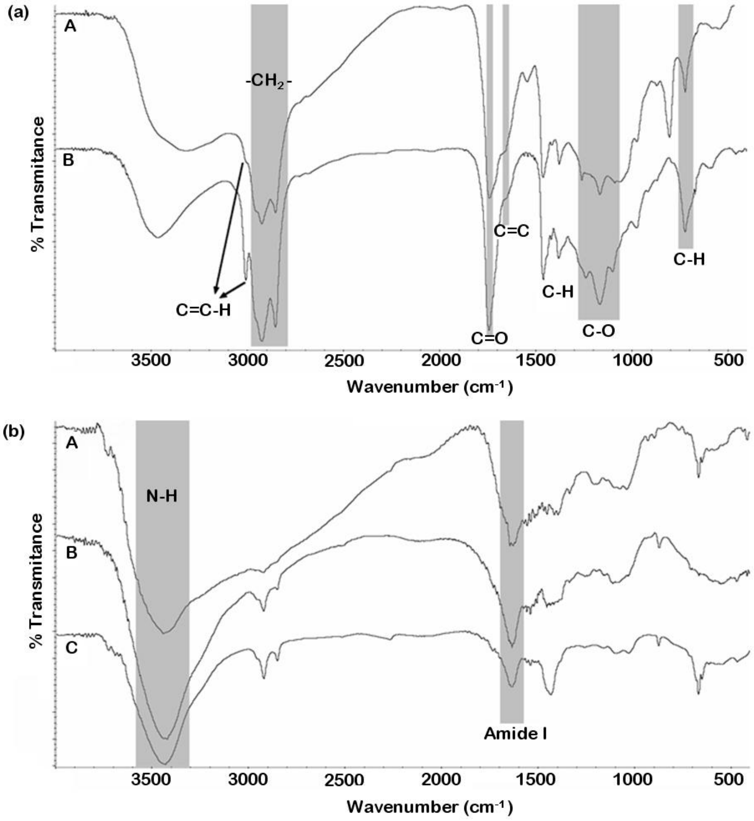


Figure 9.1. *FTIR spectra obtained for in dichloromethane (a): A — sample and B — standard oil (triglyceride) and in methanol (b): A — sample and B, C — two types of fish glue (collagen).*

On the other hand, proteins (Figure 9.1b) were characterized by the amide I and II bands at 1650 and 1550 cm^{-1}, sometimes, the amide III band at 1450 cm^{-1}, and finally, the stretching N–H band at 3350 cm^{-1}.

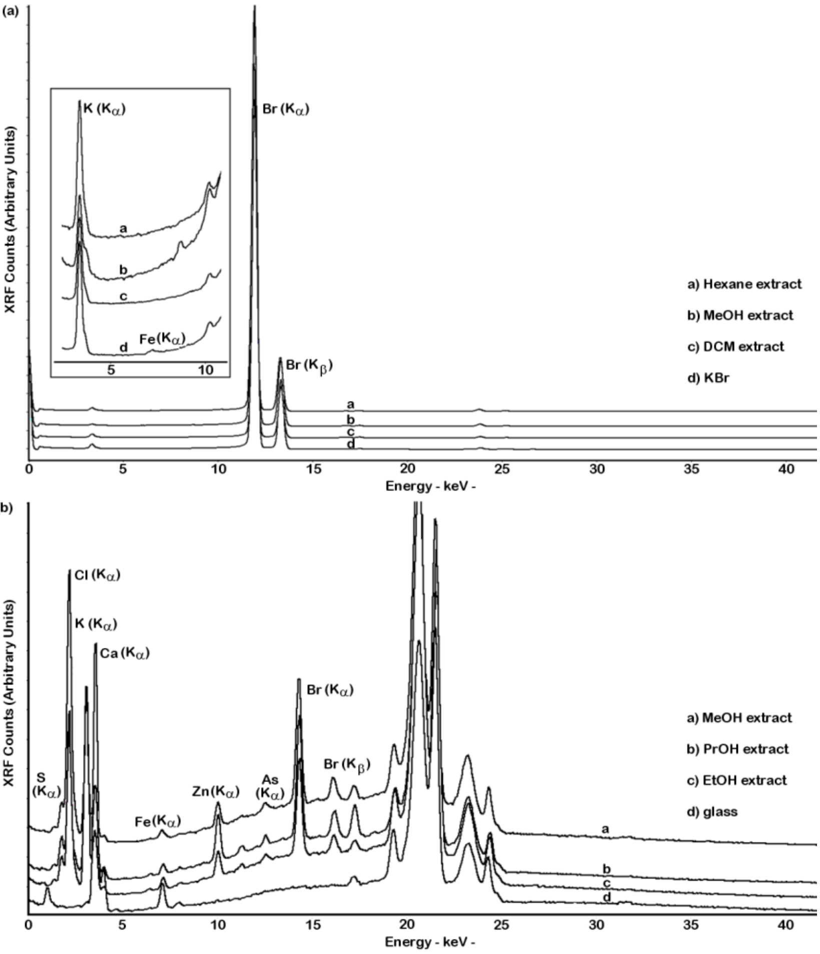


Figure 9.2. *XRF spectra obtained for different extracting agents.*

Finally, two samples of the XRF spectra obtained in each case are shown in Figure 9.2 considering the raw signal: (a) organic liquid fraction concentrated in KBr pellets and (b) organic dry residue concentrated in a glass. With the purpose of excluding the noise, the final spectral range (830–1024 channels, 40.28–50 KeV) was not included. As can be seen, the extracts obtained using MeOH or isopropanol showed qualitatively the presence of metals like As, Fe or Zn. This fact did not happen when dichloromethane or hexane was used as clean-up solvents. For that reason, the alcohols tested were discarded as extracting agents in the further optimization procedures.

9.2.2. Optimization of the microwave-assisted digestion for the analysis of metals in mussel tissue

According to the conclusion obtained from XRF and IR analysis, it can be assumed that dichloromethane or hexane could be promising alternatives as lipid extractant agents as they allowed the quantitative extraction of lipid compounds without extracting metals.

In order to optimize the lipid extraction procedure, the influence of several variables (i.e., % hexane:dichloromethane, solvent volume, agitation speed and extraction time) was assessed using an experimental design approach. The efficiency of the clean-up step was defined as the maximum recovery of the lipid compounds in the extraction procedure, i.e., the maximum dry residue weight obtained from the evaporated extract at 65 °C. This procedure was based on that proposed by NOAA [Wade et al., 1993] to treat approximately 1.0 g of mussel tissue sample.

In a first approach, in order to find the main factors affecting the clean-up step a two-level full factorial design was carried out using the Unscrambrer® software. The factor space was defined by 2^4 assays where the hexane % varied from 25 % to 75 %, the volume from 8.7 to 12.6 ml, the agitation speed from 3 to 7 rpm and the extraction time from 30 to 90 min. Three replicates of the central point were also performed. According to these results, it was concluded that the time of lipid extraction was not significant at 95% of confidence level (p-level > 0.05). However, since the rest of the variables were significant at the studied interval, the previous design was extended to a central composite design in order to build up the response surfaces and to obtain the instrumental conditions that define the maximum response for the clean-up step. Table 9.2 shows the extension to central composite design defined by The Unscrambler® software.

Table 9.2. *Experimentation proposed by central composite design to optimize the clean-up step.*

Levels	-α	-1	0	+1	+α
% Hexane	0	25	50	75	100
rpm	1	3	5	7	9
t (min)	5	33.7	62.5	91.2	120
V (ml)	5	8.7	12.5	16.2	20

The response surfaces were built with The Unscrambler® and the significant effect of variables were also reassured ($p<0.05$). Since the time was not significant it was fixed at the minimum value (5 min). The response surfaces were built as a function of the most significant variables for maximum fat dry residue. From these plots, it can be simultaneously deduced the optimum conditions within the factor space, i.e. without any extrapolation. In this sense, and using the response surfaces plotted in Figure 9.3, the optimum clean-up conditions were established for further experiments: the mussels tissues will be treated with a minimum solvent volume (5.0 ml) of pure dichloromethane (100%) with a minimum agitation speed (1.0 rpm) during 5 min.

After that clean up step, the fatty-free mussel tissue obtained (0.2 g) was acid digested by a microwave assisted system. The variables affecting the digestion step were first evaluated using two-level full factorial design. The design matrix consisted of 2^3 experiments and three replicates of the central point which allowed the evaluation of the effects of three variables within the following ranges: HNO_3 concentration (3–8% v/v), microwave power (448–952 W) and digestion time (8–17 min).

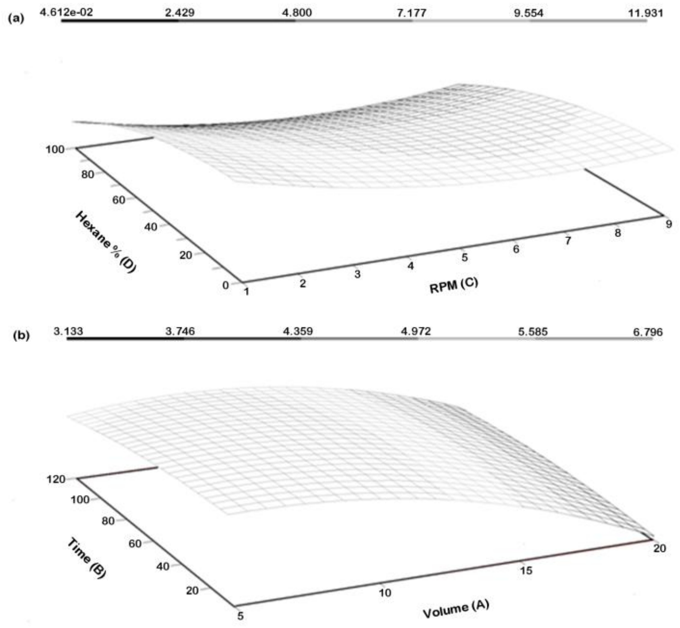


Figure 9.3. *Response surfaces defined by %hexane vs. rpm (a) and volume vs. time (b) for solid residue obtained in the extraction step.*

The concentration of each extracted metal was considered as the response of the experimental approach, and all the assessed variables were significant in the studied range at 95% of confidence level (p-level < 0.05) [Miller and Miller, 2010]. Hence, as it was performed in the previous case, the optimum values of each parameter were determined by means of a central composite design as it is detailed in Table 9.3.

Table 9.3. *Experimentation proposed by central composite design to optimize the microwave assisted digestion step.*

Levels	**-α**	**-1**	**0**	**+1**	**+α**
P (W)	280	448	700	952	1120
t (min)	5	8	12.5	17	20
C (% v/v))	1	2.8	5.5	8.2	10

According to the response surfaces built for each element, and taking into account the analysis of variance, all the studied variables were significant and have a positive effect in the extraction process. Figure 9.4 shows the response surfaces obtained for As, Cr and Zn when extraction time and acid concentration was varied and the microwave power was fixed at 980 W. Similar surface responses were obtained for the rest of the target analytes. As it can be seen, maximum local responses were obtained at the following conditions, which were set as optimum for the subsequent experiments: digestion time of 18 min using HNO_3 concentration of 7.0% v/v.

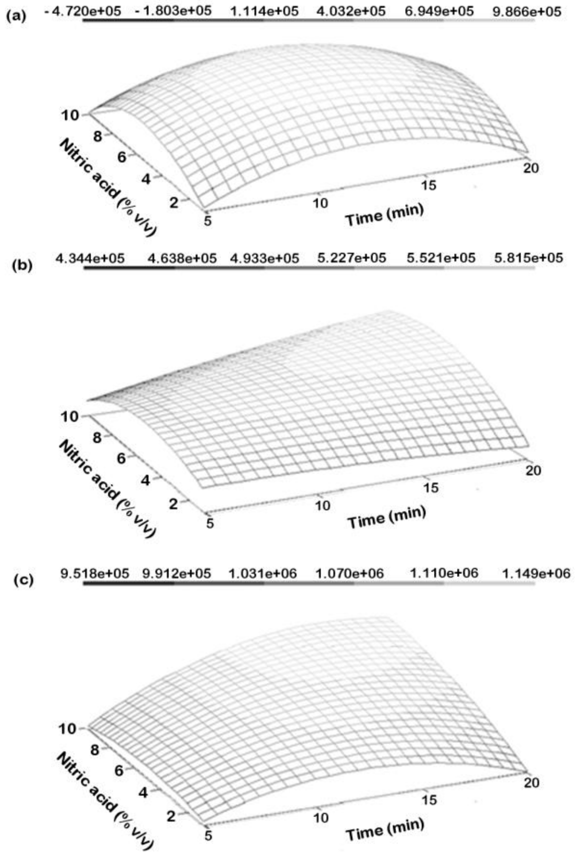


Figure 9.4. *Response surfaces defined by nitric acid concentration and digestion time for the As (a), Cr (b) and Zn (c) responses followed.*

9.2.3. Analytical performance

Using the optimized conditions, analytical figures of merit including detection limit and precision of replicate measurements were determined. The limit of detection (LOD) was calculated following the IUPAC rules, defined as blank signal+3·σ, where σ is the standard deviation of 5 measurements of a blank. The LODs estimated for the mussel samples were Cd, Co, Cr, Cu, Ni and V (<10 $ng \cdot g^{-1}$), As, Sb, Se and Sn (<20 $ng \cdot g^{-1}$), Hg (<25 $ng \cdot g^{-1}$), Mn (<0.5 $\mu g \cdot g^{-1}$), Pb (<0.1 $\mu g \cdot g^{-1}$) and Zn (<2.0 $\mu g \cdot g^{-1}$). Precision of the method (%RSD) within-a-day (n=6) and among days (n=6) was lower than 5.0% for all metals and concentrations tried.

The accuracy of the analytical procedure was verified analyzing the certified material NIST SRM 2977 (mussel tissue). These experimental data were compared to other data collected from those measured by Bartolome with the same SRM 2977 [Bartolome et al., 2010b]. This analytical method defined by Bartolome included microwave acid digestion but without any clean-up step. The results obtained are shown in Table 9.4 for each metal and a comparison with the corresponding certified values was performed by using the Z score defined in the equation 9.1. This parameter quantifies the number of standard deviations a measured value is outside the certified value (defined by mean ± σ).

$$Z = \frac{Certified\ value - Experimental\ value}{\sigma}$$

Equation 9.1. *Z score defining trueness when comparing a certified value with an analysis of it.*

In the equation 9.1, the σ parameter is defined by equation 9.2,

$$\sigma = \sqrt{\frac{S_{exp}^2}{n} + S_{cert}^2 + S_{longterm}^2}$$

Equation 9.2. *Definition of the σ parameter in equation 9.1.*

being S_{exp} the standard deviation of the analysis, S_{cert} the standard deviation of the certified value and $S_{longterm}$ is the standard deviation obtained in different days.

Due to the $S_{longterm}$ value non availability, $2 \cdot S_{exp}$ was used [Walker and Lumbey, 1999]. As it is known, the lack of trueness is considered if the Z score values are out from $-2<Z<2$.

Table 9.4. *Results obtained (mg·kg^{-1}) in the metal analysis of NIST 2977. A: collected in [Bartolome et al., 2010b]; B: using clean up procedure.*

	Cert.	**2977A**		**2977B**	
		Exp.	**Z**	**Exp.**	**Z**
As	8.8 ± 0.9	14 ± 2	-1.2	9.0 ± 0.1	-0.2
Cd	0.18 ± 0.01	0.32 ± 0.01	-6.1	0.17 ± 0.01	0.4
Co	0.5 ± 0.1	0.44 ± 0.01	0.6	0.49 ± 0.01	0.1
Cr	3.9 ± 0.5	4.6 ± 0.5	-0.6	4.00 ± 0.02	-0.2
Cu	9.4 ± 0.5	13 ± 3	-0.6	9.5 ± 0.1	-0.2
Hg	0.10 ± 0.01	0.12 ± 0.09	-0.1	0.11 ± 0.01	-0.4
Mn	23.9 ± 0.3	23 ± 2	0.2	23.86 ± 0.02	0.1
Ni	6.1 ± 0.2	6.2 ± 0.3	-0.2	6.07 ± 0.02	0.1
Pb	2.3 ± 0.1	2.0 ± 0.3	0.5	2.22 ± 0.04	0.6
Sb	0.048	---	---	0.07 ± 0.02	-0.5
Se	1.8 ± 0.2	---	---	1.6 ± 0.2	0.4
Sn	1.5 ± 0.3	---	---	1.2 ± 0.3	0.4
V	1.1	1.1 ± 0.1	0	1.09 ± 0.03	0.2
Zn	135 ± 5	163 ± 21	-0.6	132 ± 1	0.5

Experimental data are in good agreement with the certified values as it can be concluded from the Z value obtained because they match the trueness criteria. However, it could be critically concluded that the concentrations obtained with a clean up step (2977B) showed a higher trueness degree than the previous experiments defined by Bartolome for all the metals (2977A). This fact was concluded since the Z values were closer to 0 values (Table 9.4). Moreover, statistical tests were performed to investigate whether the differences obtained between the experimental and the certified values were significant from the raw experimental results (X_{mean}, S_{exp} and µCRM, σCRM). The t-test indicated that both results were similar ($p<0.05$) and, therefore, the results of metals could be assumed to be accurate. This fact assessed a recovery closer to 100% for all the metals considered and confirmed the initial qualitative spectroscopic analysis where no metal losses were detected in the clean up step. Finally, one additional improvement was obtained in the uncertainty term in the instrumental analysis by using a clean-up step because a better standard deviation of instrumental replicates was obtained in all cases (Table 9.4).

9.2.4. Application to real samples

Samples collected and used in a previous study [Bartolome et al., 2010a] were re-analyzed again with this new analytical method including the clean step. Metal body concentrations were calculated and expressed on a dry tissue weight basis (i.e. mg·kg^{-1} dry weight) with correction of the extract content determined in each sample for adequate comparison with other experimental results.

Employing the new methodology developed in this work, similar tendencies were obtained in the monitoring profiles that those obtained previously [Bartolome et al., 2010a]. Therefore, the maximum values in each sampling point were obtained during spring-summer levels for all metals except for As and Hg. Additionally, the mean concentrations obtained for the metals were similar in both studies (Figure 9.5). It is remarkable that an important improvement was obtained in the uncertainty of the instrumental measurements because the relative standard deviations of the replicates for each sample were around 10% lower than those obtained by using the analytical method without clean up [Bartolome et al., 2010a]. This fact could be explained by the reduction of the inaccuracies caused by the fat compounds, which were ranged (as determined in 9.1.3) from 10 to 25% in the analyzed samples, producing an important background effect for ICP-MS determinations.

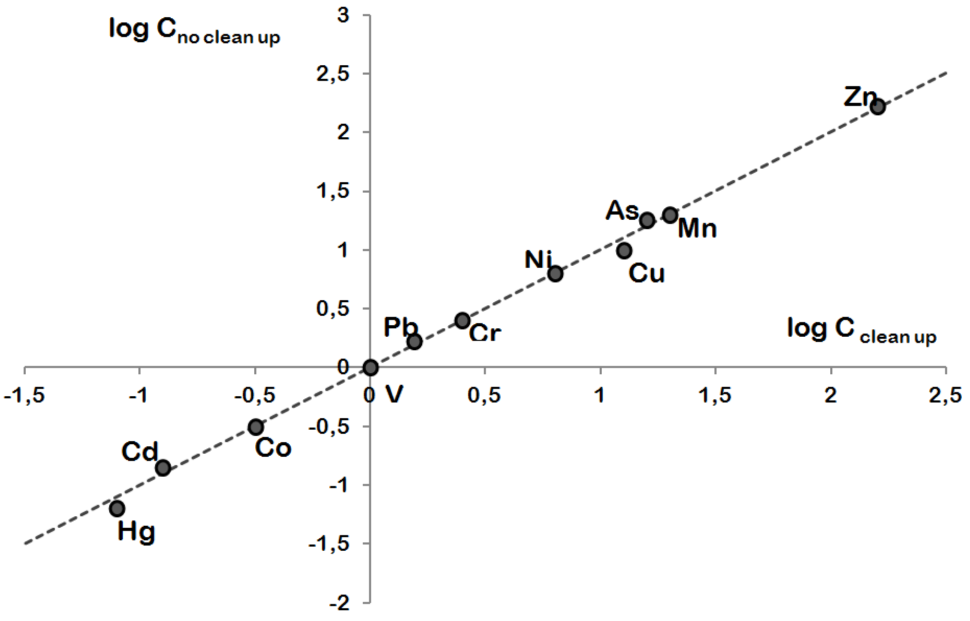


Figure 9.5. *Mean trace metal concentration (log C) obtained with clean up and without clean up analytical methods from the same stations defined by Bartolome [Bartolome et al., 2010a].*

9.3. Conclusions

A new friendly and low time consuming methodology for metal determination in mussel tissues was established in this work by submitting the freeze-dried, grinded and homogenized samples to an extraction step of fatty compounds (clean-up) previous to microwave assisted digestion of the mussel tissue and ICP-MS quantification.

The use of the clean-up step was proven to be a feasible and recommendable approach, reducing the volume of the reagents and the amount and variety of digestion residues as inaccuracy sources in the ICP-MS analysis.

Comparing with the procedure reported previously [Bartolome et al., 2010a], the present analytical procedure lead to reduce the complexity of the extracting solution, avoid the use of concentrated reagents, reduce the possibility of sample contamination, and reduce the errors associated to the dilution steps.

Another advantage of the proposed procedure is the use of low acid nitric contents.

The use of diluted acids was proven to be a feasible and recommendable alternative, reducing the volume of the reagents and the amount and variety of digestion residues. The method had been validated by determining the metals in CRM 2977. The proposed procedure can be applicable for the determination of metals in mussel samples with similar detection limits and accuracy but with better precision.

Last but not least, the optimized clean-up allows reducing costs and time consuming in maintenance operations (cleaning of cones, lenses, etc.) by significantly increasing the matrix tolerance for the direct analysis of the acid extracts. The combination of a friendly clean-up step and acid digestion optimized conditions simplifies matrix effect on the ICP-MS analysis while delivering consistently accurate results and superior performance in routine applications such as metal monitoring using mussel samples.

9.4. References

[Bartolome et al., 2010a] Bartolome L, Navarro P, Raposo JC, Arana G, Zuloaga O, Etxebarria N, Soto M. *Occurrence and Distribution of Metals in Mussels from the Cantabrian Coast*. Arch. Environ. Contam. Toxicol. 2010; 59, 235 – 243.

[Bartolome et al., 2010b] Bartolome L, Etxebarria N, Martınez I, Raposo JC, Usobiaga A, Zuloaga O, Raingeard D, Cajaraville MP. *Distribution of Organic Microcontaminants, Butyltins, and Metals in Mussels From the Estuary of Bilbao,* Arch. Environ. Contam. Toxicol. 2010; 59, 244 – 254.

[Davies, 1993] Davies L. *Efficiency in Research, Development and Production: The Statistical Design and Analysis of Chemical Experiments,* The Royal Society of Chemistry, Cambridge, 1993.

[do Amaral et al., 20005] do Amaral MCR, Rebelo MF, Machado JP, Pfeiffer WC. *Bioaccumulation and depuration of Zn and Cd in mangrove oysters (Crassostrea rhizophorae, Guilding, 1828) transplanted to and from a contaminated tropical coastal lagoon.* Mar. Environ. Res. 2005; 59, 277 – 285.

[EPA, 2007] EPA 6020A, *Inductively coupled plasma mass spectrometry, Environmental pollution agency*, USA, 2007.

[Esbensen, 1994] Esbensen KH, *Multivariate Data Analysis, An introduction to multivariate data analysis and experimental design.* CAMO AB, Oslo, 1994.

[Evans and Giglio, 1993] Evans EH, Giglio JJ. *Interferences in inductively coupled plasma mass spectrometry.* J. Anal. At. Spectrom. 1993; 8, 1 – 18.

[Franco et al., 2002] Franco J, Borja A, Solaun O, Pérez V. *Heavy metals in molluscs from the Basque Coast (Northern Spain): results from an 11-year monitoring programme.* Mar. Pollut. Bull. 2002; 44, 956 – 972.

[Gonzalez et al., 2009] Gonzalez MH, Souza GB, Oliveira RV, Forato LA, Nobrega JA, Nogueira AR. *Microwave-assisted digestion procedures for biological samples with diluted nitric acid: identification of reaction products.* Talanta. 2009; 79, 396 – 401.

[Hattendorf and Günther, 2001] Hattendorf B, Günther D. *Experimental evidence for the formation of doubly charged oxide and hydroxide ions in inductively coupled plasma mass spectrometr.* Fresenius J. Anal. Chem. 2001; 370, 483 – 487.

[Hirata et al., 2001] Hirata S, Ishida Y, Aihara M, Honda K, Shikino O. *Determination of trace metals in seawater by on-line column preconcentration inductively coupled plasma mass spectrometry,* Anal. Chim. Acta. 2001; 438, 205 – 214.

[Jayaraman et al., 2001] Jayaraman S, Pruell RJ, McKinney R. *Extraction of organic contaminants from marine sediments and tissues using microwave energy,* Chemosphere. 2001; 44, 181 – 191.

[Khadim, 1990] M.A. Khadim. *Methodologies for monitoring the genetic effects of mutagens and carcinogens accumulated in the body of marine mussels.* Rev. Aquat. Sci. 1990; 2, 83 – 107.

[May and Wiedmeyer, 1998] May TW, Wiedmeyer RH. *A table of polyatomic interferences in ICP-MS.* Atom. Spectrosc. 1998; 19, 150 – 155.

[Miller and Miller, 2010] Miller JC, Miller JN, *Statistics for Analytical Chemistry, 6th Edition,* Pearson Education, UK, 2010.

[Montaser, 1998] Montaser A (Ed.). *Inductively Coupled Plasma Mass Spectrometry,* Wiley-VCH, New York, 1998.

[Navarro et al., 2010] Navarro P, Bartolomé L, Raposo JC, Zuloaga O, Arana G, Etxebarria N, *Preparation of a reference mussel tissue material for polycyclic aromatic hydrocarbons and trace metals determination.* Anal. Chim. Acta. 2010; 675, 91 – 96.

[Poperechna and Heumann, 2005] Poperechna N, Heumann KG, *Species-specific GC/ICP-IDMS for trimethyllead determinations in biological and environmental samples.* Anal. Chem. 2005; 77, 511 – 516.

[Pratt et al., 1988] Pratt KW, Kingston HM, MacCrehan WA, Koch WF, *Application of microwave digestion technique for elemental analysis*. Anal. Chem. 1988; 60, 2024 – 2027.

[Soto-Ferreiro et al., 1991] Soto-Ferreiro RM, Casais Laino C, Bermejo-Barrera P. *Comparative Study of Sample Preparation Methods for Zn, Fe and Cu Determination in Mussels by Flame Atomic Absorption Spectrometry.* Anal. Lett. 1991; 24, 2277 – 2292.

[Szoboszlai et al., 2009] Szoboszlai N, Polgári Z, Mihucz VG, ZárayG. *Recent trends in total reflection X-ray fluorescence spectrometry for biological applications.* Anal. Chim. Acta. 2009; 633, 1 – 18.

[Vaidya and Rantala, 1996] Vaidya OC, Rantala RTT. *A comparative study of analytical methods: determination of heavy metals in mussels (Mytilus edulis) from Eastern Canada.* Int. J. Environ. Anal. Chem. 1996; 63, 179 – 185.

[Viarengo et al., 2000] Viarengo A, Burlando B, Giordana A, Bolognesi C, Gabrielides GP, *Networking and expert-system analysis: next frontier in biomonitoring.* Mar. Environ. Res. 2000; 49, 483 – 486.

[Wade et al., 1993] Wade TL, Wong JM, Brooks JM, Sweet ST. *Sampling analytical methods of the National status and trends program national benthic surveillance and mussel watch projects, Vol. II*, Ed. NOAA, USA, 1993.

[Walker and Lumbey, 1999] Walker R, Lumbey L. *Pitfalls in terminology and use of reference materials*. TraC Trends Anal. Chem. 1999; 18, 594 – 616.

[Yang et al., 2004] Yang JH, Jiang SY, Wang XR, Wang Y. *Heavy metals in coastal waters from Sanya Bay, Hainan Island: implication for human impact on the estuarine coastal environments.* J. Coast. Res. 2004; 43, 171 – 183.

[Yoshinaga et al., 1999] Yoshinaga J, Morita M, Edmonds JS. *Determination of copper, zinc, cadmium and lead in a fish otolith certified reference material by isotope dilution inductively coupled plasma mass spectrometry using off-line solvent extraction.* J. Anal. Atom. Spectrom. 1999; 14, 15989 – 15992.

10. Concluding remarks

10. Concluding remarks

Environmental analysis is one of the most important areas of the analytical chemistry due to the interaction of the human being with the nature. Thus, the easing of the analysis of pollution in the human surroundings is a priority on the wellness of human beings.

In this work and following the objectives proposed at the beginning, some methodologies have been developed or enhanced over the existing processes, which can assist obtaining comprehensive results in order to have a better understanding of environmental processes.

Firstly, the development of a methodology easing direct analysis of sediments using Raman spectroscopy can expedite the characterization of toxic metal species and evaluation of the potential risks that metal pollution on an area can cause. The distinction between using or not the new Raman pre-treatment methodology, is the difference between finding a better resolved Raman spectra and thus, identifying unequivocally a potentially toxic compound or not.

In this work, some natural and anthropic solid species have been found showing the potential of the developed method whilst, before the use of the developed method, only clay or mica spectra were found on the gathered sediments. This easy and quick methodology removes surface masking organic matter or clay without altering target species and reveals the inorganic species that cannot be found without applying this clean up step. The applicability of this methodology has been largely recognized in the literature since it has been cited in more than 20 scientific research works, including a review, dealing with many scientific areas such as metal monitoring, natural compounds identification, geochemical and geological researches (minery contamination, soil, silt and minerals identification), nanostructures and nanomaterials analysis and planetary science.

Although the previous methodology can be powerful to find out potential harmful compounds in sediment samples, the availability of metals through mobilization processes is subjected to both sediment matrix and water body characteristics. Taking in mind that the physicochemical characteristics of environmental compartments can greatly change from one to another scenario (e.g. riverine and estuarine environments), the availability of metals in environmental bodies must be studied in such conditions. In order to afford this goal, a new methodology has been proposed. The use of real water samples instead of those solvents proposed by official methods to mobilize metals assists getting closer results to what is happening in the open environmental system. Although more parameters are necessary to be taken into account in order to mimic all the processes involved in the different environmental compartments, the suggested approach has successfully demonstrated that the addition of non-naturally occurring salts (as proposed by official methodologies) can provide results that may alter the real naturally ocurring processes and thus, the conclusions obtained. In fact, the physicochemical parameters of water and sediment samples are such important that can alter not only the specie formation, but also the movement of the metals through the environmental compartments. The proposed methodology has also been considered in more than 7 research works published in the literature dealing with metal mobility in estuarine environments, metal availability in lakes, metal speciation and behavior in mine wastes, seasonal variation and evaluation of metal contaminations and metal effects and ecological evaluation of petrochemical contaminations.

This approach has been also useful to assess the mobilization of arsenic in different environmental scenarios. Besides, the combination of different methodological approaches can provide more evidences about what is really happening in the environment. In this since, the analytical and thermodynamical speciation of arsenic together with the mobilization studies and molecular speciation through spectroscopic techniques has allowed having a comprehensive overview of arsenic behavior in river and estuarine systems with tidal influence. This working scheme can be translated to other research works involving other metals as well as environmental compartments as it can be supported in the literature. In fact, this work has been included in a review as well as cited in more than 19 research works including a review and a PhD work, dealing with different aims and scopes such as arsenic speciation and mobilization studies, environmental evaluation of mines and estuaries, geochemical and animal studies for environmental quality and impact assessment, various geochemical studies, organometallic compounds investigations and specific arsenic studies in sediments and waters.

All the research works performed over river and estuaries indicate that organic matter and more specifically, humic substances, have an important role on the contaminant behavior and thus, in the pair water-sediment condition. The study of these substances can elucidate or give more answers over the behavior of the contaminants in the environment.

In this sense, a new time-saving and easy methodology has been developed in order to get a quantification of these substances in the pair water-sediment of the estuaries. The methodology has been designed joining all the forward-steps made by different researchers through the years and it has been fully optimized in order to get the best accuracy and precision.

This methodology has been applied to different sediments of the Nerbioi-Ibaizabal estuary gathered for a period of one year. In this sense, some findings have been found such as the good state of the organic matter of the whole estuary or the physicochemical interaction with these substances, causing deposition or mobilization in some specific areas of the estuary. These findings have been confirmed by mimicking the behavior of these substances with the developed mobility methodology in both high and low tide. Moreover, this methodology has been used also in other research works performed in our research group dealing with the assessment of actual state of estuaries and rivers of the Cantabrian Coast as well as the assessment of river systems affected by minery works and mining runoff disposals.

Once all these evaluations of the system have been made, metal bioavailability can be checked by the analysis of certain sentinel organisms such as mussels. However, the analysis of metals in such complicated matrix is still being an analytical challenge. The main problems arise often from the high amount of fatty compounds present in the samples. The implementation of a clean up step prior to the metal analysis have shown to provide more reliable results in terms of both accuracy and precision, as demonstrated in this PhD. Hence, this methodology has been further used in research works performed in our research group as well as in the General Services of Analysis of the Faculty of Science and Technology of the UPV/EHU for the accurate analysis of metals in mussels.

In brief, the presented methodologies in this PhD thesis have being developed as easy and inexpensive as possible, to the extent that all the methodologies developed in here can be performed with the equipment available in most laboratories. In this sense, although many of the environmental analyses are conducted often by public researchers, consultancies or governments through different environmental organisms, the proposed methodologies can approach environmental analyses to small laboratories and industries and can help all of them in their responsibility of making decisions over polluted areas.

Nevertheless, since the perfect analytical method is still a dream, the use of a multianalytical approach, combining the goodness of each technique or method, and the use of chemometric techniques to ease the data handling and understanding seem to be a must. Moreover, when pollution monitoring should be carried out, the determination of environmental threshold values, which varies site by site, is still an evident necessity in order to assure the real impacts on the environment.

Besides, taking into account that chemical analyses support the advance of fundamental knowledge in many environmental areas, and in order to make sense to all the data it should be handled, it is needed to share information among all the areas researchers taking part in environmental comprehensive investigations in order to get a complete view of the system.

With these conclusions and having met all the aimed objectives, this PhD thesis work is considered completed.