ENVIRONMENTAL HEALTH PERPERTIVES FOR COAL POLLUTION: A VIEW FROM THE SHIPPING PORTS TO DIRECT CONTACT WITH COAL DUST OR THEIR EXTRACTS

Ph.D. Thesis by
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In partial fulfillment of the requirements for the degree of Doctor of Philosophy in the subject of Environmental Toxicology

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PREFACE

This PhD thesis is submitted to Ph.D. Program in Environmental Toxicology, School of Pharmaceutical Science, University of Cartagena, and is a result of my Ph.D. project. The workhas mainly been conducted at the Environmental and ComputationalChemistry Group. The Ph.D. study was funded by Colciencias (Grant No. 567-2012 and 110756933952-2013) and Ph.D. Program in Environmental Toxicology, School of Pharmaceutical Science, University of Cartagena.

The objective of this Ph.D. was to examine the toxicological effects of coal dust on several models such as cell line, mice and zebrafish. The work conducted during this PhD includes, sample campaigns coal dust preparation, and laboratory studies. The major parts of this work have been included in the thesis as papers and paper drafts. This thesis includes an introduction that provides background information for the scientificquestions that have been addressed in the studies of this Ph.D. along with a chapter book published in the book of the Office of the Comptroller General of the Republic of Colombia, and three papers. Of these papers, two have been published in peer-reviewed journals (Papers 1 and 3, both journals have an impact factor greater than 3), and one paper in preparation which will be submitted to peer-reviewed journals (Papers 3), which are listed below:

Publications included in this thesis

1. Chemical and toxicological characterization of sediments along a Colombian shoreline impacted by coal export terminals.
   Caballero-Gallardo, K., Guerrero-Castilla, A., Johnson-Restrepo, B., de la Rosa, J., Olivero-Verbel, J.
   2015
   Chemosphere.138, 837-846.
   DOI: 10.1016/j.chemosphere.2015.07.062

2. Effects of aqueous coal dust extract on developmental toxicity and gene expression profiling of zebrafish (*Danio rerio*).
   Caballero-Gallardo, K., Wirbisky, S., Olivero-Verbel, J., Freeman, J.
   2016
   Papers in preparation

3. Mice housed on coal dust-contaminated sand: A model to evaluate the impacts of coal mining on health.
   Caballero-Gallardo, K., Olivero-Verbel, J.
   2016
   Toxicology and Applied Pharmacology. 294, 11–20.
   DOI: 10.1016/j.taap.2016.01.009

Chapter book

4. Olivero-Verbel, J., **Caballero-Gallardo, K.,** Guerrero-Castilla, A. (2013). **Chapter 5.** *Implications of the coal mining on the environment and human health.* **Book** *Mining in Colombia: Institutionality and territory, paradoxes and conflicts*.Comptroller General of the Republic of Colombia. National Press of Colombia. ISBN 978-958-9351-92-5. 229-251 pp.

Presentations at national conferences

1. **Caballero-Gallardo, K.,** Olivero-Verbel, J. Gene expression and histopathological changes in mice exposed to sand contaminated with coal dust with size less than 38 µm. First Colombian Congress of Biochemistry and Molecular Biology. Bogotá, Colombia. June. 2014.

Presentations at international conferences

2. **Caballero-Gallardo, K.,** Olivero-Verbel, J. An animal model to evaluate the toxic effects of coal dust. XIV International Congress of Toxicology and the X Mexican Congress of Toxicology. Merida-Mexico October 2-6, 2016.
3. **Caballero-Gallardo, K.,** Sara Wirbisky., Jennifer Freeman., Olivero-Verbel, Effects of an aqueous coal dust extract on developmental toxicity and gene expression profiling of zebrafish (*Danio rerio*). The Society of Toxicology (SOT) Annual Meeting. New Orleans. March, 2016.
4. **Caballero-Gallardo, K.,** Olivero-Verbel, J. Molecular, cellular and histological changes in mice living on sand contaminated with coal dust (<38um) under laboratory conditions. Health and Disease: Science, Culture, and Policy Research Poster Session. March 5, 2015. Purdue Memorial Union. West Lafayette (USA).

5. **Caballero-Gallardo, K.**, De la Rosa, J., Johnson-Restrepo, B., Garcia-Cantillo, A., Guerrero-Castilla, A., Olivero-Verbel, J. Metals, PAHs, and Toxicity of Sediments from Santa Marta, a Coal Port in Colombia. The Society of Toxicology (SOT) Annual Meeting. Phoenix, Arizona. March, 2014.
6. **Caballero-Gallardo, K.,** Carranza-Lopez L, Olivero-Verbel, J. Molecular, Cellular, and Histological Changes in Mice Living on Sand Contaminated with Coal Dust under Laboratory Conditions. The Society of Toxicology (SOT) Annual Meeting. San Antonio, TX. March, 2013.

Awards

- **SOT Travel award.** San Antonio. TX. March, 2013. Work "Molecular, Cellular, and Histological Changes in Mice Living on Sand Contaminated with Coal Dust under Laboratory Conditions".
- **SOT/AstraZeneca Travel Fellowship Awards .** San Antonio. TX. March, 2013. Work "Molecular, Cellular, and Histological Changes in Mice Living on Sand Contaminated with Coal Dust under Laboratory Conditions".
- **COLCIENCIAS Scholarship**. National Ph.D. Programs (2012). Bogotá, Colombia. Sixth Place nationwide.

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I would also want to thank Prof. Jennifer Freeman, an excellent scientific, she works in the area of genetic and epigenetic mechanisms of toxicity of environmental stressors with current focus on pesticides, metals, radiation, and emerging contaminants. I thank you for giving me the opportunity to work in her laboratoryat the Purdue University, and her PhD students, especially Sara Wirbisky, who helped me during experiments with zebrafish.

I would like to thank the University of Cartagena and Colciencias for financial support (Grant: 567-2012 and Grant: 110756933952-2013); Society of Toxicology (SOT) of of the United States for the graduate student travel award (2013); and SOT/AstraZenecatravel award (2013).

Finally, I also express my gratitude to the assessment committee for evaluating this thesis.

Karina Rocio Caballero Gallardo. March 2016

DEDICATION

For all those people working in coal mining, a workplace where their lives could be in danger, due to inhalation of coal dust, as well as ecosystems affected by this activity.

RESUMEN EN ESPAÑOL (SUMMARY IN SPANISH)

El carbón constituye uno de sus principales recursos económicos para países en vía de desarrollo que cuenten con reservas de este mineral, el cual proporciona materias primas para la industria y genera una considerable cantidad de oportunidades de empleo para la población que vive en zonas de influencia minera. La extracción de los recursos minerales también aporta otros beneficios a las comunidades cercanas tales como escuelas, hospitales, construcción, y medios de transporte, todo esto debido a que el país recibe regalías de los ingresos mineros por la extracción, lo que permite que la inversión en diversos programas de bienestar para la mejora de la situación socioeconómica general de la ciudadanía. Sin embargo, a pesar de que las actividades mineras constituyen un impulso para el crecimiento económico y el desarrollo, también son responsables de una gran cantidad de efectos adversos, dado que conducen a la degradación del medio ambiente y los recursos naturales.

Por otro lado, los recursos minerales son no renovables, su extracción tiene importantes implicaciones para la equidad intergeneracional. Externalidades negativas de la extracción, como la degradación del medio ambiente y de los recursos naturales y su agotamiento, son significativos, lo que puede contrarrestar los beneficios de la minería. El impacto es generado sobre las actividades agrícolas, la salud humana y los ecosistemas que en su mayoría caen sobre la población local. La minería de carbón a cielo abierto, emiten una cantidad considerable de polvo y otras partículas en la atmósfera que afectan otras actividades económicas en la región donde se ejerce la actividad, así como la salud humana de las personas que viven alrededor de estas.

Aunque la lista de contaminantes generados por la industria del carbón es extensa, el estado del arte señala que el material particulado conocido con el nombre de polvo o polvillo de carbón, es una de los principales agentes de contaminación con efectos sobre la salud.

El polvo de carbón es generado como resultado de la explotación del mineral, la fricción de las rocas, el transporte del mineral a los puertos, su pulverización para facilitar el embarque, entre otras fuentes. Es igualmente conocido que este material heterogéneo es portador de hidrocarburos aromáticos polinucleares (HAPs) y metales que lo hacen altamente tóxico. Ante los pocos estudios en el país que detallen los efectos derivados de la exposición al carbón, este trabajo tuvo como objetivo estudiar su impacto sobre los organismos, cubriendo aspectos relacionados con los posibles impactos derivados del cargue del mineral

en las costas colombianas, hasta el reconocimiento de los mecanismos moleculares involucrados en efectos derivados de la exposición directa al polvo o a su extracto acuoso, tratando de reproducir los posibles efectos que puedan presentarse en la naturaleza.

Con base en lo anterior, el trabajo contempla tres fases, descritas a continuación. En la primera, los sedimentos marinos colectados en una zona reconocida por representar las áreas de carga del mineral para exportación, como lo es el área de la Bahía de Santa Marta y sus alrededores, fueron utilizados para evaluar su composición química y toxicidad frente a células hepáticas con capacidad metabólica.

Partiendo del hecho que el polvo de carbón dispuesto sobre el sedimento marino libera al agua los contaminantes presentes en su superficie, la segunda fase del proyecto contempló la obtención de un extracto acuoso de una muestra de carbón con tamaño de partícula inferior a 38 μm, la cual fue liofilizada y el material sólido resultante, es decir, la sustancia extraída con agua, se empleó para determinar posibles efectos en peces. Para tal propósito fue empleado el pez cebra (*Danio rerio*), especie sobre la que fue estudiada la toxicidad aguda, la capacidad de alterar el desarrollo embrionario y su perfil de expresión génica.

Hasta el momento, es claro que la presencia de material particulado de carbón en los sedimentos oceánicos genera diversos perfiles de toxicidad, tanto a nivel celular como en organismos multicelulares. Surge entonces la siguiente pregunta, ¿es posible que estos efectos también ocurran en el suelo, cuando los organismos de la biota tienen contacto directo con el polvo de carbón presente en el mismo? Para responder esta pregunta fue desarrollado un modelo de exposición a polvo de carbón en arena, en donde ratones (*Mus musculus*) estuvieron expuestos al mineral por un período de ocho semanas. Los resultados de estos experimentos determinaron que la minería del carbón genera efectos toxicológicos en organismos expuestos a polvo de carbón.

En síntesis, estos resultados permitieron mostrar que el embarque de carbón en los puertos colombianos impacta negativamente los sedimentos marinos, y que estos efectos son cuantitativamete medibles en modelos celulares, lo cual sugiere que estos residuos no son en lo absoluto inertes e interaccionan con la biota. En este mismo sentido, el extracto acuoso del carbón, de igual forma a como lo harían las partículas en los sedimentos, también es bioactivo en un modelo de toxicidad con peces, en donde produce alteraciones genómicas en los mismos.

SUMMARY

Coal is one of the main economic resources to developing countries who have reserves of this mineral, which provides industrial raw materials and generates a considerable amount of employment opportunities for people living in areas of mining influence. The extraction of mineral resources also brings other benefits to nearby communities such as schools, hospitals, construction, and transportation, all this due to the fact that countries receive royalties from mining revenues for extraction, allowing investment in various welfare programs for improving the overall socio-economic situation of citizens. However, despite that mining activities are a boost for economic growth and development, they are also responsible for a lot of adverse effects, since lead to degradation of the environment and natural resources.

On the other hand, mineral resources are non-renewable, their extraction has important implications for intergenerational equity. Extraction negative externalities such as environmental and natural resources degradation and depletion are significant, which can offset the benefits of mining. The impact is generated on agricultural activities, human health and ecosystems, what mostly fall on the local population. Opencast coal mining emits a considerable amount of dust and other particles in the atmosphere that affect other economic activities in the region where the activity is carried out, as well as human health of people living around these.

Although the list of pollutants generated by the coal industry is extensive, state of the art indicates that the particulate matter known as dust or coal dust, is one of the main agents of pollution health effects.
Coal dust is generated as a result of the mineral exploitation, friction of the rocks, transport of coal to ports, spraying to facilitate boarding, among other sources. It is also known that this heterogeneous material carries Polynuclear Aromatic Hydrocarbons (PAHs) and metals that make it highly toxic. Given that there are few studies in the country detailing the effects resulting from exposure to coal, this study is focusing on its impact on organisms, covering aspects regarding the possible impacts of the mineral load in the Colombian coast. Also, this research brings up good discussion points about the recognition of molecular mechanisms involved on health effects associated with direct exposure to coal dust or its aqueous extract, trying to reproduce the possible effects that may arise in nature.

Based on the above, the research includes three phases, described below. In the first, the marine sediments collected in the Bay of Santa Marta and

its surroundings, which are recognized as representing the areas of load mineral for export, were used to evaluate their chemical composition and their toxicity on liver cells with metabolic capacity.

Based on the fact that coal dust is disposed on the marine sediment, and it releases contaminants present on her surface to the water, the second phase of the project involves the preparation of an aqueous extract of a coal sample with particle size below 38 μm, which was lyophilized and the resulting solid material, in other words, the substance extracted with water, was used to determine possible effects on fish. For this purpose was used zebrafish (*Danio rerio*), species on which was studied its acute toxicity, the ability to alter embryonic development and its gene expression profile.

So far, it is clear that the presence of particulate coal matter in ocean sediments, generates different toxicity profiles, both at the cellular level and in multicellular organisms. Then the question arises: is it possible that these effects also occur in the soil, when biota organisms have direct contact with coal dust present therein? To answer this question was developed a model of exposure to coal dust in sand, where mice (*Mus musculus*) were exposed to the mineral for a period of eight weeks. The results of these experiments determined that coal mining generates toxicological effects on organisms exposed to coal dust.

In summary, these results allowed to show that the shipment of coal in Colombian ports has a negative impact on marine sediments, and that these effects are quantitatively measurable in cellular models, suggesting that these residues aren't at all inert and interact with biota. In this sense, the aqueous extract coal, in the same way as sediment particles would do, it is also bioactive in a fish toxicity model, what produces genomic alterations in them.

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CHAPTER 1

1. CHAPTER 1. BACKGROUND

1.1. Rationale

Coal, the second source of primary energy, is mostly used for power generation (IEA, 2016), it is widely used in cement, electricity, steel, transport, y combustion products. Coal reserves are available in almost every country worldwide, with recoverable reserves in around 70 countries. The biggest reserves are in the USA, Russia, China and India, and there are an estimated 892 billion tonnes of proven coal reserves worldwide (WCA, 2016). Colombia ranks eleventh as coal producer worldwide according to data reported by GG 2014 (IEA, 2016). Colombia is the main. In South America, Colombia and Brazil contain the main coal reserves worldwide (WEC, 2016). In Colombia, coal reserves are mainly located in the Caribbean region, in the departments of La Guajira and Cesar, according to data from Colombian Mining Information System (SIMCO), the largest coal production for 2015 was reported in these two departments (SIMCO, 2016).

By definition mining is the extraction of valuable minerals or other geological materials from the earth. These deposits have been there for millions of years, acting as a natural reservoir within the biogeochemical cycles of each particular item. As these are removed, an alteration of these cycles occurs and disposal or transportation processes can generate in situ contamination or remote areas where minerals will have to be processed or used (Olivero-Verbel, 2011). Coal mining contributes to both the social and economic development of the region (Koppe and Costa, 2002); however, mining large areas of land results in drastic structural and biological changes, triggering many environmental concerns. Changes within the environment by mining processes include increased soil acidification, compaction, erosion, and air and water pollution (de Quadros et al., 2016).

The coal mining industry has had a significant impact in mining areas where the original landscape is disturbed by hundreds of coal-waste dumps, subsidence caused by underground mining, mine construction and coal transportation (Nádudvari and Fabiańska, 2015). In addition, the coal operations, which include stockpiles, shunting, conveyer belts and ship loading, create dust which is a major problem in the harbour and surrounding areas as it tends to coat all exposed surfaces (Naidoo and Chirkoot, 2004). Some of the main pollutants associated with coal mining and transport includes polycyclic aromatic hydrocarbons (PAHs) and heavy metals (Miller and Maccalman, 2010; Masto et al., 2015), both with mutagenic and carcinogenic effects (Singla et al., 2012). Once in the

environment, these contaminants interact with sediments, water column, and organisms, and after several physical and chemical processes, those may be released, immobilized, or transformed into reactive forms or byproducts, which are more effectively available to organisms (NRC, 2003).

Polycyclic aromatic hydrocarbons (PAH) comprise of many carcinogenic substances and are ubiquitous in the environment. The U.S. Environmental Protection Agency regulated 16 priority pollutant PAH (16 EPA-PAH) spanning from 2–6 condensed aromatic rings, which are commonly analyzed (Achten and Hofmann, 2009). In sediment, PAHs can be free, absorbed in a mineral matrix (bioavailable) or can be strongly sorbed and accumulated (trapped) in sedimentary organic macromolecules. In recent years, increasing interest has been shown in the level of heavy metals in sediment, because many of these are toxic to organisms. Twenty-six trace elements in coal, proposed by Swaine (Swaine, 2000), could lead to potential environmental impacts, including As, Cr, Cd, Hg, Pb, Se, B, Mn, Ni, Cu, V, Zn, Co, Sn, Cl, F, Mo, Bo, P, Th, U, Ba, I, Ra, Sb and Tl. Particularly, elements As, Cd, Cr, Hg, Pb and Se are of most environmental importance (Swaine, 2000). The ecotoxicological impact of coal particles in soils and sediments has not yet been studied in detail. In this case, the first step of this thesis project consisted on the determination of these contaminants in marine sediments from coal port.

Ever since the commencement of industrial-scale coal mining, substantial environmental impacts have been recorded as arising from both the mined voids and from the wastes left behind at the surface (Younger, 2004). Trace elements are not expected to cause problems during underground mining, except for minor local effects where some soluble elements may be present in the mine water. However, after mining has ceased, there may be problems from the weathering of coal around the surface of the mine area, especially from the breakdown of pyrite that gives acidic leachates. Such run-off contaminates nearby waterways with an unsightly brown slurry and adds some unwanted trace elements (Swaine, 2000). In addition, the transport and export of coal in the port can also be a major source of metal contamination in water bodies. In the second step of this thesis, an *in vivo* evaluation of the an aqueous extract of a sample of coal was used to determine possible effects on fish. For this purpose was used the zebrafish (*Danio rerio*) and studied acute toxicity, the ability to alter embryonic development and gene expression profile by microarrays.

The coal mining operations generate significant amount of total particulate matter (PM), which is known as coal dust and disperses in the surrounding atmosphere (Gautam et al., 2012; Chaulya, 2006; Chakraborty et al., 2002). Several studies have reported adverse health effects due to increased PM concentration from the mining operations (Pless-Mulloli et al., 2001; Ramanathan and Subramanian, 2001; Love et al., 1997). Inability of the body to remove progressive build-up of coal dust leads to inflammation, fibrosis and in the worst case, necrosis (Davis and Mundalamo, 2010). Respiratory diseases such as black lung, asthma and cardiovascular diseases are a very common health problem in and around coal mining area (Banks et al., 1998; Hendryx, 2009; Yudovich and Ketris, 2005). Therefore and based on the existing data regarding the relationship between coal dust exposure and several pathologies, we hypothesized that contact with soil contaminated with coal dust could cause toxicological effects in exposed organisms. Accordingly, in the third step of this thesis, an animal model to evaluate the toxic efects of coal dust was proposed.

Finally, when using the terms "coal dust", "coal mining" and "Colombia", as descriptors search in the database PubMed (www.pubmed.gov), which is the most important globally in terms of scientific articles related to health, as well as the database Scopus (www.scopus.com), which is of general topics in science. This, with the aim of conducting a review of articles published both internationally and in Colombia on mining and health. A preliminary review shows very little research, the most numerous aspects related lung problems in mining, and there are very few that describe the molecular effects and quantify the presence of metals and polynuclear aromatic hydrocarbons. In this context, this thesis was posed the following research question: *¿Is it possible that the coal dust with size less than 38 microns generates toxic effects on organisms exposed*?.

1.2. Hypothesis

Exposure to coal dust with size less than 38 microns generates toxicological effects at cellular, molecular and histological level.

1.3. Pertinence

Mining is site specific and the method of extraction depends on the occurrence of the coal deposits (Maiti, 2013). Its activities change the

land use of the area, which include deforestation, topsoil removal followed by excavation of overburden and coal, resulted the creation of deep voids, external dumps and internal overburden dumps (backfilled) (Maiti, 2006). Other activities like timber felling during construction of the approach road, houses and other infrastructure facilities, such as school, hospital and residential colony, which cause migration of population due to the creation of job opportunities that increase anthropogenic or biotic pressure in the periphery of the open cast project (OCP) and creates different types of land uses (Ahirwal and Maiti, 2016). Negative externalities from extraction, such as environmental and natural resource degradation and depletion, are significant, which can offset the benefits from the mining (Hilson, 2002). These adverse effects on agricultural activities (Fang et al., 2015), human health (Cohen et al., 2015; Pemberton, 1956), and ecosystems (Bian and Lu, 2013) are mostly generated in the local people.

Coal mining activities emit a substantial amount of dust and other particles into the atmosphere that affect other economic activities in the region, as well as human health. They increase the concentration of local pollutants in the atmosphere, such as suspended particulate matter (SPM), respirable suspended particulate matter (RSPM), ozone, sulfur oxides, and nitrogen oxides, which have serious implications on the health of the people living around the mining regions (Hota and Behera, 2015). In addition, coal mining is a source of pollutants such as polycyclic aromatic hydrocarbons (PAHs), heavy metals, and fluorine (Miller, 2005).

Coal dust is a complex mixture of several minerals, trace metals and organic materials different levels. This is the result of the collision, abrasion, crushing and pulverizing coal (Kania et al., 2014), which come mainly from mining. In Colombia, coal production has increased considerably in recent years, which is reflected in its exports.

The development of mining activity has been linked to the manifestation of some diseases, epidemiological studies have shown that inhalation of particulate matter increases morbidity and mortality due to cardiovascular diseases. In the US, for example, populations living close to mining areas of influence have shown an increase in the rates of such diseases. Additionally, coal dust has been shown to produce free radicals and increases oxidative stress in rats and humans (Setiawan et al., 2013). This has been confirmed by results of studies conducted by our research group, which revealed significant increases in the expression of biomarker genes of oxidative stress in the liver of wild mice captured in

mining areas of coal, compared with those who lived in areas without this activity (Guerrero-Castilla et al., 2014).

In this thesis several models were employed to elucidate the toxicological effects of exposure to coal dust. The importance of each is detailed below:

In vitro assays

One of the best models used for toxicological studies is the HepG2 cell line, or line of human hepatocarcinoma cells, because this line has been well characterized, being used in the study of the cytotoxicity of nanoparticles, as well as damage to DNA (Lordan et al, 2011; Jarvis et al, 2014). As reported by Kang et al. (2010), this cell line is a good candidate to better understand human exposure to dust, because this model allows evaluate possible adverse effects on human health through ingestion of dust. This line has a series of enzymatic pathways characteristics of human hepatocytes.

In vivo assays

In this thesis two models in vivo (zebrafish and laboratory) were used to assess the effects of exposure to coal dust.

Recently zebrafish (*Danio rerio*) has become a preferred toxicity model due to its rapid life cycle, high fecundity, transparent development, and because the embryos are amenable to genetic manipulation using transgenic approaches and morpholino gene knockdowns (Sipes et al. 2011). This fish in comparison to the human reference genome shows that approximately 70% of human genes have at least one obvious zebrafish orthologue (Howe et al. 2013). Studies related to coal mining using this model have not been reported. However, some studies have been performed for heavy metals (MacDonald, 2015; Avallone et al., 2015; da Silva Acosta et al., 2016) and PAHs (Gao et al., 2015; Sogbanmu et al., 2016), which are pollutants that are present in coal dust. The impact of mining on surface waters raises questions of impacts on biota in those waters. Mining disturbance causes a decline in stream biodiversity with consequences that extend well beyond the limits of the mining permits, according to the above, study the impacts of mining on aquatic biota is a key point.

The mouse has developed into the premier mammalian model system for genetic research. Scientists from a wide range of biomedical fields have gravitated to the mouse because of its close genetic and physiological

similarities to humans, as well as the ease with which its genome can be manipulated and analyzed (Spencer, 2002). It is important for toxicological pathologists working with mice to be aware of the background changes commonly seen in laboratory mice, and the stock and strain differences that occur in their background pathology. Several studies have shown genotoxic effects in wild mice captured level in coal mining areas (León et al., 2007; Cabarcas-Montalvo et al., 2012; Guerrero-Castilla et al., 2014). It is important to note that there are few studies on the impact of coal mining where mice have been used as a laboratory model for assessing toxicological effects, so this thesis generated new knowledge in this area.

Despite the existence of these studies, the research in this field should be increased because of the need for a better understanding of the intracellular mechanisms of toxicity involved in the processes of exposure to coal dust, allowing thereby generate biomarkers early exposure, and so that the authorities may have grounds to issue rules to protect the health of people. Therefore, conducting this type of study is essential.

1.4. Objectives of study

The purpose of the PhD project was to evaluate toxicological effects of coal mining in export terminals and contact exposure of this mineral. To achieve this object, models *in vitro* and *in vivo* were used.

The specific studies conducted during this PhD project focused on the following objectives:

1. Assess the levels of PAHs and metals in marine sediments from a coast withintensive coal export operations and measure theexpression of genes related to oxidative stress, DNA damage andxenobiotic metabolism in HepG2 cells exposed to extracts fromthose sediments.

2. Evaluate the effects caused by exposure to aqueous leachate generated from coal dust, trying to reproduce what may be happening directly in the mines, especially during the rainy season, using zebrafish as a model.

3. Design a model to replicate the conditions under which mice interact with pollutants in areas that receive permanent atmospheric depositions from coal mining activities.

1.5. Outline of this Thesis

The dissertation is arranged as follows:

Chapter 1 provides the rationale, hypothesis, pertinence, objectives and structure of this thesis.

Chapter 2 of the thesis gives an introduction to the topics of coal mining. Here the main focus is on coal dust.

Chapter 3 presents the levels of polycyclic aromatic hydrocarbons (PAHs) and trace elements in marine sediments from a coast with intensive coal export operations, as well as the expression of genes related to oxidative stress, DNA damage and xenobiotic metabolism in HepG2 cells exposed to extracts from those sediments (**Paper 1**).

Chapter 4 the main purpose of this step was evaluates the effects of exposure to aqueous leachate generated from coal dust. In the case, we wanted to reproduce what may be happening directly in the mines, especially during the rainy season; in this study was used zebrafish (*Danio rerio*) as a model system (**Paper 2 in redaction)**.

Chapter 5 shows the toxic effects associated with exposure to sand contaminated with coal dust particles below 38 μm in diameter, obtained from a mineral sample collected in the largest coal mine in South America, La Loma, Cesar, Colombia (**Paper 3**).

Chapter 6 concludes the research, provides a summary of key findings, and gives suggestions for future research.

1.6. References

Achten, C., Hofmann, T., 2009. Native polycyclic aromatic hydrocarbons (PAH) in coals - a hardly recognized source of environmental contamination. Sci. Total Environ. 407, 2461–2473.

Ahirwal, J., & Maiti, S. K. (2016). Assessment of soil properties of different land uses generated due to surface coal mining activities in tropical Sal (Shorea robusta) forest, India. Catena, 140, 155-163.

Avallone, B., Agnisola, C., Cerciello, R., Panzuto, R., Simoniello, P., Cretì, P., & Motta, C. M. (2015). Structural and functional changes in the zebrafish (*Danio rerio*) skeletal muscle after cadmium exposure. Cell Biology and Toxicology, 1-11.

Banks, D.E., Wang, M.L., Lapp, N.L., 1998. Respiratory health effects of opencast coalmining: a cross sectional study of current workers. Occup. Environ. Med. 55 (4), 287–288.

Bian, Z., & Lu, Q. (2013). Ecological effects analysis of land use change in coal mining area based on ecosystem service valuing: a case study in Jiawang. Environmental Earth Sciences, 68(6), 1619-1630.

Chakraborty, M.K., Ahmad, M., Singh, R.S., Pal, D., Bandopadhyay, C., Chaulya, S.K., 2002. Determination of the emission rate from various open-cast mining operations. Environ. Modell. Softw. 17, 467–480.

Chaulya, S.K., 2006. Emission rate formulae for surface iron ore mining activities. Environ. Model. Assess. 11 (4), 361–370.

Cabarcas-Montalvo M, Olivero-Verbel J, Corrales-Aldana H. 2012. Genotoxic effects in blood cells of Mus musculus and Iguana iguana living near coal mining areas in Colombia, Science of The Total Environment, 416, 208-214.

Guerrero-Castilla A, Olivero-Verbel J, Marrugo-Negrete J. 2014. Heavy metals in wild house mice from coal-mining areas of Colombia and expression of genes related to oxidative stress, DNA damage and exposure to metals, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 762, 24-29.

Cohen, R. A., Petsonk, E. L., Rose, C., Young, B., Regier, M., Najmuddin, A., ... & Green, F. H. (2015). Lung Pathology in US Coal Workers with Rapidly Progressive Pneumoconiosis Implicates Silica and Silicates. American journal of respiratory and critical care medicine, (ja).

da Silva Acosta, D., Danielle, N. M., Altenhofen, S., Luzardo, M. D., Costa, P. G., Bianchini, A., ... & Dafre, A. L. (2016). Copper at low levels impairs memory of adult zebrafish (Danio rerio) and affects swimming performance of larvae. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 185, 122-130.

Davis, C.T., Mundalamo, R.H., 2010. Environmental health impacts of dispersed mineralization in South Africa. J. Afr. Earth Sci. 58, 652–666.

de Quadros, P. D., Zhalnina, K., Davis-Richardson, A. G., Drew, J. C., Menezes, F. B., Flávio, A. D. O., & Triplett, E. W. (2016). Coal mining practices reduce the microbial biomass, richness and diversity of soil. *Applied Soil Ecology*, *98*, 195-203.

Fang, T., Liu, G., Zhou, C., & Lu, L. (2015). Lead in soil and agricultural products in the Huainan Coal Mining Area, Anhui, China: levels, distribution, and health implications. Environmental monitoring and assessment, 187(3), 1-10.

Gao, D., Wu, M., Wang, C., Wang, Y., & Zuo, Z. (2015). Chronic exposure to low benzo [a] pyrene level causes neurodegenerative disease-like syndromes in zebrafish (Danio rerio). Aquatic Toxicology, 167, 200-208.

Gautam, S., Patra, A.K., Prusty, B.K., 2012. Opencast mines: a subject to major concern for human health. Int. Res. J. Geol. Min. 2 (2), 25–31.

Guerrero-Castilla, A., Olivero-Verbel, J., Marrugo-Negrete, J. 2014. Heavy metals in wild house mice from coal-mining areas of Colombia and expression of genes related to oxidative stress, DNA damage and exposure to metals. Mutation Research/Genetic Toxicol. Environ. Mutagen. 762: 24-29.

Hendryx, M., 2009. Mortality from heart, respiratory and kidney disease in coal mining areas of Appalachia. Int. Arch. Occup. Environ. Health 82, 243–249.

Hota, P., & Behera, B. (2015). Coal mining in Odisha: An analysis of impacts on agricultural production and human health. The Extractive Industries and Society, 2(4), 683-693.

Howe, K., M. D. Clark, C. F. Torroja, J. Torrance, C. Berthelot, M. Muffato, J. E. Collins, S. Humphray, K. McLaren, and L. Matthews. 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496:498-503.

International Energy Agency (IEA). http://www.iea.org/publications/freepublications/publication/KeyCoalTrends.pdf Último acceso. Junio del 2016.

International Energy Agency (IEA). http://www.iea.org/topics/coal/ Último acceso. Junio del 2016.

Kania, N., Setiawan, B., Widjadjanto, E., Nurdiana, N., Aris Widodo, M., Chandra Kusuma, H.M. 2014. Subchronic inhalation of coal dust particulate matter 10 induces bronchoalveolar hyperplasia and decreases MUC5AC expression in male Wistar rats. Exp. Toxicol. Pathol. 66, 383-389.

Koppe, J.C., Costa, J.F.C.L., 2002. Mineração. In: Teixeira, E.C. (Ed.), Meio Ambiente E Carvão: Impactos Da Exploração E Utilização. FEPAN, Porto Alegre, Brazil, pp. 15–27

León, G., Pérez, L. E., Linares, J. C., Hartmann, A., & Quintana, M. (2007). Genotoxic effects in wild rodents (Rattus rattus and Mus musculus) in an open coal mining area. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 630(1), 42-49.

Love, R.G., Miller, B.G., Groat, S.K., Hagen, S., Cowie, H.A., Johnston, P.P., Hutchison, P.A., Soutar, C.A., 1997. Respiratory health effects of opencast coalmining: a cross sectional study of current workers. Occup. Environ. Med. 54 (6), 416–423.

MacDonald, T. (2015). Effects of inorganic mercury on developing zebrafish (Danio rerio) larvae.

Maiti, S.K., 2006. Properties of mine soil and its affects on bioaccumulation of metals in tree species: case study from a large opencast coalmining project. Int. J. Min. Reclam. Environ. 20, 96–110.

Maiti, S.K., 2013. Ecorestoration of the Coalmine Degraded Lands. Springer, New York.

Masto, R.E., Sheik, S., Nehru, G., Selvi, V.A., George, J., Ram, L.C., 2015. Environmental soil quality index and indicators for a coal mining soil. Solid. Earth Discuss. 7(1), 617–638.

Miller, B.G., Maccalman, L., 2010. Cause-specific mortality in British coal workers and exposure to respirable dust and quartz. Occup. Environ. Med. 67, 270–276.

Miller, G. 2005. The effect of coal usage on human health and the environment. En: Coal Energy Systems. Elsevier Inc. 77-122.

Nádudvari, Á., & Fabiańska, M. J. (2015). Coal-related sources of organic contamination in sediments and water from the Bierawka River (Poland).International Journal of Coal Geology, 152, 94-109.

Naidoo, G., & Chirkoot, D. (2004). The effects of coal dust on photosynthetic performance of the mangrove, Avicennia marina in Richards Bay, South Africa.Environmental Pollution, 127(3), 359-366.

National Research Council (NRC), 2003. Bioavailability of Contaminants in Soils and Sediments: Processes, Tools, and Applications. National Academies Press.

Pemberton, J. (1956). Chronic Bronchitis, Emphysema, and Bronchial Spasm in Bituminous Coal-Workers. An Epidemiologie Study. Arch. Indust. Health,13(6, Sect. 6), 529-44.

Pless-Mulloli, T., Howel, D., Prince, H., 2001. Prevalence of asthma and other respiratory symptoms in children living near and away from opencast coal mining sites. Int. J. Epidemiol. 30 (3), 556–563.

Ramanathan, A.L., Subramanian, V., 2001. Present Status of Asbestos Mining and Related Health Problems in India—A Survey. Ind. Health 39, 309–315.

Setiawan, B., Darsuni, A., Muttaqien, F., Adiputro, D.L., Kania, N., Nugrahenny, D., Widodo, M.A. 2013. The effects of combined particulate matter 10 coal dust exposure and high-cholesterol diet on lipid profiles, endothelial damage, and hematopoietic stem cells in rats. J. Exp. Integr. Med. 3:219-223.

Singla, V., Pachauri, T., Satsangi, A., Kumari, K.M., Lakhani, A., 2012. Characterization and mutagenicity assessment of PM2.5 and PM10 PAH at Agra, India. Polycycl. Aromat. Comp. 32, 199–220.

Sipes, N. S., S. Padilla, and T. B. Knudsen. 2011. Zebrafish—As an integrative model for twenty-first century toxicity testing. Birth Defects Research Part C: Embryo Today: Reviews 93:256-267.

Sistema de Información Minero Colombiano (SIMCO). http://www.upme.gov.co/generadorconsultas/Consulta_Series.aspx?idModulo=4&tipoSerie=121&grupo=371 Último acceso. Junio del 2016.

Sogbanmu, T. O., Nagy, E., Phillips, D. H., Arlt, V. M., Otitoloju, A. A., & Bury, N. R. (2016). Lagos lagoon sediment organic extracts and polycyclic aromatic hydrocarbons induce embryotoxic, teratogenic and genotoxic effects in Danio rerio (zebrafish) embryos. Environmental Science and Pollution Research, 1-13.

Spencer, G. (2002). Background on mouse as a model organism. The Mouse Genome and The Measure of Man. National Human Genome Research Institute, Bethesda. http:// www. genome. gov, 10005834.

Swaine, D. J. (2000). Why trace elements are important. Fuel Processing Technology, 65, 21-33.

Swaine, D.J. Why trace elements are important. Fuel Process. Technol. 2000, 65–66, 21–23.

World Coal Association (WCA). 2016. http://www.worldcoal.org/coal/where-coal-found Último acceso. Junio del 2016.

World Energy Council (WEC). https://www.worldenergy.org/data/resources/region/latin-america-the-caribbean/coal/ Último acceso. Junio del 2016.

Younger, P. L. (2004). Environmental impacts of coal mining and associated wastes: a geochemical perspective. Geological Society, London, Special Publications, 236(1), 169-209.

Yudovich, E.Y., Ketris, M.P., 2005. Arsenic in coal: a review. Int. J. Coal Geol. 61, 141–196.

CHAPTER 2

2. CHAPTER 2. INTRODUCTION

Coal mining is one of the oldest occupational activities that was and still is performed on a large scale (Born, 2002), and one of the most important economic activities in many countries. Coal mines are generally located far away from the final utilization location. Thus, throughout the entire coal mining, handling and transport process coal dust emissions are produced. Opencast coal mines or surface coal mining use large-scale mechanization and release huge quantities of dust and gases, which adversely affect human health. Surface coal mining has increased globally during the past 30 years. The use of coal is expected to increase by 60% over the next 20 years (http://www.worldcoal.org). This increase in coal usage raises a number of environmental challenges. In the case of underground mines, which allow coal companies to extract deeper deposits of coal, is viewed as less destructive than strip mining. However, underground mining causes huge amounts of waste earth and rock to be brought to the surface, waste that often becomes toxic when it comes into contact with air and water, mines collapse, damage to buildings, change the flow of groundwater and streams (**Figure 2.1**). Dust fall rate and its chemical constituents are required in quantitative as well as qualitative terms to study the dust pollution of a particular region. Managing dust from coal mines is important as it can impact local and regional air quality, adversely affect local amenity and pose a risk to public health.

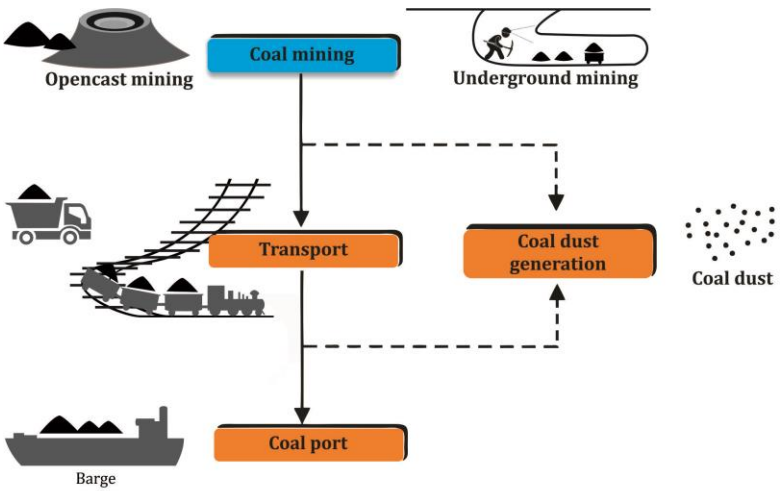

Figure 2.1. Generation of coal dust in mining activities.

Coal dust emissions depend upon different parameters related to the specific activity or to the characteristics of the material, such as particle size distribution, coal type, moisture content, pile configuration, dumping height, as well as weather related parameters (wind speed, and relative humidity among others) (Fabiano et al., 2014).

Together with the concern in terms of pollutant content, coal dust emissions are capable of producing a considerable environmental impact in both coal workers and people in nearby residential areas (Petsonk et al., 2013). Inhalation of coal dust may cause a variety of lung diseases, including coal workers pneumoconiosis (CWP) (Heppleston, 1992; Mo et al., 2014), progressive massive fibrosis (PMF) (Cohen et al., 2008), lung function loss (Stansbury et al., 2013), chronic bronchitis (Wouterset al., 1994) and emphysema (Ruckley et al., 1984; Leigh et al., 1994; Omland et al., 2014) (**Figure 2.2**). Relationships between exposure to coal particles and the occurrence of cardiovascular disease have also been identified (Nadadur et al., 2009). In humans and animal models of acute and chronic exposure to coal dust, inflammatory effects and oxidative damage to the lung parenchymal tissue are characterized by activation of antioxidant enzymes, such as superoxide dismutase (SOD); increased markers of lipid peroxidation and decreased antioxidant defenses (Pinho et al., 2004).

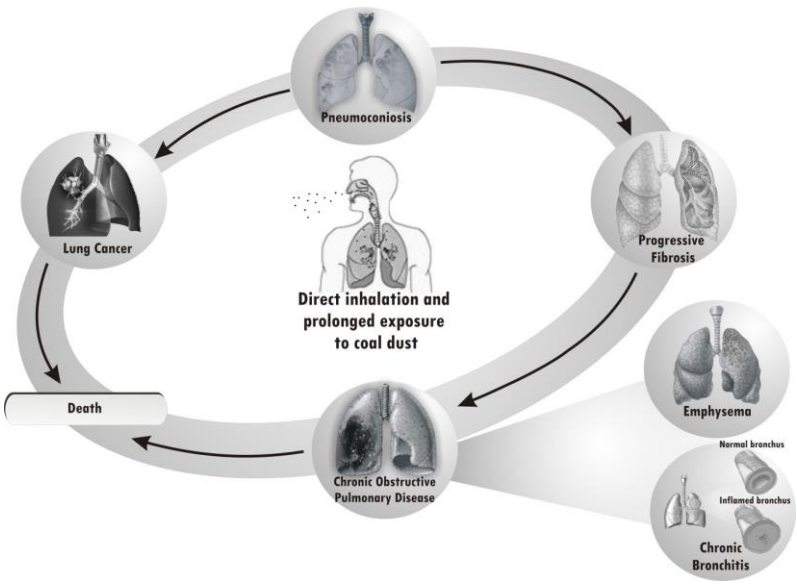

Figure 2.2. Generated diseases by direct inhalation and prolonged exposure to coal dust.

At the global level, several studies have documented that coal dust exposure in biota (Zocche et al., 2010, 2014) and humans (Rohr et al., 2013a) living near coal mining areas produces genotoxicity and diseases related to inhalation of coal dust (Petsonk et al., 2013). In Colombia, researchers have also found genotoxic damage as a possible effect of coal dust exposure (Leon et al., 2007; León-Mejía et al., 2011, 2014). Guerrero-Castilla et al. (2014) reported that hepatic concentrations of Cd, Cu and Zn in *Mus musculus* specimens were significantly higher in animals living near mining areas when compared to a reference site, as well as the mRNA expression of NAD(P)H dehydrogenase, quinone 1 (*Nqo1*),metallothionein 1 (*Mt1*), superoxide dismutase 1 (*Sod1*), metallothionein 2 (*Mt2*), and DNA-damageinducible transcript 3 (*Ddit3*). However, there is no direct evidence that coal dust is contributing to these effects.

The expansion of the coal mining industry has negative effects on the ecosystem. This is reflected in erosion, destruction of water resources, land subsidence, air pollution, declining biodiversity, landscape fragmentation, release of contaminated water, generation of solid waste and the loss of agricultural land (Keating, 2001; Mamurekli, 2010), among other problems.

In this context, this chapter were revised some of the most important implications of coal mining on the environment and human health.

2.1. Coal

Coal varies considerably in composition and consists largely of carbon, hydrogen, and oxygen with smaller amounts of sulfur, nitrogen, trace elements, and metals. Coal originated from mostly organic material that was long ago buried by sediments. Heat and pressure converted the plant remains over geologic time to coal, the process thought to have led to peat, lignite, bituminous coal, and anthracite in turn (Crelling et al., 2000) (**Figure 2.3**). Coal, as extracted, contains many minerals in various proportions, including quartz, clays, carbonates, and sulfides. These minerals can be intrinsic to the coal, as in silica grains within the coal matrix, or may lie in pockets or layers. Although there are different methods for classifying coal—each developed for a specific geological or economic purpose—all tend to relate to the age of the coal.

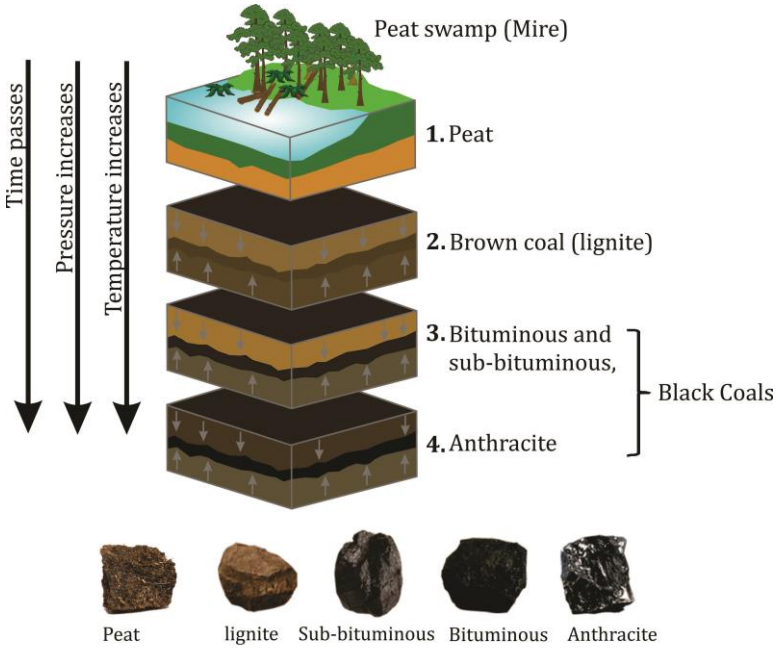

Figure 2.3. Different stages of coal formation (Whitehurst 1978).

Coal structure is a three dimensionally cross-linked macromolecular system containing extractable compounds of relatively low molecular weight. The macromolecular network possesses aromatic and hydroaromatic ring structures joined by hydrogen bonds and covalent linkages consisting of short chain of carbon, oxygen, sulfur, or nitrogen atoms. The cross-linkages in the network generate pores being most of the coal surface area enclosed in micropores of less than 1 mm in diameter (Şimşek, Bilgesü, & Olcay, 1995). The molecular weight of coal as mined is indeterminate. Alkylated coal products have molecular weights from 500 to 2000 (low- to high-rank coal) (Speight, 1994; Speight, 2012). There are an abundance of structures that capture, to a certain degree, the structural features of coal, with >134 structures being generated over the last 70 years the field has been active, yet only a few structures are well known. The field has been dominated by the representations of bituminous coal (Mathews and Chaffee, 2012).

The mineral has polluting effects thanks to the inorganic fraction, although it represents only a small fraction by weight of carbon (Ward, 2002). Various inorganic components are salts, they are found in the pores of the mineral, inorganic elements incorporated in organic

compounds (especially trace elements) and minerals of various kinds (Ward, 2002; Arbuzov et al., 2011).

On the other hand, the mining and use of coal does have pronounced effects on the environment, and are principal causes of environmental concern. A summary of the effects of the use of coal on the environment is shown in **Figure 2.4**. These have a direct influence upon the geological investigation and exploration for coal. During the removal of coal from underground, as well as transportation and shipment, various physical phenomena allow the formation of small particles whose size is varied, ranging diameters less than one micron to several millimeters, commonly known as “coal dust”. This pulverized material called "particulate material" and the rocky material, in contact with water or by mechanical friction, can leach contaminants present on its surface. Among these compounds can be mentioned polycyclic aromatic hydrocarbons (PAHs), heavy metals and fluorine (Miller and MacCalman, 2010), and many possible elements foundin rocks. The group of metals with a toxicological concern, usually are lead (Pb), mercury (Hg), nickel (Ni), vanadium (V), beryllium (Be), cadmium (Cd), barium (Ba), chromium (Cr), copper (Cu), molybdenum (Mo), zinc (Zn), selenium (Se), arsenic metalloid (as), and some radioactive isotopes of natural origin, such as radium (Ra), uranium (U) and thorium (Th). These contaminants may also be released during the combustion of coal, affecting several ecosystems (Keating, 2001) (**Figure 2.4**).

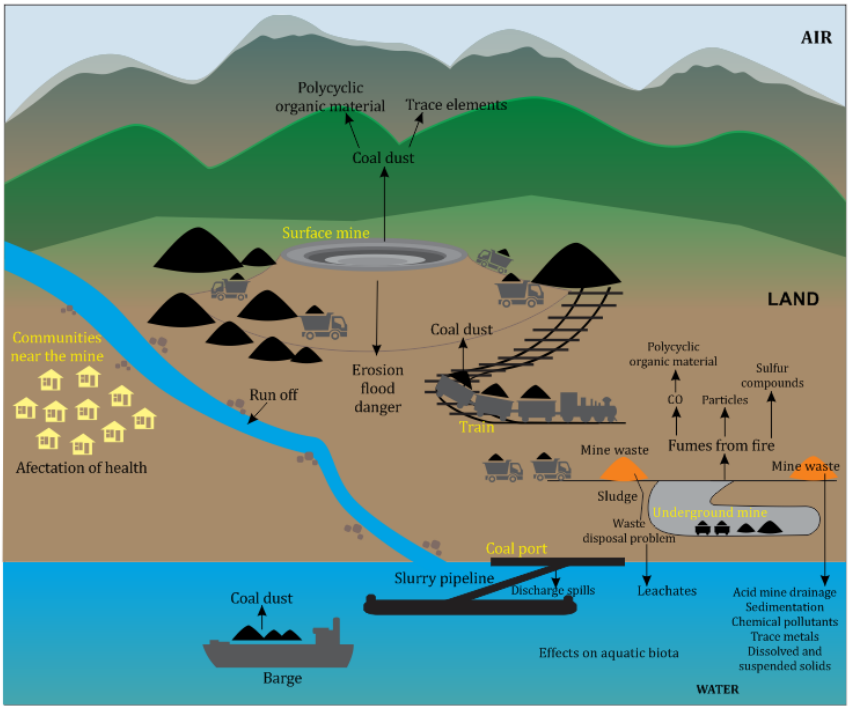

Figure 2.4. Physical and chemical effects on the environment due to coal mining. Adapted from Thomas (2013).

2.1.1. Toxic effects and mode of action of coal dust

Coal dust is defined, for the purpose of establishing a threshold value, as the respirable fraction of the dust produced during prospecting, extraction and processing of coal. It is equivalent to the dust produced during transport and storage of coal. This fraction is comprised of particles of run-of-mine coal, particles of the minerals present in the coal seam and particles of the associated rock. The mineral fraction can contain quartz in proportions depending on the kind of rock and stratigraphic horizon of the deposits.

The mechanism of action of coal mine dust is not yet understood. However, a correlation between the exposure to coal mine dust and the

development of pathological effects in the lung has been demonstrated. The information available to date on the mechanism of action of coal dust is summarized in **Figure 2.5**. With increasing levels of deposited dust, the alveolar macrophages and epithelial cells become activated. This results in increased release of inflammation mediators, reactive oxygen species (ROS), enzymes (elastase, protease, collagenase), cytokines [e.g. Tumor necrosis factor (TNF), interleukin, macrophage inflammatory protein (MIP)] and growth factors [platelet-derived growth factor (PDGF), transforming growth factor (TGF)]. These in turn are responsible for the stimulation of the various kinds of pulmonary fibrosis and emphysema.

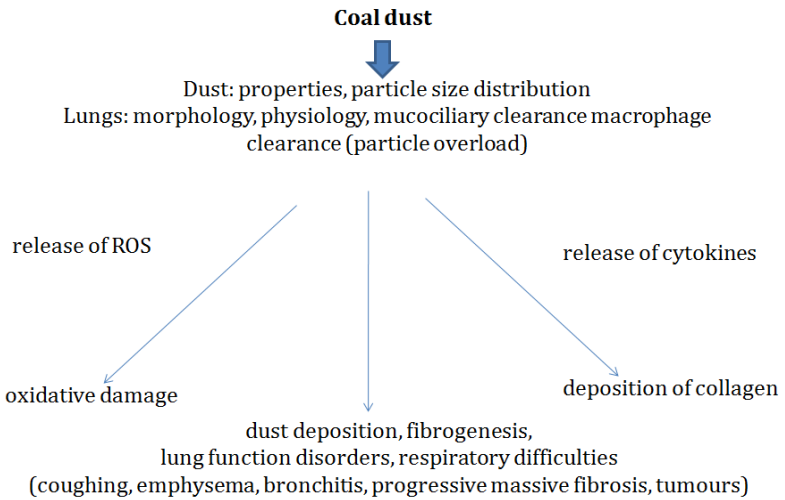

Figure 2.5. Physical and chemical effects on the environment due to coal mining. Adapted from Thomas (2013).

Coal mine dust contains quartz and metal compounds, as well as low levels of PAH. The complex PAH mixtures contain substances such as benzo[a]pyrene which has been shown to cause bronchial epithelial damage and to have carcinogenic effects on the stomach mucosa (Triolo et al. 1977). The effect of hydroxyl radicals on the development of pneumoconiosis in coal miners has been studied (Dalal et al. 1995). It was demonstrated that higher levels of Iron in coal mine dust are involved in the generation of hydroxyl radicals, which could play an important role in the development of CWP. Also the findings of Tourmann and Kaufmann (1994a, 1994b) supported the hypothesis that the iron compounds in coal mine dust can have damaging effects. In

addition, increased release of iron was demonstrated in persons with lung fibrosis.

2.2. Environmental implications of coal mining

The impact of coal mining on the environment varies depending on different factors. For example, if the mine is active or abandoned, the extraction methods used, environmental conditions, climatic and geological location, proximity to urban areas, among others. In all cases, it will have harmful effects on plant organisms, animals and humans, either by habitat destruction or release of pollutants into the environment (Keating, 2001). In general, the spectrum of environmental impacts of coal mining is extremely complex, both ways to mine coal (opencast and underground) have their own impact to the environment and health. An approach to it is presented in **Figure 2.6**, which includes environmental pollution problems and alterations in the landscape, among many other impacts (Mamurekli, 2010). Although each mine can have different environmental effects, there are a number of factors that strongly influence the environment. Not only will the mine be assessed but also the effect on the surrounding landscape, water courses, and native flora and fauna, as well as social effects on the local community.

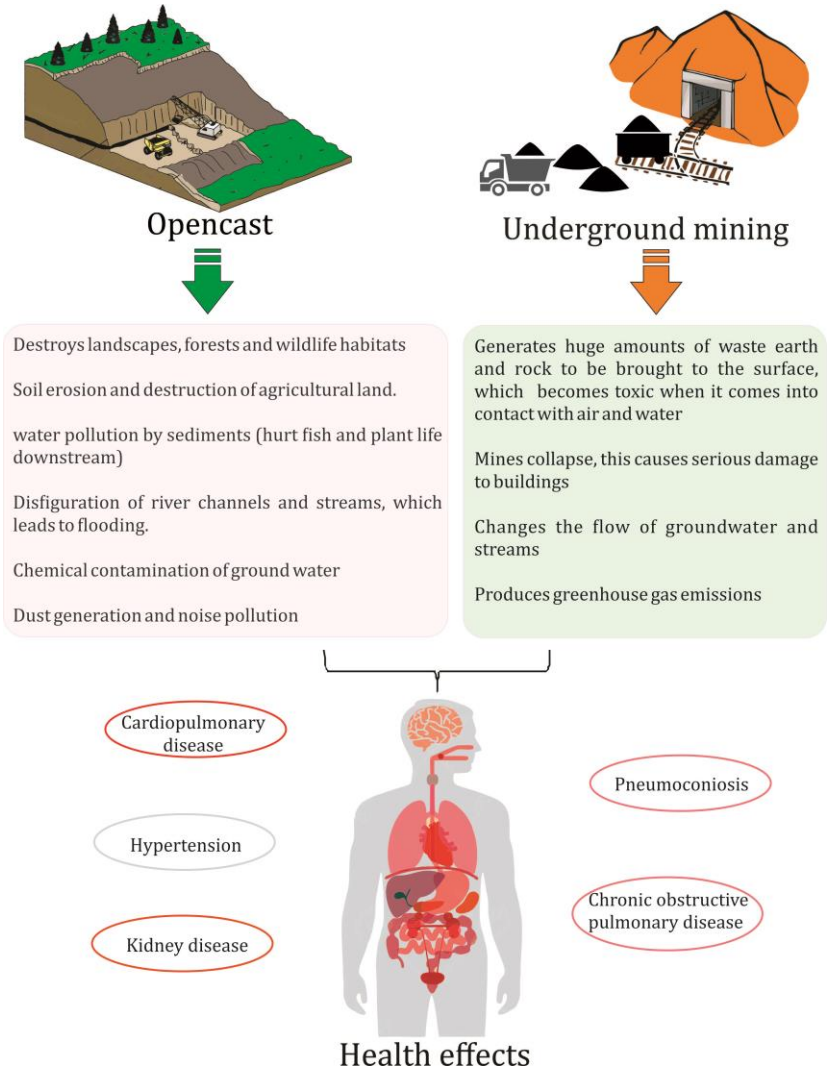

Figure 2.6. Coal mining industry has negative effects on the ecosystem and human health (Lapp and Parker, 1992; Hendryx and Zullig, 2009).

2.3. Environmental pollution

One of the most renowned coal mining aspects, particularly the opencast mining, in its various forms, is air pollution, which is mainly due to

particulate emissions from coal and other materials, including also gases such as methane, sulfur dioxide and nitrogen oxides (Bian et al., 2010). During the process of extraction and transportation, mineral particles are generated of all sizes, with a significant fraction of them incorporated into the air. A percentage of these particles form the fraction known as "total suspended particles (TSP)", they can stay in the air a considerable time before deposition on the ground. During this time, TSP can travel great distances, affecting distantareas from the extraction areas. There are different types of TSP, according to their size (diameter): PM 10 (<10 microns), MP 2.5 or fine particles (<2.5 micron) and PM 0.1 particles or ultrafine (<0.1 micron). All these types of particles have potential adverse health effects after being inhaled, so it permissible limits have been established to ensure the quality of breathing air (Ming-Ho, 2005). In Colombia, TSP measurements made in the mining region coal from the north coast have shown readings that exceed current standards in near coal mines populations, so the biota are exposed to polluted air with particles of high toxicological potential (Huertas et al, 2012a;. Huertas et al, 2012b.). Similarly, for different reasons unexplained, Colombian law does not regulate the presence of highly dangerous particles, such as MP 2.5, and is limited only to the PM 10.

A significant number of coal mines releases acid drainage, which is producedby the reaction of pyrite with air and water (FeS2) (Rivers et al, 2008;.. Oliveira et al, 2012a, b), forming sulfuric acid and dissolved iron. Associated with these drains, have been reported a lot of suspended solids and a high content of dissolved metals, Al, Mn, Zn, Cu, Pb, Fe, etc., which ultimately are deposited into rivers (Silva et al. , 2013). It is important to note that these drains may remain indefinitely once the mine has been abandoned or improperly closed. In some cases the drains can be alkaline, and they have implications on the releasing of other metals, they can affect ecosystems and humans. In any case, the release of toxic metals is a problem, and in Colombia there isn't enough information.

The pollutants released by coal mining have a high potential to enter the soil (Costa and Zocche, 2009), sources of water, food chain (O'Shea, 2001) or the atmosphere (Silva et al., 2009), becoming thus a potential danger for biota (Silva et al, 2010;. 2011). Among the affected wildlife, fish are often vulnerable to contaminants in these drains, emphasizing that also are important economic and human consumption as a source of protein component. Some reports in Asian fish have shown bioaccumulation of certain metals toxic trace (Cr, Cd, Cu, Fe, Mn, Zn, Ni, and Pb) in different vital organs of fish (*Heteropneustes fossilis*), resulting from the exposure to mine effluents coal (Bharti and Banerjee, 2011). Likewise, in Brazil, in animals from area of influence of coal mining,

various metals have been detected in amphibians tissues (blacksmith tree frog), associating the presence of these with DNA damage in blood cells (Zocche et al., 2013).

In some regions of coal mining are frequently found high concentrations of dangerousmetals, for example As (metalloid), Mn, Zn, Pb and Ti, in soil samples collected in areas near mining areas, creating a potential risk for fishes, amphibians and local residents (Liu et al, 2013b; Shi et al, 2013; Bhuiyan et al, 2010; Wang et al., 2010). Other reports indicate that these metals are effectively absorbed by exposed persons. Thus, serum levels of cadmium considered toxic (>0.5 mg/dL), have been found in 85% of children living in surrounding towns to coal mines, as a result of direct or indirect exposure to soil and aquatic ecosystems contaminated with products and/or mining waste (Yapici et al., 2006).

One of the consequences of mining and burning coal is the mobilization of trace elements corresponding to virtually all the elements of the periodic table (Finkelman, 1999). Among these fluorine (F), an element that appears frequently in moderately high concentrations in coal, particularly in China (Qi et al, 2000; Luo et al, 2001; Chen and Tang, 2002), can induce severe healthproblems (Guijian et al, 2007) such as dental fluorosis and in extreme cases, skeletal (WHO, 2002); high doses also have been linked to cancer (Marshall, 1990), increased bone fractures, decreased birth rates, kidney stones, thyroid dysfunction, and learning problems in children (Alarcon-Herrera et al., 2001; Committee on Fluoride in Drinking Water, National Research Council, 2006). That is why it is considered as an element of environmental interest (Swaine, 1990) and potentially dangerous (Greta et al., 2013). Most studies are related to fluorosis resulting from excessive exposure to coal combustion (Ling et al, 2012;. Liu et al, 2013.).

Although the potential impact on coal mining regions of Colombia is being reviewed, a preliminary sampling conducted to a group of children in Loma Jagua, Cesar, showed that there isn't a significant frequency of occurrence of fluorosis.

In Colombia, the information related to the impacts of coal mining on ecosystems is beginning to be published, although there is some important material, most of the data are thesis of universities, whose data haven't been submitted to journals. A review in PubMed (http://www.ncbi.nlm.nih.gov/PubMed) using the terms "Colombia and coal mining" yielded less than ten items, which shows that the studies to knowledge of the effects of this activity are scarce. Some of these reports show genotoxic damage of coal and its associated contaminants on wildlife and human organisms, by standard tests such as the comet assay

and micronucleus determination and respiratory effects in humans (**Table 2.1**).

Table 2.1. Studies in Colombia in relation to coal mining.

Site	Experimental unit	Test	Effects	References
Laboratory	*Lemna minor* (aquatic plant)	Toxicity by exposure to ethanol extracts of coal dust	Chlorosis, reduced leaf size, leaf abscission and roots, presence of necrotic tissue.	Coronado-Posada et al. (2013)
Cesar department (La Loma and La Jagua de Ibirico)	*Mus musculus* (wild mouse) and *Iguana iguana*(Wild Iguana)	Genotoxicity by comet assay and micronuclei in peripheral blood cells	High percentages of DNA damage for both species compared with the reference group (unexposed).	Cabarcas-Montalvo et al. (2012)
Guajira department (opencast mine "El Cerrejón")	Mine workers according to activities such as transportation, equipment maintenance, extraction and coal shipment	Genotoxicity by comet assay and micronuclei in lymphocytes	High rates of DNA damage for different activities of workers compared with the reference group (unexposed).	León-Mejía et al. (2011)
Cordoba department (town ship of Puerto Libertador)	*Rattus rattus* (Wild rat) and *Mus musculus* (Wild mouse)	Genotoxicity by comet assay in peripheral blood	Both species were shown to be sensitive indicators of environmental genotoxicity caused by coal mining activities	León et al. (2007)

2.4. Changes in the landscape

One of the most complex impacts of coal mining is the transformation of the landscape. This transformation is usually permanent, and it is an anthropogenic trace to be handled by several, perhaps dozens of generations. The problem isn't just the fragmentation of ecosystems, changes in the local landscape due to the elimination of native vegetation, decreased agricultural territories, accumulation of mining waste (Bian et al., 2010), is a matter of total loss of territory. It means that there isn't possibility to use in the future a crater with several kilometers of diameter with liquid toxic inside it. Agriculture, livestock and basically any other human activity will disappear.

Before the abandonment of mines, of course, the collapse of soil over them (subsidence) is another environmental impact that can affect the security of organizations and individuals in the area, and it can affect in many ways the field (Bian et al., 2010). It has recently been reported that subsidence in coal mines alters the distribution of metals in the soil, because concentrations of heavy metals such as Cd and Zn were found higher in areas of subsidence, relative to unaffected areas (Zhang et al ., 2012). In this context, there is always the possibility of leaching of contaminants into water sources, mainly underground.

There are a lot of economic expectations generated by coal mining in countries like Colombia, but can't be denied that this activity has implications that go beyond pollution and alterations in the landscape (Angen, 2008; Lei et al., 2009) they vary undoubtedly for the type of mining operation, extraction methods used and geological conditions (Zhengfu et al, 2010;. If et al., 2010). The point is that regardless of the process, the use of subsoil leads to the extinction of floor areas, which can even be seen from space. This means playing threats against food security and displacement of populations, which, as in the case of some of Cesar, they can stay for years in this process, abandoned against poverty, social injustice and multiple conflicts social (Morrice and Colagiuri, 2013), which usually are not quantified in establishing cost-benefit relationship of these activities.

2.5. Effects of coal mining on human health

There is evidence of the health effects of coal mining workers and populations near mines. Pneumoconiosis is the main and most studied disease in coal miners, linked with exposure to particular material (Karkhanis and Joshi, 2013) and specifically attributed to the content of silica and iron (McCunney et al, 2011;. McCunney et al., 2009). Moreover, cardiovascular diseases and cancer have also been linked to the extraction of this mineral in near mines (Palmer et al., 2010) populations. There is even evidence of a possible association between coal production and the occurrence of neural tube defects (NTDs) in infants

born to mothers exposed (Liao et al., 2010). NTDs are a group of diseases that occur in the unborn child to the brain and spinal cord.

2.6. Pulmonary effects associated with exposure to coal dust

The particular material exposure is one of the most serious health problems of many workers in the mining sector threats. The toxicity of these particles depends on their size and arises from several factors, among them, their chemical nature, when they contain toxic substances such as Pb, Cd, Ni, Hg, As or radionuclides. Also, the particles can absorb chemicals with various toxic effects and increase its harmful effect, either by increasing the availability of income by inhalation or by prolonging its residence time in the respiratory system. Moreover, if there are high concentrations of particles in the air can be generated overloading of the mucociliary apparatus, which in turn decreases their removal and generates its retention in the lungs triggering an inflammatory response (Ghose, 2007; Ghoseand Majee 2007; Ming-Ho, 2005).

Exposure to coal dust induces alveolar inflammation that can lead to chronic lung diseases, such as coal workers pneumoconiosis (Miller and MacCalman, 2010) and other related activities such as obstructive lung disease, emphysema, cancer and chronic bronchitis pathologies (Coggon et al, 2010;. Cohen et al, 2008;.Vallyathan et al, 2011). Even some researchers have suggested that there is progression of pneumoconiosis in workers in coal mines even after his retirement (Kimura et al., 2010).

Pneumoconiosis is a disease caused by the inhalation and lung deposition of coal dust, which usually contains small amounts of iron and crystalline silica (quartz) and is mainly characterized by fibrous degeneration (fibrosis) caused after an inflammatory process (Borm et al ., 2011). In 2013, China reported a prevalence of pneumoconiosis 6.02% among workers in the coal industry, a high rate compared to developed countries like the United Kingdom and the United States that record data of 1% and 3.2%, respectively (Mo et al., 2013). In Colombia, meanwhile, this problem has already been reported in miners from Boyacá department (Jimenez et al., 2009).

Pneumoconiosis development depends on the type of coal, anthracite type being the highest cytotoxicity and pathogenicity. It has been estimated that required 10 years to visualize small opacities on chest X-rays in coal mining workers (Farzaneh et al., 2010). Pneumoconiosis is incurable and incapacitating and its most severe form is associated with high mortality. These features make so important the regulation and control of exposure to coal, to

prevent the development of lung disease (Santo Tomas, 2011). The progress of this disease in miners is associated with complications such as chronic obstructive pulmonary disease (COPD), hemoptysis, pneumothorax, pleural disease, tuberculosis, autoimmune disease, chronic interstitial pneumonia and malignancy (cancer) (June et al., 2013).

In models of acute and chronic exposure, in rats exposed to coal dust, have been observed inflammatory effects and oxidative damage in the lung parenchymal tissue, characterized by activation of the enzyme superoxide dismutase (SOD), increased markers of lipid peroxidation and decreased antioxidant defense (Pinho et al., 2004). Similar effects have been seen in humans, in whom exposure to coal dust stimulates the inflammatory response by increasing the release of cytokines (set of proteins that regulate cell interactions of the immune system) such as TNF-alpha, so these molecules have been proposed as biomarkers of pneumoconiosis (Ates et al., 2011). In addition to alterations in markers of inflammation and oxidative stress, when analyzing whole blood and serum from individuals occupationally exposed to coal dust, it has found cellular damage and metabolic abnormalities, evidenced by increased creatinine, ferritin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), g-glutamyltransferase (g-GT), lactate dehydrogenase (LDH), glutathione reductase (GR), triglycerides, very low-density lipoprotein (VLDL), carbonyl protein and malondialdehyde (Tuluce et al., 2011).

Reactive oxygen species (ROS) have been proposed as directly involved in the development of pneumoconiosis, lung carcinogenesis and other diseases caused by exposure to coal dust (Vallyathan et al., 1998). Pyrite (FeS_2) is the main component of coal dust that is directly related to the ability to generate ROS. This mineral, specifically the iron atom, spontaneously form hydrogen peroxide and hydroxyl radical in aqueous medium which on contact with biomolecules, for example, adenine, causing oxidation and consequent alteration in cellular genetic material (Cohn et al, 2010;. Cohn et al, 2006a;. Cohn et al, 2006b).

As a mechanism of toxicity of coal dust, it is thought that initially the particles interact with epithelial cells and alveolar macrophages, stimulating epithelial cells the components secretion of extracellular matrix (ECM) to initiate fibrosis and cytokine secretion by alveolar macrophages that stimulate the migration of phagocytic cells capable of generating an amplification in the local production of cytokines and ROS. Additionally, the carbon particles oxidize cell membranes and allow the output of intracellular protease type enzymes, which damage the pulmonary alveoli. At this point, the generation of ROS outnumber the antioxidant defense, while the lipid peroxidation occurs with the subsequent lung damage (Schins, 2002; Schins and Borm, 1999; Shi et al., 2001; Vallyathan et al., 1998; van et al., 2012). The increase of ROS may also alter cell

proliferation after activation of nuclear transcription factors that produce the synthesis of growth factors, induction of oncogene expression and generation of mutations of tumor suppressor genes (Lim and Seow, 2012).

In summary, the carbon particles with an average diameter of 0.5-10 micrometers (PM 10) can penetrate the alveoli and through mechanisms such as generation of oxidative stress and release of pro-inflammatory cytokines induces hauling fibroblast fibrosis of the lung tissue and cellular transformation that causes cancer (Karkhanis and Joshi, 2013). In the case of pneumoconiosis the most advanced form of the disease called progressive massive fibrosis (Cohen et al., 2008), the latter lung function is compromised due to extensive scarring and emphysema. Once the lungs are committed to fibrosis, they are more susceptible to acquiring COPD in their common forms known as chronic bronchitis and emphysema (Coggon and Newman Taylor., 1998; Wang et al, 1999; Isidro Monteset al., 2004).

2.7. Cardiovascular effects associated with coal mining

Each stage in the cycle of coal mining, extraction, processing, transportation, storage and disposal, is associated with serious health problems. Particularly, air pollution has been linked to cardiovascular morbidity and mortality. Some investigations by Hendryx et al. (2007-2010) have suggested a possible association between coal production and the high prevalence and increased mortality from cardiopulmonary diseases, cancer and kidney diseases, living close to the coal mines (Hendryx and Ahern, 2008; Hendryx, 2009; Hendryx and Zullig, 2009; Hendryx and Ahern, 2009; Hendryx et al, 2012a;. Hendryx and Fedorko, 2011; Hendryx et al, 2010a, 2010b;.Hendryx and Luo, 2012; Hendryx et al, 2012b.). In particular, mortality from chronic cardiovascular diseases has proved to be significantly higher in the mining areas compared to non-mining areas (Esch and Hendryx, 2011), and the number of hospitalizations for hypertension, whose value increases according to the mineral production (Hendryx et al., 2007). In fact, diseases with cardiovascular consequences such as type II diabetes, has been statistically related to the presence of abandoned coal mines, after finding that individuals of the surrounding towns have higher hemoglobin A1c (HbA1c), an important biomarker status and risk of diabetic patients (Liu et al., 2013a).

Proposed mechanisms in thesecardiovascular effects involve the endothelium, a tissue present in the inner surface of blood vessels. A endothelium in individuals exposed to particulate matter, such as that generated by the mining industry, have been found altered cell permeability and disruption in

intracellular signaling mechanisms that maintain the proper functioning of this tissue (Nadadur et al., 2009). In addition, exposure to carbon particles from mining activities can cause systemic microvascular dysfunction in various organs, as a result of vasodilation mediated by nitric oxide and involves disruption of signaling pathways that have not yet been fully identified (Knuckles et al ., 2013).

2.8. Mortality associated with coal mining accidents

An important aspect that involves coal mining and all the minerals, are accidents in mines, they are able to produce gas leaks, landslides, fires and explosions. This is also a global problem. In 2012 China reported 1384 deaths from accidents in coal mines (China Labour Bulletin, 2013), figures that exceed other important countries such as the United States, whose security reports indicate that in the last five years has been 24 deaths per year approximately (Mine Safety and Health Administration, 2013).

In Colombia, the National Mining Agency reported in 2011 a total of 127 people died in mining emergencies. In 2012 the accidents generated 101 deaths and in the first quarter of 2013 were reported 14 deaths of miners. Because of that, 90% of deaths were caused for accidents in coal mines (National Agency of Mining, 2013). Therefore, mining accidents can't be considered a problem of "independent" health and the occupational health conditions must be evaluated.

Some studies that have been developed around the world and some of the impacts that coal mining can generate in the population are presented in Table 2.2

Table 2.2 Examples of international studies regarding the impact of coal mining.

Country	Research	Findings	References
Brazil	Analysis of genotoxicity in mining and unexposed persons, using the micronucleus test in buccal cells and determination of metals in blood.	Compared to unexposed people, miners were found significantly higher frequencies of basal cells, micronucleus in basal cells and differentiated cells, as well as binucleated cells. No correlation between DNA damage and concentration of metals in the blood of the miners was found.	Rohr et al. (2013)
Brazil	Evaluation of the content of metals and damage to DNA in specimens of blacksmith tree frog (amphibian) from mining areas compared with those obtained for the same species in unpolluted areas.	Waste coal mining aregenotoxic for amphibians. In samples from the area of coal mining metal concentrations in organisms followed the order: Fe> Cu> Al> Zn>Rb>Mn.	Zocche et al. (2013)
Czech Republic	Evaluation of the risk of incidence of lung cancer, stomach, colon, bladder and kidneys miners removed from the coal mines for the period 1992-2006.	Compared to the general population, there is an increased risk of developing lung cancer in coal miners with pneumoconiosis, but not for those without the disease. These results allowed the inclusion of lung cancer associated with pneumoconiosis in the list of occupational diseases in the Czech Republic.	Tomaskova et al. (2012)
Turkey	Genotoxicity assays using the micronucleus test and chromatid exchange , in patients with pneumoconiosis and mine workers exposed to coal dust.	High and significant percentages of DNA damage in patients with neumoniosis in coal miners and the unexposed group.	Ulker et al. (2008)
Laboratory assays		**Findings**	**References**
Effects of inhalation of coal dust on the lipid profile, hematopoietic stem cells and circulating		Subchronic inhalation of coal dust with particle size of 10 microns (MP10) has a double effect on the lipid	Setiawan et al. (2013)

endothelial cells in Wistar rats.	profile of exposed rats. In the first causes a decrease of cholesterol and low density lipoproteins and the second effect is increased triglyceride levels.	
Heteropneustes fossilis specimens (Asian catfish) were exposed to effluents from coal mines	Fish experienced weight loss, condition factor and somatic organ indices as well as decrease in content of certain energy reserves, which prevented survival after 26 days of exposure.	Bhatu y Banerjee (2013)
The nematode *Caenorhabditis elegans* was exposed to water samples and sediments impacted by coal mining in West Virginia, USA	Water and stream sediment impacted by coal mining inhibits the growth of *C. elegans.* In general, osmotic stress generated water in the nematode, while the sediments produce toxicity attributable to the presence of metals or metalloids.	Turner et al. (2013)

According to the nature of coal, toxicdad mechanism for PAHs and heavy metals present in the ore it is shown in **Figure 2.7**. PAHs induce CYP1A1, which is used as a biomarker of the toxic effects of these compounds. Induction of CYP1A1 is mediated by the aromatic hydrocarbon receptor (AhR). PAHs to be AhR ligands, bind to this receptor, forming a ligation product (PAH-AhR) which is transported to the nucleus. In the nucleus, AhR dimerizes with nuclear protein traslocadora receptor hydrocarbons (ARNT), forming the complex responsible for the recognition of xenobiotic response element (XRE), that located in the promoters of genes such as CYP1A1 and other enzymes other enzymes involved in the metabolic activation of PAHs induce transcription (Baird, Hooven, & Mahadevan, 2005).

In the case of heavy metals, induction of gene expression can be given through the pathway Keap1/Nrf2/ARE. Nrf2 is a transcription factor, which under basal conditions repressor binds to its Keap1 in the cytoplasm. Oxidative stress induced by exposure to heavy metals leads to activation of the pahtway Keap1/Nrf2/ARE. The transduction pathway Keap1/Nrf2/ ARE signals controls the expression of multiple proteins cytoprotective various characteristics in response to exogenous stressors. The expression of these molecules can be quantified and used as a biomarker for Nrf2 activation. For example, the evaluation of NAD(P)H dehydrogenase (quinone 1) (NQO1); Glutation-S-transferasas (GSTs); Hemoxigenasa 1 (HO-1 ó HMOX1) y Metalotioneínas (MTs), for the specific case of pollutants associated coal dust, this allows to propose mechanisms of toxicity and selecting biomarkers for further progress in elucidating pathological effects on the biota exposed (Kensler, Wakabayashi, & Biswal, 2007; Nguyen, Nioi, & Pickett, 2009).

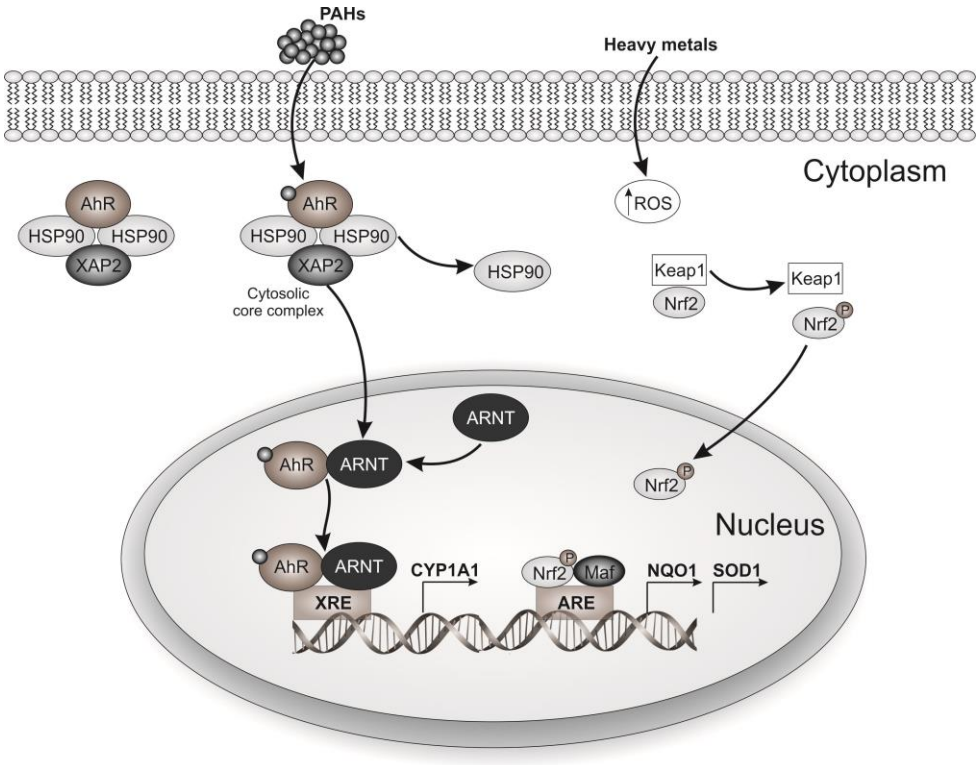

Figure 2.7. Mechanism of toxicity of the compounds present in coal dust.

2.9. Conclusions

Coal is not an inert mineral. Its chemical structure is complex and the mineral and the particles that may be generated during extraction and transport, are capable of releasing environmental pollutants.

The most recognized environmental pollutants from coal mining are respirable particles, polynuclear aromatic hydrocarbons and heavy metals.

Each of these contaminants, individually or in combination, generates various adverse effects on the health of organisms and humans who live in the areas of mining influence as a result of chronic exposure to them, particularly respiratory, cardiovascular disease and cancer, among others.

Studies on the environmental impact and human health of coal mining in Colombia are just emerging. Pneumoconiosis and chromosomal damage has

been detected in some miners, while in wild organisms inhabiting near the mines has been documented chromosomal, cell and tissue damage.

The impacts of mining on the health of nearby populations are also associated with poverty and socio-economic neglect.

2.10. References

Lapp, N. L., & Parker, J. E. (1992). Coal workers' pneumoconiosis. Clinics in chest medicine, 13(2), 243-252.

Hendryx, M., & Zullig, K. J. (2009). Higher coronary heart disease and heart attack morbidity in Appalachian coal mining regions. Preventive Medicine,49(5), 355-359.

Whitehurst, D. D. (1978). A primer on the chemistry and constitution of coal. In: Larsen, J.W. (Ed.), Organic chemistry of coal (pp. 1–35). ACS Symposium Series 71, American Chemical Society, Washington, DC.

CHAPTER 3

3. CHAPTER 3. HAPS, TRACE ELEMENTS IN MARINE SEDIMENTS FROM COAL EXPORT TERMINALS AND ITS TOXICOLOGICAL EFFECTS ON HepG2 CELLS (PAPER 1)

3.1. Introduction

The first work of this thesis was to study the coal ports. These sites, provides jobs and drives local and regional economy. However, its ongoing operation also leads to negative externalities. Because, port activities generate contamination as a result of coal dust emissions from storage piles, spills in the ocean during transportation to tankers, or due to coal barges that capsize. Moreover, studies on the respiratory health effects of living nearby sources of industrial pollution, which mainly emit particulate matter such as coal dust, suggest that proximity to those is a major cause of respiratory morbidity.

Paper 1 describes the effects of port activities in Santa Marta City area (Northern Colombia), a touristic place, capital of the Department of Magdalena and one of the largest coal exporting. The main objective was to assess the levels of pollutants (PAHs and metals) in marine sediments from this place and to measure the expression of genes related to oxidative stress, DNA damage and xenobiotic metabolism in HepG2 cells exposed to extracts from those sediments.

3.2. Materials and methods

3.2.1. Study area

This study did not involve endangered or protected species and no specific permissions were required. Sampling locations around the coastal area of the city of Santa Marta, Colombia, are shown in **Figure 3.1**. This city is a touristic and historical place with 1.013.389 inhabitants according to projections of the The National Administrative Department of Statistics -DANE for 2016 (DANE, 2016). It possesses coal port activities within the urban area, although other coal ports are located south the city. Surface marine sediments were manually collected within 40–50 m in front of the shoreline at nine sampling points (S) along the coastal area around Santa Marta District (**Fig. 3.1**), including the Tayrona National Natural Park (S1), and zones with beaches devoted to intense

tourism and coal port activities (S2–S8). A sample was also collected close to the area where coal is loaded into large vessels (S9), 300 m from the beach. The total distance between sampling sites was 55.8 km. The Santa Marta area is characterized by a tropical climate with average temperature of 27 C and the mean annual precipitation varies between 19 and 975 mm/month due to alternating dry (December to April) and rainy (May to November) periods.

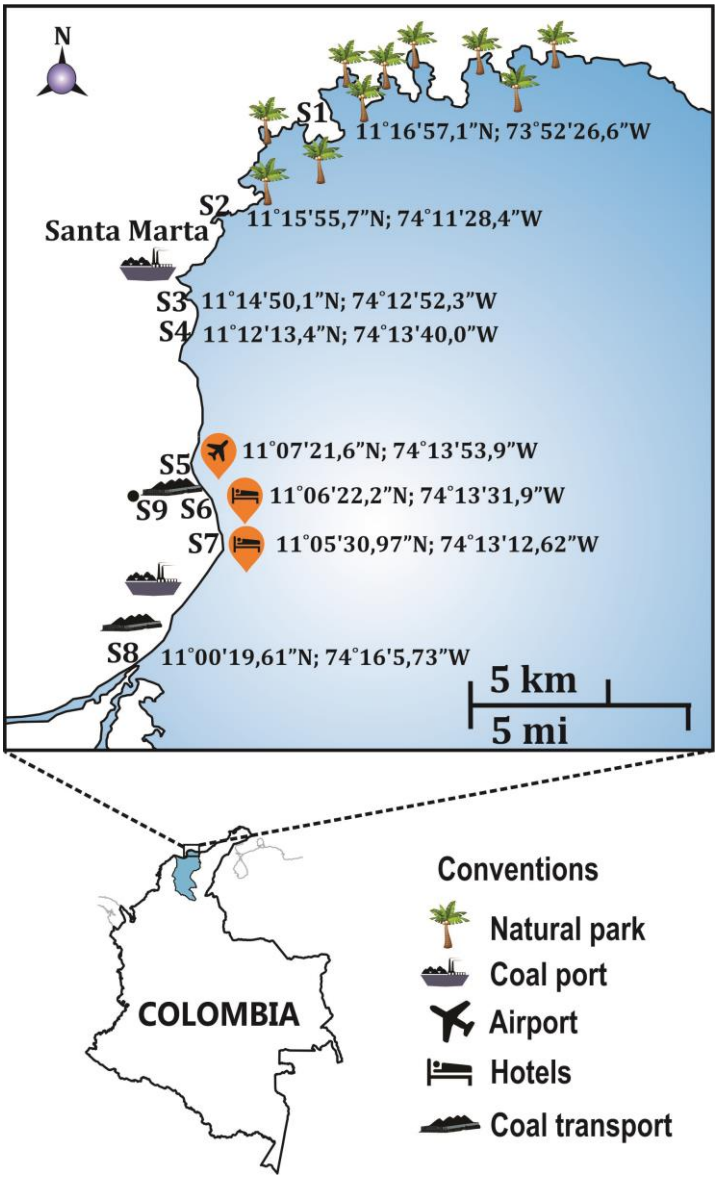

Figure 3.1. Map of Santa Marta, Colombia, showing sampling locations.

3.2.2. Samplings

In April (Dry season, Campaign 1, C1) and September (Rainy Season, C2) 2011, a total of 17 sediment samples were obtained. At each station four subsamples were collected producing a composite sample of approximately 500 g. Samples were placed in plastic bags, labeled and packed in ice, transported to the lab, and kept at -20 °C. In the laboratory all samples were freeze dried (Labconco Freezone 2.5) at -50 °C for 20 h, samples were homogenized to produce a particle size of 0.50 mm. Granulated samples were kept in glass bottles previously cleaned and stored at -20 °C until their analysis. 2.3.

3.2.3. PAH analysis

The U.S. EPA's 16 prioritary PAHs were analyzed in sediments following the methods described elsewhere (Gu et al., 2013; Johnson-Restrepo et al., 2008; Kannan et al., 2005). Sediment samples were Soxhlet-extracted with hexane and dichloromethane, extracts were fractioned and cleaned-up, concentrated to 1 mL under nitrogen stream, and analyzed by gas chromatography coupled to mass spectrometry (GC– MS). The entire analytical procedure, including the extraction, cleanup, and fractionation steps were evaluated by measurement of the absolute recoveries of spiked compounds. Limits of detection for individual PAHs ranged from 0.2 to 4.5 ng/g.

3.2.4. Determination of trace metals by ICP/MS

Seventeen sediment samples were tested for trace-element content. Freeze-dried samples were pulverized using standard methods. Fourty six trace elements (Li, Be, Sc, V, Cr, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Y, Zr, Nb, Mo, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Hf, Ta, W, Tl, Pb, Bi, Th and U) were analyzed on an AGILENT 7700 ICP-MS at the Central Laboratory of the University of Huelva, Spain, following digestion of 0.1 g in a HF + HNO_3 (8 mL:3 mL) solution, drying, and a second dissolution in HNO3 (3 mL) and HCl (3 mL). All the acids used were High Purity Acid Suprapur®, Merck. Three multi-elemental solutions Spec® 1 (rare earth elements, REE), Spec® 2 (alkalis, earth alkalis, and metals) and Spec® 4 (Nb) were employed to construct an external calibration curve. The lower detection limit (LDL) for most of elements in solution was 0.01 ppb. The average precision and accuracy for analyzed elements fall in the range of 5–10%, and were controlled by

repeated analysis of the SARM-1 (granite) and SARM-4 (norite) international rock standard of the South Africa Bureau of Standards.

3.2.5. Preparation of sediment extracts

The extracts of sediment samples were obtained in accordance with procedures suggested in the literature (Banjoo and Nelson, 2005). Briefly, lyophilized samples of sediments were mixed with DMSO (1 part of lyophilized sediment and 2 parts of DMSO), and then sonicated for four periods of 15 min each, with rest intervals of 5 min between sonication periods. The suspensions weredecanted, the supernatant filtered with Whatman disposable syringefilters 0.45 μm, and the filtrates stored at -25 °C. Coal dust(size of ~75 μm) obtained from a coal sample isolated from a minein La Loma, at the Department of Cesar, Colombia, was extracted inthe same manner as sediments and used as positive control.

3.2.6. Effects of sediments extracts in HepG2 cells

HepG2 cell line was obtained from the American Type Culture Collection (HB-8065/LOT:5 8987012), and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum at 37 C under 5% CO_2. Cells were counted and seeded (5 x 10^5 cells/well) to 6-well tissue culture plates. After 24 h, cells were exposed to 1% sediment extracts in DMSO for 12 h, utilizing DMSO (1%) and coal extract at 1%, as vehicle and positive controls, respectively. RNA was isolated from HepG2 cells using RNeasy®Mini Kit (Quiagen, California, USA) as described by the manufacturer. The concentration of RNA was determined by spectrophotometry (A260) and purity was assessed by the A260:A280 ratio (1.9–2.0). The integrity of RNA was checked by visual inspection of 28S and 18S ribosomal RNA on an agarose gel. Aliquots of RNA samples were stored at 80 C until analysis. For each sample, 1 μg of total RNA was reverse transcribed using QuantiTect® Reverse Transcription Kit (QiagenInc, Valencia, CA, USA). The resultant cDNA was used as the template in a 20 μL PCR reaction containing 10 pmol each of forward and reverse gene-specific primers. Real time-PCR was conducted utilizing a StepOne®System (Applied Biosystems, Foster City, CA). The reactions were performed in MicroAmp optical 48-well reaction plates (Applied Biosystems) employing SYBR® Green PCR Master Mix (Applied Biosystems). Conditions for PCR were as follows: Initial denaturation and enzyme activation for 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing/extension). In total, 4 genes were analyzed, including markers of oxidative stress (NQO1), DNA damage (GADD45B), xenobiotic metabolism

(CYP1A1) and lipid metabolism (PPARA). Gene names, accession numbers, forward and reverse primer sequences, as well as amplicon sizes are listed in **Table 3.1** (Du et al., 2014; Guerrero-Castilla and Olivero-Verbel, 2014). Changes in gene expression were determined using HMBS (Hydroxymethylbilane synthase) and B2M (Beta-2-microglobulin) and reference genes (housekeeping), and the comparative CT (ΔΔCT) method was utilized to determine the relative mRNA amount of the target genes. All experiments were run by duplicates and negative controls contained no cDNA (Arya et al., 2009; Valasek and Repa, 2005). A flowchart of the entire protocol is shown in **Figure 3.2.**

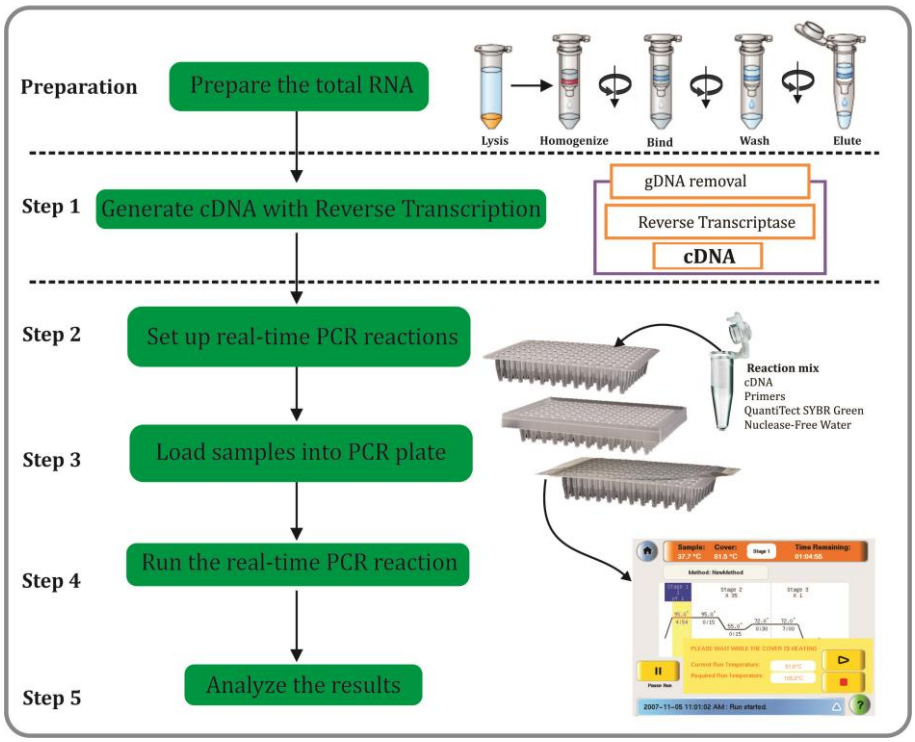

Figure 3.2. Flowchart complete protocol for RT-PCR including sample preparation RNA.

Table 3.1. RT-PCR primer sequences.

Gene name	Gene symbol	Entrez Gene ID	Forward (5' → 3')	Reverse (5' → 3')	Amplicon Size (pb)
			Oxidative stress		
NAD(P)Hdehydrogenase, quinone 1	NQ01	1728	TGGCTTCCAAGTCTTAGAACCT	AGTGTGCCCAATGCTATATGTC	49
			DNA damage		
Growth arrest and DNA-damage-inducible, beta	GADD45B	4616	TACGAGTCGGCCAAGTTGATG	GGATGAGCGTGAAGTGGATTT	115
			Xenobiotic metabolism		
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	1543	TCCTGGAGACCTTCCGACACT	CTTTCAAACTTGTGTCTCTTGTTGTG	78
			Other		
Peroxisomeproliferator-activatedreceptor alpha	PPARα*	5465	AGAGATTTCGCAATCCATCGG	ACTGGTATTCCGTAAAGCCAAAG	62
			Housekeeping		
Hydroxymethyl-bilane synthase	HMBS	3145	TGCAACGGCGGAAGAAAA	ACGAGGCTTTCAATGTTGCC	113
Beta-2-microglobulin	B2M	567	ATGAGTATGCCTGCCGTGTGA	GGCATCTTCAAACCTCCATG	97

*. Primer sequences for PPARα were obtained from Du et al. (2014).

3.2.7. Data analysis

Data are presented as mean ± standard errors, and for statistical purposes those were checked for normality and variance homogeneity, using Kolmogorov–Smirnov and Bartlett's test, respectively. Wilcoxon matched-pairs signed-ranks test was used to compare total PAHs between sampling periods. Multivariate methods were utilized to evaluate relationships between variables and sampling stations. Spearman's rank correlations were conducted for assessing relationships between abiotic and biochemical variables. We employed single linkage cluster analysis using the constrained Ward's method to identify interrelationships between the investigated sites depending on metal sediment content, being the linkage distance reported as Dlink/Dmax, a ratio between the linkage distance for a specific case (D_{link}) divided by the maximal distance (D_{max}), multiplied by 100, standardizing the Dlink. The selection of the number of significant clusters in the dendrogram was performed considering the Sneath index, which considers significance based on two levels of distance measure, 33% and 66% of maximum distance (D_{max}). In this case, the latest one was used for interpretation. Statistical analyses were performed by using Statistica 10. For all statistical purposes, the criterion of signifi- cance was set at $P<0.05$.

3.3. Results

3.3.1. PAHs in marine sediments

The concentrations of PAHs are reported in **Table 3.2**. The sum of PAH levels in sediment samples ranged from below detention limits (S5-C1) to 89.9 ng/g (S9-C1). The stations which had the highest concentrations of total PAHs were S9 (89.9 ng/g) and S3 (62.8–72.8 ng/g). PAH concentrations showed high variability between stations, and, excluding S9, total PAH content was statistically greater during the rainy season than in the dry season (**Table 3.2**, Wilcoxon matched-pairs signed-ranks test, P=0.008). Sampling stations S2 (C2), S3 (C2), S8 (C2) and S9 (C1) were the stations with the larger number of PAHs above detection limits, with 8, 9, 6, and 6 PAHs, respectively.

PAH distribution in sediments according to ring number is presented in **Fig. 3.3.** The most common PAHs present at measurable concentrations were naphthalene (2-rings), phenanthrene (3-rings) and fluoranthene (4-rings). Naphthalene, a low-molecular weight compound (2-rings), was found in 3 out

of 17 analyzed samples, with greatest concentrations detected at S9 (52.8 ng/g) and S8 (12.6 ng/g), all during C1, being this PAH the one with the highest registered concentration among all sampling sites (52.8 ng/g). Fluoranthene was present in all sampling stations, although only during one sampling period (C1) at S1, S4 and S5. Maximum levels of fluoranthene were detected at S3 (34.3 ng/g) and S8 (11.8 ng/g). In the case of phenanthrene, greatest values were measured in S9 (19.4 ng/g) and S3 (18.6 ng/g) stations, with S1 and S5 stations with concentrations below detection limits. It is worth it to mention that PAHs content in S9 accounted for over 50% of the total value obtained after adding all sediment concentrations measured in the different stations. Moreover, S2 and S3 had the highest concentrations of PAHs with five rings (B(b)F + B(k)F, B(a)P, Db(ah)A, Fa). The PAHs with concentrations below detection limits were acenaphthylene, anthracene, dibenzo(a,h)anthracene, and benzo(g,h,i)perylene. Finally, only a small percentage of 6-rings PAHs (IP, B(ghi)P) were recorded in the samples from stations S2 and S3.

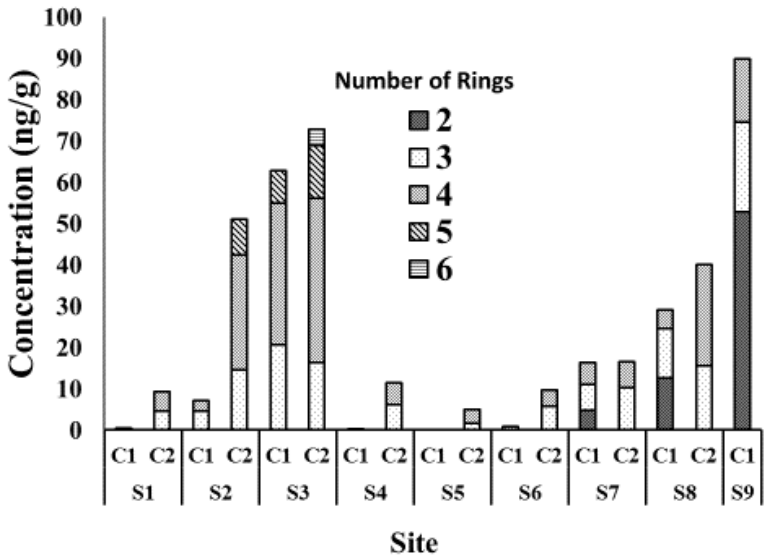

Figure 3.3. Distribution of PAHs in sediments according to the number of rings.

Table 3.2. PAHs in marine sediments (ng/g, dry weight) from Santa Marta shoreline, Colombia.

HAPs	Number of Rings	S1		S2		S3		S4		S5		S6		S7		S8		S9
		C1[a]	C2[b]	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1
Np	2	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	4.7	<2.5	12.6	<2.5	52.8
Ace	3	<3.5	2.6	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5
Fl	3	<0.8	1.9	<0.8	2.8	2.1	3.8	<0.8	1.7	<0.8	1.7	<0.8	2.2	<0.8	2.9	1.7	3.3	2.4
Phe	3	<4.5	<4.5	<4.5	11.8	18.6	12.6	<4.5	4.5	<4.5	<4.5	<4.5	3.6	6.4	7.4	10.2	12.2	19.4
Fa	4	<0.7	1.7	2.7	11.7	34.3	13.9	<0.7	1.8	<0.7	0.8	0.9	1.2	4.4	3	3.5	11.8	8.5
Pyr	4	0.4	3.1	<0.2	7.2	<0.2	11.2	0.3	3.5	<0.2	2.4	<0.2	2.6	0.9	3.2	1.0	8.4	0.4
B[a]A	4	<3.5	<3.5	<3.5	4.3	<3.5	7.3	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	2.3	<3.5
Chry	4	<3.5	<3.5	<3.5	4.7	<3.5	7.3	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	2.1	6.4
B[b]F+B[k]F	5	<3.5	<3.5	<3.5	4.4	7.8	7.8	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5
B[a]P	5	<3.5	<3.5	<3.5	4.2	<3.5	4.9	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5
Ip	6	<3.5	<3.5	<3.5	<3.5	<3.5	4	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5
Σ HAPs		**0.4***	**9.3**	**2.7**	**51.1**	**62.8**	**72.8**	**0.3**	**11.5**	**<DL**	**4.9**	**0.9**	**9.6**	**16.4**	**16.5**	**29**	**40.1**	**89.9**

Abbreviations: Naphthalene (Np); Acenaphthene(Ace); Acenaphthylene (Acy); Fluorene (Fl); Phenanthrene (Phe); Anthracene (An); Fluoranthene (Fa); Pyrene (Pyr); Benzo(a)anthracene (B[a]A); Chrysene (Chry); Benzo(b)fluoranthene (B[b]F) + Benzo(k)fluoranthene (B[k]F); Benzo(a)pyrene (B[a]P); Indeno(1,2,3-cd)pyrene (Ip); Dibenzo(a,h)anthracene (Db[ah]A) and Benzo[g,h,i]perylene (B[ghi]P). Acy, An, Db[ah]A and B[ghi]P were always below detection limits.
a Sampling campaigns, April 2011 (C1), b September 2011 (C2).* Wilcoxon matched-pairs signed-ranks test for total PAHs between C1 and C2, P = 0.008. Spearman correlation coefficient for the pairing, R = 0.714; P = 0.03.

3.3.2. Trace elements in marine sediments

The concentrations of metals found in marine sediments are shown in **Table 3.3.** Metal concentrations for some metals showedhigh variability within stations. Levels ranged between 9.15 and47.6 µg/g for Li, from 30.5 to 141.2 µg/g for Cr, from 7.09 to46.1 µg/gfor Ni, from 3.53 to 29.9 µg/g for Cu, from 63.9 to193.7 µg/gfor Ga, from 2.42 to 11.2 µg/g for As, from 15.7to 59.0 µg/gfor Rb, from 4.83 to 33.0 µg/g for Zr, from0.27 to 1.13 µg/gfor Mo, from 0.06 to 0.37 µg/g for Sb, from< 0.01 to 2.21 µg/g for Cs, from 260.8 to 796.2 µg/g forBa, from 6.49 to 32.6 µg/gfor La, from 14.2 to 60.9 µg/g forCe, from 1.98 to 7.21 µg/gfor Pr, from 0.27 to 1.06 µg/gfor Hf, from 0.35 to 0.80 µg/gfor Ta, from 0.15 to 0.85 µg/gfor W, from 5.7 to 12.2 µg/g for Pb, from 0.03 to 0.13 µg/g forBi, from 1.64 to 22.4 µg/g for Th and from 0.57 to 3.57 µg/gfor U. For most metals, S1, S2, S5 and S6 had the lowest concentrationvalues, whereas the largest were found in S9. The highest concentrationsfor Li, Be, V, Zn, As, Rb, Sr, Zr, Mo, Sb, Cs, Hf, W, Tl, Pb,and Bi were recorded at S9. Anomalously levels of Th and U wererecorded at S4, whereas the highest values for Ni and Cu werefound in S2 and S5, respectively.

The concentrations obtained for some selected trace metals, specifically Cd, Cr, Cu, Pb, Zn and As, were compared to differentsediment quality guidelines, including the American MarineSediment Quality Standards, SQS; the Sediment Primary andSecondary Standard Criteria, Chinese Marine Sediment QualityStandards, GB 18668-2002; and the Marine Sediment Pollution Index (MSPI). These comparisons are presented in **Table 3.4**. Cd levels were below all guidelines. Cr, Cu, Pb and Zn concentrationswere below the American Marine Sediment Quality Standards forall sampling stations, but for the Sediment Primary StandardCriteria (GB 18668-2002) only S2 presented greater values for Crand S9 for Zn.

In terms of contamination, according to MSPI, in this study allsampling stations are in excellent condition based on Cd and Asconcentrations, except S9 that is in poor condition for As.Regarding to Cr, most samples are in poor condition, but S2, S8and S9 are in bad condition. In the case of Cu, sediment quality variesconsiderably, from good condition at S4, S6 and S7, to near poorcondition at S5. For Pb, most samples are between average conditionand poor condition, whereas for Zn, sediment quality is similarto that observed for Pb, with the exception of S9 that showed poorcondition.

Table 3.3. Trace element concentrations (µg/g, dry weight) in sediments from Santa Marta shoreline, Colombia.

Element	Sampling site									
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**Average**
Li	9.15 ± 1.94	9.30 ± 0.25	14.0 ± 1.7	13.4 ± 4.3	14.5 ± 0.3	11.6 ± 0.3	13.8 ± 0.5	**31.7 ± 13.8**	**47.6 ± 2.4***	18.3 ± 4.3
Be	0.76 ± 0.01	0.51 ± 0.01	0.96 ± 0.09	0.92 ± 0.03	0.89 ± 0.05	0.86 ± 0.07	0.85 ± 0.12	1.04 ± 0.06	**1.39 ± 10.1**	0.9 ± 0.1
Sc	11.6 ± 3.1	19.6 ± 2.1	15.4 ± 2.2	15.3 ± 1.9	12.7 ± 1.6	13.4 ± 0.8	14.0 ± 2.8	16.9 ± 4.3	14.2 ± 0.5	14.8 ± 0.8
V	84.3 ± 17.7	135.1 ± 13.9	124.2 ± 13.3	110.3 ± 14.7	101.6 ± 11.1	99.5 ± 6.4	112.8 ± 23.0	134.1 ± 8.5	141.0 ± 1.4	115.9 ± 6.4
Cr	30.5 ± 1.8	**141.2 ± 7.2**	48.8 ± 3.5	44.9 ± 6.3	39.7 ± 4.2	38.8 ± 2.8	33.7 ± 5.0	64.9 ± 3.5	67.1 ± 1.2	56.6 ± 11.4
Co	7.35 ± 0.90	**13.3 ± 0.4**	7.60 ± 0.05	7.28 ± 1.09	7.03 ± 0.77	6.61 ± 0.32	7.45 ± 1.08	12.6 ± 0.9	11.3 ± 0.4	8.9 ± 0.9
Ni	10.0 ± 0.5	**46.1 ± 1.0**	9.51 ± 0.94	10.7 ± 2.3	9.34 ± 0.61	8.68 ± 0.35	7.09 ± 0.48	24.7 ± 5.8	**25.9 ± 1.0**	16.9 ± 4.3
Cu	13.3 ± 6.7	**22.0 ± 0.1**	9.77 ± 3.50	4.16 ± 1.18	**29.9 ± 35.9**	3.53 ± 0.22	4.03 ± 0.39	**21.4 ± 7.0**	**22.1 ± 0.8**	14.5 ± 3.2
Zn	46.8 ± 3.4	59.2 ± 2.0	69.7 ± 3.3	54.9 ± 4.7	53.7 ± 0.5	48.9 ± 0.5	58.3 ± 7.2	89.3 ± 18.8	**106.0 ± 0.6**	65.2 ± 6.7
Ga	143.7 ± 18.3	63.9 ± 26.8	**193.7 ± 10.1**	120.3 ± 10.7	127.0 ± 6.0	132.4 ± 4.2	112.5 ± 11.4	106.2 ± 6.8	114.0 ± 0.8	123.7 ± 11.5
Ge	2.86 ± 0.16	1.94 ± 0.17	4.12 ± 0.89	3.20 ± 0.28	2.46 ± 0.28	2.67 ± 0.10	2.73 ± 0.39	4.17 ± 1.24	3.39 ± 3.11	3.1 ± 0.2
As	2.66 ± 0.15	3.09 ± 0.09	3.18 ± 0.24	3.00 ± 0.46	2.53 ± 0.21	2.76 ± 0.18	2.42 ± 0.08	**7.42 ± 2.25**	**11.2 ± 1.1**	4.3 ± 1.0
Se	1.98 ± 0.43	1.91 ± 0.35	2.63 ± 0.57	2.24 ± 0.18	1.75 ± 0.24	1.92 ± 0.04	2.05 ± 0.43	2.97 ± 0.97	2.21 ± 3.63	2.2 ± 0.1
Rb	34.5 ± 6.0	15.7 ± 2.6	29.9 ± 1.3	21.6 ± 5.0	26.2 ± 0.1	22.0 ± 1.4	24.3 ± 0.7	44.6 ± 15.0	**59.0 ± 0.7**	30.9 ± 4.5
Sr	318.4 ± 9.5	216.4 ± 35.1	408.1 ± 27.4	399.5 ± 14.9	406.5 ± 23.3	435.5 ± 35.8	406.9 ± 83.1	317.3 ± 107.0	464.0 ± 1.5	374.7 ± 25.5
Y	15.7 ± 3.9	14.9 ± 3.2	21.7 ± 3.9	18.1 ± 1.8	13.9 ± 2.0	15.5 ± 0.7	16.8 ± 3.7	23.89 ± 9.04	16.7 ± 1.9	17.5 ± 1.1
Zr	8.03 ± 0.74	5.63 ± 0.66	8.21 ± 1.13	5.89 ± 0.13	4.83 ± 0.54	5.35 ± 0.13	5.86 ± 1.45	**19.1 ± 5.3**	**33.0 ± 2.4**	10.7 ± 3.2
Nb	6.65 ± 0.60	4.10 ± 0.69	9.27 ± 1.66	6.67 ± 0.33	5.40 ± 0.69	5.02 ± 0.01	5.36 ± 0.97	8.32 ± 1.14	8.42 ± 1.97	6.6 ± 0.6
Mo	0.70 ± 0.29	0.48 ± 0.06	0.75 ± 0.04	0.42 ± 0.17	0.27 ± 0.07	0.29 ± 0.05	0.29 ± 0.01	0.80 ± 0.03	**1.13 ± 0.48**	0.6 ± 0.1
Cd	0.08 ± 0.02	0.11 ± 0.01	**0.15 ± 0.01**	0.13 ± 0.00	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.02	**0.16 ± 0.02**	0.13 ± 4.13	0.1 ± 0.0
Sn	1.11 ± 0.44	1.62 ± 0.55	1.88 ± 0.01	1.51 ± 0.53	1.10 ± 0.14	1.13 ± 0.04	1.08 ± 0.12	1.42 ± 0.02	1.49 ± 1.85	1.4 ± 0.1
Sb	0.06 ± 0.01	**0.21 ± 0.02**	0.16 ± 0.02	0.07 ± 0.02	0.06 ± 0.00	0.10 ± 0.06	0.05 ± 0.00	**0.19 ± 0.09**	**0.37 ± 2.00**	0.1 ± 0.0
Cs	<0.01	<0.01	**0.10 ± 0.06**	0.04 ± 0.06	0.12 ± 0.07	<0.01	0.05 ± 0.03	**1.24 ± 0.96**	**2.21 ± 0.26**	0.4 ± 0.3
Ba	561.1 ± 44.1	260.8 ± 82.8	**796.2 ± 50.0**	474.8 ± 56.0	499.1 ± 36.7	514.0 ± 36.7	431.2 ± 53.9	414.7 ± 14.6	447.8 ± 0.5	488.9 ± 47.6
La	16.1 ± 0.9	6.49 ± 0.15	**32.6 ± 14.5**	21.0 ± 2.7	12.8 ± 1.1	15.4 ± 0.6	12.8 ± 1.7	24.6 ± 7.6	21.6 ± 1.4	18.2 ± 2.6
Ce	34.0 ± 0.7	14.2 ± 1.2	**60.9 ± 22.0**	40.9 ± 5.3	26.8 ± 3.0	32.1 ± 0.1	27.9 ± 3.9	50.9 ± 17.2	43.4 ± 0.7	36.8 ± 4.7
Pr	4.26 ± 0.05	1.98 ± 0.16	**7.21 ± 2.28**	5.04 ± 0.50	3.49 ± 0.36	4.09 ± 0.06	3.80 ± 0.67	6.48 ± 2.23	5.27 ± 0.90	4.6 ± 0.5
Nd	17.4 ± 0.4	9.01 ± 1.07	27.5 ± 7.3	20.4 ± 1.8	14.5 ± 1.8	16.7 ± 0.6	16.3 ± 3.0	26.9 ± 9.9	21.0 ± 0.8	18.9 ± 2.0
Sm	3.61 ± 0.37	2.40 ± 0.39	5.22 ± 1.11	4.24 ± 0.41	3.11 ± 0.39	3.55 ± 0.10	3.64 ± 0.75	5.74 ± 2.08	4.33 ± 0.82	4.0 ± 0.3
Eu	1.02 ± 0.16	0.83 ± 0.15	1.38 ± 0.25	1.17 ± 0.09	0.94 ± 0.13	1.03 ± 0.06	1.09 ± 0.23	1.58 ± 0.67	1.07 ± 1.73	1.1 ± 0.1
Gd	3.34 ± 0.49	2.71 ± 0.52	4.72 ± 0.94	3.96 ± 0.39	2.93 ± 0.38	3.29 ± 0.09	3.44 ± 0.72	5.36 ± 2.02	3.90 ± 1.01	3.7 ± 0.3
Tb	0.50 ± 0.10	0.45 ± 0.10	0.69 ± 0.13	0.58 ± 0.06	0.43 ± 0.06	0.49 ± 0.01	0.52 ± 0.11	0.78 ± 0.29	0.56 ± 0.56	0.6 ± 0.0

Dy	3.01 ± 0.71	2.83 ± 0.62	4.08 ± 0.76	3.45 ± 0.34	2.63 ± 0.33	2.92 ± 0.11	3.14 ± 0.66	4.64 ± 1.75	3.26 ± 0.75	3.3 ± 0.2
Ho	0.60 ± 0.15	0.58 ± 0.13	0.82 ± 0.15	0.68 ± 0.07	0.52 ± 0.07	0.58 ± 0.03	0.63 ± 0.13	0.91 ± 0.35	0.63 ± 0.70	0.7 ± 0.0
Er	1.76 ± 0.45	1.66 ± 0.36	2.40 ± 0.45	2.00 ± 0.20	1.54 ± 0.21	1.68 ± 0.08	1.84 ± 0.40	2.62 ± 0.97	1.81 ± 1.18	1.9 ± 0.1
Tm	0.25 ± 0.07	0.23 ± 0.05	0.35 ± 0.06	0.29 ± 0.03	0.22 ± 0.03	0.24 ± 0.01	0.27 ± 0.06	0.37 ± 0.13	0.26 ± 1.41	0.3 ± 0.0
Yb	1.65 ± 0.46	1.44 ± 0.29	2.27 ± 0.42	1.83 ± 0.18	1.44 ± 0.21	1.55 ± 0.07	1.71 ± 0.36	2.34 ± 0.85	1.67 ± 1.00	1.8 ± 0.1
Lu	0.24 ± 0.06	0.20 ± 0.04	0.33 ± 0.06	0.26 ± 0.02	0.21 ± 0.03	0.22 ± 0.01	0.25 ± 0.06	0.34 ± 0.12	0.24 ± 1.21	0.3 ± 0.0
Hf	0.40 ± 0.01	0.27 ± 0.04	0.58 ± 0.08	0.40 ± 0.02	0.32 ± 0.03	0.36 ± 0.01	0.41 ± 0.09	0.68 ± 0.10	**1.06 ± 1.84**	0.5 ± 0.1
Ta	0.49 ± 0.12	0.35 ± 0.08	**0.80 ± 0.14**	0.49 ± 0.03	0.38 ± 0.05	0.39 ± 0.01	0.37 ± 0.04	0.56 ± 0.08	0.60 ± 1.23	0.5 ± 0.0
W	0.41 ± 0.10	**0.65 ± 0.14**	0.25 ± 0.07	0.23 ± 0.09	0.20 ± 0.06	0.17 ± 0.04	0.15 ± 0.02	**0.63 ± 0.02**	**0.85 ± 1.83**	0.4 ± 0.1
Tl	0.17 ± 0.00	0.14 ± 0.01	0.26 ± 0.03	0.16 ± 0.04	0.18 ± 0.01	0.15 ± 0.01	0.18 ± 0.01	**0.32 ± 0.11**	**0.47 ± 1.89**	0.2 ± 0.0
Pb	6.32 ± 0.33	6.58 ± 0.31	11.0 ± 0.3	6.59 ± 0.17	7.08 ± 1.04	6.52 ± 0.30	5.70 ± 0.62	9.98 ± 1.67	**12.2 ± 1.2**	8.0 ± 0.8
Bi	0.03 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.10 ± 0.02	0.13 ± 1.46	0.1 ± 0.0
Th	2.77 ± 0.04	1.64 ± 0.11	7.55 ± 2.61	**22.4 ± 26.2**	2.81 ± 0.16	3.00 ± 0.41	2.14 ± 0.15	4.07 ± 0.24	5.52 ± 1.40	5.8 ± 2.2
U	0.57 ± 0.08	0.58 ± 0.03	2.22 ± 0.33	**3.57 ± 3.45**	0.88 ± 0.14	0.93 ± 0.01	1.01 ± 0.22	1.25 ± 0.05	2.05 ± 0.78	1.5 ± 0.3

* Sampling stations with values greater than 150% the average obtained for all stations are written in bold.

Table 3.4. Trace element concentrations (μg/g, dry weight) in sediments from Santa Marta shoreline, Colombia, compared to marine Sediment Quality Standards.

Station/Indexname		Cd	Cr	Cu	Pb	Zn	As	Reference
S1		0.1 ± 0.0	30.5 ± 1.8	13.3 ± 6.7	6.3 ± 0.3	46.8 ± 3.4	2.7 ± 0.2	Thisstudy
S2		0.1 ± 0.0	141.2 ± 7.2	22.0 ± 0.1	6.6 ± 0.3	59.2 ± 2.0	3.1 ± 0.1	Thisstudy
S3		0.2 ± 0.0	48.8 ± 3.5	9.77 ± 3.5	11.0 ± 0.3	69.7 ± 3.3	3.2 ± 0.2	Thisstudy
S4		0.1 ± 0.0	44.9 ± 6.3	4.16 ± 1.2	6.6 ± 0.2	54.9 ± 4.7	3.0 ± 0.5	Thisstudy
S5		0.1 ± 0.0	39.7 ± 4.2	29.9 ± 35.9	7.1 ± 1.0	53.7 ± 0.5	2.5 ± 0.2	Thisstudy
S6		0.1 ± 0.0	38.8 ± 2.8	3.5 ± 0.2	6.5 ± 0.3	48.9 ± 0.5	2.8 ± 0.2	Thisstudy
S7		0.1 ± 0.0	33.7 ± 5.0	4.0 ± 0.4	5.7 ± 0.6	58.3 ± 7.2	2.4 ± 0.1	Thisstudy
S8		0.2 ± 0.0	64.9 ± 3.5	21.4 ± 7.0	10.0 ± 1.7	89.3 ± 18.8	7.4 ± 2.3	Thisstudy
S9		0.1 ± 4.1	67.1 ± 1.2	22.1 ± 0.8	12.2 ± 1.2	106.0 ± 0.6	11.2 ± 1.1	Thisstudy
American Marine Sediment Quality Standards, SQS		5.1	260	390	450	90-260		WDOE (1995)
Sediment Primary Standard Criteria*		0.5	80	35	60	150		Zhang et al. (2007)
SedimentSecondary Standard Criteria*		1.5	150	100	130	350		Zhang et al. (2007)
Marine sediment pollution index (MSPI)**.	Percentile 0–20	0.6	2.0	3.0	3.3	15.4	7.0	Shin and Lam (2001)
	Percentile 21–40	1.0	5.0	6.0	5.0	34.0	8.0	
	Percentile 41–60	1.5	9.2	12.0	8.0	57.0	10.2	
	Percentile 61–80	2.9	19.6	30.6	18.2	101.6	21.0	
	Percentile 81–100	8.0	63.0	191.0	69.0	507.0	58.0	

* Chinese Marine Sediment Quality Standards (GB 18668-2002) (SEPA, 2002).** The sediment quality based on MSPI is rated on the basis of the percentile in the dataset, as follows: MSPI 0-20: sediment in excellent condition; MSPI 21-40: sediment ingood condition; MSPI 41-60: sediment in average condition; MSPI 61-80: sediment in poor condition; MSPI 81-100: sediment in bad condition.

3.3.3. Quantification of mRNA in HepG2 cells exposed to sediment extract

The results of gene expression in HepG2 cells for sedimentextracts obtained during sampling campaigns C1 and C2 are presentedin **Tables 3.5 and 3.6**, respectively. During sampling 1 (C1,April, 2011), HepG2 cells exposed to S3 and S9 extracts showedstatistically significant over-expression of CYP1A1 (11.4 to61.5-fold increase). The S1, S2 and S8 sediments, although didnot produce a significant over expression of CYP1A1, were ableto generate about 2-fold increase in the relative expression of thisgene. NQO1 expression was augmented in HepG2 exposed to S6‑S9 samples (2.4 to 4.3-fold increase). Expression of GADD45B andPPARa did not change with extract exposure.During sampling 2 (C2, September, 2011), no significantchanges were observed in the mRNA expression of the evaluatedgenes. However, S1 and S8 generated some over-expression (values>2-fold) of NQO1 and CYP1A1, respectively, but this was notstatistically significant.

Table 3.5. Relative quantification of mRNA of CYP1A1 (Cytochrome P450, family 1, subfamily A, polypeptide 1), NQO1 (NAD(P)H dehydrogenase quinone 1), GADD45B (DNA damageinduciblegene 45 b) and PPARa (Peroxisome proliferator-activated receptor alpha) in HepG2 Cells treated with 1% marine sediment extracts (Campaign 1).

	RELATIVE QUANTIFICATION OF mRNA							
	CYP1A1		***NQO1***		***GADD45B***		***PPARA***	
SAMPLE	***HMBS***	***B2M***	***HMBS***	***B2M***	***HMBS***	***B2M***	***HMBS***	***B2M***
C-	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ±0.1	1.0 ± 0.2	1.0 ± 0.2
CE (500 ppm)	176.4 ± 23.0*	189.9 ± 31.8*	1.9 ± 0.0	2.6 ± 0.6	6.8 ± 1.7*	7.3 ± 1.8*	1.1 ± 0.3	1.2 ± 0.4
CE (1000 ppm)	307.2 ± 40.5*	231.9 ± 73.0*	3.9 ± 0.4*	4.0 ± 0.1*	30.2 ± 9.5*	19.0 ± 4.0*	1.6 ± 0.9	0.8 ± 0.0
S1	3.6 ± 0.4	2.7 ± 0.1	1.8 ± 0.4	1.3 ± 0.1	1.4 ± 0.3	1.0 ± 0.0	0.6 ± 0.1	0.5 ± 0.0
S2	2.6 ± 0.9	1.6 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	0.9 ± 0.2	0.7 ± 0.2	0.4 ± 0.1
S3	**61.5 ± 13.1***	**36.6 ± 2.4***	2.1 ± 0.2	1.7 ± 0.0	1.4 ± 0.2	0.8 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
S4	1.2 ± 0.2	1.7 ± 0.3	1.0 ± 0.3	1.2 ± 0.1	0.7 ± 0.3	0.8 ± 0.3	0.5 ± 0.1	0.8 ± 0.2
S5	0.5 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	2.7 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	0.8 ± 0.1	1.6 ± 0.1
S6	1.1 ± 0.4	1.5 ± 0.5	3.3 ± 0.9*	3.4 ± 0.4*	0.1 ± 0.0	0.1 ± 0.0	1.4 ± 0.2	1.8 ± 0.9
S7	1.1 ± 0.3	1.3 ± 0.4	4.3 ± 1.3*	3.0 ± 0.4*	0.1 ± 0.0	0.2 ± 0.0	0.9 ± 0.1	1.6 ± 0.2
S8	2.2 ±0.2	3.3 ± 0.7	2.4 ±0.1*	3.5 ± 0.5*	0.1 ± 0.0	0.2 ± 0.0	0.8 ± 0.1	1.2 ± 0.3
S9	**11.9 ± 4.5***	**11.4 ± 3.2***	3.7 ±0.9*	3.6 ±0.1*	0.2 ± 0.0	0.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.2

Gene expression was normalized with HMBS and B2M. C-, Negative control; CE, Coal Extract (Positive control); S1‑S9, marine sediment extracts.* Significant difference ($P < 0.05$) compared to the Negative control.

Table 3.6. Relative quantification of mRNA of CYP1A1 (Cytochrome P450, family 1, subfamily A, polypeptide 1), NQO1 (NAD(P)H dehydrogenase quinone 1), GADD45B (DNA damageinduciblegene 45 b) and PPARa (Peroxisome proliferator-activated receptor alpha) in HepG2 Cells treated with 1% marine sediment extracts (Campaign 2).

	RELATIVE QUANTIFICATION OF mRNA							
	CYP1A1		***NQO1***		***GADD45B***		***PPARA***	
SAMPLE	***HMBS***	***B2M***	***HMBS***	***B2M***	***HMBS***	***B2M***	***HMBS***	***B2M***
C-	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.2
CE (500 ppm)	176.4 ± 23.0*	189.9 ± 31.8*	1.9 ± 0.0	2.6 ± 0.6	6.8 ± 1.7*	7.3 ± 1.8*	1.1 ± 0.3	1.2 ± 0.4
CE (1000 ppm)	307.2 ± 40.5*	231.9 ± 73.0*	3.9 ± 0.4*	4.0 ± 0.1*	30.2 ± 9.5*	19.0 ± 4.0*	1.6 ± 0.9	0.8 ± 0.0
S1	0.4 ± 0.1	0.4 ± 0.1	2.9 ± 0.8	2.4 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	1.5 ± 0.6	1.5 ± 0.2
S2	1.3 ± 0.3	1.3 ± 0.6	1.7 ± 0.0	3.4 ± 0.4	0.1 ± 0.0	0.1 ± 0.0	0.8 ± 0.2	1.4 ± 0.2
S3	1.3 ± 0.0	2.5 ± 0.4	1.2 ± 0.3	3.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.2	1.1 ± 0.0
S4	0.3 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.5 ± 0.1
S5	0.4 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.1
S6	1.3 ± 0.2	1.0 ± 0.3	0.8 ± 0.1	0.6 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.1
S7	1.0 ± 0.1	1.1 ± 0.6	0.5 ± 0.0	0.8 ± 0.2	0.1 ± 0.1	0.1 ± 0.0	0.4 ± 0.1	0.5 ± 0.1
S8	2.3 ± 0.3	4.9 ± 1.2	0.8 ± 0.0	1.9 ± 0.7	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.7 ± 0.2

Gene expression was normalized with HMBS and B2 M. C-, Negative control; CE, Coal Extract (Positive control); S1‑S9, marine sediment extracts.* Significant difference ($P < 0.05$) compared to the Negative control.

3.4. Discussion

In this investigation, PAHs and metal content in sediments, aswell as toxicity data from sediment extracts were obtained alongthe shoreline of Santa Marta, Colombia. This study is the first topresent a chemical and toxicological approach to characterizeone of the most important coal ports in Latin America.

3.4.1. PAHs in sediments

PAH concentrations in sediments ranged from undetectable to 89.9 ng g_1 (**Table 3.2**). The most commonly found PAHs in sedimentsamples were fluoranthene, followed by phenanthrene and pyrene.These three PAHs have been found as the most abundant in coalwaste material from coal field exploitations (Ribeiro et al., 2012).However, both fluoranthene and pyrene are quite common in portsediments, as they have been reported as highly abundant in sedimentsfrom Spanish Mediterranean coast (Leon et al., 2014) andRio de la Plata Estuary, Argentina (Colombo et al., 2006). In termsof total PAHs, samples collected during the rainy season (C2) hadgreater PAH content, as also reported by other authors (Jawardet al., 2012; Nouira et al., 2013), although a significant correlationwas observed for both campaings ($R = 0.714$; $P = 0.03$).

The largest total PAH concentration was registered at S9(89.9 ng/g). S9 is located in the path followed by barges thattransport coal from coastal ports to large vessels. S3, on the otherhand, corresponds to the port of Santa Marta. This port is used toload coal and many other products on mostly diesel-based vessels. Accordingly, the process of coal loading seems to be an importantsource of PAHs. Coal is loaded directly into the vessels and the dustformation may reach the sediments. Although PAHs concentrationsin coal may vary between 1 to about 2500 μg/g (Achten andHofmann, 2009), sediment PAHs observed in Santa Marta shorelineare most likely to be the result of both diesel burning from theships and coal dust. The greatest PAH concentration was observedfor naphthalene (52.8 ng/g) at S9 and fluoranthene (34.3 ng/g) at S3. Naphthalene was also detected in S8 and S7, stations that areinfluenced by the major coal cargo transport in the area. This lowmolecular weight PAH may be released from many types of coal (Achten and Hofmann, 2009; Van Kooten et al., 2002; Tripp et al., 1981). Therefore, its presence in sediments could be associatedwith coal dust spills and accidental releases occurred in the site.Moreover, resuspension of sediments from ocean andship-generated waves can result in increased bioavailability andtoxicity of PAHs (Kammann, 2007).

As many of the PAH levels detected in marine sediments werebelow detection limits, it is not worthy to use PAH ratios to presumableidentify sources of these

compounds. Still, for some stationsit is possible to generate some data. For instance, a valuebetween 1.0‑1.2 for B[a]A/Chry has been attributed to coal (Socloet al., 2000; Gschwend and Hites, 1981), and for S2, S3 and S8(C2), those values were 0.91, 1.0, and 1.1, respectively.

3.4.2. Trace elements in sediments

Element concentrations in sediments (**Table 3.3**) clearly suggestthat despite the sampling period, Santa Marta Bay (S3) andTaganga Bay (S2), as well as other stations near coal cargo ports(S8, S9), are impacted by anthropic pollution. Equally problematicis the data suggesting that even the Tayrona Park (S1) may bereceiving some pollution impact, but still it is the most pristinelocation among sampling sites.

Swaine (2000) has reported that 26 trace elements in coalwould lead to potential environmental impacts, and these havebeen classified into three classes: I (As, Cr, Cd, Hg, Pb, Se), II (B,Cl, F, Mn, Mo, Ni, Be, Cu, P, Th, U, V, Zn), and III (Ba, Co, I, Ra, Sb,Sn, Tl). In this study, 46 trace elements were analyzed andsome are discussed in detail, in particular several belonging toClasses I and II, as critical chemical factors to evaluate the impactby coal cargo activities in this shoreline.

Arsenic is a ubiquitous metalloid element in the environment, and its high toxicity often threatens human health and even globalecosystems (Nriagu et al., 2007). Moriarty et al. (2014) reportedthat coal mining increases levels of As in sediments, and usuallyit is mainly associated with pyrite (Ward, 2002; Dai et al., 2005; Kang et al., 2011). Sediments from S8 and S9 had As concentrationsthat doubled the average found in all stations. S9, as mentioned, islocated along the pathway followed by barges to transport coalfrom the coast to cargo vessels; whereas S8 is southern the siteand probably receives impacts due to wind patterns.

The highest concentration of Cr was found at S2 (141.2±7.2 μg/g), with values almost threefold greater thanthe average for all sampling stations. S8 and S9 also had Cr concentrationsgreater than the average. Cr may enter the aquatic mediumthrough effluent discharges from tanneries, textiles,electroplating, mining, dyeing, printing industries, photographicand pharmaceutical industries (Ahmed et al., 2013). According tothe American Marine Sediment Quality Standards (SQS) and theChinese Marine Sediment Quality Standards (GB 18668-2002), Crlevels in this station notably decrease the environmental qualityof the sediments. Stations S8 and S9 also had Cr concentrationsgreater than the average. Cr toxicity is related to reactive oxygenspecies (ROS)

formation and alteration of antioxidant enzymeactivities (Rai et al., 2004; Dazy et al., 2008).

The concentration of Ni was greatest in S2, followed by S8 andS9. It has been reported that this metal is associated with bothinorganic and organic matter in coal and exists as sulfide minerals (Finkelman, 1995). On the other hand, greater Cu concentrations were found in S5, followed by S2, S9 and S8. Copper is a commoningredient in antibiofouling paints which is applied on the surfaces of ships and in offshore engineering (Pan and Wang, 2012).

The highest concentrations of Zn and Pb were recorded at S9.Lead is used as a pigment and an anti-corrosion agent in paintsand as an antiknock factor in gasoline and diesel fuels(Thompson et al., 2005); therefore, this could be a factor thatincreases Pb in this station. Zinc is reported to be associated withinorganic matter, and it is present in pyrite and sphalerite(Finkelman, 1995; Swaine and Goodarzi, 1995).An important finding in this study was that compared to theaverage value obtained for all sampling stations, S9 had fivefoldgreater concentration of Ce, followed by S8 (fourfold) and S3 (twofold). Both S8 and S9 represent stations impacted by coal transporttoward large vessels, while S3 is used to load coal and other productsin Santa Marta port. A plausible source of Ce in these locationsmay be diesel combustion. Ce has been used as a fuel additive; inparticular as diesel engine catalyst that lowers the amount of dieselexhaust particles, decreases fuel consumption as well as greenhousegas emissions (Park et al., 2008). It is also known that ceriumoxide (CeO_2) nanoparticles are emitted in the diesel exhaust, beingable to induce pulmonary fibrosis (Ma et al., 2012; Cassee et al., 2011).

Another interesting observation revealed in this work was thatfor some metals, significant correlations could be detected, forinstance, Ni-W, Ce-Nb, and Ce-La, among others. This could beexplained mostly considering similar sources, such as corrosionresistant coatings based on Ni-W; or catalysts to control pollutantemissions, in the case of Ce-Nb or Ce-La.

Trace metals in sediments come from aqueous phase depositionfrom several physical, chemical or biological pathways. Those canalso be released through resuspension, dissolution and biodegradation.Furthermore, they are good indicators of historical and presentenvironmental pollution (Gao et al., 2014). Sediments act asa metals deposit and also as a source of contamination for theaquatic environment, as trace elements may be transformed intosoluble, and potentially more toxic species through degradationor reactions (Torres et al., 2007). This is why sediment characterizationin terms of metal content is a highly used method todescribe their environmental health status. Several marine sedimentquality indexes were used in this study as a general measureof marine sediment contamination. Although they offer someguidelines for

cathegorization, these standard criteria are usefulfor protecting habitats for marine life or human recreation andsports, regulating general industrial use and coastal tourism anddefining harbor rs and special use for ocean exploration (Zhanget al., 2007), among other uses. Some of them, for instance, theMSPI, has also shown correlation with benthic and toxicity data (Shin and Lam, 2001). In general, sediment concentrations of sometoxicologically relevant elements, such as Cr, Cu, Pb and Zn, areabove some international guidelines, and require careful monitoringto prevent further environmental damage.

Hierarchical cluster analysis was performed to identify similarities between sampling stations according to metal content insediments. The dendrogram resulting for all sampling campaigns (C1‑C2) and locations (S1‑S9) is shown in **Figure 3.4.** Three different clusters were obtained. One corresponding to S2, another to S3and one cluster grouping the other stations (OS). S2 may beimpacted by coal cargo operations and by a submarine outfall thatis located between S2 and S3, whereas S3 is the main cargo port inSanta Marta city. The cluster formed by OS showed three distinctminor clusters. One formed exclusively by S1, located at TayronaNational Natural Park, a protected area, had high similarity inmetal composition during the two sampling periods. The othertwo comprises sampling stations S4‑S9.

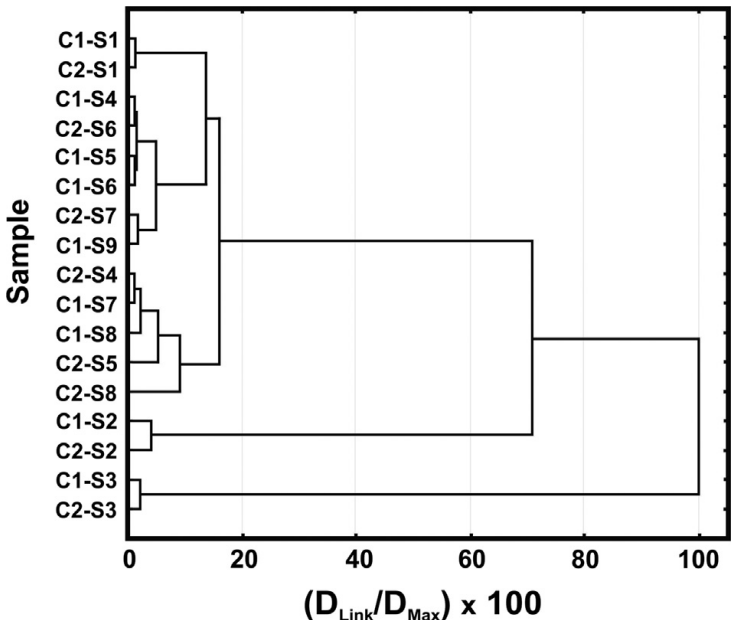

Figure 3.4. Dendrogram depicting associations between sampling sites (S1–S9) and campaigns (C1–C1).

Results presented here clearly suggest that despite the samplingperiod, Santa Marta Bay (S3), Taganga Bay (S2) and the area of coalcargo (S3) are highly impacted by anthropic pollution. Surprisingly, although the Tayrona Park (S1) may be receiving some impact, itstill is the most pristine location among sampling sites.

3.4.3. Sediment extracts toxicity

In vitro bioassays may be considered an extension of chemicalanalyses, as they allow the estimation of the biological activity ofchemicals acting through different mechanisms, visualizing thepotential hazards associated with contaminants present in particularsamples (Vondrac ̌ek et al., 2001). In this study, the expressionof some marker genes was evaluated in HepG2 cells exposed toDMSO extracts prepared from marine sediments sampled aroundthe coastal area of the city of Santa Marta (Colombia), site with anumber of coal ports. DMSO was employed as the extraction solventas it has low cytotoxicity and it is capable of extracting bothorganic and inorganic chemicals from solid samples.

Exposure to PAHs induces a variety of molecular responses inthe organisms such as oxidative stresss, enzyme activation, and/orchanges in gene expression. Many of these effects are mediated bythe aryl hydrocarbon receptor (AhR)/aryl hydrocarbon nucleartranslocator (ARNT) signaling pathway. These signaling cascadeshave been studied, particularly with respect to the induction ofgenes involved in activation and detoxification, such as CYP1A1(Burczynski and Penning, 2000; Shimada, 2006) and NQO1(Shimada, 2006). In this study, exposure of HepG2 cells to coalextract affected the expression of these genes, particularly in locations S3 and S9 during C1 (**Table 3.5**). These samples had a pattern ofgene expression with some similarities to that generated by a coaldust extract (positive control). The high levels of PAH in S3 and S9 samples (**Table 3.2**) may explain the expression of CYP1A1. Thisstrong CYP1A1 induction may correspond to a mixture of coal dustand diesel combustion residues that have been deposited in thesediments by processes related to coal loading and transportation, although other port activities may also contribute as a source ofPAHs.

In the case of NQO1, S6-S9 samples (C1) induced thisoxidative-stress related gene (**Table 3.5**). This may have some explanationon elevated values for several metals in S8-S9 samples (**Table 3.3**), but additional pollutants or a combination with PAHscould also participate in the overexpression on NQO1 evoked byS6-S7 (C1) samples. Bioactivity was mostly observed during thedry season, whereas greater PAH values appeared during the rainyseason. This difference in bioavailability could have been related tochanges in

environmental factors driven by rain-related processes,such as sediment composition, presence of humic substances thatmay bind and occlude PAHs (Liang et al., 2007), or perhaps therewere other chemicals carried into the sediments by runoff thatworked as AhR antagonists or endocrine disruptos, crosstalkingwith AhR-related mechanisms.

CYP1A1 and NQO1 overexpression may indicate cellularresponses against xenobiotic stressors (Fujii-Kuriyama andKawajiri, 2010; Simmons et al., 2009). In HepG2, CYP1A1 is a classicalPAH-responsive gene (Castorena-Torres et al., 2008; Vakhariaet al., 2001). Achten and Hofmann (2009) have stated that unburnthard coal may release several PAHs to the aquatic environment, making them available to organisms. These compounds includethe 16 EPA-PAH, benzo[e]pyrene, perylene, coronene, methylatedchrysenes, methylated picenes, tetrahydrochrysenes, hydropicenesand methylphenanthrenes. These chemical mixtures have thepotential to generate sustained induction of CYP1A1 in HepG2 cells (Gabelova et al. (2013); and as presented here, HepG2 cellsexposed to DMSO extracts of polluted estuarine sediments haveshown over-expression of CYP1A1. The overexpression of CYP1A1gene has been consistently correlated to organic contaminants instudies performed with organisms exposed to contaminated estuarinesediments (Costa et al., 2011). Although S3 had the greatestPAH concentrations, the PAH source may be the result of both coalshipment and heavy vessel transport taking place in this port.Increased expression of NQO1 induced primarily through activationof the Nrf2 serves to limit oxidative damage generated byexposure to pro-oxidant pollutants such as heavy metals, PAHsand particulate material (Baird and Dinkova-Kostova, 2011). ThePAHs have been reported as bifunctional inducers of phase Iand phase II detoxification gene expression, via AhR and Nrf2pathways, respectively. This is explained by the different chemicalreactivity of the PAHs, which allows relative abilities to activateone or both cellular responses (Burczynski and Penning, 2000).

The present study deals with the distribution and risk assessment of PAHs and heavy metals in marine sediments from SantaMarta shoreline, Colombia. Many of these pollutants are knownto be mutagenic and carcinogenic substances that may impactthe ecosystem (Carreira et al., 2013) and human health (Maertens et al., 2008). Data presented on metal and PAH contentin sediments, together with observed cellular effects driven by sedimentextracts exposure, suggest that some gene expressionresponses, in particular those related to oxidative stress andxenobiotic metabolism, could be explained by the presence of bothtypes of pollutans. Such findings have also been published for toxicitymodels using different organisms such as Ruditapesdecussates (Carreira et al., 2013), Senegalese sole (Goncalves et al., 2013), and Sepia officinalis (Rodrigo et al., 2013), as well as HepG2 cells(Pinto et al., 2014a,b; Costa et al., 2014) exposed tosediment-bound toxicants or extracts.

3.5. Conclussions

Finally, considering that the overall demand for coal is drivingstrong growth in Colombia´s mining industry, matched byincreases in port and shipping activities leading to pollution ofcoastal sediments, threatening marine biodiversity, specially inplaces such as Santa Marta Bay (S3) and the area impacted by coalcargo (S9), it is necessary to carefully monitor and reduce/regulatethe anthropogenic sources of pollution that may continue causingecosystem damage.

3.6. Referencias

Achten, C., Hofmann, T., 2009. Native polycyclic aromatic hydrocarbons (PAH) in coals - a hardly recognized source of environmental contamination. Sci. Total Environ. 407, 2461-2473.

Ahmed, M.K., Kundu, G.K., Al-Mamun, M.H., Sarkar, S.K., Akter, M.S., Khan, M.S., 2013. Chromium (VI) induced acute toxicity and genotoxicity in freshwater stinging catfish, Heteropneustes fossilis. Ecotoxicol. Environ. Saf. 92, 64-70.

Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N., Patel, H.R., 2009. Basic principles of real-time quantitative PCR. Exp. Rev. Mol. Diagn. 5, 209-219.

Baird, L., Dinkova-Kostova, A.T., 2011. The cytoprotective role of the Keap1-Nrf2 pathway. Arch. Toxicol. 85, 241-272.

Banjoo, D.R., Nelson, P.K., 2005. Improved ultrasonic extraction procedure for the determination of polycyclic aromatic hydrocarbons in sediments. J. Chromatogr. A 1066, 9-18.

Burczynski, M.E., Penning, T.M., 2000. Genotoxic polycyclic aromatic hydrocarbon ortho-quinones generated by aldo-keto reductases induce CYP1A1 via nuclear translocation of the aryl hydrocarbon receptor. Cancer Res. 60 (4), 908-915.

Carreira, S., Costa, P.M., Martins, M., Lobo, J., Costa, M.H., Caeiro, S., 2013. Ecotoxicological heterogeneity in transitional coastal habitats assessed through the integration of biomarkers and sediment-contamination profiles: a case study using a commercial clam. Arch. Environ. Contam. Toxicol. 64, 97- 109.

Cassee, F.R., van Balen, E.C., Singh, C., Green, D., Muijser, H., Weinstein, J., Dreher, K., 2011. Exposure, health and ecological effects review of engineered nanoscale cerium and cerium oxide associated with its use as a fuel additive. Crit. Rev. Toxicol. 41, 213-229.

Castorena-Torres, F., Bermudez de Leon, M., Cisneros, B., Zapata-Perez, O., Salinas, J.E., Albores, A., 2008. Changes in gene expression induced by polycyclic aromatic hydrocarbons in the human cell lines HepG2 and A549. Toxicol. In Vitro. 22, 411-421.

Colombo, J.C.1., Cappelletti, N., Lasci, J., Migoya, M.C., Speranza, E., Skorupka, C.N., 2006. Sources, vertical fluxes, and equivalent toxicity of aromatic hydrocarbons in coastal sediments of the Rio de la Plata Estuary, Argentina. Environ. Sci. Technol. 40, 734-740.

Costa, P.M., Pinto, M., Vicente, A.M., Goncalves, C., Rodrigo, A.P., Louro, H., Costa, M.H., Caeiro, S., Silva, M.J., 2014. An integrative assessment to determine the genotoxic hazard of estuarine sediments: combining cell and whole-organism responses. Front. Genet. 5, 1-12.

Costa, P.M., Miguel, C., Caeiro, S., Lobo, J., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., DelValls, T.A., Costa, M.H., 2011. Transcriptomic analyses in a benthic fish exposed to contaminated estuarine sediments through laboratory and in situ bioassays. Ecotoxicology 20, 1749-1764.

Dai, S., Ren, D., Tang, Y., Yue, M., Hao, L., 2005. Concentration and distribution of elements in Late Permian coals from western Guizhou Province, China. Int. J. Coal Geol. 61, 119-137.

Dazy, M., Beraud, E., Cotelkle, S., Meux, E., Masfaraud, J.F., Ferard, J.F., 2008. Antioxidant enzyme activities as affected by trivalent and hexavalent chromium species in Fontinalis antipyretica Hedw. Chemosphere 73, 281-290.

Departamento Administrativo Nacional de Estadística (DANE). (2016). Demografía y población - proyecciones de población. Estimación y proyección de población nacional, departamental y municipal total por área 1985-2020. Disponible en: http://www.dane.gov.co/index.php/poblacion-y-demografia/proyecciones-de-poblacion. Último acceso Mayo del 2016.

Du, Y., Wang, J., Jia, J., Song, N., Xiang, C., Xu, J., Hou, Z., Su, X., Liu, B., Jiang, T., Zhao, D., Sun, Y., Shu, J., Guo, Q., Yin, M., Sun, D., Lu, S., Shi, Y., Deng, H., 2014. Human hepatocytes with drug metabolic function induced from fibroblasts by lineage reprogramming. Cell Stem. Cell 14, 394-403.

Finkelman, R.B., 1995. Modes of occurrence of environmentally-sensitive trace elements of coal. In: Swaine, D.J., Goodarzi, F. (Eds.), Environmental Aspacts of Trace Elements of Coal. Kluwer Academic Publishers, Netherlands, pp. 24-50.

Fujii-Kuriyama, Y., Kawajiri, K., 2010. Molecular mechanisms of the physiological functions of the aryl hydrocarbon (dioxin) receptor, a multifunctional regulator that senses and responds to environmental stimuli. Proc. Jpn. Acad. Ser. B 86, 40-53.

Gabelova, A., Polakova, V., Prochazka, G., Kretova, M., Poloncova, K., Regendova, E., Luciakova, K., Segerback, D., 2013. Sustained induction of cytochrome P4501A1 in human hepatoma cells by co-exposure to benzo[a]pyrene and 7H-dibenzo[c, g]carbazole underlies the synergistic effects on DNA adduct formation. Toxicol. Appl. Pharmacol. 271, 1-12.

Gao, X., Zhou, F., Chen, C.T.A., 2014. Pollution status of the Bohai Sea: an overview of the environmental quality assessment related trace metals. Environ. Int. 62, 12- 30.

Goncalves, C., Martins, M., Costa, M.H., Caeiro, S., Costa, P.M., 2013. Ecological risk assessment of impacted estuarine areas: integrating histological and biochemical endpoints in wild Senegalese sole. Ecotoxicol. Environ. Safety. 95, 202-211.

Gschwend, P.M., Hites, R.A., 1981. Fluxes of polycyclic aromatic hydrocarbons to marine and lacustrine sediments in the northeastern United States. Geochim. Cosmochim. Ac. 45, 2359-2367.

Gu, Y.G., Lin, Q., Lu, T.T., Ke, Ch.L., Sun, R.X., Du, F.Y., 2013. Levels, composition profiles and sources of polycyclic aromatic hydrocarbons in surface sediments from Nan’ao Island, a representative mariculture base in South China. Mar. Pollut. Bull. 75, 310-316.

Guerrero-Castilla, A., Olivero-Verbel, J., 2014. Altered gene expression in HepG2 cells exposed to a methanolic coal dust extract. Environ. Toxicol. Pharmacol. 38, 742-750.

Jaward, F.M., Alegria, H.A., Galindo Reyes, J.G., Hoare, A., 2012. Levels of PAHs in the waters, sediments, and shrimps of Estero de Urias, an estuary in Mexico, and their toxicological effects. ScientificWorldJournal 2012, 687034.

Johnson-Restrepo, B., Olivero-Verbel, J., Lu, S., Guette-Fernandez, J., Baldiris-Avila, R., O’Byrne-Hoyos, I., Aldous, K.M., Addink, R., Kannan, K., 2008. Polycyclic aromatic hydrocarbons and their hydroxylated metabolites in fish bile and sediments from coastal waters of Colombia. Environ. Pollut. 151, 452-459.

Kammann, U., 2007. PAH metabolites in bile fluids of dab (Limanda limanda) and flounder (Platichthys flesus): spatial distribution and seasonal changes. ESPR 14, 102-108.

Kang, Y., Liu, G., Chou, C.L., Wong, M., Zheng, L., Ding, R., 2011. Arsenic in Chinese coals: distribution, modes of occurrence, and environmental effects. Sci. Total Environ. 412-413, 1-13.

Kannan, K., Johnson-Restrepo, B., Yohn, S., Giesy, J., Long, D., 2005. Spatial and temporal distribution of polycyclic aromatic hydrocarbons in sediments from Michigan inland lakes. Environ. Sci. Technol. 39, 4700-4706.

Leon, V.M., Garcia, I., Martinez-Gomez, C., Campillo, J.A., Benedicto, J., 2014. Heterogeneous distribution of polycyclic aromatic hydrocarbons in surface sediments and red mullet along the Spanish Mediterranean coast. Mar. Pollut. Bull. 87 (1-2), 352-363.

Liang, Y., Tse, M.F., Young, L., Wong, M.H., 2007. Distribution patterns of polycyclic aromatic hydrocarbons (PAHs) in the sediments and fish at Mai Po Marshes Nature Reserve, Hong Kong. Water Res. 41, 1303-1311.

Ma, J.Y., Mercer, R.R., Barger, M., Schwegler-Berry, D., Scabilloni, J., Ma, J.K., Castranova, V., 2012. Induction of pulmonary fibrosis by cerium oxide nanoparticles. Toxicol. Appl. Pharmacol. 262 (3), 255-264.

Maertens, R.M., Yang, X., Zhu, J., Gagne, R.W., Douglas, G.R., White, P.A., 2008. Mutagenic and carcinogenic hazards of settled house dust I: polycyclic aromatic hydrocarbon content and excess lifetime cancer risk from preschool exposure. Environ. Sci. Technol. 42, 1747-1753.

Moriarty, M.M., Lai, V.W., Koch, I., Cui, L., Combs, C., Krupp, E.M., Feldmann, J., Cullen, W.R., Reimer, K.J., 2014. Speciation and toxicity of arsenic in mining affected lake sediments in the Quinsamwatershed, British Columbia. Sci. Total Environ. 466-467, 90-99.

National Research Council (NRC), 2003. Bioavailability of Contaminants in Soils and Sediments: Processes, Tools, and Applications. National Academies Press. Nouira, T., Tagorti, M.A., Budzinski, H., Etchebert, H., Boussetta, H., 2013. Polycyclic aromatic hydrocarbons (PAHs) in surface sediments of Monastir Bay (Tunisia, Central Mediterranean): distribution, origin and seasonal variations. Int. J. Environ. Anal. Chem. 93, 1470-1483.

Nriagu, J.O., Bhattacharya, P., Mukherjee, A.B., Bundschuh, J., Zevenhoven, R., Loeppert, R.H., 2007. Arsenic in soil and groundwater: an introduction. In: Bhattacharya, P.,

Mukherjee, A.B., Bundschuh, J., Zevenhoven, R., Loeppert, R.H. (Eds.), Arsenic in soil and groundwater environment: biogeochemical. Interactions, health effects and remediation. Trace metals and other contaminants in the environment, vol. 9. Elsevier, Amsterdam, pp. 1-58 (Series Editor Nriagu, JO).

Palmer, M.A., Bernhardt, E.S., Schlesinger, W.H., Eshleman, K.N., Foufoula-Georgiou, E., Hendryx, M.S., Lemly, A.D., Likens, G.E., Loucks, O.L., Power, M.E., White, P.S., Wilcock, P.R., 2010. Science and regulation, Mountaintop mining consequences. Science 327, 148-149.

Pan, K., Wang, W.X., 2012. Trace metal contamination in estuarine and coastal environments in China. Sci. Total Environ. 421, 3-16.

Park, B., Donaldson, K., Duffin, R., Tran, L., Kelly, F., Mudway, I., Morin, J.P., Guest, R., Jenkinson, P., Samaras, Z., Giannouli, M., Kouridis, H., Martin, P., 2008. Hazard and risk assessment of a nanoparticulate cerium oxide-based diesel fuel additive - a case study. Inhal. Toxicol. 20 (6), 547-566.

Patrolecco, L., Ademollo, N., Capri, S., Pagnotta, R., Polesello, S., 2010. Occurrence of priority hazardous PAHs in water, suspended particulate matter, sediment and common eels (Anguilla anguilla) in the urban stretch of the River Tiber (Italy). Chemosphere 81 (11), 1386-1392.

Pinto, M., Costa, P.M., Louro, H., Costa, M.H., Lavinha, J., Caeiro, S., Silva, M.J., 2014a. Determining oxidative and nonoxidative genotoxic effects driven by estuarine sediment contaminants on a human hepatoma cell line. Sci. Total Environ. 478, 25-35.

Pinto, M., Costa, P.M., Louro, H., Costa, M.H., Lavinha, J., Caeiro, S., Silva, M.J., 2014b. Human hepatoma cells exposed to estuarine sediment contaminant extracts permitted the differentiation between cytotoxic and pro-mutagenic fractions.Environ. Pollut. 185, 141-148.

Rai, V., Vajpayee, P., Singh, S.N., Mehrotra, S., 2004. Effect of chromium accumulation of photosynthetic pigments, oxidative stress defense system, nitrate reduction, proline level and eugenol content of Ocimum tenuiflorum L. Plant Sci. 167, 1159-1169.

Ribeiro, J., Silva, T., Mendonca, Filho, J.G., Flores, D., 2012. Polycyclic aromatic hydrocarbons (PAHs) in burning and non-burning coal waste piles. J. Hazard. Mater, 105-110. 199-200.

Rodrigo, A.P., Costa, P.M., Costa, M.H., Caeiro, S., 2013. Integration of sediment contamination with multi-biomarker responses in a novel potential bioindicator (Sepia officinalis) for risk assessment in impacted estuaries. Ecotoxicology 22, 1538-1554.

SEPA (State Environmental Protection Administration of China), 2002. Marine Sediment Quality (GB 18668-2002). Standards Press of China, Beijing. Sette, C.B., Pedrete, Tde A., Felizzola, J., Nudi, A.H., Scofield, Ade L., Wagener, Ade L., 2013. Formation and identification of PAHs metabolites in marine organisms. Mar. Environ. Res. 91, 2-13.

Shimada, T., 2006. Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons. Drug Metabol. Pharmacokinet. 21, 257-276.

Shin, P.K.S., Lam, W.K.C., 2001. Development of a marine sediment pollution index. Environ. Pollut. 113, 281-291.

Simmons, S.O., Fan, C.Y., Ramabhadran, R., 2009. Cellular stress response pathway system as a sentinel ensemble in toxicological screening. Toxicol. Sci. 111, 202-225.

Singla, V., Pachauri, T., Satsangi, A., Kumari, K.M., Lakhani, A., 2012. Characterization and mutagenicity assessment of PM2.5 and PM10 PAH at Agra, India. Polycycl. Aromat. Comp. 32, 199-220.

Soclo, H.H., Garrigues, P.H., Ewald, M., 2000. Origin of polycyclic aromatic hydrocarbons (PAHs) in coastal marine sediments: case studies in Cotonou (Benin) and Aquitaine (France) areas. Mar. Pollut. Bull. 40, 387-396.

Swaine, D.J., Goodarzi, F. (Eds.), 1995. Environmental Aspects of Trace Elements in Coal. Kluwer Academic Publishers, Netherlands, pp. 1-4.

Swaine, D.J., 2000. Why trace elements are important. Fuel Process. Technol. 65-66, 21-33.

Thompson, K.C., Wadhia, K., Loibner, A.P. (Eds.), 2005. Environmental toxicity testing. Blackwell Publishing Ltd., CRC Press, Oxford, United Kingdom, p. 388.

Torres, D.P., Vieira, M.A., Ribeiro, A.S., Curtius, A.J., 2007. Slurry sampling for arsenic determination in sediments by hydride generation atomic absorption spectrometry. J. Braz. Chem. Soc. 18, 728-732.

Tripp, B.W., Farrington, J.W., Teal, J.M., 1981. Unburned coal as a source of hydrocarbons in surface sediments. Mar. Pollut. Bull. 12, 122-126.

Vakharia, D.D., Liu, N., Pause, R., Fasco, M., Bessette, E., Zhang, Q.Y., Kaminsky, L.S., 2001. Polycyclic aromatic hydrocarbon/metal mixtures: effect on PAH induction of CYP1A1 in human HepG2 cells. Drug Metab. Dispos. 29, 999-1006.

Valasek, M.A., Repa, J.J., 2005. The power of real-time PCR. Adv. Physiol. Educ. 29, 151-159.

Van Kooten, G.K., Short, J.W., Kolak, J.J., 2002. Low-maturity Kulthieth Formation coal: a possible source of polycyclic aromatic hydrocarbons in benthic sediment of the Northern Gulf of Alaska. Environ. Forensics. 3, 227-241.

Vondrac˘ek, J., Machala, M., Minksova, K., Blaha, L., Murk, A.J., Kozubik, A., Hofmanova, J., Hilscherova, K., Ulrich, R., Ciganek, M., Nec˘a, J., Švrc˘kova, D., Holoubek, I., 2001. Monitoring river sediments contaminated predominantly with polyaromatic hydrocarbons by chemical and in vitro bioassay techniques. Environ. Toxicol. Chem. 20, 1499-1506.

Ward, C.R., 2002. Analysis and significance of mineral matter in coal seams. Int. J. Coal Geol. 50, 135-168.

WDOE, 1995. Sediment Management Standards. Olympia, WA: Washington State Department of Ecology (Chapter 173-204-320) WAC.

Zhang, L., Ye, X., Feng, H., Jing, Y., Ouyang, T., Yu, X., Liang, R., Gao, C., Chen, W., 2007. Heavy metal contamination in western Xiamen Bay sediments and its vicinity, China. Mar. Pollut. Bull. 54, 974-982.

CHAPTER 4

4. CHAPTER 4. EFFECTS OF AQUEOUS COAL DUST EXTRACT ON DEVELOPMENTAL TOXICITY AND GENE EXPRESSION PROFILING OF ZEBRAFISH (Danio rerio) (PAPER 2)

4.1. Introduction

Coal and its by-products (e.g. fly ash) and coal mining waste (e.g. acid mine drainage) are complex mixtures, which contain a variety of chemical elements that have an impact on the ecosystem, especially hydrocarbons and metals (Caballero-Gallardo et al., 2015). The different contaminants found in coal as well as by-products and waste are considered serious environmental contaminants due to their mobilization and bioaccumulation in the food chain (Arnold et al., 2014), contamination of the water (Tiwary, 2001), apart from their toxicity to biota (Zocche et al., 2014).

It has been documented that coal dust inhaled particles induce acute and chronic response involving a variety of cell types, including epithelial alveolar, interstitial fibroblasts, resident and recruited macrophages and other cells of the immune system (Kania et al., 2012). A reason for this, several studies have been conducted with the purpose of establishing the genotoxic effect of this mineral and pollutants associated with the development of chronic diseases in coal mining. The experiments carried out have included comet assay and micronucleus determination (León-Mejía et al., 2011; León, Pérez, Linares, Hartmann, & Quintana, 2007). However, details of the intracellular mechanisms are poorly understood, and there are only a few references about this topic.

Globally it has been reported in connection with the study of coal dust, particularly with exposure in mines (Liu et al., 2013; Mittal, 2013), genotoxicity (Rohr et al., 2013), metals and PAHs (Caballero-Gallardo et al., 2015), diseases caused by inhalation of coal dust in mines (Petsonk et al., 2013), among others.

In Colombia, several studies have shown the potential genotoxic effects (León-Mejía et al., 2011; León et al., 2007).

In this context, the elucidation of the mechanisms that might be happening in humans are still unknown. Recently zebrafish (*Danio rerio*) has become a preferred toxicity model due to its rapid life cycle, high fecundity, transparent development, and because the embryos are amenable to genetic manipulation using transgenic approaches and morpholino gene knockdowns (Sipes, Padilla, & Knudsen, 2011). This fish in comparison to the human reference genome shows that approximately 70% of human genes have at least one obvious zebrafish orthologue (Howe et al., 2013). The goal of Paper 2 was evaluate the effects caused by exposure to aqueous leachate generated from coal dust with a particle size of 38 µm, trying to reproduce what may be happening directly in the mines, especially during the rainy season, using zebrafish as a model.

4.2. Materials and methods

4.2.1. Coal dust preparation

Bituminous gross coal obtained from a mine, located in La Loma, Department of Cesar (Colombia), was used to prepare the coal dustsample. The coal was ground and passed through an Rx-812 sieveshaker to obtain particle sizes of less than 38 µm in diameter (Mesh No. 400, Serial No. 15917). This particle size was employed in theexperiments since it roughly corresponds to the fraction of totalsuspended particles (TSP) (Cyrys et al., 2005; EPA, 2010) that could be formed during opencast mining (Chaulya, 2004), a practice that allowscoal particles to reach neighboring mining areas. The sample was splitand stored in sterile glass vials under laboratory conditions (26±2 °C and relative humidity 70–85%).

4.2.2. Preparation of coal dust aqueous extract

50 g of coal dust (<38 µm) was extracted for 36 h with Milli-Q water in a Soxhlet apparatus (Maharaj et al., 2014). The samples were filtrated with a syringe filter (0.22 µm) and stored at −80 °C until, freeze-dried in a lab-scale freeze dryer (Labconco Equipment). Coal dust aqueous extract was characterized by ICP/MS at the Center for Research in Sustainable Chemistry (CIQSO), University of Huelva, University Campus El Carmen, E21071 Huelva, Spain (36°16´14.14´´ N and 6°55´26.69´´ W).

4.2.3. Zebrafish maintenance

Adult zebrafish (*D. rerio*) of the wild-type (AB strain) were housed in a Z-Mod System (Aquatic Habitats, Apopka, FL), under standard laboratory conditions of 28 ^{o}C, a 14:10 h light:dark photoperiod, pH of 7.2 and conductivity range of 470–520 μS. The zebrafish were fed twice daily a mixture of live brine shrimp, Golden Pearls, and Zeigler adult zebrafish food powder following established laboratory protocols and guidance of the Zebrafish Book (Peterson, Zhang, Weber, & Freeman, 2011; Westerfield, 2007). Embryos were obtained from spawning adults bred in cages, and rinsed with system water. Fish care and experiments were conducted according the Purdue University's Institutional Animal Care and Use Committee Guidelines.

Zebrafish (*Danio rerio*) are a small tropical freshwater fish native to the rivers of India and South Asia. Females are highly prolific egg layers with one individual capable of laying 100-200 eggs in a day, on a weekly basis. The females spawn in the dawn hours and only in the presence of the males, laying unfertilized eggs into the water, which are externally fertilized by the males. In the laboratory, the timing of egg-laying can be controlled using a regulated light-dark cycle. Its trophic level is 3.1 (Fishbase, 2016). The zebrafish male is more yellowish in color, with slimmer, longer body contour. The zebrafish female has a more rounded body (because her belly is full of eggs) and small genital papilla in front of the anal fin.

For optimum breeding and collection of eggs, males and females are maintained in a tank with shallow water overnight, and the newly laid eggs are separated from the adults by a mesh to prevent them from eating the eggs.

Over 800 labs worldwide now routinely use the zebrafish in fundamental and applied research (www.zfin.org) and there is an increasing interest in its use as a model for understanding the genetic basis of behavior.

The development of the zebrafish is very similar to embryogenesis in higher vertebrates, including humans (Raterink et al., 2013). Detailed descriptions of developmental stages of zebrafish embryos are described in Kimmel et al. (1995). These descriptions are summarized below with the life cycle illustrated in **Figure 4.1.**

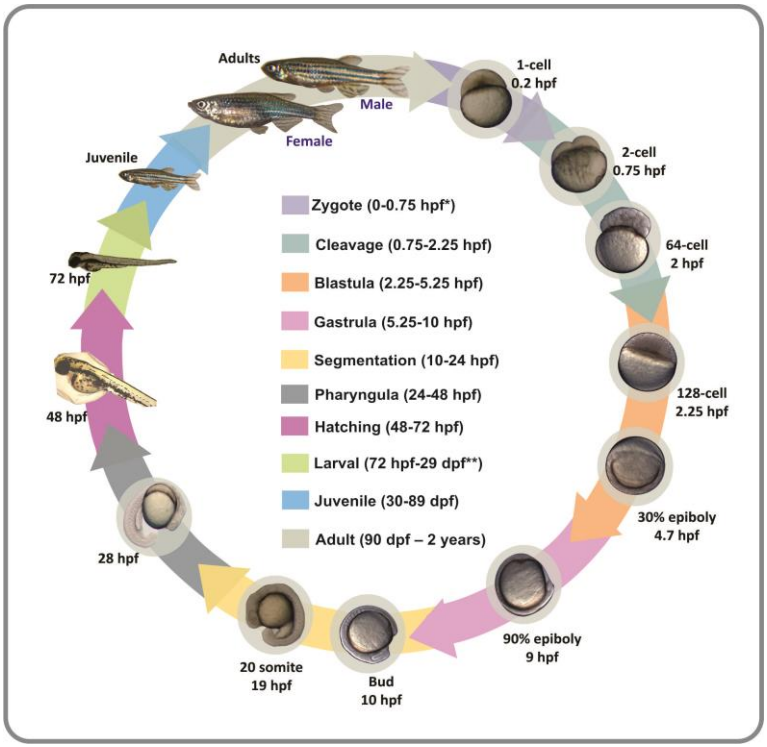

Figure 4.1. Developmental stages of zebrafish (Adapted from Kimmel et al., 2013). * hpf: hours post fertilization. ** dpf: days post fertilization.

Zebrafish are genetically similar to humans with approximately 70 % of human protein-coding genes having orthologs in zebrafish and with over 80 % of human disease-related genes having a zebrafish counterpart (Howe et al., 2013). The zebrafish shares high similarity to human developmental processes, physiology, and behavior (Yu et al., 2008).

The zebrafish has become a preferred biological model for experimentation over the past three decades (Giacomotto and Ségalat, 2010).

4.2.4. Acute toxicity test

For each solution to be tested, serial 1000, 100, 10, 1, and 0.1 ppm dilutions with fish water were prepared. Fish water was used as the negative control. Zebrafish embryos at 1hour post fertilization (hpf) were exposed to coal dust

aqueous extracts and a negative control. The exposures were carried out in glass Petri dishes with 50 embryos (considered as subsamples) cultured in 20 mL solution in each Petri dish. Three replicates were performed for each coal dust extract concentration and negative control. The surviving embryos/larvae were observed and recorded throughout the exposure period (through 72 hpf), as well as the hatching success examined and recorded at 48 and 72 hpf. Dead embryos/larvae were removed and discarded according to animal care protocols. All exposures were performed in a temperature-controlled room with light-dark cycle controlled as previously described for rearing of adults.

4.2.5. Morphological assessment

Zebrafish embryos were exposure to coal dust aqueous extracts (0.1, 1, 10 and 100 ppm) and a negative control from 1 to 72 hpf in groups of 50 in a Petri dish. The concentrations were selected based on the results of the acute toxicity test. Twenty larvae from each Petri dish (considered subsamples) were analyzed with light microscopy using a Nikon SMZ1500 dissecting microscope with NIS Elements imaging software (Melville, NY) to measure endpoints such as head length, eye diameter, and total larvae length (measured snout to tail)as previously described (Weber, Sepúlveda, Peterson, Lewis, & Freeman, 2013; Wirbisky, Weber, Lee, Cannon, & Freeman, 2014). Three replicates were performed for each coal dust extract concentration and negative control.

4.2.6. RNA extraction and cDNA synthesis

Zebrafish embryos were exposed to a 0, 1, 10, and 100 ppm coal dust extract treatment beginning at 1 hpf with 50 embryos per Petri dish (considered as subsamples) and collected at 72 hpf. Total RNA was isolated from frozen, pooled embryos by homogenization in Trizol (Life Technologies, Carlsbad, CA) followed by extraction with chloroform and ethanol and final purification using RNeasy Mini Spin Columns (Qiagen Sciences, Maryland, USA) following established protocols (Peterson and Freeman (2009). RNA concentrations were measured by using a NanoDrop 1000 spectrophotometer and the quality was verified by absorbance ratio 260/280 nm. RNA was then stored at -80 °C until use. cDNA was obtained using the Invitrogen Super Script First-Strand Systemas described in Peterson and Freeman (2009).Three replicates were completed (n = 3).

4.2.7. Microarray

For sample preparation and array processing, the Agilent protocol "One-Color Microarray-Based Gene Expression Analysis"was used. Briefly, the recommended volume of control RNAs (Agilent One-Color RNA Spike-In Kit) was added to 100 ng of total RNA from the each simple (negative control, 1, 10 and 100 ppm). Thereafter, Cy3-labeled cRNA was produced using the Agilent Low Input Quick Amp Labeling (one-color), purified with the RNeasy Mini Kit, fragmented using the Hybridization Kit (Agilent Technologies Inc.), A custom zebrafish 4x180K expression platform (Agilent Technologies, Inc.) was used for the microarray analysis. This microarray is a multiplex format of 4 arrays each consisting of 180,000probesinterrogating 36,000 targets with approximately 3-5 probes per target and is based on the Ensemble and UCSC Genome Databases. Hybridized slides were scanned at 5 microns (G4900DA scanner, Agilent Technologies Inc.), and data were obtained using Agilent Feature Extraction software (version 10.7.1) with defaults for all parameters.

Microarray data analyses were performed using GeneSpring GX (version 12.5) software (Agilent Technologies Inc.). Expression values of less than 1 were substituted by 1, and 75th percentile normalization was performed using GeneSpring normalization algorithms. Reliability of each expression value was represented by a flag based on the default setting of GeneSpring (Detected, Marginal and Not Detected). Genes referred to in the results and discussion sections are reported as the human homologs of the genes identified to be altered by microarrays.

4.2.8. Quantitative polymerase chain reaction (qPCR) validation of microarray data

Zebrafish microarray data were validated by qPCR with the control and 100 ppm samples (n=3). qPCR was performed on 8 target genes: *ACD2, CDHR, CHSY1, HPX, MAPK3*, *TRIM59, TRPM3* and *UPF3A*. Gene expression was normalized to *ELF1α*. Similar to as conducted in previous studies e.g., Wirbisky et al. (2014) and Weber et al. (2013), several housekeeping genes were fist assessed for lack of change following coal dust aqueous extract exposure. *ELF1α* was found to have the most consistent expression (data not shown). Probes specific to gene targets were designed using the Primer3 Website (**Table 4.1**). qPCR was performed following similar methods as described previously (Peterson et al., 2011) following the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009). The BioRad CFX Connect TM Real Time PCR Detection System was used with the SSOAdvance SYBR Green Supermix according to

manufacturer recommendations (Bio-Rad, Hercules, CA) (Wirbisky et al., 2014). All samples were run in triplicate technical replicates and efficiency and specificity were checked with melting and dilution curve analysis and no-template controls.

Table 4.1 qPCR primer sequences.

Gene name	Gene symbol	Entrez Gene ID	Forward	Amplicon Size (pb)
Angiotensin I converting enzyme 2	*ACD2*	XM_005169360.2	F:TCCCAGAGATGAGACGTACTGT R:CTGAGTTTGTCACCAGCTTTGG	192
cadherin-related family member 2	*CDHR2*	XM_009291246.1	F:CTACTGTGGGCTTCGTACTGTT R:AGTGGCCGCAACTATTGAAAAC	131
Chondroitin sulfate synthase 1	*CHSY1*	NM_212678.1	F:GGAGATGATTAACGCCAATGCC R:TGTCATAGTCTTCCCCTTGTGC	154
Hemopexin	*HPX*	NM_001111147.1	F:TCTGGTGCTCTGCTCAAATCAT R:TGTACATTAGTCTGCACGCCAT	100
Mitogen-activated protein kinase 3	*MAPK3*	NM_201507.1	F:GCAACTCAGCAACGACCATATC R:TATCCTCGCCAACCCAAAATCA	163
Tripartite motif containing 59	*TRIM59*	XM_009291407.1	F:ACGTACATGCACTTGAAACTGC R:GTGTGTCTTGTTTGTGCCCTAC	152
Transient receptor potential cation channel, subfamily M, member 3	*TRPM3*	XM_009301562.1	F:CAAGCCTGACCTTTCCAACATG R:TCTACTGCCATGTCAGTGCATT	166
UPF3 regulator of nonsense transcripts homolog A (yeast)	*UPF3A*	XM_005167851.2	F:AGAAGAGGAGAGACAAAAGCGG R:ATCGTCTCGGTCACACTTCTTC	100
Housekeeping				
Eukaryotic translation elongation factor 1 alpha 1, like 1	*ELF1α*	NM_131263.1	F:GCCAAGACCAAGTGAATTTCCC R:GTCCTTAAGTAGAGTGCCCAGG	105

5. *. 5’ → 3’.

A scheme of the methodology is shown in **Figure 4.2**.

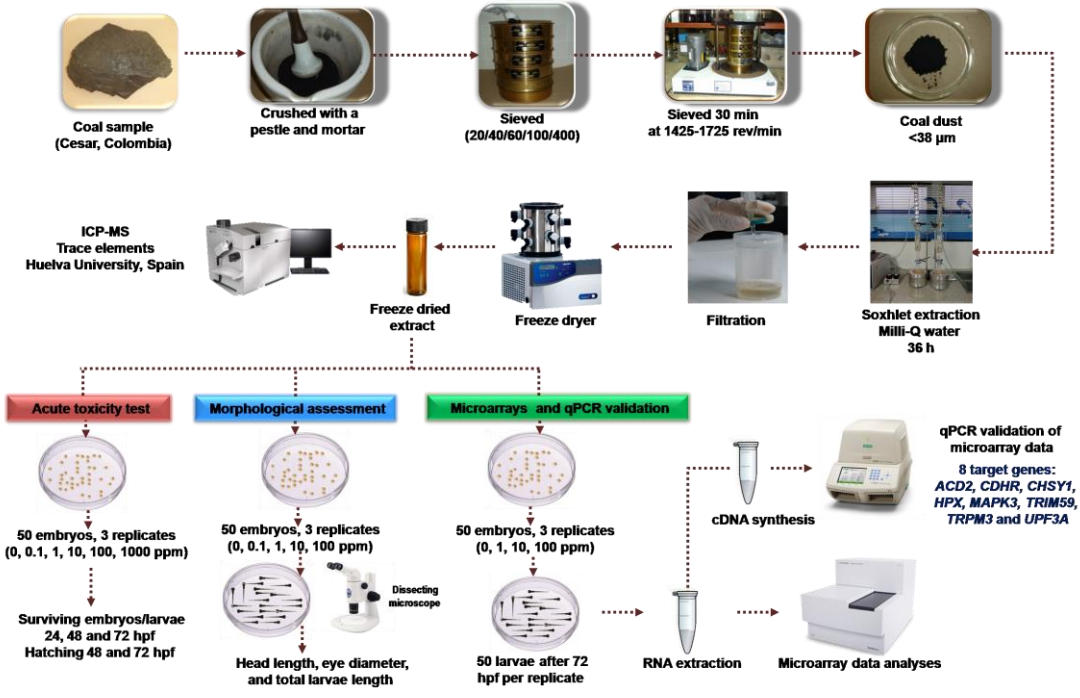

Figure 4.2. Diffrents assays with zebrafish.

4.2.8.Statistical analysis

The data presented in this study were checked for normality and homogeneity of variances with Kolmogorov-Smirnov and Bartlett tests, respectively. Survival, hatching rate, and morphological measurements were analyzed with an analysis of variance(ANOVA) and a post-hoc least significant difference (LSD)test($p < 0.05$) with SAS software (SAS Institute Inc., Cary, NC) when a significant ANOVA was observed. Expression microarray analysis was completed in GeneSpring GX (version 12.5) software (Agilent Technologies Inc.). with an ANOVA and a Tukey's post-hoc test when a significant ANOVA was observed (α=0.05). In addition, a mean absolute $\log_2$ expression ratio of at least 0.585 (50% increase or decrease in expression) was used as the cut off for inclusion in the microarray analysis. A Student's t-test was used to calculate differences between the control and 100 ppm samples for the qPCR analysis.

4.3. Results

Element content in coal dust aqueous extract

The concentrations of trace elements in coal dust aqueous extract are presented in Table 2. The highest concentrations were found for Sr (600,25 ± 3,13 ppb), Zn (87,67 ± 0,30 ppb), Ba (66,94 ± 0,34 ppb), As (19.74±1.20 ppb), Cu (17,65 ± 2,49 ppb), Se (11.56±0.05 ppb), Li (9.39±0.00 ppb), Ni (4.44±0.06 ppb), Sb (2.45 ± 1.58 ppb), Rb (2.40±0.03ppb), Co (2.24± 0.01ppb) and Cr (1.29±0.02 ppb). In order to assess the level of concern for analyzed elements, their average concentrations in the coal dust aqueous extract sample were compared to the classification of the US National Research Council (NRC) (**Table 4.2**), which are based on known adverse health effects or because of their abundances in coal. In this case, trace elements such as As, Se, Mo, Pb, and Cd were presented in the extract.

Table 4.2. Trace element concentrations (ppb) from coal dust aqueous extract.

Elements	Concentration (ppb)
Sr[c]	600.25 ± 3.13
Zn[b]	87.67 ± 0.30
Ba[c]	66.94 ± 0.34
As[a]	19.74 ± 1.20

Cu[b]	17.65 ± 2.49
Se[a]	11.56 ± 0.05
Li[c]	9.39 ± 0.00
Ni[b]	4.44 ± 0.06
Sb[c]	2.45 ± 1.58
Rb	2.40 ± 0.03
Co[c]	2.24 ± 0.01
Cr[b]	1.29 ± 0.02
Mo[a]	0.86 ± 0.01
Tl[e]	0.71 ± 0.02
V[b]	0.51 ± 0.01
Sc	0.48 ± 0.01
Pb[a]	0.36 ± 0.31
Bi	0.26 ± 0.23
Cs	0.17 ± 0.01
Be	0.15 ± 0.08
Zr	0.14 ± 0.00
Ge[c]	0.12 ± 0.00
U[d]	0.11 ± 0.00
Cd[a]	0.11 ± 0.00
Sn[e]	0.10 ± 0.01
Ga	0.09 ± 0.00

Classification of the trace elements by level of concern based on known adverse health effects (NRC, 1980): a. Elements of greatest concern, b. Elements of moderate concern, c. Elements of minor concern, d. Radioactive elements, e. Elements of concern, but present only in very low concentrations.

Zebrafish mortality following exposure to coal dust aqueous extract

During the 72-h exposure of zebrafish embryos to coal dust aqueous extract,

mortality was monitored and recorded at 24, 48 and 72 hpf. No significant difference was observed in mortality during experiment (**Figure 4.3**) with survival near 90% in all treatments.

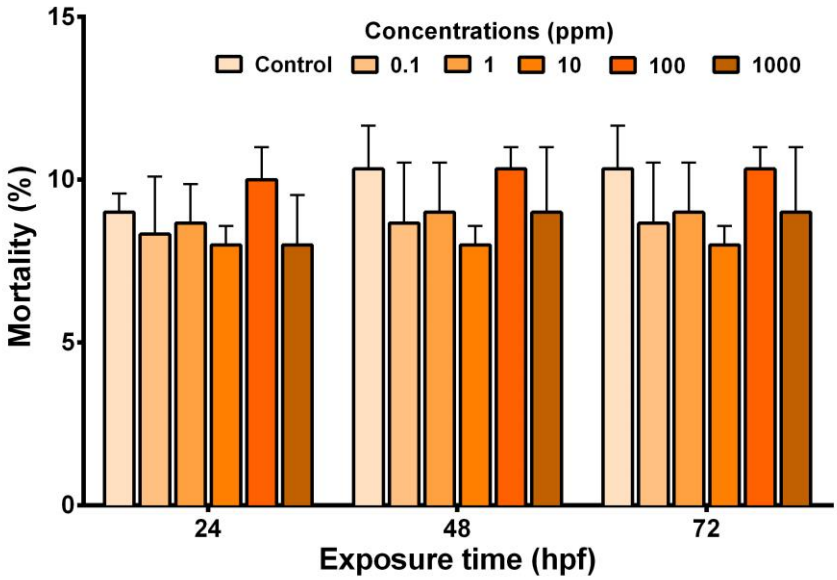

Figure 4.3. Mortality of zebrafish embryos exposed to various concentrations of coal dust aqueous extract for different periods. The error bars represent the standard error (SE) of the mean.

Hatching success following exposure to coal dust aqueous extract

In the present study, the hatching rates at 48 and 72 hpf (defined as the number of hatched embryos at a specific exposure time as divided by the surviving individuals) were recorded to evaluate the influence of coal dust aqueous extract on the hatching process (**Figure 4.4**). Hatching began around 48 hpf in all treatments with a significant increase in the percent hatched in the highest two treatment concentrations (100 and 1000 ppm) at 48 hpf. Importantly, the highest concentration of the aqueous extract, the hatching was inhibited. Alternatively, a significant decrease in the percent of embryos hatched was observed in the 0.1 and 1000 ppm treatment groups at 72 hpf.

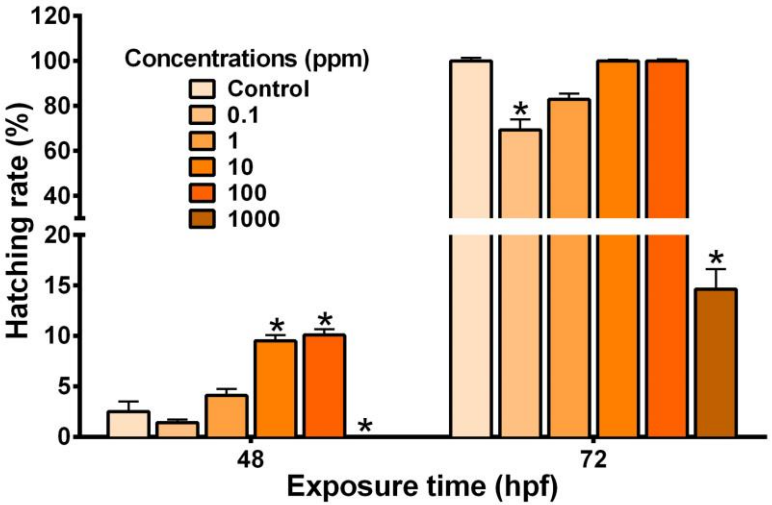

Figure 4.4. Hatching rates of zebrafish embryos exposed to various concentrations of coal dust aqueous extract for different periods in relation to the surviving individuals. The combined data of three replicates is shown. The error bars represent the standard error (SE) of the mean. The asterisks indicate significant differences from the negative control group at **P*<0.05.

Morphological assessments

The total body length, head length and eye diameter were measured in the larvae at 72 hpf. No significant differences were observed in any of the developmental measurements in any of the treatments (**Table 4.3**).

Table 4.3. Morphological analysis at 72 hpf.

Concentrations (ppm)	Body length (μm)*	Head length (μm)	Eye diameter (μm)
0	3.265,64 ± 51,79	593,24 ± 3,54	545,55 ± 2,93
0.1	3.290,12 ± 13,03	591,06 ± 2,70	548,84 ± 3,51

1	3.275,13 ± 16,67	587,39 ± 3,84	552,75 ± 3,52
10	3.324,38 ± 17,80	593,05 ± 3,31	547,65 ± 3,43
100	3.302,69 ± 13,68	591,52 ± 2,91	546,77 ± 2,96

*. Data are representative of three independent replicates of 20 subsamples each ± standard error.

Transcriptome profiles of larvae zebrafish exposed to coal dust aqueous extract

In order to gain further understanding of possible mechanisms involved in the developmental exposure to coal dust aqueous, we investigated the transcriptomic response of the zebrafish lavae to each treatment after an exposure of 72 hpf using microarrays. Results from the microarrays revealed probes differentially expressed in all exposures consisting of 573, 567, and 4933 unique probes representing 77, 61, and 1376 genes in the 1, 10, and 100 ppm treatments, respectively (**Figure 4.5**).

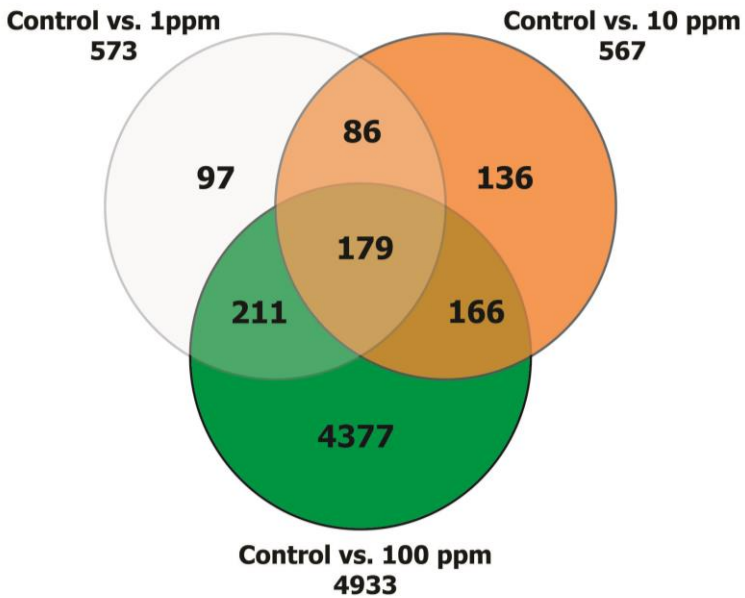

Figure 4.5. Venn diagram representing the probes that were differentially expressed by coal dust aqueous extract on larvae zebrafish for three different exposure concentrations.

The exposure of zebrafish to coal dust aqueous extract has distinct roles in regulating gene expression during development. A majority of the probes significantly altered were found in the 100 ppm treatment (4933) compared to treatments 1 ppm (573) and 10 ppm (567). In the case of 1 ppm treatment was observed a mixture of up (54%) and downregulated (46%) probes representing 37 and 40 genes, respectively, as well as 10 ppm treatment showed up (66%) and downregulated (34%) probes representing 37 and 24 genes, respectively. However, a majority of the gene up (520) and downregulated (856) significantly altered in the 100 ppm exposures showed an increase in transcript expression. Of these differentially expressed genes, 19 were common among all three concentrations are shown in **Table 4.4**. The results showed that the most notable biological functions were associated with cellular processes (**Table 4.4**).

Table 4.4. Genes differentially expressed by coal dust aqueous extract on larvae zebrafish in all three exposure concentrations.

Gene symbol	Biological process	Direction of change in expression
AIFM3	Apoptosis	Down
ARHGEF37	GTPase Activity	Up
BC051665	Proteolysis	Up
BTG4	Cell Cycle	Down
DZIP1L	Cilium Assembly	Down
FAM19A4	Membrane Potential	Up
FOS	Cellular Processes	Up
FOSB	Cellular Processes	Up
HPX	Iron Ion Homeostasis	Down
JDP2	Transcription	Down
MAPK3	MAPK Signaling	Down
MEF2D	Cell Signaling/Transcription	Down
NPAS4	Cellular Response to Stimuli/Transcription	Up
PTEN	Cellular Function	Down
PTPRC	Cellular Processes	Up

SOCS3	Cytokine Signaling	Up
SPRY1	Neurotrophin/TRK Signaling	Down
TGM1	Cellular Processes	Down
TMEM216	Cellular Organization	Up

Gene ontology analysis was performed to physiological system development and function (1, 10 and 100 ppm, also for common genes in the three treatments). Additionally, gene enrichment of diseases and disorders, as well as molecular and cellular function in all three coal dust aqueous extract treatments was conducted. In the case of physiological system development and function, 1 ppm showed enrichment for genes corresponding to embryonic development, connective tissue development and function, organismal development, skeletal and muscular system development and function. In addition, altered genes were also associated with behavior to 10 and 100 ppm coal dust aqueous extract treatments. Of the genes common in all three treatments differentially expressed gene lists, the most notable pathways enriched were associated with hematological system development and function, tissue morphology, embryonic development, connective tissue development and function, and tissue development.

In the others hand, significantly altered gene lists for each of the common genes in the three treatments showed enrichment for genes associated with carcinogenesis and tumorigenesis including prostate, bladder, and renal cancers, as well as hyperplasia. This analysis also showed several genes enriched for inflammatory disease such as chronic inflammatory disorder, airway hyperresponsiveness and inflammation of the liver. Moreover, the data also showed connective tissue and skeletal and molecular disorders. In addition, altered genes were also associated with immunological disease including rheumatoid arthritis, splenomegaly and thymic lymphoma (**Table 4.5**). The differentially expressed gene list from three treatment with enrichment of genes with established roles in molecular and cellular function such as cell cycle, cellular development, cell death and survival, cellular growth and proliferation, and gene expression (**Table 4.6**).

Table 4.5. Gene Enrichment table of diseases and disorders in 72 hpf zebrafish larvae in all three coal dust aqueous extract treatments.

Diseases and Disorders	**p-value[a]**	**Number of Genes[b]**
CONNECTIVE TISSUE DISORDERS	1.34E-03 – 1.00E-	**7**

Arthritis	06	6
Rheumatoid arthritis	5.50E-04	5
Osteoporosis	9.23E-04	2
	1.34E-04	
IMMUNOLOGICAL DISEASE	7.14E-03 – 1.00E-	**7**
Rheumatoid arthritis	06	5
Splenomegaly	9.23E-04	4
Thymic lymphoma	1.15E-06	2
	1.34E-03	
INNFLAMTORY DISEASE	7.14E-03 – 1.00E-	**8**
Chronic inflammatory disorder	06	6
Airway hyperresponsiveness	5.55E-04	3
Inflammation of the liver	1.33E-04	3
	2.93E-03	
SKELTAL AND MUSCULAR DISORDERS	6.25E-03 – 1.00E-06	**8**
Arthritis		6
Rheumatoid arthritis	5.50E-04	5
Arthritis of the ankle joint	9.23E-04	2
	1.59E-05	
CANCER	7.14E-03 – 5.38-	**10**
Hyperplasia	06	5
Prostate cancer and tumors	2.53E-04	5
Bladder cancer	9.23E-04	4
Development of tumor	2.32E-03	4
	3.94E-04	

a. Derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.
b. Classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

Table 4.6. Gene Enrichment table of molecular and cellular function in 72 hpf zebrafish larvae in all three coal dust aqueous extract treatments.

Molecular and Cellular Function	p-value[a]	Number of Genes[b]
CELLULAR DEVELOPMENT	7.14E-03 – 3.09E-	13
Differentiation of cells	06	11
Differentiation of connective tissue	8.15E-06	7
Differentiation of bone cells	8.23E-06	6
Differentiation of blood cells	3.09E-06	5
	1.64E-03	
CELLULAR GROWTH AND	7.14E-03 – 4.21E-	11

PROLIFERATION	06	
Proliferation of cells		11
Formation of cells	1.21E-03	6
Proliferation of fibroblasts	6.95E-04	5
	2.49E-05	
CELL CYCLE	6.25E-03 – 7.57E-	7
Cell cycle progression	06	7
Mitosis	1.07E-04	4
Senescence of cells	1.85E-03	3
	1.55E-03	
CELL DEATH AND SURVIVAL	7.14E-03 – 7.57E-	12
Necrosis	06	10
Apoptosis	2.72E-04	10
Cell Survival	3.51E-04	8
Cell viability	8.91E-05	6
	2.68E-03	
GENE EXPRESSION	5.36E-03 – 1.52E-	9
Transcription of RNA	05	8
Binding of DNA	7.80E-04	6
Transactivation of RNA	1.52E-05	5
	2.85E-04	

a. Derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.
b. Classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

Quantitative polymerase chain reaction (qPCR) confirmation of microarray data.

Eight genes with differential expression in the microarray analysis were confirmed independently by qPCR. The data were statistically significant with genes such as *TRIM59, HPX* and *TRPM3*, while no significant change in the expression of *ACD2*, *CDHR2, CHSY1, MAPK3,* and *UPF3A* was observed.

4.4. Discussion

Coal dust has become one of the major issues that are produced in the course of coal mining activities, transportation and storage of coal, which seriously affecting production safety and causing occupational h azards (Erol, Aydin, Didari, & Ural, 2013; Rout, Masto, Ram, George, & Padhy, 2013), environmental

pollution (Kurth et al., 2014; Pandey, Agrawal, & Singh, 2014) and economic losses (Gowrisankaran, He, Lutz, & Burgess, 2015).

In the current study, zebrafish embryos were exposed to coal dust aqueous extract concentrations ranging from 0 to 1000 ppm and evaluated mortality and hatching rates. Results showed no significant differences in mortality, but hatching rates was observed a decrease at 72 hpf and an inhibition of 100% at 48 both effects were both effects were observed at the highest concentration of coal dust aqueous extract. This behavior is similar to endocrine disruptors, which have an unconventional dose-response relationships called non-monotonic dose- response (Lagarde et al., 2015).

HPX puede estar implicado en la protección de las células del estrés oxidativo. En este estudio, la baja regulación de este gen podría ser una respuesta a la presencia de metales en el carbón. Asi mismo, la alteración en la expression de MAPK3 explica algunos procesos biológicos relacionados con crecimiento y proliferación celular, supervivncia y desarrollo encontrados en los análisis de Enriquecimiento de términos de ontología de genes.

Our study is innovative in that it is the first to examine the effects of coal dust aqueous extract exposure on zebrafish.

4.5. Conclusions

Zebrafish exposed to coal dust aqueous extract experienced molecular and cellular changes. These included inhibition in the hatching, changes in gene expression profiling expression of genes related to oxidative stress, cellular processes, and apoptotic process, among others.

4.6. References

Arnold, M., T. T. Lindberg, Y. Liu, K. Porter, H. Hsu-Kim, D. Hinton, and R. Di Giulio. 2014. Bioaccumulation and speciation of selenium in fish and insects collected from a mountaintop removal coal mining-impacted stream in West Virginia. Ecotoxicology 23:929-938.

Maharaj, S. V. M., Orem, W. H., Tatu, C. A., Lerch III, H. E., & Szilagyi, D. N. (2014). Organic compounds in water extracts of coal: links to Balkan endemic nephropathy. Environmental geochemistry and health, 36(1), 1-17.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, and G. L. Shipley. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry 55:611-622.

Caballero-Gallardo, K., A. Guerrero-Castilla, B. Johnson-Restrepo, J. de la Rosa, and J. Olivero-Verbel. 2015. Chemical and toxicological characterization of sediments along a Colombian shoreline impacted by coal export terminals. Chemosphere 138:837-846.

Cao, J., C. Yang, J. Li, R. Chen, B. Chen, D. Gu, and H. Kan. 2011. Association between long-term exposure to outdoor air pollution and mortality in China: a cohort study. Journal of hazardous materials 186:1594-1600.

Cyrys, J., M. Hochadel, U. Gehring, G. Hoek, V. Diegmann, B. Brunekreef, and J. Heinrich. 2005. GIS-based estimation of exposure to particulate matter and NO2 in an urban area: stochastic versus dispersion modeling. Environ. Health Persp 113:987-992.

Chaulya, S. 2004. Assessment and management of air quality for an opencast coal mining area. J. Environ. Manag 70:1-14.

Chen, B., C. Hong, and H. Kan. 2004. Exposures and health outcomes from outdoor air pollutants in China. Toxicology 198:291-300.

EPA, U. 2010. Basic Concepts in Environmental Sciences, Module 3: Characteristics of Particles. http://www.epa.gov/apti/bces/module3/category/category.htm. EPA.

Erol, I., H. Aydin, V. Didari, and S. Ural. 2013. Pneumoconiosis and quartz content of respirable dusts in the coal mines in Zonguldak, Turkey. International Journal of Coal Geology 116:26-35.

Gowrisankaran, G., C. He, E. A. Lutz, and J. L. Burgess. 2015. Productivity, Safety, and Regulation in Coal Mining: Evidence from Disasters and Fatalities. National Bureau of Economic Research.

Howe, K., M. D. Clark, C. F. Torroja, J. Torrance, C. Berthelot, M. Muffato, J. E. Collins, S. Humphray, K. McLaren, and L. Matthews. 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496:498-503.

Kania, N., B. Setiawan, E. Widjadjanto, N. Nurdiana, M. A. Widodo, and H. C. Kusuma. 2014. Subchronic inhalation of coal dust particulate matter 10 induces bronchoalveolar hyperplasia and decreases MUC5AC expression in male Wistar rats. Experimental and Toxicologic Pathology 66:383-389.

Kania, N., B. Setiawan, E. Widjajanto, N. Nurdiana, M. A. Widodo, and H. C. Kusuma. 2012. Peroxidative index as novel marker of hydrogen peroxide involvement in lipid peroxidation from coal dust exposure. Oxidants and Antioxidants in Medical Science 1:209-215.

Kurth, L., A. Kolker, M. Engle, N. Geboy, M. Hendryx, W. Orem, M. McCawley, L. Crosby, C. Tatu, and M. Varonka. 2014. Atmospheric particulate matter in proximity to mountaintop coal mines: sources and potential environmental and human health impacts. Environmental geochemistry and health 37:529-544.

Lagarde, F., C. Beausoleil, S. M. Belcher, L. P. Belzunces, C. Emond, M. Guerbet, and C. Rousselle. 2015. Non-monotonic dose-response relationships and

endocrine disruptors: a qualitative method of assessment. Environ Health.
León-Mejía, G., L. Espitia-Pérez, L. S. Hoyos-Giraldo, J. Da Silva, A. Hartmann, J. A. P. Henriques, and M. Quintana. 2011. Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay. Sci. Total Environ 409:686-691.
León, G., L. E. Pérez, J. C. Linares, A. Hartmann, and M. Quintana. 2007. Genotoxic effects in wild rodents (Rattus rattus and Mus musculus) in an open coal mining area. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 630:42-49.
Liu, Q., Y. Hu, C. Bai, and M. Chen. 2013. Methane/coal dust/air explosions and their suppression by solid particle suppressing agents in a large-scale experimental tube. Journal of Loss Prevention in the Process Industries 26:310-316.
Mamurekli, D. 2010. Environmental impacts of coal mining and coal utilization in the UK. Acta Montanistica Slovaca 15:134-144.
Miller, G. 2005. The effect of coal usage on human health and the environment. En: Coal Energy Systems. . Elsevier Inc.:77-122.
Mittal, M. 2013. Limiting oxygen concentration for coal dusts for explosion hazard analysis and safety. Journal of Loss Prevention in the Process Industries 26:1106-1112.
NRC. 1980. US National Research Council. Trace-element Geochemistry of Coal Resource Development Related to Environmental Quality and Health. National Academy Press.
Pandey, B., M. Agrawal, and S. Singh. 2014. Coal mining activities change plant community structure due to air pollution and soil degradation. Ecotoxicology 23:1474-1483.
Peterson, S. M., J. Zhang, G. Weber, and J. L. Freeman. 2011. Global gene expression analysis reveals dynamic and developmental stage-dependent enrichment of lead-induced neurological gene alterations. Environmental Health Perspectives 119:615-621.
Petsonk, E. L., C. Rose, and R. Cohen. 2013. Coal mine dust lung disease. New lessons from an old exposure. American journal of respiratory and critical care medicine 187:1178-1185.
Rohr, P., J. da Silva, F. R. da Silva, M. Sarmento, C. Porto, R. Debastiani, C. E. dos Santos, J. F. Dias, and K. Kvitko. 2013. Evaluation of genetic damage in open-cast coal mine workers using the buccal micronucleus cytome assay. Environmental and molecular mutagenesis 54:65-71.
Rout, T. K., R. Masto, L. Ram, J. George, and P. K. Padhy. 2013. Assessment of human health risks from heavy metals in outdoor dust samples in a coal mining area. Environmental geochemistry and health 35:347-356.
Sipes, N. S., S. Padilla, and T. B. Knudsen. 2011. Zebrafish—As an integrative model for twenty-first century toxicity testing. Birth Defects Research Part C: Embryo Today: Reviews 93:256-267.

Tiwary, R. 2001. Environmental impact of coal mining on water regime and its management. Water, Air, and Soil Pollution 132:185-199.

Weber, G. J., M. S. Sepúlveda, S. M. Peterson, S. S. Lewis, and J. L. Freeman. 2013. Transcriptome alterations following developmental atrazine exposure in zebrafish are associated with disruption of neuroendocrine and reproductive system function, cell cycle, and carcinogenesis. toxicological sciences 132:458-466.

Westerfield, M. 2007. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio). (fifth ed.)Eugene, Oregon

Wirbisky, S. E., G. J. Weber, J.-W. Lee, J. R. Cannon, and J. L. Freeman. 2014. Novel dose-dependent alterations in excitatory GABA during embryonic development associated with lead (Pb) neurotoxicity. Toxicology letters 229:1-8.

Zocche, J. J., L. A. da Silva, A. P. Damiani, R. Á. Mendonça, P. B. Peres, C. E. I. dos Santos, R. Debastiani, J. F. Dias, V. M. de Andrade, and R. A. Pinho. 2014. Heavy-metal content and oxidative damage in Hypsiboas faber: The impact of coal-mining pollutants on amphibians. Archives of Environmental Contamination and Toxicology 66:69-77.

CHAPTER 5

5. CHAPTER 5. DESIGN OF AN ANIMAL MODEL TO EVALUATE THE IMPACTS OF COAL DUST (Paper 3)

5.2. Introduction

The expansion of the coal mining industry has negative effects on the ecosystem. This is reflected in erosion, destruction of water resources, land subsidence, air pollution, declining biodiversity, landscape fragmentation, release of contaminated water, generation of solid waste and the loss of agricultural land (Keating, 2001; Mamurekli, 2010), among other problems. However, there is little information on how the health effects on animals living near areas of coal mining activity.

When animal models are employed in the study of human disease, they are frequently selected because of their similarity to humans in terms of genetics, anatomy, and physiology. Also, animal models are often preferable for experimental disease research because of their unlimited supply and ease of manipulation. For example, to obtain scientifically valid research, the conditions associated with an experiment must be closely controlled.

Rodents are the most common type of mammal employed in experimental studies, and extensive research has been conducted using rats, mice, gerbils, guinea pigs, and hamsters. Among these rodents, the majority of genetic studies, especially those involving disease, have employed mice, not only because their genomes are so similar to that of humans, but also because of their availability, ease of handling, high reproductive rates, and relatively low cost of use. The laboratory mouse is a major model organism for basic mammalian biology, human disease, and genome evolution. About 99% of mouse genes have a homolog in the human genome, and for 80% of these genes, the best match in the human genome has, in turn, its best match against the orthologous mouse gene in the conserved syntenic interval (Guénet, 2005).

The goal of Paper 2 was to expose mice to soil containing coal dust, aiming to replicate the conditions under which mice interact with this pollutant in areas that receive permanent atmospheric depositions from coal mining activities. This allows the comparison between biochemical and cellular effects published

for field-collected animals and those housed with coal dust under laboratory conditions.

5.3. Materials and methods

5.3.8. Coal dust preparation

The coal dust was obtained in accordance with the methodology detailed in Chapter 4.

5.3.9. Coal dust characterization

The coal dust sample used in the experimentwas tested for trace elementscontent, analyzing forty-six trace elements (Li, Be, Sc, V, Cr, Co,Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Y, Zr, Nb, Mo, Cd, Sn, Sb, Cs, Ba, La, Ce,Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Hf, Ta, W, Tl, Pb, Bi, Th, and U). Briefly, 0.1 g coal dust sample was digested with a HF + HNO_3 (8 mL:3 mL) solution, drying, and a second dissolution in HNO_3(3 mL) and HCl (3 mL). All the acids used were Suprapur® High PurityAcid, Merck. Three multi-elemental solutions Spec® 1 (rare earthelements, REE), Spec® 2 (alkalis, earth alkalis, and metals) and Spec®4 (Nb) were employed to construct an external calibration curve readon an AGILENT 7700 ICP/MS at the Central Laboratory of the Universityof Huelva, Spain. The average precision and accuracy for most of theseelements fall in the range of 5–10%, and were controlled by repeatedanalysis of the SARM-1 (granite) and SARM-4 (norite) internationalrock standard of the South African Bureau of Standards. The lowerdetection limit (LDL) for most elements in the solution was 0.01 ppb.Total Hg in coal dustwas analyzed employing a direct mercury analyzer (Tri cell DMA-80) from Milestone, Italy. Operational conditions wereestablished according to EPA method 7473 (EPA 7473, 2007). The sensitivity and accuracy of the DMA was monitored by analyzing certified reference material. The detection limit (DL) was 0.003 μg/g.

5.3.10. Preparation of coal dust in sand mixture used for bedding

Sandwas purchased froma local store and passed through a sieve toobtain a particle size less than 1 mm. Subsequently, the sand waswashed eight times with tap water, three with distilled water and twowith ultrapure water (Milli-

Q), followed by sterilization in an autoclaveat 120 °C × 1 h and 15 min, and finally dried in anHDF-120 oven at 70 °C for 24 h. The sand was stored in sterile glass bottles and maintained atroom temperature.

5.3.11. Animals

Female ICR (Institute of Cancer Research) mice, 6 weeks old werepurchased from the National Institute of Health, Bogota (Colombia).Animals weighing between 19 and 20 g each were housed six perpolycarbonate cage (32 cm long × 20 cm wide × 21 cm high) andmaintainedunder standard laboratory conditions, 26±2 °C, 70–85% relativehumidity, and dark/light cycle 12/12 h. Animals were acclimated to thelab for oneweek prior to the experiment. Standard diet and water wereprovided *ad libitum*. Animal care and experimental procedures wereapproved by the Institutional Ethics Committee of the University of Cartagena.

5.3.12. Experimental protocol

Thirty-six mice were randomly and equally divided into six groups.The non-exposure group was caged on sand only as bedding. Animalsin treatment groups were housed during eight weeks on coal dustcontaminatedsand at different concentrations (0.25, 0.5, 1, 2, and 4%w/w) (**Figure 5.1**), varying from a levelwhere sand looks completely black in color (4%) to that where theblack color from dust particles is not distinguished from the sandalone (0.25%). Moreover, as seen in **Table 5.1**,metal concentrations reported for several coal mining sites (da Silva et al., 2000a; Niu, Gao, & Zhao, 2015; Pandey, Agrawal, & Singh, 2016; Reza, Baruah, Singh, & Das, 2015), are greater than those obtained after incorporating coal dust at thehighest concentration (4%) to the sand.

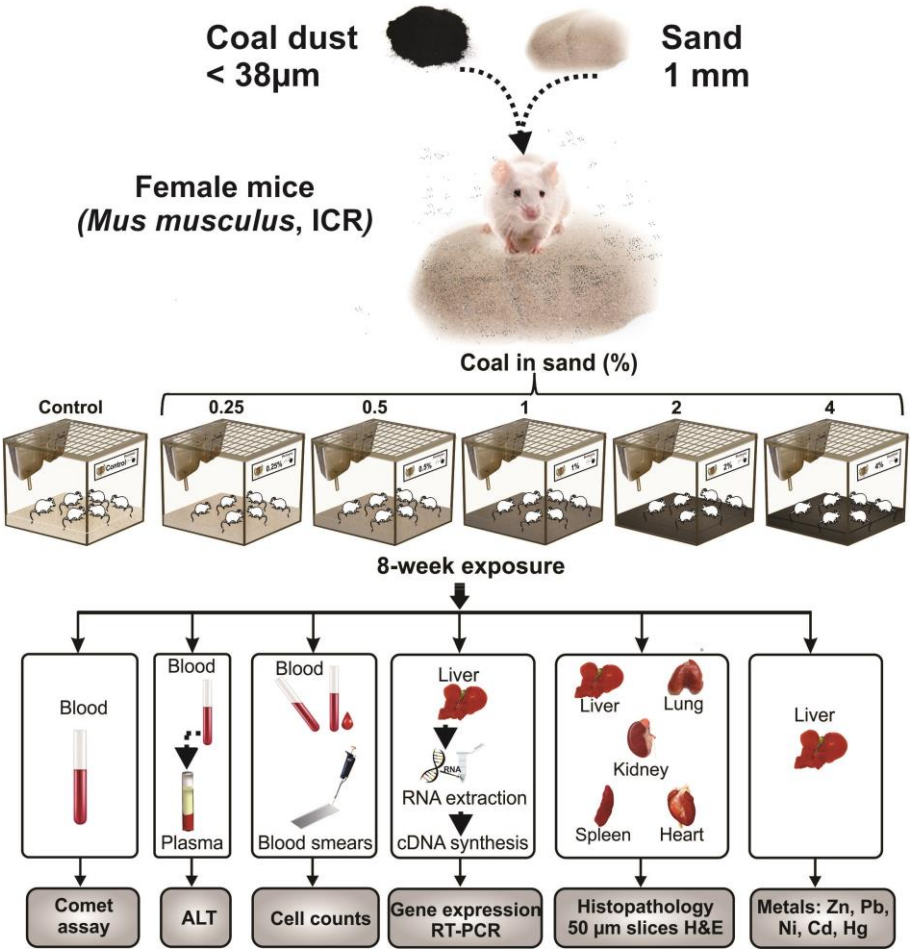

Figure 5.1. Schematic overview of the experimental design.

This approach was carried out based on the following criteria: first, rodents living near or within coal mine areas are in permanent contact with sand, material where dust particles are deposited from the air. Therefore, we simulated that specific environment. Second, several authors have shown that wildlife from coal mining areas, including mice (Leon, Perez, Linares, Hartmann, & Quintana, 2007), rats (Leon et al., 2007), fish (Holm et al., 2003), and collared tuco-tuco(da Silva et al., 2000a), experience different types of toxic stress, effect that we have monitored at the gene expression level in mice(Guerrero-Castilla, Olivero-Verbel, & Marrugo-Negrete, 2014). Taken together, the basis for these experiments was to replicate what occurs inthe wild but under laboratory conditions, allowing the mice to be incontact with

dust particles present in the sand, through dermal, inhalation and ingestion exposure.

Bedding was changed weekly. Food remains that fell onto the sandand excreted stools were removed daily. Mice were weighed everythree days over the course of the study. The experiment was repeatedonce. In selecting the concentrations of coal dust that the mice wouldbe exposed to, we took into account several studies that reported levelsof metals in soil from areas of coal mining (**Table 5.1**) (da Silva et al., 2000a; Niu et al., 2015; Pandey et al., 2016; Reza et al., 2015). These data showed that the concentrations usedin this study were lower than those found in the environmental dustlevels near coal mines.

Table 5.1. Trace element concentrations in soil from coal mining area.

Metal	Concentration in soil (ppm)	Reference	This study Coal dust (coal dust in sand 4%)
Zn	187.2	da Silva et al. (2000a)	8.99 (0.36)
	65.4	Niu et al. (2015)	
	210.0	da Silva et al. (2000a)	
	127.0	Pandey et al. (2016)	
	199.0	da Silva et al. (2000a)	
Ni	4.4	da Silva et al. (2000a)	0.87 (0.035)
	43.1	Niu et al. (2015)	
	6.1	da Silva et al. (2000a)	
	64.1	Pandey et al. (2016)	
	87.5	Reza et al. (2015)	
Pb	25.2	da Silva et al. (2000a)	0.71 (0.03)
	27.8	Pandey et al. (2016)	
	28.2	da Silva et al. (2000a)	
	48.3	Niu et al. (2015)	
	183.1	Reza et al. (2015)	
Cd	34.6	da Silva et al. (2000a)	0.04 (0.002)
	0.1	da Silva et al. (2000a)	
	2.6	Reza et al. (2015)	
	1.01	Niu et al. (2015)	
	0.4	Pandey et al. (2016)	
V	0.1	da Silva et al. (2000a)	1.17 (0.012)
	3.2	da Silva et al. (2000a)	
	10.3	da Silva et al. (2000a)	
	8.6	da Silva et al. (2000a)	
	2.0	da Silva et al. (2000a)	
Cu	17.0	da Silva et al. (2000a)	4.26 (0.17)
	23.6	Niu et al. (2015)	
	66.3	Pandey et al. (2016)	
Cr	3.6	da Silva et al. (2000a)	1.07 (0.04)
	112.3	Reza et al. (2015)	
	188.2	Niu et al. (2015)	
	43.0	Pandey et al. (2016)	

5.3.13. Tissue and blood collection

At the end of the exposure period, animals were weighed andanesthetized with an IP injection of sodium pentobarbitalat a dose of 60 mg/kg. Once anesthesia was induced, the animals were dissected and the blood was collected via vena cava flow, stored in tubeswith sodium citrate to a final concentrationof 0.76%, andmaintained under refrigeration for a maximumof 24 hprior to analysis. An aliquot of the bloodwas usedwithin the next 8 h forgenotoxicity assays and another for enzyme activity measurements.During necropsy, tissue samples from lung, liver, kidney, and spleenwere removed. The left lobule of the liver was immersed in RNAlater® (Qiagen, California, USA) for 30 min and stored at −80 °C. A portionof liver tissue was kept at−20 °C for metal content analysis. Additionalfragments of each organ were sliced and placed in 10% bufferedformalin, and 24 h later transferred to 70% ethanol.

5.3.14. Metal analysis in mice liver

Cadmium, Ni and Pb content in mice liver was measured by atomicabsorption spectroscopy (AAS) with a Graphite Furnace, whereas Znwas determined by flame AAS, using a Thermo Fisher Scientific iCE3000. Atomic Absorption Spectrophotometer standard solutions for Cd, Pb, Ni, and Zn were purchased from Merck (NIST traceable). Suprapur nitric acid was used for the preparation of the standard andsample solutions. Quantification was carried out utilizing calibrationcurves for each metal. The measurements were performed accordingto the peak height of the metal analyzed as well as its the absorbance.
A direct mercury analyzer (Tri cell DMA-80) from Milestone, Italy, wasemployed for Hg analysis in liver tissue samples by atomic absorptionafter sample ashing and Hg pre-concentration in a gold trap. Operationalconditions were established according to EPA method 7473 (EPA 7473, 2007). The analytical validation of themethod was demonstratedthrough the analysis of certified reference materials, DORM-3 from theNational Research Council of Canada. The detection limit (DL) was0.0004 μg/g.

5.3.15. Enzyme analysis

Plasma alanine aminotransferase (ALT) activity, amarker of hepatocellulardamage (Schomaker et al., 2013) was measured using a clinicalchemistry analyzer (Roche Cobas C111).

5.3.16. Comet assay

Blood samples were harvested after exposure to coal dust contaminated sand and subjected to a comet assay following themethod proposed by Trevigen (Trevigen; Gaithersburg, MD) withsome modifications (Yang et al., 2011). An aliquot of blood was mixedwith low-melting temperature agarose. After lysis, electrophoresiswas performed at 300 mA for 60 min in the dark. Slides were stainedwith SYBR green dye for 30 min. One thousand randomly selectednucleated cells per sample were captured under a Nikon fluorescentmicroscope and digital fluorescent images were obtained using theNis-Elements F3.0 software. Based on tail size, comets were categorizedin four classes. The damage index (DI) was calculated multiplying thepercentage of nucleoids in each class by the corresponding factor (Amaia Azqueta, Pachón, Cascante, Creppy, & de Cerain, 2005), and the subtotal frequency of nucleoids(DF_{2+3+4}) with medium, high, and completely damaged DNA was estimated as the sum of cells in classes 2, 3, and 4.

In this thesis, comet assay was used to assess genotoxic damage, and its procedure is shown in **Fig. 5.2.** Basically, this occurs in the following steps: (1) Cells are mixed with low melting point agarose and (2) immobilised on CometSlides™. (3) Cells are lysed to remove membranes and DNA associated proteins before (4) alkaline treatment to unwind and denature DNA. (5) During electrophoresis, damaged, unwound, relaxed DNA migrates out of the cell and can be visualized using SYBR® Green.

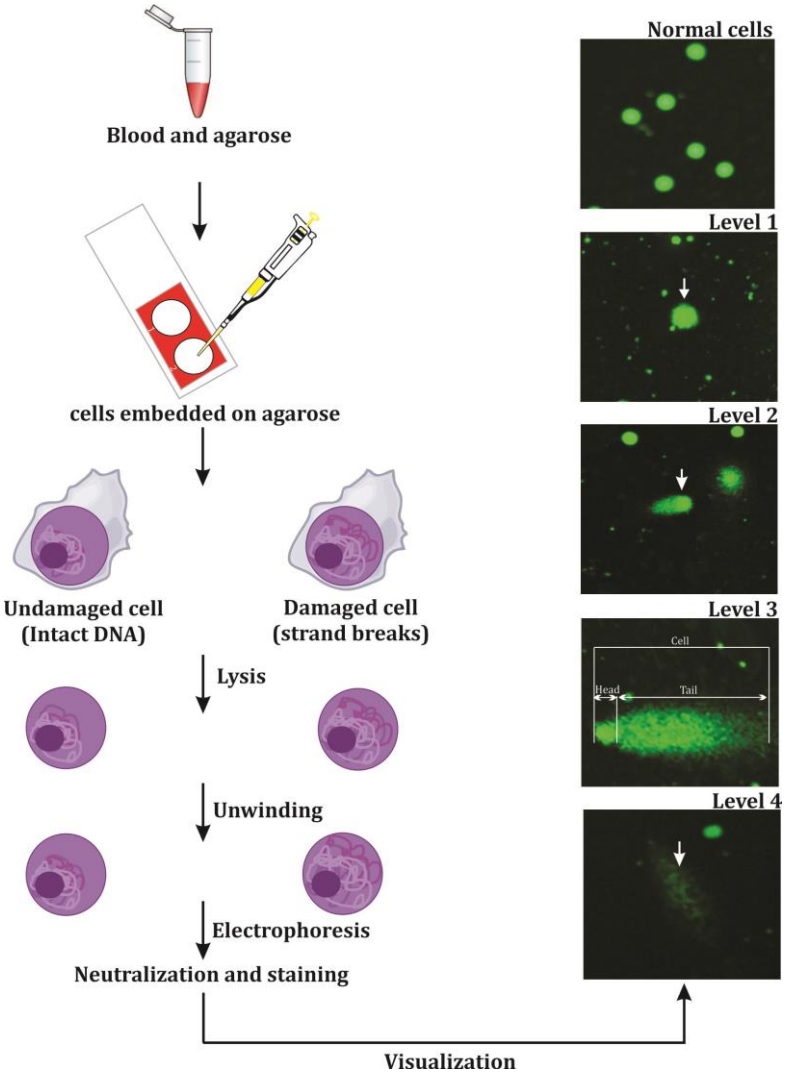

Figure 5.2. Comet assay procedure. Adapted from Azqueta and Collins (2011).

5.3.17. Micronucleus (MN) and cell counts in peripheral blood smears

The MN assay was carried out as reported elsewhere Lynch, 1990; Weilbauer et al., 2009). Briefly, blood smears were deposited on cleanglass slides, air-dried and stained with Wright. At least five bloodsmears were prepared per animal.

The frequency of erythrocytes with MN, microcytes, and polychromatic erythrocytes were scored by randomlyexamining 10 fields per slide corresponding to approximately 2000 cells examined per slide. The percentage of white blood celltypes (lymphocytes, reactive lymphocytes, immature cells, monocytes, neutrophils, eosinophils, binucleated lymphocytes and basophils) wascounted by randomly examining 100 cells per slide. All measurementswere performed under a 100× objective using a Nikon ECLIPSE E-100microscope.

5.3.18. Gene expression

The total RNA was extracted from liver tissue using the RNeasy®Mini Kit (Quiagen, California, USA) based on themanufacturer's instructions.RNA concentration was determined by spectrophotometry(A260) employing a NanoDrop 2000 Spectrophotometer (ThermoScientific), and the purity assessed by measuring the A260/A280 ratio(1.9–2.0). Agarose (1.2%) gel electrophoresis was utilized to verify thequality of the RNA. Subsequently, cDNA was synthesized from 1.5 μgtotal RNA using the QuantiTect® Reverse Transcription Kit (QiagenInc., Valencia, CA, USA), and used as the template in a 20 μL PCR reactioncontaining 10 pmol each of forward and reverse gene-specific primers.Real-time polymerase chain reaction (RT-PCR) was conducted on aStepOne® System (Applied Biosystems, Foster City, CA). Reactionswere carried out in MicroAmp optical 48-well reaction plates (AppliedBiosystems) utilizing QuantiTect SYBR Green PCR kit (Qiagen). Theamplification was performed under the following conditions: 95 °C for15 min to activate the DNA polymerase, then 40 cycles of 94 °C for15 s, 50–60 °C for 30 s and 72 °C for 30 s. In total, 16 genes wereanalyzed, including markers of heavy metal exposure, xenobioticmetabolism, nuclear receptor modulation, oxidative stress, DNAdamage, inflammation, and lipid metabolism. Accession numbers andprimer sequences for the target genes are listed in Table S1 (Supplementaryinformation). Changes in gene expression were determinedusing Gapdh, Rps19 and Actb as reference genes (housekeeping), andthe comparative CT (ΔΔCT) method was utilized to determine therelative target quantity. All experiments were conducted in duplicate, and negative controls were without template cDNA (Arya et al., 2005; Valasek and Repa, 2005; Wang et al., 2010).

5.3.19. Histological examination

Tissue sections from different organs were fixed with 10% neutralbuffered formalin and dehydrated in ethyl alcohol. Samples wereembedded in paraffin, sectioned at 5 μm and stained with hematoxylinand eosin (H&E) (Olivero-Verbel et al., 2011). The images for theanalysis of different tissue sections were captured using a NikonECLIPSE E-100 microscope.

5.3.20. Statistical analysis

The data are presented as the mean ± standard error, and forstatistical purposes those were checked for normality and variancehomogeneity using the Kolmogorov–Smirnov and Bartlett's tests, respectively.ANOVA was used to evaluate mean differences betweengroups exposed to different coal dust concentrations and the Dunn'stest was employed as a post-hoc test. In the absence of normality, Kruskal–Wallis followed by Mann–Whitney testwas used. The criterionof significance was set at $P<0.05$. All statistics were performed bymeans of GraphPad Prism 5.0.

5.4. Results

5.4.8. Body weight

Weight gain was similar in all experimental groups. No significantdifference was found between the body weight of control mice andthe other treated groups (**Table 5.2**).

Table 5.2. Effect of coal dust treatment on body weight (g) of experimental mice after an exposure of 8 weeks*.

	Coal dust concentrations %					
	0	0.25	0.5	1	2	4
Beginning body weight	22.57±0.97	23.77±1.14	22.40±0.98	24.21±0.91	24.50±1.05	23.71±1.14
Terminal body weight	31.00±1.21	31.20±1.50	31.80±0.96	30.37±0.59	32.10±0.93	32.03±1.81
Weight gain	8.43±1.13	7.43±1.06	9.40±0.83	5.84±0.96	7.60±0.45	8.33±0.98

*. Values are expressed as mean ± standard error for 12 animals in each group.

5.4.9. Element content in coal dust

The concentrations of 47 trace elements in coal dust, classifiedaccording to the description by Ketris and Yudovich (2009) and NRC (1980) are listed in Table 5.3. The highest concentrations were found forSr (34.91 ± 3.00 μg/g), Ba (57.20 ± 0.17 μg/g), Zn (8.99 ± 3.74 μg/g), As (4.38 ± 2.76 μg/g), Cu (4.26 ± 2.14 μg/g), Se (1.28 ± 0.35 μg/g), V(1.17±0.74 μg/g) and Cr (1.07±0.27 μg/g). In order to assess the levelof concern for analyzed elements, their average concentrations

in thecoal dust sample were compared to the classification of the US National Research Council (NRC) (**Table 5.3**), which is based on known adversehealth effects or abundance of the elements in coal. Arsenic was foundwithin the trace elements of concern with concentrations greater than4 μg/g, as well as Se, Pb, Hg, Cd and Mo with concentrations of 1.28,0.71, 0.04, 0.04, and 0.90 μg/g, respectively.

The concentrations of trace elements in coal dust were also comparedwith reports of coal contents from Canada (Goodarzi et al.,2008), Poland (Smolińskiet al., 2014), and China (Dai et al., 2007,2008) as well as world average values reported by Ketris andYudovich (2009) (**Table 5.3**). The results showed metal concentrationsin the Colombia coal sample from this study are generally low whencompared to other reported coal metal concentrations. Theconcentration of arsenic was similar to that observed for coal inCanada (Goodarzi et al., 2008), noting that both are bituminous coals.

Table 5.3. Element concentrations (μg/g) in coal dust compared with other studies.

Elements	This study	Coal Clarke Ketris and Yudovich (2009)	China Dai et al. (2007) Dai et al. (2008)	Poland* Smoliński, Rompalski, Cybulski, Chećko, and Howaniec (2014)	Canada Goodarzi, Huggins, and Sanei (2008)
Ba[c]	**57.20±0.17**	150	nd	246.24	nd
Sr[c]	**34.91±3.00**	110	nd	133.22	nd
Zn[b]	**8.99±3.74**	23	41.4	78.52	nd
As[a]	**4.38±2.76**	8.3	nd	2.03	4.39
Cu[b]	**4.26±2.14**	16	17.5	17.62	nd
Se[a]	**1.28±0.35**	1.3	2.47	nd	nd
V[b]	**1.17±0.74**	25	35.1	30.08	nd
Cr[b]	**1.07±0.27**	16	15.4	18.34	51.71
Ce	0.97±0.00	23	nd	nd	nd
Mo[a]	0.90±0.03	2.2	3.08	nd	nd
Ni[b]	0.87±0.43	13.0	13.7	17.01	15.24
Zr	0.77±0.40	36	nd	nd	nd
Li[c]	0.75±0.31	12.0	nd	nd	nd
Pb[a]	0.71±0.34	7.8	15.1	16.58	9.70
Y	0.61±0.06	8.4	nd	nd	nd
Co[c]	0.55±0.06	5.1	7.08	6.30	nd
La	0.50±0.01	11	nd	nd	nd
Nd	0.36±0,02	12	nd	nd	nd
Ga	0.29±0.01	5.8	6.55	nd	nd
W	0.28±0.05	1.1	nd	nd	nd
Tl[e]	0.19±0.10	0.63	nd	nd	nd
Rb	0.16±0.06	14.0	nd	15.96	nd
Nb	0.15±0.06	3.7	nd	nd	nd
Hf	0.12±0.03	1.2	nd	nd	nd

Sn[e]	0.11±0.03	1.1	nd	nd	nd
Pr	0.11±0.00	3.5	nd	nd	nd
Sm	0.10±0.01	2.0	nd	nd	nd
Dy	0.10±0.01	2.1	nd	nd	nd
Sb[c]	0.09±0.03	0.92	nd	0.85	nd
Gd	0.08±0.01	2.7	nd	nd	nd
Ge[c]	0.08±0.01	2.2	nd	nd	nd
Th[d]	0.07±0.04	3.3	nd	nd	nd
Er	0.06±0.01	0.93	nd	nd	nd
Yb	0.05±0.01	1.0	nd	nd	nd
Be	0.05±0.03	1.6	nd	nd	nd
U[d]	0.04±0.02	2.4	nd	nd	nd
Cd[a]	0.04±0.01	0.22	0.25	0.41	0.47
Hg[a]	0.04±0.01	0.10	nd	0.02	0.18
Ta	0.04±0.02	0.28	nd	nd	nd
Eu	0.03±0.00	0.47	nd	nd	nd
Bi	0.02±0.00	0.97	nd	nd	nd
Tb	0.02±0.00	0.32	nd	nd	nd
Ho	0.02±0.00	0.54	nd	nd	nd
Tm	0.01±0.00	0.31	nd	nd	nd
Lu	0.01±0.00	0.20	nd	nd	nd
Cs	0.01±0.00	1.0	nd	nd	nd
Sc	0.01±0.00	3.9	nd	nd	nd

nd, no data.

Classification of the trace elements by level of concern based on known adverse health effects (NRC, 1980): a. Elements of greatest concern, b. Elements ofmoderate concern, c. Elements ofminor concern, d. Radioactive elements, e. Elements of concern, but present only in very low concentrations.

*Mean values for mines from the central zone of the Upper Silesian Coal Basin, Poland.

In the case of Hg, the concentration of the Colombia coal sample was higher (0.04 µg/g) than that reported in Poland (0.02 µg/g) and lowerthan that registered in Canada (0.18 µg/g), and in coal worldwide(0.10 µg/g). Levels of Se in coal dust (1.3 µg/g) were similar to thosereported for coal worldwide (1.3 µg/g) and lower than that fromChina (2.47 µg/g).

5.4.10. Metal content in mice liver

Liver samples frommice exposed to coal dust (control, 2 and 4%w/w)were analyzed for Hg, Ni, Zn, Cd and Pb content, and the results areshown in **Table 5.4**. Mercury, Pb and Cd levels were similar for treatedand control groups. Nickel was significantly greater for the 4% w/wgroup when compared to control, and Zn was significantly different betweenthe non-exposed group to both the 2 and 4% w/w groups.

Table 5.4. Trace element concentrations (µg/g) in hepatic tissue.

Metal	n	Control	2 %	4 %
Hg	6	<DL	<DL	<DL
Pb	5	<DL	<DL	<DL
Ni	6	0.07±0.003	0.07±0.001	0.09±0.001*
Cd	6	0.07±0.001	0.06±0.001	0.07±0.002
Zn	6	78.92±0.10	80.22±0.20*	87.74±0.59*

*. Significant difference (P< 0.05) when compared to non-exposed group (sand withoutcoal dust). DL=0.0004 µg/g fw for Hg; 0.013 µg/g dw for Ni; 0.002 µg/g dw for Cd; 0.038µg/g dw for Pb and 0.002 µg/g dw for Zn.

5.4.11. Comet assay

The comet assay was performed from the same blood samples usedto make smears for the MN assay. The results of the comet assayperformed in peripheral blood samples from mice exposed to sandcontaminated with coal dust are shown in **Table 5.5**. Groups exposed to2 and 4% coal dust in sand showed significant increases in the damageindex (DI) values, as compared to the control (P<0.05). Mice exposedto a 4% concentration of coal dust had the highest DI and the highestfrequency of nucleoids to 2, 3, and 4 [DF_{2+3+4} (16.87 and 5.38, respectively]. A dose-dependent increase in DNA damagewas observedwhen coal dust concentration increased in sand.

Table 5.5. DNA damage measured by the comet assay in peripheral blood leukocytes isolated frommice exposed to coal dust.

Group (coal dust % in sand)	n	GDI± SD	F_{2+3+4}± SD
0	6	0.96±1.02	0.09±0.16
0.25	6	2.72±4.29	0.07±0.16
0.5	6	6.27±6.80	0.53±1.30
1	5	6.90±0.51	0.63±0.81
2	6	12.98±3.45*	2.50±0.11*
4	6	16.87±5.13*	5.38±1.00*

*. Significant difference (P<0.05) when compared to non-exposed group (sand without coal dust).

5.4.12. MN and cell counts in peripheral blood smears

The results of the MN assay in peripheral blood smears are presented in **Figure 5.3.** The presence of MN in erythrocyteswas significantly increasedin the group exposed to the maximum coal dust concentration (4%) compared to the control group. Both microcytes and polychromaticerythrocytes were significantly augmented when animals were exposedto 2 and 4% coal dust in sand compared to the control group. In all cases, a clear concentration-dependent relationship was observed.A similar increase was also significant and concentration-related forthe percent of reactive lymphocytes, immature cells and monocytes,but the percent of normal lymphocytes showed a significant decreasein the mice exposed to the maximum coal dust concentration whencompared to control group (**Figure 5.4**). In the case of neutrophils, eosinophils, binucleated lymphocytes and basophils, no significant changeswere observed with treatment (**Figure 5.5**).

5.4.13. Plasma ALT activity

Hepatic injury was determined by measuring ALT activity in plasmaand results are presented in **Figure 5.6.** The ALT activity followed a modestconcentration-response relationship, with significant differences, when compared to the control, only for mice exposed to 4% coal dustcontaminatedsand.

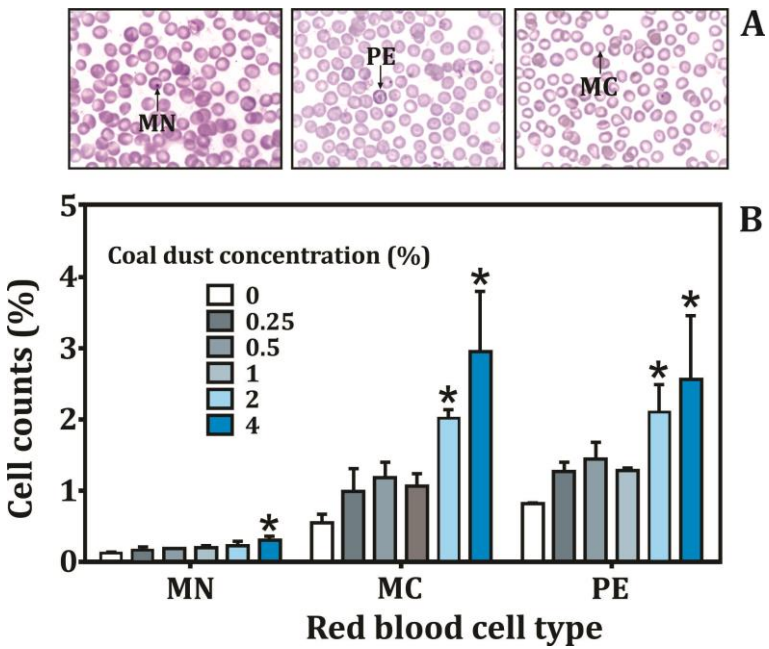

Figure 5.3. Microphotographs of red blood cells and their distribution in blood smears. Panel A.Micronucleus in red blood cells (MN), microcytes (MC) and polychromatic erythrocytes (PE) in blood smears, Panel B. Quantitative results for abnormal cell counts in bloodsmears. *Significant difference (P<0.05) when compared to control group.

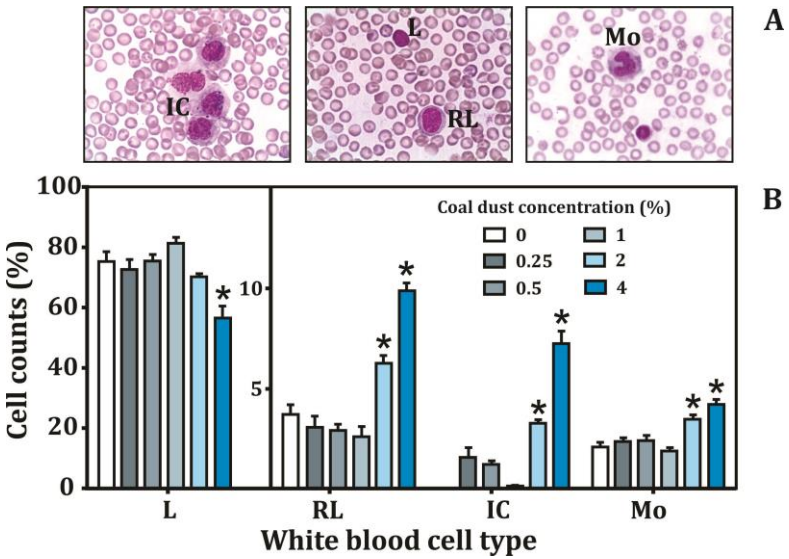

Figure 5.4. Microphotographs of some leukocytes and their distribution in blood smears. PanelA. Immature cells (IC), reactive lymphocytes (RL), lymphocytes (L), and monocytes (Mo). Panel B. Cell counts on treatment groups. *Significant difference (P<0.05 when comparedto control.

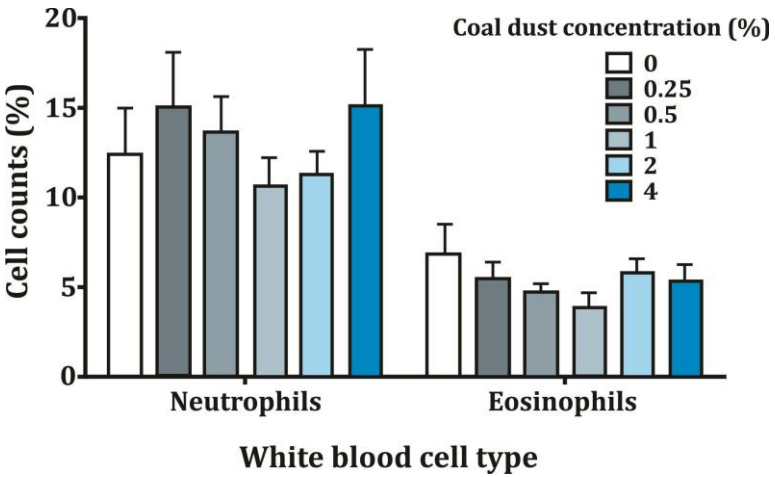

Figure 5.5. Neutrophils and eosinophils in blood smears.

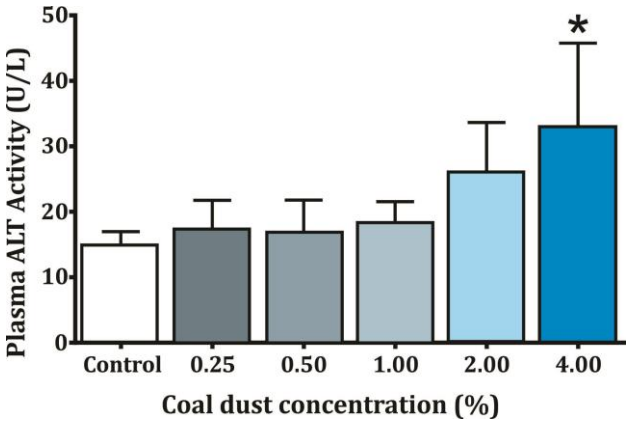

Figure 5.6. Plasma ALT activity in mice exposed to coal dust.

5.4.14. Hepatic gene expression profiles

Sixteen genes belonging to several representative mechanisms oftoxicity were evaluated to visualize their expression profile. Genesthat changed significantly with treatment are shown in **Figure 5.7.** HepaticmRNA expression of *Cyp1a1*and Scd1 were upregulated, and *Sod1*and *Nqo1*were significantly downregulated at the highest testedconcentrations (2–4%), suggesting a role for activation of xenobiotic/lipid metabolism and alterations in the oxidative stress pathways, asoutcomes from the exposure to this pollutant.

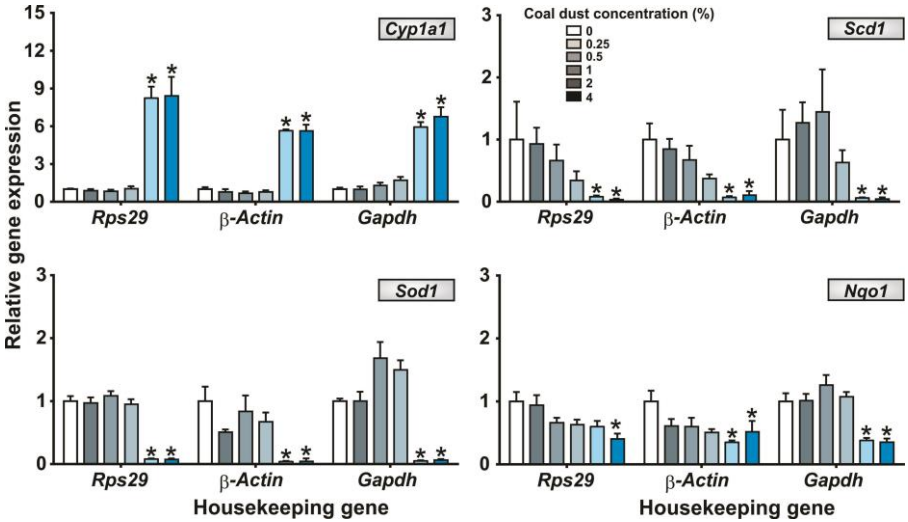

Figure 5.7. mRNA expression of selected genes. Expression was normalized against Rps29, β-Actin and Gapdh (Housekeeping genes). *Significant difference (P b 0.05) when compared to control.

5.4.15. Histopathological analysis

Representative images of lung and liver sections from *M. musculus*are presented in **Figures. 5.8** and **5.9**, respectively. Lung tissue containedinflammation-related features such as perivascular and interstitialchronic inflammation. Liver tissues from coal dust exposed animals (2and 4%) displayedvacuolization/steatosis, inflammation and hepatocyteenlargement. No histological alterations were observed in thespleen, heart, and kidney of the control group. However, the heart andspleen showed evidence of melanomacrophage centers, while the presenceof inflammation was detected in the kidney in the groups exposedto 2 and 4% coal dust in sand (**Figure 5.10**).

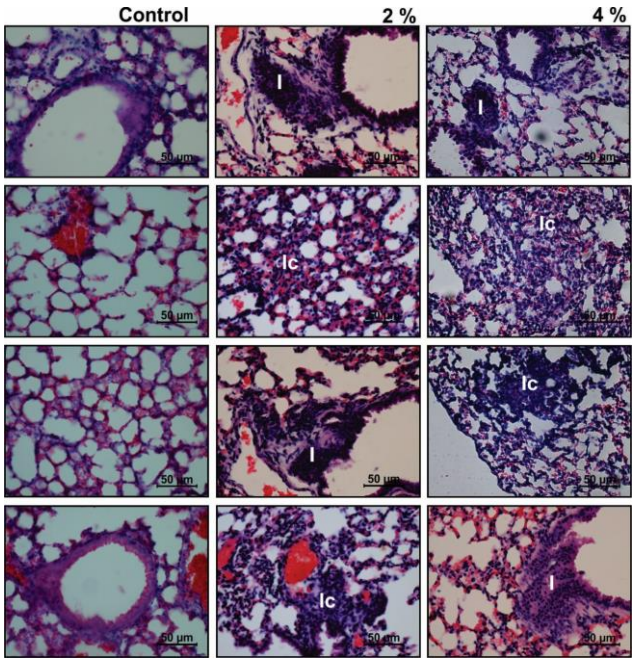

Figure 5.8. Morphologic alterations by exposure to coal dust in the lung tissue of mice after 8 weeks. Stain H&E (40×). Control: normal anatomy, peribronchial tissue. Peribronchial inflammation (I), pulmonary parenchyma with scattered massive inflammatory cells (Ic).

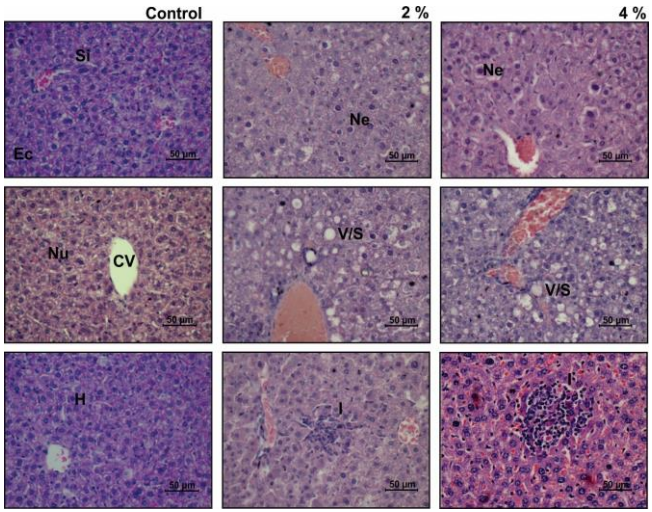

Figure 5.9. Morphological alterations in the liver tissue of mice after 8weeks of exposure to sand contaminated with coal dust. Stain H&E (40×). Control: Hepatocytes (H) polyhedral in shape with eccentrically placed rounded euchromatic prominent nucleoli (Nu) and endothelial cells (Ec) lining the sinusoids (Si) and central vein (CV). 2 and 4%: unequal size of hepatocytes and their nuclei enlargements (Ne), steatosis, vacuolization (V/S), and inflammatory cells (I).

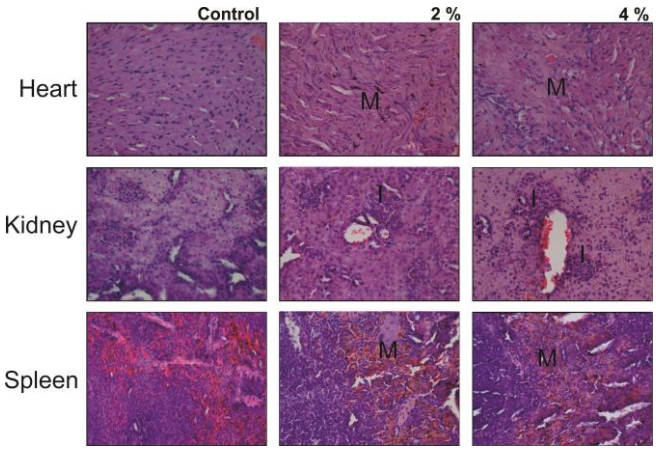

Figure 5.10. Histopathology of heart, kidney and spleen tissues of ICR mice after exposure to coal dust in sand. Control group shown normal tissue architecture; M, melanomacrophage and I,

inflammation. Cut sections of these tissues were stained with hematoxylin and eosin (H&E) and viewed under a light microscopy.

5.5. Discussion

The release of coal dust is one of the major environmental problemsencountered with coal mining. However, information on the toxicity ofthis material is scarce. In this study, mice were exposed to coal dustpresent as part of their bedding, mimicking what animals may beexperiencing in the wild near coal mining areas.

5.5.8. Trace elements in coal dust

Trace elements such as As, Hg, Se, Pb, Cd, and Cr, are consideredelements of concern because they can cause environmental pollutionand human health problems during coal mining (Fernandez et al.,2013), utilization (Xiang et al., 2012), and combustion (Frandsen et al.,1994). In addition, humans and animals may be exposed to thesetrace elements from the atmospheric deposition, and leaching fromwastes (Xiang et al., 2012) resulting from coal mining practices. In thisstudy, As concentration in coal was two-fold greater than that shownby Smoliński et al. (2014) in hard coal (anthracite) from Poland andsimilar to that reported for milled coal (bituminous) from Canada(Goodarzi et al., 2008). It has been demonstrated that As is highly hazardousto the environment when released from coal during miningand combustion (Finkelman et al., 1999). Bioaccumulation of thismetaloid has been found in aquatic macrophytes in lakes affected bycoal mining (Mishra et al., 2008). It also has contaminated drinkingwater sources in coal mining areas of central Appalachia (USA)(Shiber, 2005). Mercury is, as a general rule, among the least abundantelements in coal, as was also observed in the coal sample used in thisstudy. Nevertheless, in recent years it has been one of the most studiedelements in coal due to its extremely toxic effects and its ability tobioaccumulate throughout the food chain (Muto et al., 2014). Lead is a neurotoxicant and a widespread metal in the environment (Toscanoand Guilarte, 2005). Although Pb abundance in coal is low comparedto other sites (**Table 5.3**), its emission may increase during coal exploitation, storage and utilization, disposal of coal gangue and/or ash, and as aresult of massive consumption (Fang et al., 2014).

5.5.9. Metals in hepatic tissue

In this study Zn levels were statistically greater inmice exposed to 2and 4%w/w coal dust in sand, when compared to control. This increasein Zn concentrations has also been reported for wildlife such as frogs(Hypsiboasfaber) (Zocche et al., 2014) and bats (Zocche et al., 2010) livingnear coal mining areas. In addition, Bharti and Banerjee (2011) reportedthe presence of metals in the liver of fish (Heteropneustesfossilis) exposed to coal mine effluents in India. In the case of Cd(0.44±0.191 μg/g, dw), and Pb (3.63±0.83 μg/g, dw), these werehigher in the fish livers than those in mice liver from this study(0.07±0.001 and 0.02±0.0003 μg/g, dw, respectively). The liver isan important organ to be considered when the effects of pollutantsare investigated, since this organ plays a central role in the metabolismand detoxification of biological substances. Alsko, most of the substancesabsorbed by the intestine passes first through the liver wheretoxins and heavy metals may then accumulate (Saïdi et al., 2013). Theincreases in Zn and Cd concentrations observed in exposed animals suggest these metals present in the coal are readily bioavailable. Coalparticles consumed together with food are subjected to acid digestionin the stomach, releasing these metals. If inhaled, coal dust particles inthe lung are phagocytized by macrophages, a process that also mayresult in the release of metals (Graham et al., 1975).

5.5.10. Comet assay

Mice exposed to 2 and 4% coal dust in sand experienced genotoxicdamage as determined by the comet assay, a method to measure DNAdamage in a wide variety of cell types following genotoxic insult. Thistest has beenwidely used in biomonitoring the health status of differentwild species living near coal mining areas, including amphibians (Yinet al., 2009) and mammals (Heuser et al., 2002; Mughal et al., 2010).In coal mining areas of Colombia (León-Mejía et al., 2011) and Brazil (Rohr et al., 2013b), evidence of comet assay-measurable genotoxicdamage has been demonstrated in humans working or living in coalmining zones. This suggests that wildlife monitoring can be used topredict possible genotoxic effects on humans.

5.5.11. MN assay and white blood cell counts in peripheral blood smears

Mice exposed to coal dust particles developed changes in the distributionof several blood components, including both erythrocytes andleukocytes.

Microcytes and polychromatic erythrocytes counts weresignificantly greater than control at 2 and 4% coal dust in sand, a trendalso observed in lymphocytes. Polychromatic erythrocytes have beenreported as positive indicators of genotoxicity, as suggested by themicronucleus test in rodents, Ctenomystorquatus, captured fromcoal regions (da Silva et al., 2000b). In the case of theMN assay, the presence of MN is a marker of genome instability (Balmus et al., 2015).Mice housedin 4% coal dust in sand exhibited significant genotoxicity when comparedto the control group using this assay. The micronucleus assay has been widely used to detect genotoxicityin rodents (da Silva et al., 2000b; Kasamoto et al., 2013) and in humans (Schlegel et al., 1986; Zúñiga-González et al., 2012). The evaluation ofmicronucleus induction is the primary in vivo assay in a battery ofgenotoxicity tests recommended by regulatory agencies around theworld to be conducted as part of product safety assessment. The assay, when performed appropriately, detects both clastogenicity (chromosomebreakage) and aneugenicity (chromosome lagging due todysfunction of mitotic apparatus) (Krishna and Hayashi, 2000).

Although micronucleated red blood cells are extremely rare in humans,partly owing to their efficient clearance by the spleen, mice are less efficientat clearing micronucleated red blood cells from the peripheralcirculation. Thus, micronuclei are more readily detected in this modelorganism (MacGregor et al., 1980). In coal mining areas of Colombia, this test has been used in both rodents (Leon et al., 2007) and humans (León-Mejía et al., 2011) to assess toxic effects fromcoal dust exposure.

5.5.12. Plasma ALT activity

The activity of plasma ALT is clinically used in the evaluation of hepatocellularinjury (Giannini et al., 2005). Injured hepatocytes release enzymesinto the blood stream leading to elevation in ALT. In this study, only mice exposed to 4% coal dust in sand had significantly elevatedALT plasma activity compared to the control. This tissue damage mayarise from hepatotoxicants present in coal dust, such as heavy metals (Cobbina et al., 2015), PAHs (Wang et al., 2007), and particles (Liuet al., 2012).

5.5.13. Gene expression

The expression of some marker genes were evaluated in mice exposedto sand contaminated with coal dust and mice not exposed tocoal dust. Exposure to this pollutant induced a clear increase in *Cyp1a1*expression, a gene responsible for the metabolism and activationof several types of procarcinogens, such as PAHs, planar aromaticcompounds with varying potencies of carcinogenicity (Humans,

2010). PAHs occur naturally in the environment in fossil fuels such as coal, oil, and tar, among others. Coronado-Posada et al. (2013) reported PAHs in a methanolic coal dust extract. Therefore, considering the nature of coal dust, the overexpressionof *Cyp1a1* was expected as a response to PAHs (Pliarchopoulou et al., 2012). *Cyp1a1* has garnered particular interest because of its involvementin the production of carcinogenic intermediate species that caninitiate lung cancer development. The transcriptional regulation of the *Cyp1a1* gene by PAHs is mediated through the ligand dependentactivation of the aryl hydrocarbon receptor (AhR) (Kawajiri and Fujii-Kuriyama, 2007). Interestingly, AhR activation negatively regulates theexpression of fatty acid synthesis genes in mice, in particular *Scd1* (Tanos et al., 2012). Therefore, through this mechanism the AhR exertshomeostatic control of fatty acid synthesis. *Scd1* is a key lipogenicenzyme of lipid metabolism catalyzing the 9-desaturation of thesaturated fatty acids, palmitate and stearate to the monounsaturatedfatty acids, palmitoleate and oleate, respectively (Scaglia et al., 2009).

As previously stated, reactive oxygen species have been implicatedin the pathogenesis of coal dust induced toxicity (Wittkopp et al., 2016). In the case of *Nqo1*and *Sod1*, the downregulation of thesegenes was an interesting finding that deserves further investigation.

Interestingly, this in vivo observation has been also documented forhuman blood of three different groups exposed to airborne contaminationassociated with coal mining activities from Brazil (Júnior et al., 2009).

5.5.14. Histopathological examination

Exposure to coal dust caused typical lung inflammation characterizedby the presence of cell infiltrates. Chronic inhalation of coal dustcan induce several lung diseases, including coalworker pneumoconiosis(CWP), chronic bronchitis, loss of lung function, emphysema, and progressivemassive fibrosis (PMF) (Armutcu et al., 2007). In experimentalanimals, the development of lung tumors has been identified as a resultof exposure to coal dust (Martin et al., 1975; Pott et al., 2000). In addition, Kania et al. (2014) reported that subchronic inhalation of coaldustPM10 induces bronchoalveolar reactive hyperplasia and rearrangementof epithelial cells. In this study, mice exposed to coal dust (2 and4%) presented small clusters of inflammatory cells in the liver, probablyas a result of exposure to metals and PAHs present in this pollutant. Thenuclei of some hepatocytes was also enlarged, findings suggesting anongoing ballooning degeneration of the hepatocytes. Compoundspresent in coal such PAHs and metals may generate oxidative signalsand cause cellular

damage (Goetz and Luch, 2008; Henkler et al., 2010), effects also observed in the lung tissue from coal dust exposedmice.

5.6. Conclusions

In short, themodel presented here suggest that mice exposed to coaldust under laboratory conditions, experience several toxicologicaleffects at the molecular, cellular and tissue level, similar to thosefound in some wildlife living near mining areas.This study demonstrated that mice exposed to coal dust in sandexperienced molecular, cellular and histological changes. These included DNA damage and changes in mRNA expression of genes relatedto oxidative stress, aswell as xenobiotic and lipid metabolism. Alterationsin the distribution of blood cells were observed and hepaticlesions were present represented by vacuolization/steatosis, necrosis and inflammation.

5.7. References

Armutcu, F., Gun, B.D., Altin, R., Gurel, A., 2007. Examination of lung toxicity, oxidant/antioxidant status and effect of erdosteine in rats kept in coal mine ambience. Environ. Toxicol. Pharmacol. 24, 106–113.

Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N., Patel, H.R., 2005. Basic principles of real-time quantitative PCR. Azqueta, A., Pachón, G., Cascante, M., Creppy, E.E., de Cerain, A.L., 2005. DNA damage induced by a quinoxaline 1, 4-di-N-oxide derivative (hypoxic selective agent) in Caco-2 cells evaluated by the comet assay. Mutagenesis 20, 165–171.

Balmus, G., Karp, N.A., Ng, B.L., Jackson, S.P., Adams, D.J., McIntyre, R.E., 2015. A highthroughput in vivo micronucleus assay for genome instability screening in mice. Nat. Protoc. 10, 205–215.

Bharti, S., Banerjee, T.K., 2011. Bioaccumulation of metals in the edible catfish Heteropneustes fossilis (bloch) exposed to coal mine effluent generated at northern coalfield limited, Singrauli, India. Bull. Environ. Contam. Toxicol. 87, 393–398.

Chaulya, S., 2004. Assessment andmanagement of air quality for an opencast coal mining area. J. Environ. Manag. 70, 1–14.

Cobbina, S.J., Chen, Y., Zhou, Z., Wu, X., Zhao, T., Zhang, Z., Feng, W.,Wang,W., Li, Q.,Wu, X., 2015. Toxicity assessment due to sub-chronic exposure to individual andmixtures of four toxic heavy metals. J. Hazard. Mater. 294, 109–120.

Azqueta, A., Collins, A.R. (2013). The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Archives of toxicology, 87(6), 949-968.

Cohen, R.A., Patel, A., Green, F.H., 2008. Lung disease caused by exposure to coal mine and silica dust. Semin. Respir. Crit. Care Med. 29, 651–661.

Coronado-Posada, N., Cabarcas-Montalvo, M., Olivero-Verbel, J., 2013. Phytotoxicity assessment of a methanolic coal dust extract in Lemna minor. Ecotoxicol. Environ. Saf. 95, 27–32.

Cyrys, J., Hochadel, M., Gehring, U., Hoek, G., Diegmann, V., Brunekreef, B., Heinrich, J., 2005. GIS-based estimation of exposure to particulate matter and NO2 in an urban area: stochastic versus dispersion modeling. Environ. Health Perspect. 113, 987–992.

da Silva, J., de Freitas, T.R., Heuser, V., Marinho, J.R., Bittencourt, F., Cerski, C.T.S., Kliemann, L.M., Erdtmann, B., 2000a. Effects of chronic exposure to coal in wild rodents (Ctenomys torquatus) evaluated by multiple methods and tissues. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 470, 39–51.

da Silva, J., de Freitas, T.R., Heuser, V.,Marinho, J.R., Erdtmann, B., 2000b. Genotoxicity biomonitoring in coal regions using wild rodent Ctenomys torquatus by comet assay and micronucleus test. Environ. Mol. Mutagen. 35, 270–278.

Dai, S., Li, D., Chou, C.-L., Zhao, L., Zhang, Y., Ren, D., Ma, Y., Sun, Y., 2008. Mineralogy and geochemistry of boehmite-rich coals: new insights from the Haerwusu Surface Mine, Jungar Coalfield, Inner Mongolia, China. Int. J. Coal Geol. 74, 185–202.

Dai, S., Zhou, Y., Ren, D., Wang, X., Li, D., Zhao, L., 2007. Geochemistry and mineralogy of the Late Permian coals from the Songzo Coalfield, Chongqing, southwestern China. Sci. China Ser. D Earth Sci. 50, 678–688.

Dalal, N.S., Newman, J., Pack, D., Leonard, S., Vallyathan, V., 1995. Hydroxyl radical generation by coal mine dust: possible implication to coal workers' pneumoconiosis (CWP). Free Radic. Biol. Med. 18, 11–20.

EPA, U., 2010. Basic Concepts in Environmental Sciences, Module 3: Characteristics of Particles. EPAhttp://www.epa.gov/apti/bces/module3/category/category.htm. EPA 7473, E.M., 2007. Mercury in Solids and Solutions by Thermal Decomposition, Amalgamation and Atomic Absorption Spectrophotometry. Environmental Protection Agency (EPA), p. 17.

Fang, T., Liu, G., Zhou, C., Yuan, Z., Lam, P.K.S., 2014. Distribution and assessment of Pb in the supergene environment of the Huainan Coal Mining Area, Anhui, China. Environ. Monit. Assess. 186, 4753–4765.

Fernandez, A., Ibanez, J., Llavona, M., Zapico, R., 2013. The Leaching of Aluminium in Spanish Clays, Coal Mining Wastes and Coal Fly Ashes by Sulphuric Acid. Essential Readings in Light Metals: Alumina and Bauxite. John Wiley & Sons, Inc, pp. 1098–1110.

Finkelman, R.B., Belkin, H.E., Zheng, B., 1999. Health impacts of domestic coal use in China. Proc. Natl. Acad. Sci. 96, 3427–3431.

Frandsen, F., Dam-Johansen, K., Rasmussen, P., 1994. Trace elements from combustion and gasification of coal—an equilibrium approach. Prog. Energy Combust. Sci. 20, 115–138.

Giannini, E.G., Testa, R., Savarino, V., 2005. Liver enzyme alteration: a guide for clinicians. Can. Med. Assoc. J. 172, 367–379.

Goetz, M.E., Luch, A., 2008. Reactive species: a cell damaging rout assisting to chemical carcinogens. Cancer Lett. 266, 73–83.

Goodarzi, F., Huggins, F., Sanei, H., 2008. Assessment of elements, speciation of As, Cr, Ni and emitted Hg for a Canadian power plant burning bituminous coal. Int. J. Coal Geol. 74, 1–12.

Graham, J.A., Gardner, D.E., Waters, M.D., Coffin, D.L., 1975. Effect of trace metals on phagocytosis by alveolar macrophages. Infect. Immun. 11, 1278–1283.

Guerrero-Castilla, A., Olivero-Verbel, J., Marrugo-Negrete, J., 2014. Heavy metals in wild house mice from coal-mining areas of Colombia and expression of genes related to oxidative stress, DNA damage and exposure tometals. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 762, 24–29.

Henkler, F., Brinkmann, J., Luch, A., 2010. The role of oxidative stress in carcinogenesis induced by metals and xenobiotics. Cancers 2, 376–396.

Heuser, V.D., da Silva, J., Moriske, H.J., Dias, J.F., Yoneama, M.L., de Freitas, T.R., 2002. Genotoxicity biomonitoring in regions exposed to vehicle emissions using the comet assay and the micronucleus test in native rodent Ctenomys minutus. Environ. Mol. Mutagen. 40, 227–235.

Holm, J., Palace, V., Wautier, K., Evans, R., Baron, C., Podemski, C., Siwik, P., Sterling, G., 2003. An assessment of the development and survival of wild rainbow trout (Oncorhynchus mykiss) and brook trout (Salvelinus fontinalis) exposed to elevated selenium in an area of active coal mining, The big fish bang. Proceedings of the 26th Annual Larval Fish Conference. Citeseer, pp. 22–26.

Humans, I.W.G.o.t.E.o.C.R.t., 2010. Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures. IARC monographs on the evaluation of carcinogenic risks to humans/World Health Organization. International Agency for Research on Cancer 92 p. 1.

Júnior, S.Á., Possamai, F., Budni, P., Backes, P., Parisotto, E., Rizelio, V., Torres, M., Colepicolo, P., Wilhelm Filho, D., 2009. Occupational airborne contamination in south Brazil: 1. Oxidative stress detected in the blood of coal miners. Ecotoxicology 18, 1150–1157.

Kania, N., Setiawan, B., Widjadjanto, E., Nurdiana, N., Widodo, M.A., Kusuma, H.C., 2014. Subchronic inhalation of coal dust particulate matter 10 induces bronchoalveolar hyperplasia and decreasesMUC5AC expression in maleWistar rats. Exp. Toxicol. Pathol. 66, 383–389.

Kasamoto, S., Masumori, S., Hayashi, M., 2013. In VivoMicronucleus Assay in Mouse Bone Marrow and Peripheral Blood, Genotoxicity Assessment. Springer, pp. 179–189.

Kawajiri, K., Fujii-Kuriyama, Y., 2007. Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor. Arch. Biochem. Biophys. 464, 207–212.

Keating, M., 2001. Cradle to Grave: The Environmental Impacts from Coal. Clean Air Task. Force, Boston (Disponible en: http://www.catf.us/resources/publications/files/Cradle_to_Grave.pdf).

Ketris, M., Yudovich, Y.E., 2009. Estimations of Clarkes for Carbonaceous biolithes: world averages for trace element contents in black shales and coals. Int. J. Coal Geol. 78, 135–148.

Krishna, G., Hayashi, M., 2000. In vivo rodent micronucleus assay: protocol, conduct and data interpretation. Mutat. Res. Fundam. Mol. Mech. Mutagen. 455, 155–166.

Leon, G., Perez, L.E., Linares, J.C., Hartmann, A., Quintana, M., 2007. Genotoxic effects in wild rodents (Rattus rattus and Mus musculus) in an open coal mining area. Mutat. Res. 630, 42–49.

León-Mejía, G., Espitia-Pérez, L., Hoyos-Giraldo, L.S., Da Silva, J., Hartmann, A., Henriques, J.A.P., Quintana, M., 2011. Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay. Sci. Total Environ. 409, 686–691.

Leon-Mejia, G., Quintana, M., Debastiani, R., Dias, J., Espitia-Perez, L., Hartmann, A., Henriques, J.A., Da Silva, J., 2014. Genetic damage in coal miners evaluated by buccal micronucleus cytome assay. Ecotoxicol. Environ. Saf. 107, 133–139.

Liu, T., Li, L., Fu, C., Liu, H., Chen, D., Tang, F., 2012. Pathological mechanisms of liver injury caused by continuous intraperitoneal injection of silica nanoparticles. Biomaterials 33, 2399–2407.

Lynch, E.C., 1990. Peripheral blood smear. In: Walker, H.K., Hall, W.D., Hurst, J.W. (Eds.), Clinical Methods: The History, Physical, and Laboratory Examinations, third ed. Butterworths, Boston, pp. 732–734.

MacGregor, J.T., Wehr, C.M., Gould, D.H., 1980. Clastogen-induced micronuclei in peripheral blood erythrocytes: the basis of an improved micronucleus test. Environ. Mutagen. 2, 509–514.

Mamurekli, D., 2010. Environmental impacts of coalmining and coal utilization in the UK. Acta Montan. Slovaca 15, 134–144.

Martin, J., Daniel, H., Le Bouffant, L., 1975. Short-and long-term experimental study of the toxicity of coal-mine dust and of some of its constituents. Inhaled Part. 4, 361–371.

Mishra, V.K., Upadhyay, A.R., Pandey, S.K., Tripathi, B., 2008. Concentrations of heavy metals and aquatic macrophytes of Govind Ballabh Pant Sagar an anthropogenic lake affected by coal mining effluent. Environ. Monit. Assess. 141, 49–58.

Mo, J., Wang, L., Au, W., Su, M., 2014. Prevalence of coal workers' pneumoconiosis in China: a systematic analysis of 2001–2011 studies. Int. J. Hyg. Environ. Health 217, 46–51.

Mughal, A., Vikram, A., Ramarao, P., Jena, G., 2010. Micronucleus and comet assay in the peripheral blood of juvenile rat: establishment of assay feasibility, time of sampling and the induction of DNA damage. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 700, 86–94.

Muto, E.Y., Soares, L.S., Sarkis, J.E., Hortellani, M.A., Petti, M.A., Corbisier, T.N., 2014. Biomagnification of mercury through the food web of the Santos continental shelf, subtropical Brazil. Mar. Ecol. Prog. Ser. 512, 55–69.

Niu, S., Gao, L., Zhao, J., 2015. Risk analysis of metals in soil from a restored coal mining area. Bull. Environ. Contam. Toxicol. 95, 183–187.

NRC, 1980. US National Research Council. Trace-Element Geochemistry of Coal Resource Development Related to Environmental Quality and Health. National Academy Press.

Olivero-Verbel, J., Roth, R.A., Ganey, P.E., 2011. Dioxin alters inflammatory responses to lipopolysaccharide. Toxicol. Environ. Chem. 93, 1180–1194.

Omland, Ø., Würtz, E.T., Aasen, T.B., Blanc, P., Brisman, J., Miller, M.R., 2014. Occupational chronic obstructive pulmonary disease: a systematic literature. Scand. J. Work Environ. Health 40, 19–35.

Pandey, B., Agrawal, M., Singh, S., 2016. Ecological risk assessment of soil contamination by trace elements around coal mining area. J. Soils Sediments 16, 159–168.

Petsonk, E.L., Rose, C., Cohen, R., 2013. Coal mine dust lung disease: new lessons from an old exposure. Am. J. Respir. Crit. Care Med. 187, 1178–1185.

Pinho, R.A., Bonatto, F., Andrades, M., Frota, M.L.C., Ritter, C., Klamt, F., Dal-Pizzol, F., Uldrich-Kulczynski, J.M., Moreira, J.C.F., 2004. Lung oxidative response after acute coal dust exposure. Environ. Res. 96, 290–297.

Pliarchopoulou, K., Voutsinas, G., Papaxoinis, G., Florou, K., Skondra, M., Kostaki, K., Roussou, P., Syrigos, K., Pectasides, D., 2012. Correlation of CYP1A1, GSTP1 and GSTM1 gene polymorphisms and lung cancer risk among smokers. Oncol. Lett. 3, 1301–1306.

Pott, F., Roller, M., Althoff, G., Rittinghausen, S., Ernst, H., Mohr, U., 2000. Lung tumors in rats after repeated intratracheal instillation of coal dusts. In: Heinrich, U., Mohr, U. (Eds.), Relationships between acute and chronic effects of air pollution. ILSI Press, International Life Sciences Institute, Washington, pp. 409–413.

Reza, S., Baruah, U., Singh, S., Das, T., 2015. Geostatistical and multivariate analysis of soil heavy metal contamination near coal mining area, Northeastern India. Environ. Earth Sci. 73, 5425–5433.

Rohr, P., da Silva, J., da Silva, F.R., Sarmento, M., Porto, C., Debastiani, R., dos Santos, C.E., Dias, J.F., Kvitko, K., 2013a. Evaluation of genetic damage in open-cast coal mine workers using the buccal micronucleus cytome assay. Environ. Mol. Mutagen. 54, 65–71.

Rohr, P., Kvitko, K., da Silva, F.R.,Menezes, A.P.S., Porto, C., Sarmento, M., Decker, N., Reyes, J.M., Allgayer, M.d.C., Furtado, T.C., 2013b. Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal.Mutat. Res. Genet. Toxicol. Environ. Mutagen. 758, 23–28.

Saïdi, S.A., Azaza, M.S., Windmolders, P., van Pelt, J., El-Feki, A., 2013. Cytotoxicity evaluation and antioxidant enzyme expression related to heavy metals found in tuna byproducts meal: an in vitro study in human and rat liver cell lines. Exp. Toxicol. Pathol. 65, 1025–1033.

Scaglia, N., Chisholm, J.W., Igal, R.A., 2009. Inhibition of stearoylCoA desaturase-1 inactivates acetyl-CoA carboxylase and impairs proliferation in cancer cells: role of AMPK. PLoS ONE 4, e6812.

Schlegel, R., MacGregor, J.T., Everson, R.B., 1986. Assessment of cytogenetic damage by quantitation of micronuclei in human peripheral blood erythrocytes. Cancer Res. 46, 3717–3721.

Schomaker, S., Warner, R., Bock, J., Johnson, K., Potter, D., VanWinkle, J., Aubrecht, J., 2013. Assessment of emerging biomarkers of liver injury in human subjects. Toxicol. Sci. 132, 276–283.

Shiber, J.G., 2005. Arsenic in domestic well water and health in central Appalachia, USA. Water Air Soil Pollut. 160, 327–341.

Smoliński, A., Rompalski, P., Cybulski, K., Chećko, J., Howaniec, N., 2014. Chemometric study of trace elements in hard coals of the upper silesian coal basin, Poland. Sci. World J. 2014.

Stansbury, R.C., Beeckman-Wagner, L.-A.F., Wang, M.-L., Hogg, J.P., Petsonk, E.L., 2013. Rapid decline in lung function in coal miners: evidence of disease in small airways. Am. J. Ind. Med. 56, 1107–1112.

Tanos, R., Murray, I.A., Smith, P.B., Patterson, A., Perdew, G.H., 2012. Role of the Ah receptor in homeostatic control of fatty acid synthesis in the liver. Toxicol. Sci. 129, 372–379.

Toscano, C.D., Guilarte, T.R., 2005. Lead neurotoxicity: from exposure to molecular effects. Brain Res. Rev. 49, 529–554.

Valasek, M.A., Repa, J.J., 2005. The power of real-time PCR. Adv. Physiol. Educ. 29, 151–159.

Wang, F., Wang, J., Liu, D., Su, Y., 2010. Normalizing genes for real-time polymerase chain reaction in epithelial and nonepithelial cells of mouse small intestine. Anal. Biochem. 399, 211–217.

Wang, J., Zhou, G., Chen, C., Yu, H.,Wang, T., Ma, Y., Jia, G., Gao, Y., Li, B., Sun, J., 2007. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. Toxicol. Lett. 168, 176–185.

Weilbauer, F., Sánchez, M., Posligua, P., 2009. Atlas de hematología. Primera edición. Sociedad Ecuatoriana de Hematología, Ecuador, pp. 20–23.

Wittkopp, S., Staimer, N., Tjoa, T., Stinchcombe, T., Daher, N., Schauer, J.J., Shafer, M.M., Sioutas, C., Gillen, D.L., Delfino, R.J., 2016. Nrf2-related gene expression and exposure

to traffic-related air pollution in elderly subjects with cardiovascular disease: an exploratory panel study. J. Expo. Sci. Environ. Epidemiol. 26(2), 141-149.
Xiang, W., Han, B., Zhou, D., Nzihou, A., 2012. Physicochemical properties and heavy metals leachability of fly ash from coal-fired power plant. Int. J. Mining Sci. Technol. 22, 405–409.
Yang, E.S., Nowsheen, S.,Wang, T., Thotala, D.K., Xia, F., 2011. Glycogen synthase kinase 3β inhibition enhances repair of DNA double-strand breaks in irradiated hippocampal neurons. Neuro-Oncology 13, 459–470.
Yin, X., Zhu, G., Li, X.B., Liu, S., 2009. Genotoxicity evaluation of chlorpyrifos to amphibian Chinese toad (Amphibian: Anura) by comet assay and micronucleus test. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 680, 2–6.
Zocche, J.J., da Silva, L.A., Damiani, A.P., Mendonça, R.Á., Peres, P.B., dos Santos, C.E.I., Debastiani, R., Dias, J.F., de Andrade, V.M., Pinho, R.A., 2014. Heavy-metal content and oxidative damage in Hypsiboas faber: the impact of coal-mining pollutants on amphibians. Arch. Environ. Contam. Toxicol. 66, 69–77.
Zocche, J.J., Leffa, D.D., Damiani, A.P., Carvalho, F., Mendonça, R.Á., Dos Santos, C.E.I., Boufleur, L.A., Dias, J.F., de Andrade, V.M., 2010. Heavy metals and DNA damage in blood cells of insectivore bats in coal mining areas of Catarinense coal basin, Brazil. Environ. Res. 110, 684-691.
Zúñiga-González, G.M., Gómez-Meda, B.C., de Lourdes Lemus-Varela, M., Zamora-Perez, A.L., Armendáriz-Borunda, J., Barros-Hernández, A., Sánchez-Díaz, A., Gallegos-Arreola, M.P., 2012. Micronucleated erythrocytes in preterm newborns exposed to phototherapy and/or oxygentherapy. J. Photochem. Photobiol. B Biol. 107, 79–83.

CONCLUSIONS, FINAL REMARKS, REFLECTIONS AND RECOMMENDATIONS

6. Conclusions

- Although in principle the environmental and health problems associated with coal mining seems exclusively linked to "typical" problems of pollution, either by exposure to an air loaded with high concentrations of particles, or drainage from the mining areas, according to studies conducted in different countries is equally true that, in general, people in areas of coal mining don't have a decent quality of life, as a rule they have high percentages of poverty, high morbidity for various diseases and very few are studying in a university, it is attributed to environmental degradation and observable socio-economic disadvantages of these populations aspects.

- Sediments from a tropical shoreline were chemically and toxicologically examined, finding that PAHs levels were greater in coal ports and sites of coal loading in vessels, as well as Cr, Cu, Pb and Zn account for most metal pollution in shoreline sediments.

- Sediment extracts from polluted sites induced gene expression markers xenobiotic metabolism (CYP1A1) and oxidative stress (NQO1).

- The aqueous extract coal dust with size less than 38 microns particle inhibited hatching of zebrafish embryos to the highest concentration, showing a behavior typical dose-response curve of endocrine disruptors. Which it is related to the nature of coal in terms of metal content. Similarly they were found changes in differential gene expression of related genes important cellular processes.

- Mice were exposed to coal dust-contaminated sand and mRNA Markers for PAH exposure, lipid metabolism and oxidative stress increased. In addition, ALT activity in plasma increased at the highest exposure to coal dust.

- Liver tissues of exposed mice showed steatosis and inflammation.

- Coal dust exposure produced changes in several blood components.

- The search for scientific knowledge about the adverse risks of coal mining on the environment and on the physical, mental and social dimensions of communities, will allow a comprehensive understanding of the whole context of the exploitation and use of coal, for so conscious and rational balance between economic gains and human health and environmental.

7. Reflections and Recommendations

- Today, the giant craters being formed with the development of opencast mining o are virtually lost territory for a long time. Environmental recovery is virtually impossible for decades, perhaps hundreds of years.

- The state should ensure that a large portion of the profits from the exploitation of coal is directly invested in improving the living conditions of the communities surrounding the mines. This action must be performed with the accompaniment of communities and strict control from the central government.

- It is imperative to establish the epidemiological and environmental status of coal mining areas in the country. Human studies should include morbidity and mortality associated with mining, workers and residents of the surrounding towns, as well as the establishment of its biochemical, cellular and tissue status.

- It is urgent that the health, environmental and mining authorities can establish clear regulations on the environmental conditions under which coal mining in Colombia is exercised, as well as the amount of particles generated during the mining process and to implement efficient controls relation to dust particles in the air breathed in the areas of influence of the coal mines, in addition to MP10, it is essential for progress in the regulation and the ability to control PM 2.5 emissions.

- Relocation plans populations in mining areas should be properly monitored and implemented promptly by the State. Arrears in these decisions increase extreme poverty and related social problems.

- The role of corporations in control and monitoring of pollutants from coal mining is invisible. In this sense, the communities do not have reliable and timely information on the quality of the environment in which they live.

- The loss or degradation of biodiversity in the areas of coal mining needs to be quantified both in biological and economic terms.