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PhD

Toxicity of the organic fraction of atmospheric particulate matter

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Abstract

Atmospheric particulate matter (PM) is a recognized risk factor for the global burden of disease in human populations and a widespread threat to all living organisms. PM is primarily emitted from combustion processes and comprises a significant fraction of organic compounds, such as polycyclic aromatic hydrocarbons (PAHs). In order to improve air quality strategies it is important to clarify the toxic potential of particulate samples of different characteristics and originated in different ambient conditions, as well as to investigate the inherent toxic mode of action of PM organic constituents. In addition, if specific tools could be developed to promptly discriminate the toxic potential of PM, based on the mode of action of its constituents rather than on PM mass concentration, which is currently used in air quality legislation, a more straightforward management approach could be accomplished. The main objective of this Thesis was to investigate the toxicity of the organic fraction of atmospheric PM and a cost-effective approach that could be easily applied to diagnose potential toxicity of exposure to this fraction. To tackle this objective, a general strategy was adopted, in which *in vitro* and *in vivo* assays and molecular tools were employed to assess biological effects elicited by different PM samples. The main toxicological assays employed in the present work were a yeast-based assay for dioxin-like activity (the aryl hydrocarbon receptor- recombinant yeast assay, AhR-RYA) and a zebrafish embryotoxicity test (ZET assay).

In Chapter 2 of this Thesis, organic extracts of particulate air samples of urban origin (Barcelona, Spain) were first screened using the AhR-RYA, and then tested with the ZET assay. The samples tested corresponded to a period of 14 months. Maximal dioxin-like activity values and phenotypical adverse effects were found for samples collected during late autumn months, correlating with high concentrations of PAHs. Vehicle- and fuel burning-related sources appeared as potentially most toxic, whereas total PM mass and mineral content appeared to be poor predictors of biological activity of the samples. Samples collected at different particle size cut-offs (10, 2.5, and 1 µm) did not differ significantly in dioxin-like PAH levels and biological activity, indicating that the sub-micron particle fraction (PM1) concentrated essentially the observed toxicity. Results of Chapter 2 support the need for a tighter control of sub-micron particle emissions and show that total PM mass and, particularly, PM10 and PM2.5, may not fully characterize the toxic potential of particulate air samples.

Chapter 3 investigated the toxic potential of urban and rural PM of different sizes and associations of observed effects with organic constituents of particles. Six PM size fractions with aerodynamic diameters ranging from >7.2 to <0.5 µm, collected in urban and rural sites during warm and cold periods of 2012/2013, were tested using the AhR-

RYA and ZET assays. Specific transcriptomic effects in fish embryos were analysed by microarray analysis and quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR). PM fraction <0.5 µm showed the highest biological activity, particularly in rural samples collected during the cold period. Transcriptomic analyses showed strong induction of the AhR signalling pathway (a.k.a. dioxin-like activity) for embryos exposed to both rural and urban extracts, correlating with PAH concentrations. Urban extracts, with contributions of traffic emissions and tobacco smoke, showed de-regulated oxidative stress-related genes, as well as pancreatic and eye-lens specific genes. Exposure to rural extracts of the cold period (high contribution of biomass burning emissions), affected genes implicated in basic cellular functions, in agreement with their strong embryotoxicity. The work presented in Chapter 3 shows that PM<0.5 µm concentrated biological and toxic activities linked to organic substances. Extracts from rural and urban samples elicited both common and specific transcriptome responses, suggesting different potentially adverse outcomes depending on PM source and composition. This study further allowed to identify a set of genes that could be useful to evaluate the submicron fraction of PM.

In chapter 4, daily and weekly patterns of biological activity were investigated, as well as their possible relation to secondary organic components of PM. Urban PM1 organic extracts were tested for dioxin-like activity (AhR-RYA), and general embryotoxicity (ZET) followed by analysis of the expression of gene markers of interest. PM1 samples were collected continuously for 12h periods during one month. This sampling scheme comprised day and night periods, weekdays and weekends. Dioxin-like activity of the extracts was linked to primary emissions from local traffic exhausts, reflecting weekday/weekend alternance. Expression levels of *cyp1a* and of gene markers for key cellular processes and development (*ier2, fos*) were also related to vehicle emissions, whereas expression of gene markers related to oxidative stress and endocrine effects (*gstal, hao1, ttr*) was strongly reduced in extracts of regional air masses with predominance of aged secondary organic species or remains of regional biomass burning emissions. Data from chapter 4 suggest that the toxic potential of PM1 organic fraction, and the biological activity of its chemical constituents, strongly depend on the emission sources and their proximity in relation to the process of aging from primary to secondary organic aerosols.

In Chapter 5, organic extracts of atmospheric PM samples obtained in different sub-basins of the Mediterranean and Black Seas were tested using the AhR-RYA and ZET assays, followed by quantitative analysis of the expression of genes of interest in exposed embryos. The dioxin-like activity correlated with the concentration of PAHs in the samples, as well as with toxic equivalent values (TEQs) predicted from their PAH

content. The ZET assay showed no major phenotypical adverse effects, whereas the expression of *cyp1a* and *fos,* among other genes, was up-regulated on treated embryos, following a geographic distribution similar to that of PAHs and dioxin-like activity. The analysis also showed a distinct geographical pattern of activation of pancreatic markers previously related to airborne pollution, probably indicating a different subset of uncharacterized PM constituents.

The work developed in the present Thesis indicates that the AhR-RYA and ZET assays could be useful and cost-effective for incorporation in the regulatory framework on ambient air quality. An approach towards effects-based analysis will allow accounting for toxic effects resulting from the complex mixture of compounds comprised in ambient PM, which may not be predicted by chemical analysis solely. Furthermore, when toxicity is detected at low concentration ranges, it allows studying the mode of action of PM organic constituents. Besides chemical composition and particle distribution, air quality assessment and management would improve if a third, biological, component for the early detection of systemic noxious effects of PM would be included, protecting human and environmental health.

Key-Words: Air pollution, Particles, Air quality, *Danio rerio*, Toxicogenomics, Aquatic Systems, Marine Environment

Resumo

A matéria particulada atmosférica (PM) é atualmente reconhecida como causadora de doença humana, assim como é uma ameaça generalizada para todos os organismos vivos. A PM é emitida principalmente a partir de processos de combustão e compreende uma fração significativa de compostos orgânicos, tais como os hidrocarbonetos aromáticos policíclicos (PAHs). A fim de melhorar as estratégias para a preservação da qualidade do ar é importante elucidar o potencial tóxico de amostras de partículas de características diferentes e originadas em diferentes condições ambientais, bem como investigar o modo de ação dos seus constituintes orgânicos. Além disso, é também importante o desenvolvimento de ferramentas específicas para discriminar o potencial tóxico de amostras de PM, com base no modo de ação dos seus constituintes e não na massa das partículas (atualmente utilizada na legislação da qualidade do ar). Estas permitiriam uma avaliação de efeitos destas amostras quimicamente complexas, fornecendo uma abordagem mais vantajosa para a gestão da qualidade do ar. Esta Tese teve por objetivos principais investigar a toxicidade da fração orgânica de partículas atmosféricas, assim como uma abordagem de baixo custo para o diagnóstico da toxicidade resultante da exposição a esta fração. Para concretizar estes objetivos, adotou-se uma estratégia geral baseada na utilização de ensaios *in vitro* e *in vivo,* e no uso de ferramentas moleculares, para avaliar os efeitos biológicos induzidos por diferentes amostras de PM. Os principais ensaios toxicológicos utilizados no presente trabalho foram um ensaio de determinação da atividade tipo dioxina utilizando leveduras (ensaio de levedura recombinante para o recetor de hidrocarboneto aromático ou arilo, AhR-RYA) e o ensaio de embriotoxicidade com o peixe-zebra (ZET).

No Capítulo 2 desta Tese extratos orgânicos de amostras de partículas de ar urbano foram testadas usando o AhR-RYA e o ensaio ZET. As amostras testadas foram recolhidas em Barcelona, Espanha, durante um período de 14 meses. Os valores máximos de atividade tipo dioxina e de efeitos adversos observados no ZET foram encontrados em amostras recolhidas no final do outono. Estes resultados apresentaram correlação com concentrações elevadas de PAHs. Fontes de PM relacionadas com a combustão de fuel óleo revelaram-se como sendo potencialmente mais tóxicas. Por outro lado, a massa total e o conteúdo mineral da PM parecem ser pobres preditores da atividade biológica das amostras. Foram também testadas amostras de PM de diferentes tamanhos (diâmetro de corte de 10, 2.5, e 1 µm), as quais não mostraram diferenças significativas entre si, relativamente aos níveis de PAHs e de atividade biológica. Estes resultados sugeriam que a fração mais fina das partículas (PM1) concentra essencialmente toda a atividade observada. Os resultados do capítulo 2

suportam a necessidade de um controlo mais apertado sobre as emissões das partículas mais finas e mostra que a massa total da PM e, em particular, PM10 e PM2.5, podem não caracterizar plenamente o potencial tóxico de amostras de matéria particulada atmosférica.

No Capítulo 3 investigou-se o potencial tóxico de PM de meio urbano e meio rural, de diferentes tamanhos, procurando-se associações dos efeitos observados com os constituintes orgânicos das partículas. Para tal, extratos orgânicos de PM pertencente a seis frações de tamanho diferente (>7.2 a <0.5 µm de diâmetro), recolhidas em meio urbano e meio rural durante as épocas quente e fria de 2012/2013, foram testados utilizando o AhR-RYA e o ensaio ZET. Alterações do transcriptoma de embriões de peixe-zebra induzidas pela exposição a estas amostras foram analisadas com a tecnologia de "microarrays" e por reação quantitativa em cadeia da polimerase via transcriptase reversa (RT-qPCR). A fração PM <0.5 µm apresentou a maior atividade biológica, em particular nas amostras de inverno do meio rural. A análise do transcriptoma mostrou uma forte indução da via de sinalização AhR (atividade tipo dioxina) nos embriões expostos aos extratos do meio rural e urbano, correlacionada com as concentrações de PAHs. A PM do meio urbano, influenciada pelo tráfego e fumo de tabaco, desregulou especificamente genes relacionados com o stress oxidativo, assim como genes relacionados com a função pancreática e com o cristalino ocular. A exposição a extratos de PM recolhidos no inverno no meio rural (elevada contribuição de combustão de biomassa) alterou a expressão de genes envolvidos em funções celulares básicas, em concordância com a elevada embriotoxicidade induzida por estas mesmas amostras. O trabalho apresentado no Capítulo 3 mostrou que a PM <0.5 µm concentrava a atividade biológica e tóxica associada a substâncias orgânicas. Os extratos de amostras do meio rural e urbano induziram respostas do transcriptoma comuns, mas também respostas específicas de cada meio, sugerindo que a origem e composição da PM pode induzir diferentes efeitos adversos. Este estudo permitiu ainda identificar um conjunto de genes de interesse para a avaliação da fração de menor tamanho da PM.

No capítulo 4, investigaram-se os padrões diários e semanais de atividade biológica e a sua relação com compostos orgânicos secundários da PM. Extratos orgânicos de PM1 de origem urbana foram testados para a atividade tipo dioxina (AhR-RYA), e embriotoxicidade geral (ZET), seguindo-se a análise da expressão de genes marcadores de interesse. Amostras de PM1 (PM<1 µm) foram recolhidas continuamente por períodos de 12h, durante um mês. Este esquema de amostragem compreendeu os períodos diurno e noturno, assim como dias da semana e de fim de semana. A atividade tipo dioxina dos extratos mostrou-se associada às emissões primárias do trafego local,

refletindo a alternância dia da semana/fim de semana. Os níveis de expressão do *cyp1a* e de genes marcadores relacionados com processos celulares vitais e de desenvolvimento (*ier2, fos*) mostraram-se também relacionados com o tráfego, enquanto que a expressão de genes marcadores relacionados com a resposta oxidativa e com efeitos endócrinos (*gstal, hao1, ttr*) mostrou-se fortemente reduzida em extratos influenciados por massas de ar regionais com espécies orgânicas secundarias envelhecidas ou com remanescentes da combustão de biomassa. Os dados do capítulo 4 sugerem que o potencial tóxico da fração orgânica da PM1, e a atividade biológica dos seus constituintes, dependerá fortemente das fontes de emissão e da sua proximidade em relação ao processo de envelhecimento das partículas primárias a secundárias.

No Capítulo 5, os extratos orgânicos de amostras atmosféricas de PM de diferentes sub-bacias dos mares Mediterrâneo e Negro foram testados utilizando os ensaios AhR-RYA e ZET seguido de análise quantitativa da expressão de genes de interesse nos embriões expostos. Foram encontradas correlações entre a atividade tipo dioxina e a concentração de PAHs nas amostras, e os valores equivalentes tóxicos (TEQ) calculados a partir das concentrações de PAHs. No ensaio ZET não se observaram alterações fenotípicas, no entanto em embriões expostos foi induzida a expressão de *cyp1a* e *fos,* entre outros genes. Esta indução seguiu um padrão geográfico de distribuição pela área em estudo semelhante à distribuição das concentrações de PAHs e à atividade tipo dioxina medida pelo AhR-RYA. A análise efetuada mostrou também que a ativação de genes ligados à função pancreática, previamente relacionados com a poluição particulada do ar, seguiu um padrão geográfico de ativação distinto, provavelmente indicando um subconjunto diferente de constituintes da PM ainda não caracterizados.

O trabalho desenvolvido na presente Tese de doutoramento mostrou o AhR-RYA e o ZET como ensaios potencialmente úteis para a análise efetiva da toxicidade da PM, para incorporação na regulamentação da qualidade do ar. Uma abordagem focada nos seus efeitos, permitindo também estudar o modo de ação dos constituintes orgânicos da PM, possibilitaria ter em consideração os efeitos tóxicos causados pelas misturas complexas de compostos químicos que constituem a PM. Além da composição química e da distribuição de partículas, a avaliação da qualidade do ar beneficiaria da inclusão de uma terceira componente, biológica, permitindo a deteção precoce dos efeitos sistêmicos nocivos da PM e protegendo a saúde humana e ambiental.

Palavras-chave: Poluição do ar, Partículas, Qualidade do ar, *Danio rerio*, Toxicogenómica, Sistemas Aquáticos, Ambiente Marinho

Scientific Publications

This thesis integrates the scientific publications listed below.

Articles in international peer reviewed journal in the Science Citation Index

Mesquita, S.R., van Drooge, B.L., Barata, C., Vieira, N., Guimarães, L., Piña, B., 2014. Toxicity of atmospheric particle-bound PAHs: an environmental perspective. Environmental Science and Pollution Research. 21, 11623-11633.

Mesquita, S.R., van Drooge, B.L., Reche, C., Guimaraes, L., Grimalt, J.O., Barata, C., Piña, B., 2014. Toxic assessment of urban atmospheric particle-bound PAHs: Relevance of composition and particle size in Barcelona (Spain). Environmental Pollution. 184, 555-562.

Mesquita, S.R., van Drooge, B.L., Oliveira, E., Grimalt, J.O., Barata, C., Vieira, N., Guimarães, L., Piña, B., 2015. Differential embryotoxicity of the organic pollutants in rural and urban air particles. Environmental Pollution. 206, 535-542.

Mesquita, S.R., Dachs J., van Drooge, B.L., Castro-Jiménez J., Barata, C., Vieira, N., Guimarães, L., Piña, B., 2016. Toxicity assessment of atmospheric particulate matter in the Mediterranean and Black Seas open waters. Science of the Total Environment. 545–546, 163-170.

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Dataset

Mesquita S.R., van Drooge B.L., Oliveira E., Grimalt J.O., Barata C., Vieira N., Guimaraes L., Piña B. (2015) Toxicogenomic analysis of rural and urban air particles. Expression profiling by array. NCBI's Gene Express Omnibus database, Series record GSE53522.

Communications in international scientific meetings

- Oral

Mesquita, S.R., van Drooge, B.L., Guimarães, L., Piña, B. (2013) Toxic assessment of particle-bound PAHs: a novel environmental perspective. 14th EuCheMS International Conference on Chemistry and the Environment (ICCE 2013), Barcelona, Spain, June 25-28, 2013. Oral presentation (Ref. OT2).

Mesquita, S.R., van Drooge, B.L., Oliveira, E., Grimalt, J.O., Barata, C., Vieira, N., Guimarães, L., Piña, B. (2015) Toxicogenomic analysis of ambient Particle Matter (PM) from rural and urban environments. SETAC Europe, 25th Annual Meeting (SETAC EU 2015), Barcelona, Spain, 3-7 May. Oral presentation (Ref. 624).

- Poster

Mesquita S.R., van Drooge B.L., Casado M., Guimarães L., Barata C., Piña B. (2014) Effects directed analysis of urban air pollution: influence of composition and particle size. SETAC Europe, 24th Annual Meeting (SETAC EU 2014), Basel, Switzerland, May 11-15. Poster presentation (Ref. WE163).

Mesquita, S.R., Dachs, J., van Drooge, B.L., Barata, C., Vieira, N., Guimarães, L., Piña, B. (2015) Biological activity and transcriptomic alterations induced by particle-bound Polycyclic Aromatic Hydrocarbons from the Mediterranean and Black Seas atmosphere. SETAC Europe, 25th Annual Meeting (SETAC EU 2015), Barcelona, Spain, 3-7 May. Poster presentation (Ref. MO101).

Mesquita, S.R., van Drooge, B.L., Oliveira, E., Barata, C., Vieira, N., Guimarães, L., Piña, B. (2015) Adverse Outcome Pathways affected on zebrafish embryos exposed to rural and urban atmospheric particles. PRIMO 18th International Symposium (PRIMO18 2015), Trondheim, Norway, 24-27 May. Poster presentation (Abstract book, page 229).

Mesquita, S.R., van Drooge, B.L., Barata, C., Vieira, N., Guimarães, L., Piña, B. (2016) Effect-directed analysis of atmospheric particulate matter. SETAC Europe, 26th Annual Meeting (SETAC EU 2016), Nantes, France, 22-26 May. Poster presentation (Ref. WE136).

Table of Contents

Index of Tables

Chapter 2 - Toxic assessment of urban atmospheric particle-bound PAHs: relevance of composition and particle size in Barcelona (Spain)

Chapter 3 - Differential embryotoxicity of the organic pollutants in rural and urban air particles

Chapter 4 - Influence of primary and secondary aerosol constituents on the toxicity of urban particulate matter

Chapter 5 - Toxicity assessment of atmospheric particulate matter in the Mediterranean and Black Sea open waters

Chapter 6 - General Discussion

Index of Figures

Chapter 1 - General Introduction

Abbreviations

AhR	Aryl hydrocarbon receptor
ahrra	Aryl-hydrocarbon receptor repressor a
AhR-RYA	Aryl hydrocarbon receptor - recombinant yeast assay
ANOVA	analysis of variance
Ant	Anthracene
AP-1	Activator protein-1
ARE	Antioxidant response element
ARNT	AhR nuclear translocator
BaA	Benzo[a]anthracene
BaP	Benzo[a]pyrene
BaPeq	Benzo[a]pyrene equivalents
BB	Biomass burning
BbF	Benzo[b]fluoranthene
BbjF	Benzo[b+j]fluoranthene
BDS	Butadiene soots
BeP	Benzo[e]pyrene
BghiPer	Benzo[ghi]perylene
BkF	Benzo[k]fluoranthene
CAA	Clean air act
CAT	Catalase
cDNA	complementary DNA
ClPAHs	Chlorinated Polycyclic aromatic hydrocarbons
Cor	Coronene
Cp	Amplification curves
Cp_{tg}	Cp values of target genes
Cry	Chrysene
Cry/Tri	Chrysene + triphenylene
Ct	Threshold cycle
ctrb1	Chymotrypsinogen B1
CYP	Cytochrome P450- monooxygenases
CYP1A	Cytochrome P4501A
dBA	Dibenzo[a,h]anthracene
DCAs	Dicarboxylic acids
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
EC	Elemental carbon
EC_{50}	50% effect concentration
EEA	European environmental agency
ela2	Elastase 2
emp2	Epithelial membrane protein 2
EU	European Union
EU-28	28 countries of the European Union
FF	Fossil fuel
Fla	Fluoranthene
fos	FBJ murine osteosarcoma viral oncogene homolog
GO	Gene ontology
GPX	Glutathione peroxidases
gpx	glutathione peroxidase
gst	glutathione S-transferase
gstal	Glutathione S-transferase, alpha-like
hao1	Hydroxyacid oxidase 1
HKGs	Housekeeping genes
hmox1	heme oxygenase 1
HMW PAHs	High molecular weight PAHs
Hop	Hopanes
hpf	Hours post fertilization
ICP-AES	Inductively coupled plasma atomic emission spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
ier2	Immediate early response 2
Ind	Indeno[123-cd]pyrene
IsoOXI	Products of isoprene oxidation
klf2a	Kruppel-like factor 2a
LC-MS	Liquid chromatography–mass spectrometry
LDH	Lactate dehydrogenase
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NAAQS	National ambient air quality standards
NH_3	Ammonia
Nic	Nicotin

NO_2	Nitrogen dioxide
NO_x	Nitrogen oxides
nqo1	NAD(P)H dehydrogenase, quinone 1
Nrf2	Nuclear factor, erythroid 2 (also known as nfe2l2a)
O_3	Ozone
OC	Organic carbon
OH·	Hydroxyl radical
PAHs	Polycyclic aromatic hydrocarbons
PC	Principal components
PCA	Principal component analyses
PCBs	Polycholorinated biphenyls
PCDD/Fs	Polychlorinated dibenzo-p-dioxins and dibenzofurans
PCR	Polymerase chain reaction
Per	Perylene
PFTE	Polytetrafluoroethylene
Phe	Phenanthrene
PinOXI	Products of α-pinene oxidation
PLS	Partial least square analysis
PM	Particulate matter
PM1	Particles with equivalent aerodynamic diameter < 1 μm
PM10	Particles with equivalent aerodynamic diameter < 10 μm
PM2.5	Particles with equivalent aerodynamic diameter < 2.5 μm
POA	Primary organic aerosols
POPs	Persistent Organic Pollutants
prdx1	peroxiredoxin 1
Pyr	Pyrene
qPCR	Quantitative Polymerase Chain Reaction
Res. & Com.	Residential/commercial
Ret	Retene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSD	Relative standard deviation
RT	Reverse transcriptase
RT-qPCR	Quantitative Reverse-Transcription Polymerase Chain Reaction
RYA	Recombinant yeast assay
SE Med	South-East Mediterranean Sea

SO_2	Sulphur dioxide
SOA	Secondary organic aerosol
SOH	Semivolatile aromatic hydrocarbons
SO_x	Sulphur oxides
SPM	Suspended particulate matter
sst2	Somatostatin 2
sultb6b1	sulfotransferase 6b1
TEFs	Toxic equivalency factors
TEQs	Toxicity equivalents
TrafficW	Traffic intensity with wind correction
ttr	Transthyretin
txnrd1	thioredoxin reductase 1
UFPs	Ultra-fine particles
USA	United States of America
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
VOCs	Volatile organic compounds
W Med	Western Mediterranean Sea
WHO	World health organisation
ZET	Zebrafish embryotoxicity test

CHAPTER 1

General Introduction

Chapter adapted from:

Mesquita, S.R., van Drooge, B.L., Barata, C., Vieira, N., Guimarães, L., Piña, B., 2014. Toxicity of atmospheric particle-bound PAHs: an environmental perspective. Environmental Science and Pollution Research. 21, 11623-11633.

1.1. Particulate Air Pollution

Poor air quality and human disease have been linked since antiquity. Indoor air pollution, from cooking and heating with open fires in poorly ventilated dwellings, is considered to have increased the risk of illness and death in ancient societies (Mosley, 2012; Montgomerie, 2013). For example, studies with mummified lung tissues from Egypt, have associated anthracosis[1], irritation and blackening of the lungs, with the combustion of fuel for cooking, heating and illumination (Walker et al., 1987; Mosley, 2012; Montgomerie, 2013). The combustion of biomass releases particulate matter (PM) to ambient air, among other pollutants. Interestingly, recent research has shown the co-presence of anthracosis and pre-mortem PM in ancient lung tissues of Egyptian mummies (Montgomerie, 2013).

In medieval times, coal was shipped to European cities and burned by tradesman in workshops, in case of wood shortage (Mosley, 2012). Around the thirteenth century, the burning of coal in London attracted criticism from citizens and made King Edward I issue a royal proclamation to reduce coal burning, because it annoyed its inhabitants and raised concern about “their bodily health” (Mosley, 2012). Nonetheless, in the sixteenth century, with the continuous growth of the population, non-fossil fuels became scarce and very expensive. Therefore, by the seventeenth century, more consumers made the transition from wood and charcoal to fossil-fuel combustion (Mosley, 2012). With the Industrial Revolution (between 1760 and 1840), the rapid urban-industrial growth made air pollution, from the burning of fossil fuels, become a major environmental problem worldwide.

Despite the rapid growth unleashed by the Industrial Revolution, sound notion that exposure to particulate air pollution could cause adverse health effects only entered the world's consciousness in the twentieth century. In Belgium, between December 1 and 5, 1930, a thick fog covered the area of the Meuse Valley and hundreds of people started to have severe respiratory symptoms (Nemery et al., 2001). More than 60 people died in three days. The event shocked the country and abroad, and the deadly fog received considerable media attention (Nemery et al., 2001). In Donora (south-western Pennsylvania, United States of America, USA), from October 27 to 30, 1948, a meteorological thermal inversion and the emission of air pollutants from metal works, coal-fired homes, cook ovens and industries resulted in 20 excess deaths by October 30, and left a third of the town's population ill (Bell and Davis, 2001; Peterman, 2009). The hospitals were full and the town had to convert the community centre into a morgue

[1] Deposition of carbon in the lungs from inhaled smoke or coal dust.

(Peterman, 2009). From December 5 to 9, 1952, a dense “smog” containing sulphur dioxide (SO_2) and PM covered London (England), resulting in over 3000 deaths over three weeks (a death rate more than three times the normal for that period). It also lead to 12000 excess deaths from December 1952 through February 1953 (Figure 1, Bell and Davis, 2001). Subsequent research compared daily mortality and PM concentrations registered on the 4-day period preceding the episode, with the data for the 4-day period ending on December 9. Researchers found an increased relative risk of mortality of 1.06 per each 100 $\mu g.m^{-3}$ increase in total suspended matter. The mean increase in PM concentrations between those two periods was 1200 $\mu g.m^{-3}$ (Schwartz, 1994). These episodes made clear that very high levels of PM can cause large increases in the daily mortality rate.

Figure 1 - Nelson's Column during the London Smog of 1952.

Since that time, several studies have reported associations between daily mortality and air pollution in many locations, suggesting the importance of airborne particles, rather than other components of air pollution (e.g. SO_2) to the adverse effects observed (Mazundar et al., 1982; Fairley, 1990; Schwartz, 1991; Dockery et al., 1992; Pope et al., 1992; Schwartz and Dockery, 1992; Schwartz, 1994; Calvo, 2013). In addition to mortality, acute respiratory symptoms and/or illness, increased need for asthma medication and school absences started to be associated with particulate exposure (Dockery et al., 1989; Pope, 1991; Schwartz et al., 1991; Pope and Dockery, 1992). With this, in the late twentieth century, confounding factors were weighted and

causality between exposure to particulate air pollution and a full range of adverse health outcomes, including mortality, was clearly established (Schwartz, 1994).

1.1.1. Legislation

As a result of the Meuse Valley, Donora and London episodes, and following research studies, regulatory efforts were made. In 1970, the Clean Air Act (CAA) was the first major American regulatory effort to study and set limits on air pollution, ultimately defining the National Ambient Air Quality Standards (NAAQS) for six air pollutants, including PM (USEPA, 2015). In the European Union (EU), the European Community' programmes of action of 1973 and 1977 determined that priority was to be given to measures against SO_2 and suspended particulates (EU, 1980). The follow-up was the Council Directive 80/779/EEC of 15 July 1980, which established air quality limit and guide values for those components of air pollution (EU, 1980). From the 70's onward, air quality standards for PM have been successively refined (USEPA, 2015a; EU, 1999). In addition to the protection of human health, the regulatory directives started to mention the need to protect also the "environment as a whole", from the adverse effects of particulate air pollution (EU, 1999).

With the developed legislation and measures for air quality improvement, the emissions of some air pollutants have been reduced, but particulate air pollution prevails as a major hazard (Figure 2, WHO, 2013; EEA, 2014). At the present time, PM is considered by the regulatory agencies as one of the most harmful air pollutants to human health (WHO, 2004; EEA, 2013). Recently particles were classified as carcinogenic by IARC (WHO, 2013). The World Health Organization (WHO) estimated that particulate air pollution is responsible for 0.80 million premature deaths and 6.4 million years of life lost, per year worldwide (WHO, 2002; WHO, 2013). These estimates consider only the impact of air pollution on mortality, but if morbidity would be considered the total burden would be much higher (WHO, 2002, WHO, 2013).

Nowadays, air quality guidelines discriminate PM by its size, namely in PM10 and PM2.5, referring to particles smaller than 10 and 2.5 µm in aerodynamic diameter, respectively (more details about sizes of particles are provided below). In the EU, the limit values for PM10 entered into force in 2005 (40 $\mu g.m^{-3}$ and 50 $\mu g.m^{-3}$, per year and per day, respectively). For PM2.5 only a target value of 25 $\mu g.m^{-3}$ per year has been defined in January 2010, and an exposure concentration obligation of 20 $\mu g.m^{-3}$ per year, to be attained by 2015, was fixed (EEA, 2014). The long term objective is to reduce PM2.5 concentrations to 8.5-18 $\mu g.m^{-3}$ per year until 2020. All these measurements refer to the PM mass.

Interestingly, WHO cautioned that even in the event of full compliance with the existing limit and target values, substantial health impacts of PM would remain (EEA, 2014). WHO defines stricter air quality guidelines than the EU air quality standards, namely 20 $\mu g.m^{-3}$ and 50 $\mu g.m^{-3}$, for PM10 per year and per day, respectively, and 10 $\mu g.m^{-3}$ and 25 $\mu g.m^{-3}$, of PM2.5 per year and per day, respectively (Figure 2, EEA, 2014). These guideline values should be considered as an acceptable and achievable objective to minimise health effects. However, no threshold has been defined for PM below which damage to health is not observed (EEA, 2014).

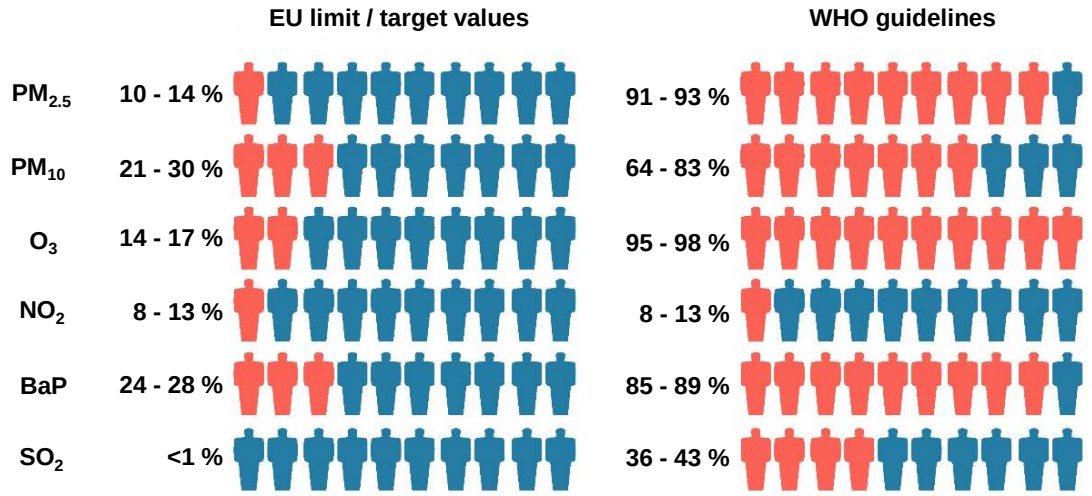

Figure 2 - Percentage of the urban population in the European Union (EU) 28 countries (EU-28) exposed to concentrations of air pollutants above EU and World Health Organization (WHO) reference levels for the period 2010–2012 (from EEA, 2014). The pollutants are ordered in terms of their relative risk for health damage, with the highest first (EEA, 2014). Up to 30% of Europeans living in cities are exposed to air pollutant levels exceeding the EU air quality standards, a value that reaches 95% accordingly to WHO more stringent guidelines (EEA, 2014). PM2.5 and PM10 refer to particles smaller than 2.5 and 10 µm, respectively, O_3, ozone; NO_2, nitrogen dioxide; BaP, benzo[a]pyrene; SO_2, sulphur dioxide.

1.1.2. Characteristics of particles

PM is defined as tiny particles of liquids or solids suspended in a gas, containing a complex mixture of chemical compounds, such as acids, organic chemicals, metals and dust. During the combustion processes, the chemical constituents of particles can distribute internally or on the particulate surface, so that particles will have a core and a shell with different constituents.

PM can be directly emitted to the atmosphere (primary PM), or formed in the atmosphere (secondary PM). Primary PM originates from natural (e.g. sea salt, dust, pollen, wildfires) or anthropogenic sources (e.g. fuel combustion from thermal power generation, incineration, domestic heating for households, fuel combustion for vehicles,

as well as wear of vehicle tyres and brakes, EEA, 2014). While primary particles are directly emitted from sources, secondary particles are formed in the atmosphere by gas-particle conversion processes (e.g. nucleation, condensation, Hallquist et al., 2009). The main precursor gases for secondary PM are ammonia (NH_3), SO_2, nitrogen dioxide (NO_2) and volatile organic compounds, VOCs (EEA, 2014).

The particles suspended in the atmosphere can be divided accordingly to their size into coarse particles (particles with aerodynamic diameter smaller than 10 μm) and fine particles (particles with aerodynamic diameter smaller than 2.5 μm, Figure 3). Ultra-fine particles, also known as UFPs have an aerodynamic diameter smaller than 0.1 μm.

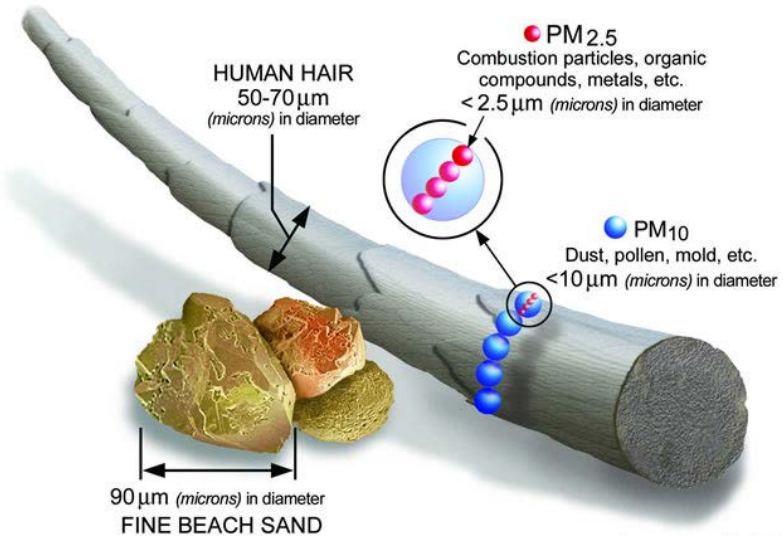

Figure 3 – Size illustration of coarse (PM10) and fine (PM2.5) particles (from United States Environmental Protection Agency).

In research studies, unless mentioned otherwise, generally PM10 corresponds to a cut-off, meaning that it contains ultrafine, fine and coarse fractions. In an environmental sample, the number and surface area of particles increases as their aerodynamic diameter decreases, but the particulate mass generally decreases with decreasing particle diameter (Figure 4, Anderson et al., 2012). Therefore, as an example, in a PM10 sample the numerical majority of particles would be ultrafine, but these would be a small portion of the sample's total PM mass (Figure 4, Anderson et al., 2012).

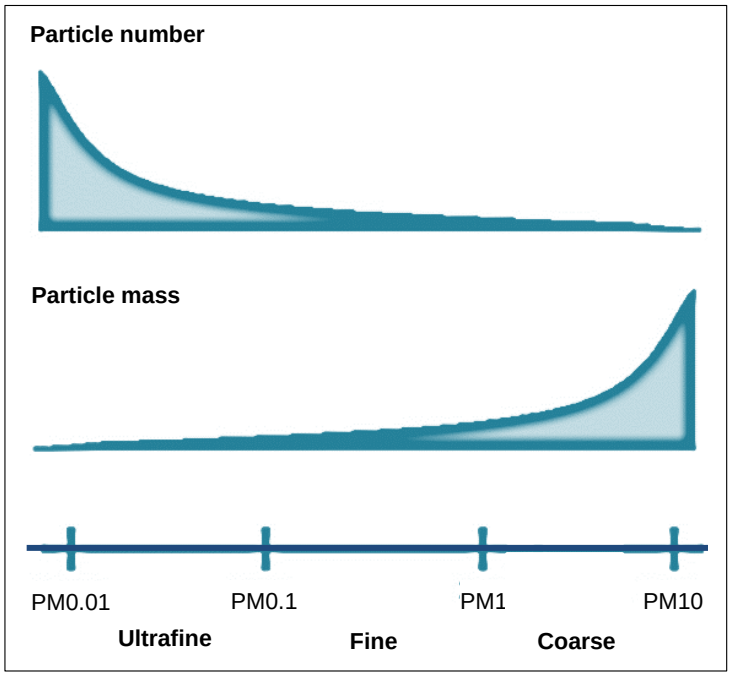

Figure 4 - Illustration of particle size distribution. PM0.01, particles with aerodynamic diameter smaller than 0.01 µm, PM0.1, particles with aerodynamic diameter smaller than 0.1 µm, PM1, particles with aerodynamic diameter smaller than 1 µm, PM10, particles with aerodynamic diameter smaller than 10 µm.

Coarse particles are typically originated from mechanical shearing and dust, while fine and ultrafine particles are in produced primarily from fuel combustion and biomass burning. Combustion processes tend to originate PM with carbonaceous cores that adsorb different organic toxicants, like polycyclic aromatic hydrocarbons (PAHs), contributing to the strong toxic potential of submicron-sized particles (de Kok et al., 2005; Zhou et al., 2005). Additionally, from a physiological point of view, while coarse particles deposit in the respiratory tract, smaller particles penetrate further in the lungs into the alveolar region, ultimately reaching the bloodstream (Squadrito et al., 2001). Therefore, considering that PM mass is the standard parameter in air quality regulations and that particulate mass generally decreases with decreasing particle diameter, there may be a lack of control on the toxic potential of the smallest particles.

The source of particles also influences their emission levels, composition and, consequently, their toxic potential (van Drooge and Ballesta, 2009; Olivares et al., 2011; Dergham et al., 2012). According to the EEA, household fuel combustion dominates the emissions of primary PM2.5 and PM10, with increasing emissions since 2003 of 11% and 13 %, respectively (Figure 5, EEA, 2014). The second-largest sources of emission of primary PM2.5 and PM10 are the transport and industry sectors, respectively (Figure 5, EEA, 2014). Traffic emissions are probably the PM source that has been more constantly associated with detrimental effects on human health (Kelly and Fussell, 2012).

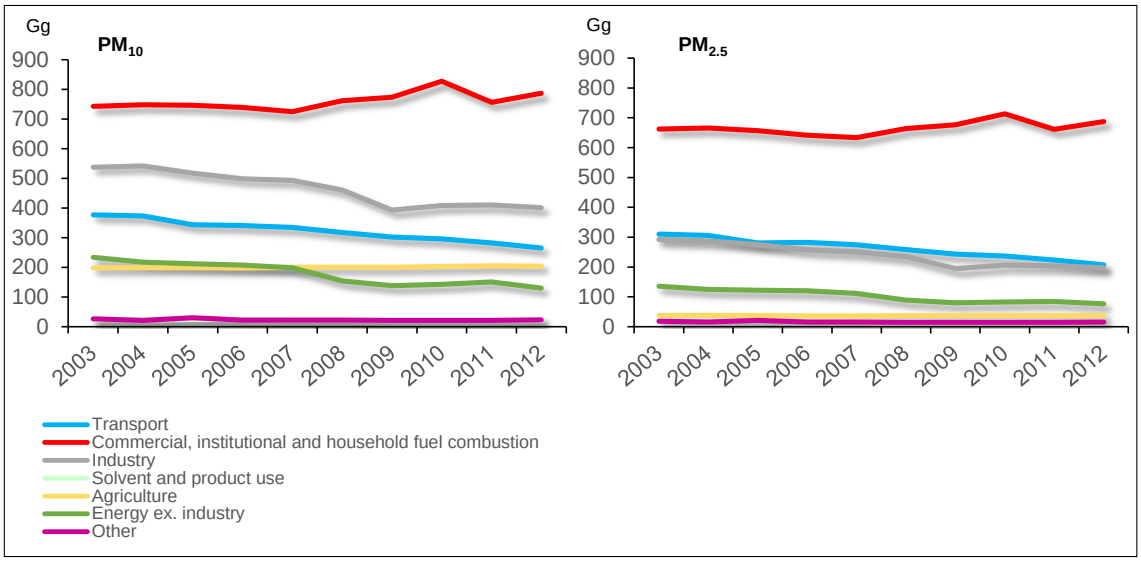

Figure 5 – Contributions of different source sectors to EU-28 emissions from 2003 until 2012 (Gg/year = 1000 tonnes/year) (from EEA, 2014).

1.1.3. PM toxicity

PM has been consistently and independently related to the most severe effects on human health, including lung cancer and cardiopulmonary mortality (WHO, 2002; Cohen et al., 2005; Anderson et al., 2012; EEA, 2014). The health effects observed in humans exposed to particulate air pollution can be divided into at least two main groups. Through direct contact, inhaled particles can induce several adverse reactions in the human trachea, bronchi and lungs (Cohen et al., 2005; Anderson et al., 2012). On the other hand, exposure to PM can induce different systemic effects, such as cardiovascular disease, neuronal impairments and neurological diseases, as well as diabetes, poor birth outcomes and health effects on neonates after prenatal exposure (Peters and Pope, 2002; Jedrychowski et al., 2004; Miller et al., 2004; Hertz-Picciotto et al., 2005; Jedrychowski et al., 2005; Calderón-Garcidueñas et al., 2008; MohanKumar et al., 2008, Wegener 2002; Cesaroni et al., 2013; Balti et al., 2014; EEA, 2014; Eze et al., 2014; Fleischer et al., 2014). Although part of these later effects could be attributable to secondary reactions started in the lungs, evidence suggests that they could result from direct action of PM toxic constituents in target tissues, either by translocation of ultra-fine particles to extrapulmonary organs, or by de-adsorption and systemic distribution through the bloodstream of particle constituents (Oberdorster et al., 2002).

Intensive research has been dedicated to understanding the biological mechanisms behind severe health effects induced by PM exposure, that ultimately lead to increased morbidity and premature mortality (de Kok et al., 2006; Pope et al., 2009; Anderson et al.; Abbas et al., 2013). It has been shown that PM induces several types

of cellular toxic effects, such as oxidative damage, enhancement of inflammatory response, cytotoxicity, genotoxicity and mutagenicity (de Kok et al., 2005; de Kok et al., 2006; Billet et al., 2008; Cavanagh et al., 2009; Abbas et al., 2010; André et al., 2010; Steenhof et al., 2011; Dergham et al., 2012). However, characterization of atmospheric PM needs further research on the properties of particles and on the toxicity of their constituents. Some of the adverse effects of PM have been attributed to its content in PAHs (de Kok et al., 2005; Abbas et al., 2009, Abbas,2013; Cavanagh et al., 2009). For example, in the highly industrialized city of Dunkerque, France, very low doses of PAH-coated onto PM2.5 were found to be involved in PM cytotoxicity to the A549 cell line[2] and in the production of deoxyribonucleic acid (DNA) bulky stable adducts in human alveolar macrophage cells (Billet et al., 2007; Abbas et al., 2010; Abbas et al., 2013).

Research studies have also described the association between prenatal exposure to airborne PAHs and adverse health effects on newborns, such as immune system alterations, aromatic-DNA adducts formation, increased frequency of gene mutations, respiratory impairments at later developmental stages and affected cognitive development (Perera et al., 2002; Miller et al.; Hertz-Picciotto et al., 2005; Jedrychowski et al., 2005; EEA, 2014). Even though the latest European Environmental Agency (EEA) report stated that prenatal exposure to airborne PAHs may adversely affect newborns (EEA, 2014), the standard regulations for PAHs are focused only on a benzo[a]pyrene (BaP) target value of 1 $ng.m^{-3}$ (WHO, 2013; EC, 2015), with no available date to become a limit value (EC, 2015). The WHO defined a BaP guideline value of 0.12 $ng.m^{-3}$, meaning that 85-89% of the European population is exposed to concentrations of BaP above it (Figure 2, EEA, 2014).

1.2. Atmospheric Particle-bound PAHs

PAHs result from the incomplete combustion of carbonaceous materials. Due to their mutagenic, carcinogenic and teratogenic properties, they represent a risk for all living organisms (IARC, 1998; EC, 2000). PAHs have been recurrently emitted from natural sources (e.g. biomass burning, volcanoes), but anthropogenic inputs from fossil fuel-burning, motor vehicle exhaust, waste incineration, home heating, oil refinery, among others, have increased exponentially since the industrial revolution (Srogi, 2007). Estimations indicate that global atmospheric emissions of United States Environmental Protection Agency (USEPA) 16 priority PAHs reached 520 giga-grams per year in the

[2] Derived from a type II-like human alveolar epithelium carcinoma.

first decade of the XXI century, with biofuel (60%), fossil fuel for transportation (13%) and wildfires (11%) pointed as major emission sources (Zhang and Tao, 2009; Shen et al., 2013).

1.2.1. PAHs in the atmosphere

Most PAHs are emitted into the atmosphere from combustion or volatilization processes (Figure 6). Fuel combustion is considered to be the most important emission source (EEA, 2014). Emission factors for PAHs depend on the fuel, the type of combustion and on ambient conditions (Galarneau, 2008). This originates a large variation in emitted PAHs and ambient air compositions. Nevertheless, there are indications that gasoline fuelled engines emit relatively more high molecular weight PAHs (HMW PAHs) than diesel vehicles (Zielinska et al., 2004). In comparison to these vehicle emissions, wood combustion emits more HMW PAHs (Schauer et al., 2001; Zhang and Tao, 2009).

Once emitted into the atmosphere, PAHs distribute in the gas phase and in the particulate phase, due to their semi-volatile character. Vapour-pressure of PAHs and atmospheric conditions, determine their behaviour in the atmosphere (Lohmann and Lammel, 2004). In general, low molecular weight (MW), volatile species (2-3 rings) remain in the gas phase, while heavier relatives tend to bind to PM (Mumtaz et al., 1996; Boström et al., 2002¸ EC, 2001). Semi-volatile 4-ring PAHs (like fluoranthene, Fla, or pyrene, Pyr) are equally distributed between both phases, depending on ambient air temperature and on the carbonaceous composition of the particles. Since PAHs have affinity to the PM carbonaceous core, they are mainly found on submicron sized particles (Miguel et al., 1998; de Kok et al., 2005; Zhou et al., 2005).

Once in the atmosphere PAHs are subjected to long-range transport, deposition, decomposition by physico-chemical transformations, and reactions with other molecules (Figure 6).

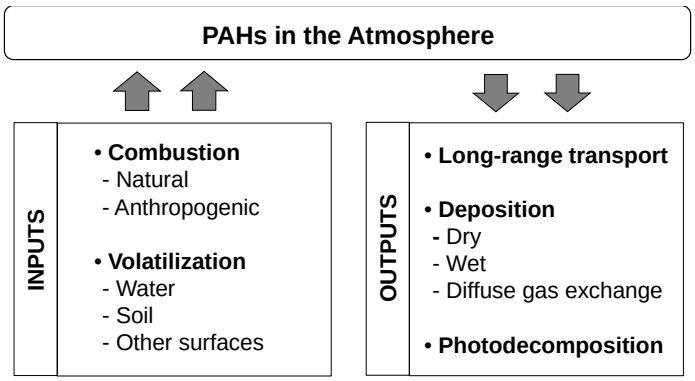

Figure 6 - Schematic diagram of PAH inputs and outputs from the atmosphere (adapted from Maliszewska-Kordybach et al. 1999).

1.2.2. Degradation and reaction with other molecules

Depending on the size and structure of the PAH and ambient conditions, PAHs become degraded by exposure to ultraviolet (UV) light, following several degradation pathways (Nielsen et al., 1983). Generally, low MW PAHs are more susceptible to direct or indirect photo-degradation than higher MW relatives (Brubaker and Hites, 1998). Hydroxyl radical (OH·) attack is the most important photo-degradation pathway for PAHs in the atmospheric gas phase, but reaction with NO_2 is also a substantial degradation pathway (Wild and Jones, 1995; Brubaker and Hites, 1998; Esteve et al., 2004; 2006). Heavier PAHs are more photo-resistant than volatile ones, due to higher chemical stability, but especially due to their tendency to adsorb onto carbonaceous particles, which reduces their exposure to light and oxidants {Mumtaz, 1996).

As mentioned, PAHs react with other co-pollutants, such as nitrogen oxides (NO_x), sulphur oxides (SO_x), O_3, chloride, free radicals and their reaction products (Nielsen et al., 1983; Arey et al., 1987; Ohura, 2007). Nitroarenes, are nitro-substituted derivatives of PAHs (arenes). These can be formed by atmospheric reaction of gas-phase PAHs with oxidants, through direct nitration during combustion processes or by heterogeneous gas–particle interaction of PAHs adsorbed onto particles with nitrating agents (IPCS, 2003). Nitroarenes are present in ambient air in the gas and particulate phases. Increasing interest has been paid to oxygenated derivatives of PAHs, particularly quinones. Quinones may be released into the atmosphere directly through the combustion of biomass processes (e.g. diesel exhaust, wood burning), or may be formed also through reaction of parental PAHs with atmospheric oxidants (OH·, NO_3 and O_3, Cho et al., 2004; Delgado-Saborit et al., 2013). Similarly to nitroarenes, quinones are present in ambient air in the gas and particulate phases.

Overall, the atmosphere does not represent a containerized storage for anthropogenic PAHs, but it rather acts as a “transporter, diluter, and reactor” for these compounds (Wild and Jones, 1995). With this, the complex mixture of pollutants comprised in air particles has an inherent toxic potential distinct from what could be expected for individual compounds.

1.2.3. Toxicity

The toxic potential of medium to high MW PAHs is very well established. There are eight PAHs typically considered as carcinogens by different regulatory bodies: benzo[*a*]anthracene (BaA), chrysene (Cry), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), BaP, dibenzo[*a,h*]anthracene (dBA), indeno[1,2,3-

cd]pyrene (Ind) and benzo[*ghi*]perylene (BghiPer, Menzie et al., 1992; WHO/IPCS., 1998). Given their properties, these compounds are expected be coated onto PM, rather than remaining in the gas phase.

Metabolic activation of PAHs

Many studies show that the toxicological effects of PAHs are strongly dependent on their metabolic activation into primary and secondary metabolites, during the organism detoxification process, whereas the toxicity of parental compounds is considered marginal and unspecific (Yan, 1985; Shimada and Fujii-Kuriyama, 2004). Figure 7 illustrates the most commonly accepted mechanism for PAHs metabolism and genotoxicity in a typical vertebrate cell. In general, most PAHs enter into the cell cytoplasm and bind to the Aryl hydrocarbon Receptor (AhR). The activation of such receptor leads to the transcription of target genes, namely genes of the Cytochrome P450- monooxygenase (CYP) family, such as CYP1A. The correspondent CYP enzymes are synthesised and will be responsible for the metabolic activation of most PAHs. The primary function of the CYP system is to transform poorly water soluble lipophilic substances into more water soluble ones, and therefore more readily excreted. However, PAH metabolites can be arbitrarily divided into water soluble groups, which can be easily excreted, and organosoluble groups such as phenols, dihydrodiols, hydroxymethyl derivatives, quinones, and epoxides. Through the catalytic activity of epoxide hydrolase, epoxide intermediates can be converted into the highly reactive diol-epoxides (Figure 7). Diol-epoxides are known mutagenic, carcinogenic and teratogenic agents (Jerina et al., 1980; Amos et al., 1992). These metabolites bind to and disrupt DNA and ribonucleic acid (RNA), leading to tumour formation and genotoxic effects (IARC, 1983, Figure 7). The described system enters into a vicious circle in which the presence of the PAH not only increases the amount of the diol-epoxides, but also multiplies the enzymatic activity responsible for their production (Nebert et al., 1993; Hankinson, 1995; Gonzalez and Fernandez-Salguero, 1998; Shimizu et al., 2000, Figure 7).

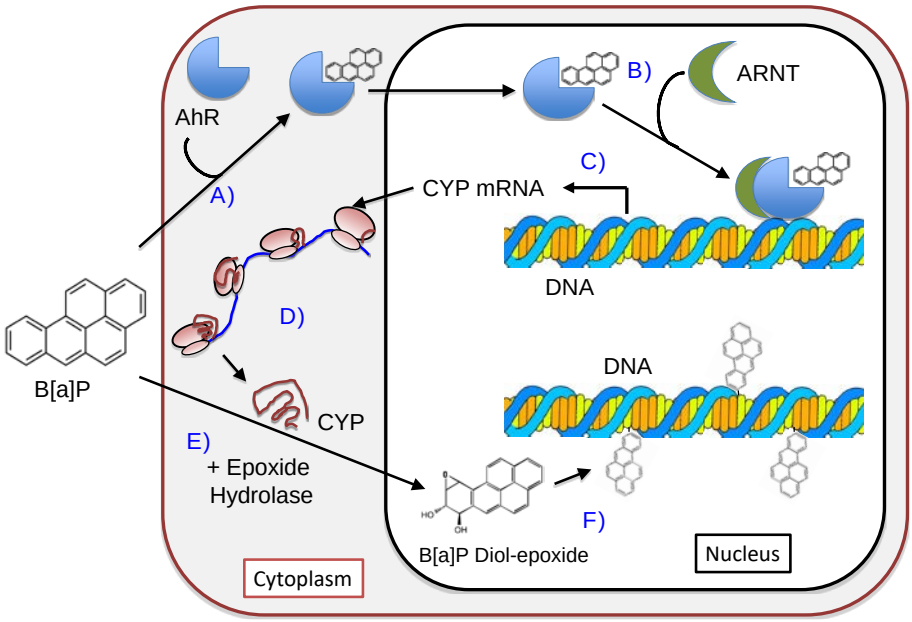

Figure 7 - Illustration of the most commonly accepted mechanism for polycyclic aromatic hydrocarbons (e.g. Benzo[a]Pyrene, BaP) metabolism and genotoxicity in a typical vertebrate cell. BaP enters into the cytoplasm and binds to the aryl hydrocarbon receptor, AhR (A). The complex enters into the nucleus, and associates to the AhR nuclear translocator (ARNT). This tertiary complex binds to specific DNA sequences on the promoter of regulated genes (B), namely genes of the Cytochrome P450- monooxygenases (CYP) family, such as CYP1A. The correspondent messenger ribonucleic acid (mRNA) is transcribed (C) and migrates to the cytoplasm, where it will serve as template for the synthesis of the corresponding proteins (D). CYP enzymes are able to chemically activate BaP into an epoxide, which is the substrate for the epoxide hydrolase (E). This enzyme catalyses the formation of dihydrodiols, which can be further activated by CYP into the highly toxic diol-epoxides. These metabolites bind to deoxyribonucleic acid (DNA), leading to mutagenic adducts formation (F), among other toxic effects.

In vitro versus in vivo studies

Several single-cell based assays, mainly with human cell lines, yeast strains or bacteria have been used to assess toxic effects of air particles or of its constituents (Cavanagh et al., 2009; Olivares et al., 2011; Turóczi et al., 2012). Cavanagh and co-workers (2009) showed that the organic content of New Zealand urban PM samples induced more toxic effects in cultured macrophages than its water soluble components (including metals). These effects were significantly correlated with the PAH content of samples. On the other hand, Bonetta and colleagues (2009) observed that the PAH content of PM seemed to have a genotoxic effect on human lung cells, whereas the presence of metals caused oxidative damage. This is in agreement with other studies that emphasize the contribution of metals to the overall PM toxicity (Bonetta et al., 2009; Steenhof et al., 2011; Dergham et al., 2012). However, these studies and, in general, most of air pollution toxicity research, rely on *in vitro* assays, which often overlook or minimize the role of detoxifying enzymes and other physiological parameters, essential for an accurate

assessment of PM toxicity. This gains particular importance in the case of PAHs due to the fact that they are metabolized *in vivo* into more soluble, but also more toxic, substances than the parental compounds. *In vitro* studies have yielded vital information on CYP regulation and function, helping to understand the PAHs mechanism of action, as well as the chemical reactions involved. However, the complex pharmacokinetics, route administration, absorption and renal clearance of a PAH *in vivo*, as well as the tissue/organ-specific CYP induction, may contribute to increase the toxicity of different PAHs, an aspect that cannot be extrapolated from *in vitro* studies (Nebert, 2006; Arlt et al., 2008). Therefore, in order to assess the toxic potential of the organic content of PM or of particle-bound PAHs, *in vivo* assays should be considered.

PAHs reaction products

In addition to the toxicity of the parental compounds, PAHs atmospheric transformation products are ubiquitous components of particulate matter, which have also been reported as highly toxic.

Nitroarenes are known *in vivo* carcinogens and genotoxic agents (Möller et al., 1990; Möller, 1994; IPCS, 2003). The metabolism of nitroarenes is complex, with at least five activation pathways that lead to mutations and genotoxic effects described both *in vivo* and *in vitro*, in bacterial and mammalian systems (Kielhorn et al., 2003). *In vivo*, cytochrome 450 enzymes are involved in their metabolism, possibly through AhR activation (Kielhorn et al., 2003). Metabolic activation of nitroarenes is another example of how endogenous detoxification mechanisms may exacerbate the toxicity of some compounds.

Whereas nitroarenes have been intensively studied and their environmental presence and risk reported (Nielsen et al., 1983; Kielhorn et al., 2003), the toxicological relevance of other particle-bound PAH products, in environmental conditions is still unclear, and their potential toxic risk largely unknown. Regarding chlorinated PAHs (ClPAHs), *in vitro* assays have shown that these compounds have AhR-mediated activity, inducing adverse effects such as mutagenicity, tumorigenicity and oncogene activation, in some cases severer than the correspondent parental PAH (Ohura, 2007).

Research studies have emphasised the overlooked importance of quinones on PM toxicity (Chung et al., 2006; Li et al., 2012). It has been hypothesised that quinones contribute to the adverse health effects caused by PM because of their high redox potency in producing reactive oxygen species (ROS, Li et al., 2012). The production of ROS by quinones has been shown to induce concentration-dependent cellular viability decrease, cellular lactate dehydrogenase (LDH) release, DNA damage in lung epithelial

cells and cause significant cell death in macrophages (Shang et al., 2012; Shang et al., 2014).

1.2.4. Toxic burden to aquatic biota

The latest commission directive addresses the need to protect the environment from adverse effects of air pollution, but mainly focus on eutrophication and acidification induced by deposition of sulphur and nitrogen compounds (EEA, 2014). Concerning airborne PAHs the information available is that BaP is considered toxic to aquatic life and birds, and with potential to bioaccumulate in invertebrates. As for PM, the EEA recognizes that it "can affect animals in the same way as humans; affect plant growth and ecosystem processes, can cause damage and soiling of buildings; and reduces visibility". Nonetheless, unlike for other atmospheric pollutants (i.e. NO_2, SO_2, O_3), no critical level, target/limit value or long-term objective, intended to protect terrestrial and aquatic organisms, has been defined for PM nor for airborne PAHs (EEA, 2014).

In order to properly evaluate the biological impact of PM, not only the effects on human health should be addressed, but also on terrestrial and aquatic environments (Jurado et al., 2004; Sheesley et al., 2005). In particular, through atmospheric deposition or soil run-off, the aquatic environment works as a natural sink to these air pollutants.
At a global scale, the net atmosphere-ocean diffuse exchange and dry deposition of PAHs is of 0.091 $Tg.month^{-1}$, which is four-fold the total PAHs inputs from the Deep Horizon oil spill[3] (Gonzalez-Gaya et al., 2016). Even though atmospheric deposition is one of the major sources of PAH contamination of aquatic systems (Franz et al., 1998; Arzayus et al., 2001; Jurado et al., 2004; Gonzalez-Gaya et al., 2016), there is a lack of information about the toxicity of particle-bound PAHs to aquatic species, as emphasised previously (Sheesley et al., 2004; 2005).

Atmospheric transport and aquatic deposition

PAHs are relatively persistent compounds that may travel long distances before they deposit on terrestrial and aquatic environments, either as vapours or adsorbed onto particles. For this reason, PAHs are covered by the Persistent Organic Pollutant (POPs)-Protocol under the United Nations Economic Commission for Europe's Convention on Long Range Transboundary Air Pollution (EC, 2001). These compounds are found even in remote Earth places, such as high mountain areas, the Antarctic Ocean or the Arctic

[3] Largest oceanic accidental oil spill that occurred in the Gulf of Mexico in 2010

atmosphere (Cripps, 1992; Ding et al., 2007; van Drooge et al., 2010). The significance of soot carbon for atmospheric transport of particle-bound PAHs has been assessed and is held to be responsible for long-range transport of these compounds (Lohmann and Lammel, 2004).

Dry or wet deposition and gas flux are the main processes of PAHs removal from the atmosphere. Particle-bound PAHs can be removed through dry deposition (i.e. gravitational settling), while more volatile species undergo diffuse gas exchange with the aquatic/terrestrial compartments (Franz et al., 1998; Maliszewska-Kordybach, 1999; Jurado et al., 2004). Wet deposition (scavenging by rain, snow, fog) can be relevant for PAHs in both gas and particulate phases (Franz et al., 1998; Maliszewska-Kordybach, 1999; Jurado et al., 2004). For example, 23-60% of the total input of atmospheric PAHs from United Kingdom and northeast USA is deposited directly in the ground and water surfaces, with greater importance for heavier particle-bound PAHs (Simonich and Hites, 1994; Wild and Jones, 1995; Maliszewska-Kordybach, 1999).

This intrusion of atmospheric PAHs into the aquatic systems (streams, rivers, seas and oceans) occurs mainly by atmospheric deposition and by diffuse gas exchange between the atmospheric boundary layer and water surface (Figure 8, Franz et al., 1998; Gigliotti et al., 2002; Nizzetto et al., 2008). Additionally, PAH-rich urban runoffs may also contribute to the load of aquatic systems with airborne PAHs (Manoli and Samara, 1999). PAH emissions from urban/industrial sources significantly affect coastal and inland surface waters, as well as open seas and oceans (Franz et al., 1998; Manoli and Samara, 1999; Gigliotti et al., 2002; Nizzetto et al., 2008). Gigliotti and co-workers (2002) observed that in the New York-New Jersey estuary the profiles of PAH concentrations in the water dissolved and particulate phases were significantly similar to the PAHs profile in the atmospheric gas and particulate phases, demonstrating the close coupling of air-water compartments (Gigliotti et al., 2002). In agreement, other studies have provided evidence that the atmospheric input of PAHs can be the most relevant source of these contaminants to estuaries and other riverine and freshwater systems (Franz et al., 1998; Arzayus et al., 2001; Patrolecco et al., 2010).

For the marine environment there are still major gaps about the factors controlling the occurrence of PAHs, particularly in non-coastal areas (Marinov et al., 2009; Berrojalbiz et al., 2011). However, it is generally acknowledged that coastal inputs have negligible importance in the overall loads of open sea and ocean waters, while atmospheric exchange and vertical sinking are the main drivers of PAHs fate (Tsapakis et al., 2006; Marinov et al., 2009; Berrojalbiz et al., 2011). In the Atlantic Ocean (to the Northwest of African coast), PAHs concentrations and profiles in the water were consistent with the correspondent atmospheric ones, suggesting the occurrence of

strong atmospheric deposition/air-water exchange of these compounds (Nizzetto et al., 2008). Recently, González-Gaya and collaborators (2015) made the first global assessment of the occurrence and atmosphere-ocean flux of 64 PAHs and semivolatile aromatic hydrocarbons (SOH, Gonzalez-Gaya et al., 2016). The authors confirmed the important role that diffuse fluxes and dry deposition of PAHs and SOHs play at a global scale, in the overall atmosphere-ocean exchange of organic compounds. The relevance of the continuous emission of PAHs, mainly due to fossil fuel combustion, to the Earth chemosphere, carbon cycle and ecotoxicological effects is largely unknown (Gonzalez-Gaya et al., 2016).

In addition to sinking in the aquatic systems, a major fraction of PAHs also undergoes volatilization, dissolution, biotic and abiotic degradation, and uptake and accumulation by the biota (Figure 8, Manoli and Samara, 1999; Tsapakis et al., 2006).

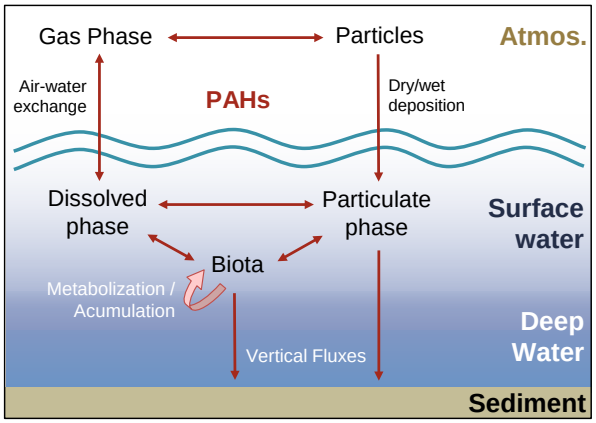

Figure 8 – General schematic representation of physical and biogeochemical processes affecting the fate of polycyclic aromatic hydrocarbons (PAHs), from atmosphere (Atmos.) to aquatic systems.

In aquatic systems, like in the atmosphere, the fate of PAHs depends on their physicochemical properties. Due to their low aqueous solubility and hydrophobic nature, PAHs bind to suspended particulate matter (SPM) or remain dissolved in the water phase (Manoli and Samara, 1999), eventually depositing in the aquatic sediment (Figure 8, Lipiatou et al., 1997; Tsapakis et al., 2003). In general, concentrations of PAHs in the water dissolved phase are low, but higher concentrations can be found in SPM and sediments (Manoli and Samara, 1999; Patrolecco et al., 2010). Total particulate PAH concentrations in the Seine River and estuary (France) ranged from 2 to 687 ng.l^{-1}, while PAH concentrations in the dissolved phase where one order of magnitude lower (Fernandes et al., 1997). Occurrence of PAHs in different matrices (water, SPM, sediment, and common eels) of an urban stretch of the River Tiber (Italy) was investigated by Patrolecco and colleagues (2010). SPM was also found to be the most

polluted matrix (Patrolecco et al., 2010). Some evidences also suggest that airborne particle-bound PAHs concentrate mostly in aquatic SPM and sediments. Indeed, in the estuary of the Chesapeake Bay (USA), PAHs concentrations in sediments correlated with the atmospheric dry deposition fluxes (Arzayus et al., 2001). Interestingly, common eels also sampled in the Chesapeake Bay were found to be contaminated with PAHs, and the PAHs bioaccumulation factors reflected the sediment PAH concentrations (Arzayus et al., 2001). The authors therefore considered that atmospheric deposition of particle-bound PAHs onto the Chesapeake Bay watershed would be contributing to potential toxic effects of these compounds in common eels. However, the fate and transformations of airborne PM in the aquatic environment needs further research, to better understand and evaluate the real exposure of aquatic organisms and its associated risk.

In the marine environment, concentrations of PAHs in particles are driven by air-water-particle interactions and modulated by the biogeochemical cycles operating in the water column (Berrojalbiz et al., 2011). Although, PAH profiles in the dissolved phase are dominated by low MW species (Dachs et al., 1997; Tsapakis et al., 2006; Nizzetto et al., 2008; Berrojalbiz et al., 2011), heavier particle-bound PAHs may also be present in significant proportions. For example, in a study developed in the Biscay Bay (Northeast Atlantic Ocean), particle-bound PAHs represented 41% of the total PAHs present in the water, with relatively high concentrations of 5 to 6-ring PAHs, like BkF, BaP, BghiPer and coronene (Cor, Nizzetto et al., 2008).

Aquatic toxicity data

Although the information available on this subject is still scarce, some studies have shown that atmospheric PM extracts can induce mutagenic effects on aquatic organisms, such as bacteria and algae, with good correlations with the PAH content of PM (Jaffé et al., 1993; Kim Oanh et al., 2002; Lin and Chao, 2002; Sheesley et al., 2004).

An important progress has been accomplished by Sheesley and co-workers (2004, 2005) who have emphasised the need to broaden the scope of toxicity studies of atmospheric PM to include potential adverse effects on aquatic organisms. Aquatic bioassays with freshwater organisms *Selenastrum capricornutum* (green algae) and *Ceriodaphnia dubia* (microcrustacean) were used to study the toxicity of ambient PM from the Lake Michigan (USA, Sheesley et al., 2004; 2005). PM extracts with high PAH concentrations were highly toxic to both organisms, inhibiting algal grow and reducing survival, respectively (Sheesley et al., 2005). The observed toxicity closely followed the fluctuations in PAH content of PM extracts (Sheesley et al., 2005).

Diamond and colleagues (2000) studied the toxicity of the organic film formed on the exterior surface of urban windows using zebrafish embryos. The authors suggested that the organic film (a combination of accumulated atmospheric organic constituents and deposited particles) would have implications as a transportation route of urban air contaminants to receiving waters, potentially affecting aquatic organisms (Diamond et al., 2000). Exposure of developing zebrafish embryos to the organic extract of a film sample induced cardiovascular, hematopoietic, neural crest related, and behavioural defects (Diamond et al., 2000).

Olivares and collaborators (2013) assessed the developmental effects in zebrafish embryos of atmospheric PM from a semi-rural area in Italy and coal burning particles. Exposure of embryos to the organic extract of PM samples, significantly induced CYP1A gene expression (Olivares et al., 2013). Exposure to extracts of coal burning particles resulted in embryo deformities, which included pericardial edema and malformations of the mouth and the spinal cord. The results correlated well with single-cell based assays for the AhR activation and with the PAH content of samples.

Regarding PAHs transformation products, data on the toxicity of arenes or quinones to aquatic organisms is scarce and the reported effect concentrations are not environmentally relevant (Kielhorn et al., 2003). Nonetheless, when evaluating the adverse effects of PM organic extracts, the transformation products of PAHs, and in general other extractable biologically active compounds, may also be contributing to the observed effects.

1.3. The Zebrafish as a model organism

Danio rerio is a cyprinid fish also known as zebrafish for the uniform, pigmented horizontal stripes on the side of the body, resembling the stripes of a zebra. It is autochthonous in some South Asian countries.

Zebrafish is considered a unique species for research, with respect to the level of knowledge, technology and approaches available (Westerfield, 2000). In particular, this species is considered an excellent model for studying the early life stages of vertebrates, since it has *ex utero* development, great transparency during embryogenesis and larval stages, and it is able to reproduce during the whole year, with high offspring number per brood. In addition, performing laboratorial assays with zebrafish embryos is in agreement with legal requisites and social tendency to replace conventional animal testing. Indeed, zebrafish embryos are not considered experimental

animals under the current European legislation, only zebrafish larvae with autonomous feeding are encompassed in the legal definition (EU, 2010).

With this, zebrafish has served as a highly informative model for human development, disease and pharmacology (Goldsmith, 2004; Xu and Zon, 2010). It has also a wide range of applications environmental sciences, where in particular, it is successfully used to elucidate the mode of action of pollutants (Scholz et al., 2008).

1.3.1. Zebrafish embryonic development

Zebrafish developmental stages are very well characterized and follow a strict temporal pattern (Kimmel et al., 1995). After fertilization, the zygote enter synchronous cell cycles, leading to the formation of a high-piled cell mass, the blastocyst, about 3.3 hours post fertilization (hpf, Figure 9). Later, around 5.25 hpf, the blastocyst turns into a cup-shaped cell multilayer of uniform thickness, starting the epiboly (Figure 9). Development continues with the beginning of the gastrula period where morphogenetic cell movements will produce the primary germ layers and the embryonic axis, completely engulfing the central yolk cell around 10 hpf (Figure 9). At the segmentation period, the somites develop, rudimental primary organs become visible, the tail bud becomes prominent and the embryo elongates (around 17.5 hpf, Figure 9). From 24 until 48 hpf the embryo is called pharingula because it possesses the classical vertebrate bauplan (Kimmel et al., 1995). The body axis straightens, the heart starts beating, pigmentation appears and the fins begin to develop. From 48 to 72 hpf morphogenesis of primary organ systems is complete and the embryo hatches. At 72 hpf the mouth of the early larva protrudes anteriorly, the swim bladder inflates and the larva has active avoidance behaviours. From 96 to 120 hpf the larva begins to swim actively and moves its jaws, opercular flaps, pectoral fins and eyes. These developments will eventually lead to swift escape responses, improved respiration and prey seeking (Kimmel et al., 1995).

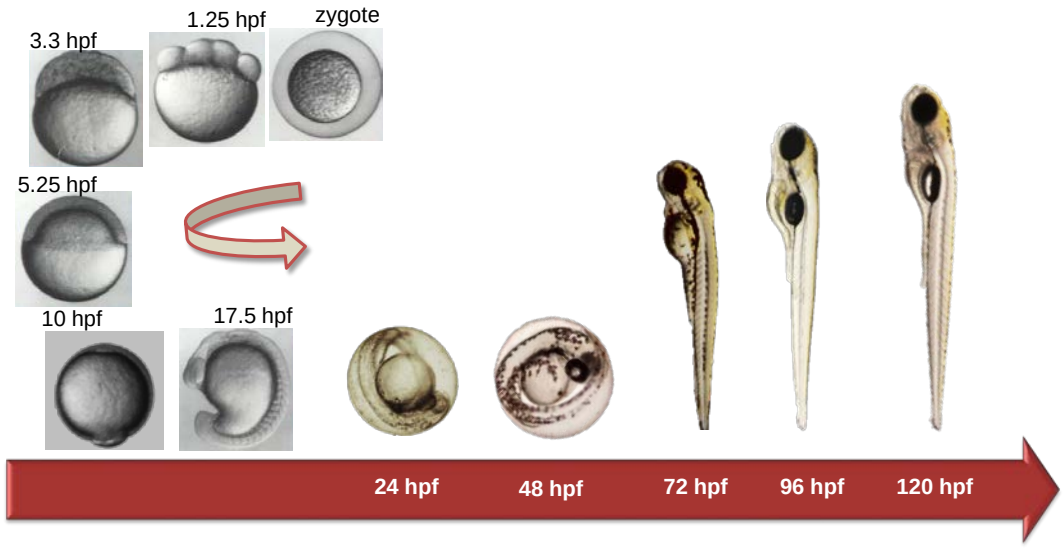

Figure 9 – General zebrafish development from the zygote period until 120 hours post fertilization.

1.4. Transcriptomic tools

In a simplified picture of the flow of information in a cell, the information contained in genes (DNA) is transcribed into messenger ribonucleic acid (mRNA), and translated into proteins, which can subsequently act on DNA, mRNA, metabolites, or other proteins. The presence in a cell of an endogenous or exogenous effector, leads to the activation of a receptor (or sensor) and its binding to specific DNA sequences, promoting de transcription of target genes (Piña et al., 2007). The correspondent mRNA information is translated to synthesise the correspondent protein molecules, constituting the molecular basis of the biological response to the presence of the effector (Piña et al., 2007).

Advances in molecular biology propelled the development of methodologies to determine, understand and predict the mode of action of compounds, also providing information on low-level toxic effects. Toxicogenomics is defined as the application of genomic technologies to study adverse effects of chemicals on human health and the environment (Simmons and Portier, 2002). Three major options are available for investigating molecular dynamics of the cell: analysing the set of proteins in the cell (proteomics), the set of mRNA transcripts that leads to the production of these proteins (transcriptomics), or the set of metabolites generated by the proteins (metabolomics) (Karakach et al., 2010). Transcriptomics has become one of the most robust tools of molecular biology, due to the relatively homogenous nature of the mRNA and the development of robust methods based on complementary base pairing.

The study of the transcriptome, the fraction of the genetic code that is transcribed into RNA molecules, allows to achieve an understanding of the mechanisms of toxicity

and to identify gene expression patterns that accurately reflect and predict specific toxicological endpoints (Robbens et al., 2007; Piña and Barata, 2011). This knowledge can be used to develop new molecular biomarkers and, in many cases, discover other target genes.

mRNA-quantification methods rely on knowledge of the exact sequence of at least a portion of the genes to be monitored (Ding and Cantor, 2004). For this it is also advantageous to use the zebrafish as a model organism because, in addition to the numerous advantages previously described, its genome is sequenced and available. Furthermore, it encompasses orthologues for approximately 70% of human genes, confirming its relevant applicability as a model with implications for human health (Howe et al., 2013).

1.4.1. Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-qPCR)

The polymerase chain reaction (PCR) was developed by Kary Mullis in 1983 (Nobel Prize in Chemistry, 1993), and it allows the exponential amplification of short DNA sequences from a longer double-stranded DNA molecule. This method has led to development of the actual mature field of specific mRNA-detection. PCR is based on the specificity of complementary sequences, the activity of a thermo-stable polymerase enzyme, and the dependence of both on temperature (Figure 10). DNA polymerases can make exact copies of any DNA molecule, only in the presence of the necessary co-factors, such as a pair of oligonucleotides complementary to sense and anti-sense strands of the DNA sequence of interest (i.e. primers), deoxyribonucleotide triphosphates and magnesium. The reaction relies on a cyclic process of temperature changes, where the separation of the double-stranded DNA molecule needs higher temperature than oligonucleotide hybridisation and polymerase extension (Figure 10). Therefore, new copies of the target sequence are synthesised in each cycle, leading to exponential amplification of the desired fragment (Figure 10). This methodology can be applied to RNA, as long as it is first reverse-transcribed into the complementary DNA (cDNA), by a reverse transcriptase (RT, Figure 10).

Semi-quantitative PCR methods estimate the amount of the original molecule by quantifying the total amount of the amplification product (amplicon), obtained at the end of the procedure. With the help of a fluorescence-based monitoring system, the progress of PCR can be monitored continuously in real time (quantitative "Real-Time" PCR, qPCR), making the quantitation of an amplified sequence more precise and reproducible (Heid et al., 1996). The RT-qPCR is highly sensitive, it allows to process a large number

of samples and can be easily applied to small organisms or to small dispensable body parts (e.g., fish scales, Piña et al., 2007).

In each RT-qPCR cycle, the fluorescence recorded is directly proportional to the number of molecules accumulated in the reaction well (Piña et al., 2007). The fluorescent dye (e.g. SYBR Green) is added to the reaction mixture to be intercalated in any double-stranded DNA. Initially the amount of fluorescence is too low for detection, but it grows exponentially until the fluorescence signal reaches a value distinguishable from the background level, called the ‘‘threshold cycle’’ (Ct, Figure 10). Ct values are directly related to the initial number of copies of the amplified sequence, as expressed in the following equation (Kubista et al., 2006; Nolan et al., 2006):

$$N=N_o(1+E)^{Ct}$$

N - Amount of amplicon molecules at the Ct (assumed identical for all samples and genes)

N_o - Initial amount of molecules

E - Efficiency of the amplification reaction (ideally 100%, E = 1).

Since many internal and external factors may influence the number of mRNA copies in a given sample and, therefore, impair adequate data comparison between samples, normalization by an internal control is required. Commonly reference genes are used, such as genes implicated in the formation and maintenance of the cell (i.e. “housekeeping genes”, HKGs). The expression of HKGs should generally be uniform across individuals and not affected by the parameters under study (Piña et al., 2007).

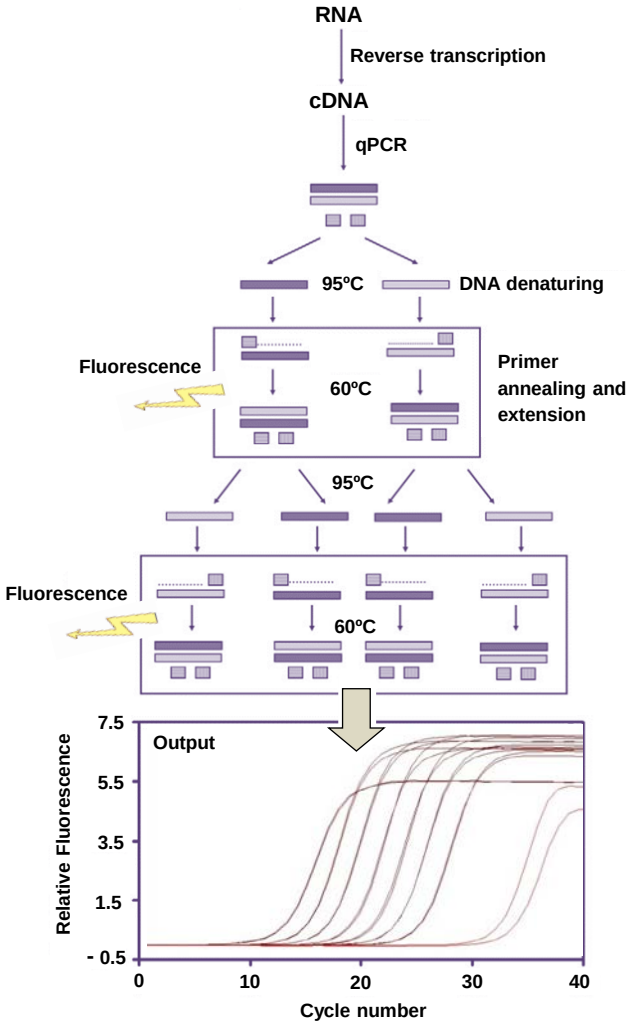

Figure 10 – Illustration of mRNA quantification by quantitative reverse-transcription polymerase chain reaction (RT-qPCR). RNA, Ribonucleic acid; cDNA, complementary DNA; qPCR, quantitative polymerase chain reaction; DNA, deoxyribonucleic acid.

1.4.2. Microarray technology

Microarrays made possible the simultaneous monitoring of thousands of nucleic acid sequences. They consist of small rigid supports made of glass or silica, onto which thousands of DNA sequences called probes, are arranged in an ordered array, in known and fixed locations ("spots"). Usually each microarray contains several thousand spots for Prokaryotes and from 10,000 to 40,000 spots for eukaryotes (Piña and Barata, 2011). The principle of the microarrays technique is based on the hybridisation of the probes with complementary target sequences of the test samples, which have been previously labelled with a fluorescent dye. In general, after RNA extraction, reverse transcription is performed using nucleotide derivatives that are either fluorescent or that can be attached to fluorescent molecules (e.g. Cy3-deoxyuridine triphosphate (dUTP) and Cy5-dUTP).

The following hybridisation and washing conditions promote the formation of strong paired strands, avoiding non-specific interactions. The fluorescence emitted by each spot on the microarray is taken as a measure of the relative concentration of the corresponding RNA molecule in the initial sample, allowing to monitor all genes present in the microarray in a single experiment.

There is a wide variety of microarray platforms differing in the type of probe and manufacturing technology, which dictate the protocol for designing and analysing the microarray experiments (Karakach et al., 2010). Namely, the probes can be labelled using single-colour versus two-colour fluorescent dyes. In the one-colour approach, all samples are labelled with the same fluorescent dye and hybridised to separate microarrays, while in the two-colour approach samples are labelled using two different fluorescent dyes, and are directly compared by mixing and hybridising the two samples on a single microarray (Figure 11). In general, it is preferable to use the two-colour approach if a control sample can be defined, since each treated sample can be hybridised against a common reference sample (Figure 11). The one-colour approach needs consistent manufacturing to minimise array-to-array variation, may not detect small intensity differences, and may introduce more variability than the two-colour method, due to processing multiple microarrays per assay. Dual-colour derived ratios are more robust against hybridisation artefacts, because they tend to affect both colour channels similarly, and the resulting detraction is minimised/equalised when calculating ratios of intensities of the two channels. Nonetheless, with the two-colour approach comparing more than two samples can be difficult, requiring hybridisation strategies that combine multiple different pairs of samples.

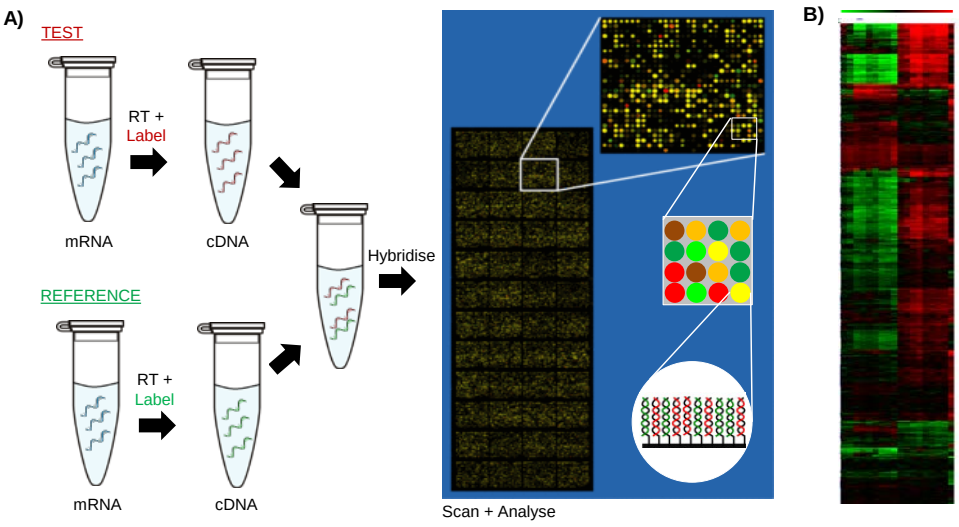

Figure 11 – A) Schematic illustration of the set-up of a microarray experiment, with messenger ribonucleic acid (mRNA) samples from two experimental conditions, coloured with two-colour fluorescent dyes. B) Scanned image of a zebrafish complementary DNA (cDNA) microarray. RT, reverse transcriptase.

Studying microarray data is a workflow of discrete steps, starting with the design of the experiment. It proceeds with samples extraction, labelling, hybridization, scanning, image processing, normalisation and ratio calculation. It ends with validation, statistical analysis, data annotation and generation of knowledge (Stoughton, 2005; Karakach et al., 2010; Piña and Barata, 2011). Validation of microarrays' data is commonly performed using RT-qPCR, to obtain quantitative, accurate and detailed results of the expression of specific genes selected among those exhibiting higher expression changes. Annotation is the process of assigning biological information (functional and/or structural) to gene products (Piña and Barata, 2011). Even though each microarray probe is intended to correspond to a given gene, the specific function of this gene may not be obvious. Interpretation of data is easier for known model species (such as zebrafish, mouse or drosophila), for which genetic and functional knowledge is available (Piña and Barata, 2011). A collaborative effort has been made to address the need for consistent descriptions of gene product attributes (function, localization, processes), across species and databases. This is referred to as Gene Ontology (GO)[4]. By linking a particular gene to a GO term, one can identify other genes with similar functions, activities or subcellular localisation.

The changes detected on levels of mRNA have to be interpreted as modifications of metabolic or regulatory pathways within the cell, in order to maintain its metabolism compatible with the presence of external stressors. The interpretation of microarray results can lead to discovery of coordinate changes of genes with common GO terms (also called GO Enrichment Analysis), indicating which cellular processes can be affected by, or respond to, toxicants. Thus, microarray technology allows to simultaneously study multiple pathways and mechanisms, providing a comprehensive view of the adverse effects of toxicants. This is particularly important since toxicity is generally preceded, not by the alteration of expression of a few genes but, by the alteration of a cascade of gene interactions.

Currently, microarrays have many applications in the field of medical diagnosis and treatment (e.g. analysis of the progression and probable outcome of diseases, prediction of possible mechanisms of toxicity for new pharmaceutical formulations). They have also been increasingly used in ecotoxicological studies, for example to determine adverse outcome pathways of environmental pollutants and identify new biomarkers as indicators of exposure and effect for risk assessment (Stoughton, 2005; Robbens et al., 2007; Piña and Barata, 2011).

[4] http://www.geneontology.org/

1.5. Objectives and Outline

From the above it is clear that a wealth of evidence points to a high burden of the organic fraction to overall toxicity of PM. Moreover, PAHs play an important role here due to their characteristics and toxic properties. The main objective of this thesis was therefore to investigate the toxicity of the organic fraction of atmospheric PM and provide a cost-effective approach that could be easily applied to diagnose potential toxicity resulting from exposure to this fraction. The specific aims were: i) to elucidate the toxicity of PM organic fraction in relation to particle size and sources; ii) to investigate differences in toxicity of PM from different environments (i.e. urban, rural, marine) to gain further insight into hazard and risk of the organic fraction and PAH content of PM; iii) to unravel relevant modes of action of PM components originating from different human activities or landscapes and identify early-warning biomarkers that discriminate the different toxic potential of particulate air samples; iv) to investigate daily and weekly patterns of biological activity and clarify their possible relation to secondary organic components of PM; and v) investigate the usefulness of a combination of *in vitro* and *in vivo* assays to the early diagnosis of the toxicity of PM organic fraction.

To tackle these objectives, a general strategy was adopted, in which toxicological and molecular tools were employed to evaluate biological effects elicited by different PM samples. A single cell-based assay was used to assess AhR activation. The Zebrafish Embryotoxicity Test (ZET) was employed to evaluate effects that could result from metabolic activation of parental contaminants in the organic fraction into more toxic metabolites. Toxicogenomic tools were used to screen molecular pathways that could be affected by exposure to the organic fraction of PM. The profiles of biological effects obtained in each study, were related to detailed chemical characterisation of the organic fraction of PM samples through multivariate statistical techniques. The chemical characterisation of organic fraction was obtained previously within the scope of multidisciplinary project TEA-PARTICLE, by analytical chemistry team members.

The presentation of the results is done according to the outline presented herein. Following this introductory Chapter 1, the studies carried out to address the research objectives are presented in Chapters 2 to 5. These are followed by a general discussion (Chapter 6), conclusions and future perspectives (Chapter 7).

In Chapter 2, dioxin-like activity and *in vivo* toxicity were determined for particulate air samples of different particle size cut-offs (10, 2.5, and 1 µm) from an urban background site, over a period of 14 months.

Chapter 3 compares the toxicity of the organic fraction of atmospheric PM samples collected in urban and rural areas. Transcriptomic analysis was used to detect changes in mRNA abundance at the whole-genome scale, providing information on the gene-mediated adverse outcome pathways affected upon exposure to PM organic constituents, and allowing to identify a set of biomarkers that facilitate the assessment of differential toxic potential of particulate air samples

Chapter 4 focused on the toxicity of PM1 organic extracts. The behaviour of the gene markers found in the previous chapter was related to primary or secondary PM organic components. Daily and weekly differences in the biological activity of PM were also determined.

In Chapter 5, the toxic potential of organic extracts of atmospheric PM samples from different sub-basins of the Mediterranean and Black Seas was investigated. The methods and approaches previously employed were useful to analyse, for the first time, atmospheric PM samples from marine open waters.

Chapter 6 comprises a general discussion of the work developed throughout the Thesis, with suggestion of an efficient and rapid approach for hazard assessment of the organic fraction of PM. Chapter 7 provides final concluding remarks and future research perspectives to be developed.

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CHAPTER 2

Toxicity assessment of urban atmospheric particle-bound PAHs: relevance of composition and particle size

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2.1. Introduction

Particulate matter (PM) is a major constituent of ambient air pollution by itself. It is considered by the regulatory agencies as one of the most harmful air pollutants to human health (EEA, 2012; WHO, 2013) and the PM mass is a widely used parameter in air quality regulations (EEA, 2012; WHO, 2013). Adverse biological effects associated to PM include oxidative damage, enhancement of inflammatory responses, cytotoxicity and mutagenicity followed by increased morbidity and mortality (de Kok et al., 2005, 2006; Billet et al., 2008; Cavanagh et al., 2009; Steenhof et al., 2011; Dergham et al., 2012). Airborne particles show a wide range of physicochemical and morphological properties that may vary with season, climate, space, and source, making it difficult to characterize their potential toxic constituents. The correct risk assessment of their toxicity requires a broad look, including effects on terrestrial and aquatic environments, in addition to the impact on human health (Sheesley et al., 2005).

Atmospheric suspended particles can be divided accordingly to their size. Traditionally, PM10 (particles with aerodynamic diameter < 10 μm) have been associated to adverse health effects, although in recent times smaller cut-offs (PM2.5, particles with aerodynamic diameter <2.5μm, or PM1, aerodynamic diameter <1 μm) have been proposed as better indexes of air pollution, due to higher lung deposition of smaller particles in combination with the presence of toxic compounds (Varghese and Gangemma, 2006; Seinfeld and Pandis, 2008). Although the results from epidemiological studies demonstrate the link between PM and adverse health effects (Pope et al. 2002), the role of particle size on these effects is still inconclusive (Perez et al. 2009).

Polycyclic aromatic hydrocarbons (PAHs) are pollutants of great environmental concern due to their mutagenic, carcinogenic and teratogenic properties, being included in the European Union list of priority pollutants (IARC, 1983; EC, 2000). These compounds originate from incomplete combustion of carbonaceous materials, both from natural (e.g. wildfires) and anthropogenic sources (e.g. traffic emissions, domestic combustion of wood or coal, industry). PAHs are resilient to environmental degradation and prone to long-range transport. They are found in all environmental compartments: water, soil and air. In the atmosphere, PAHs can be found in both gas and particulate phases, being particularly relevant constituents of PM (Pankow and Bidleman, 1992). Since PAHs have affinity to the PM carbonaceous core, they are mainly found on small sized particles (Miguel et al., 1998; de Kok et al., 2005; Zhou et al., 2005). Therefore, it

is important to clarify the contribution of PAHs to the toxicity of PM, taking into account the size of particles.

Some of the adverse effects caused by PAH exposure are mediated by their binding to cytosolic nuclear receptors, such as the aryl hydrocarbon receptor (AhR). AhR activation, translocation of the complex into the nucleus, and the subsequent transcription of target genes, precede the proliferative, oxidative, and carcinogenic effects of PAHs (Nebert et al., 1987; 1993). Hence, monitoring the ectopic activation of AhR, also known as dioxin-like activity, is a useful tool to assess contamination by dioxin-like compounds in environmental samples (Noguerol et al., 2006a; Olivares et al., 2011). The recombinant yeast assay (RYA) consists of genetically modified yeast strains, capable of detecting ligands for several receptors (Noguerol et al., 2006b). The AhR-RYA assay can detect and quantify the receptor-binding activity of dioxin-like compounds, such as PAHs, by expressing the human AhR and AhR nuclear translocator (ARNT) in the yeast strain (Miller, 1997).

PM toxicological studies frequently use human or rat cell lines designed to evaluate the toxicity of PM or its components *in vitro* (Obot et al., 2004). Nevertheless, the assessment of alterations at the individual level gives us information on how the contaminants interact with the organism's physiological systems and with which toxic outcomes. However, until date, most laboratorial studies that use whole living organisms to study PM toxicity rely on bacterial assays or rodent intratracheal instillation (Hannigan et al., 1994; Zhang et al., 2011). The zebrafish embryotoxicity (ZET) assay is a suitable method to assess the effects of chemical compounds, including PAHs, in the early life stages of a vertebrate species. With simple maintenance features, high number of offspring per brood, ex utero development, and great transparency during embryogenesis and larval stages, zebrafish (*Danio rerio*) is an excellent model for toxicology studies (Fraysse et al., 2006; Hermsen et al., 2011).

A comprehensive risk assessment of PM toxicity requires the analysis of the effects on the environment, in addition to the impact on human health (Sheesley et al., 2005; Mesquita et al., 2014). The aquatic environment works as a natural sink to these air pollutants, through both atmospheric deposition and soil run-off. Whereas oil spills are major drivers of local PAH pollution in aquatic systems, atmospheric deposition is the main responsible for the widespread and continuous distribution of PAHs in lakes, rivers, estuaries, and seas (Franz et al., 1998; Arzayus et al., 2001; Jurado et al., 2004). However, the information about the toxicity of particle-bound PAHs to aquatic species is scarce.

This work determined the dioxin-like activity and in vivo toxicity of PM10, PM2.5 and PM1 organic extracts using the AhR-RYA and ZET assays. The results were

compared with the chemical composition of samples, especially taking into consideration their PAH content. The working hypothesis is that the PAHs content of PM should elicit the activation of the AhR and further induce *in vivo* toxicity in such a sensitive test as the ZET assay, providing valuable information on the general toxicity of urban PM. In addition, the results should also give insights on the potential embryotoxic effects of the particulate contamination in aquatic systems.

2.2. Methods

2.2.1. Sampling, extraction and chemical analysis

Filter samples were collected from October 2008 to December 2009, at an urban background site in Barcelona (41°23′05″N; 02°07′09″E; 68m a.s.l.). The methodology used for sampling, extraction and chemical analysis has been described elsewhere (Reche et al., 2012). Briefly, PM samples were collected on 150mm quartz micro-fibre filters (*Munktell*) twice a week using a HiVol sampler (MCV; 30 $m^3.h^{-1}$) for 24 hours. In addition to the standard 10 µm cut-off filters (i.e. PM10), PM2.5 and PM1 samples were collected simultaneously in the period from October 2008 to March 2009. One half of each quartz micro-fibre filters was acid digested (2HF:HNO_3:$HClO_4$), for the chemical analysis using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) for the determination of the major elements and Mass Spectrometry for the trace elements (ICP-MS). The elemental composition of filter samples was used for source apportionment purposes as described (Escrig et al., 2009); a detailed discussion on these results has been published elsewhere (Reche et al., 2012). For PAHs analysis, one-eighth section of the quartz micro-fibre filters were used. Filter samples were spiked with surrogate standards, anthracene-D, benz[a]anthracene-D, benzo[k]fluoranthene-D and benzo[ghi]perylene-D, and extracted in a mixture of hexane and dichloromethane (DCM, 3 x 5 mL; 9:1 v/v; Merck, Germany), then concentrated and passed over an aluminium column to separate the PAHs from other components in the extracts. The final extract was analysed on a gas-chromatograph coupled with a mass-spectrometer.

Toxicity equivalents (TEQs) were calculated from the content of different samples in the parental PAHs phenanthrene (Phe), anthrancene (Ant), fluoranthene (Fla), pyrene (Pyr), benz[a]anthracene (BaA), chrysene+triphylene (Cry/Tri), benzo[b+j]fluoranthene (BbjF), benzo[k]fluoranthene (BkF), benzo[e]pyrene (BeP), benzo[a]pyrene (BaP), perylene (Per), indeno[123-cd]pyrene (Ind), and benzo[ghi]perylene (BghiPer), using

previously reported toxicity equivalence factors (Nisbet and LaGoy, 1992). These factors reflect the total (human) toxicity of the different PAHs, irrespectively to their ability to bind the AhR.

2.2.2. Biological and toxicity analysis

One-eighth of filter samples (corresponding to 90 m^3 of air) were extracted for both RYA and ZET assays by sonication with a mixture of dichloromethane: methanol (Merck, Germany, 2:1 v/v, 3 x 10 mL, 15 min). The extract was then filtered in a syringe with 0.45um polytetrafluoroethylene (PFTE) membrane (Puradisc, Whatman, USA), concentrated on Rotovap to 1 mL, further concentrated to almost dryness with a gentle nitrogen gas stream, and dissolved in 1 mL of methanol. Then, 0.5 mL of extract were reserved at -20ºC for RYA, and the other 0.5 mL were exchanged into Dimethyl sulfoxide (DMSO, Sigma–Aldrich Chemical, Germany), and reserved at -20ºC for the ZET assay.

AhR-RYA

The yeast strain YCM4 (provided by Charles A. Miller from the University of New Orleans) harbours two chromosomally integrated constructs, one coexpressing AhR and ARNT human genes under the GAL1-10 promoter, and the reporter construct pDRE23-z, which contains three XRE5 sequences and the CYC1-LacZ fusion (Miller, 1997; Noguerol et al., 2006b). Air samples extracts (in duplicate) and positive (1µM β-Naphthoflavone), negative (5% of vehicle, methanol), and inhibitory (sample extract plus 1µM β-Naftoflavone) controls were tested in 96-well polypropylene microtiter plates (NUNC™, Roskilde, Denmark). β-galactosidase activity was measured by fluorescence in a Synergy 2 spectrofluorometer (BioTec, USA) at 355 nm excitation and 460nm emission wavelengths, using 4-methylumbelliferone β-D-galactopyranoside (MuGal, Sigma–Aldrich Chemical, Germany) as a fluorogenic substrate (Noguerol et al., 2006b). β-galactosidase activity values were calculated as the rate of increase in time in the arbitrary units of fluorescence, by means of standard linear regression models. AhR-ligand activity of samples is represented as BaP equivalents (BAPeq), calculated as the 50% effect concentration (EC_{50}) of each sample divided by the calculated EC_{50} for BaP, 3.4 $\mu g.L^{-1}$ (Noguerol et al., 2006b; Misaki et al., 2007). Final results are expressed in BaPeq $.m^{-3}$.

ZET

Wildtype zebrafish purchased from a local dealer were maintained at 27 ± 1.5ºC in a 12h light and 12h dark cycle and reconstituted water (Instant Ocean 90 mg/L; 0.58 M calcium sulfate dihydrate in reverse osmosis purified water). Fish were fed twice a day with dry flakes (TetraMin, Tetra, Germany) and once a week with *Artemia salina* nauplii to stimulate optimal egg production. On the evening before spawning, two males and three females were placed on a 2L-breeding chamber with a mesh bottom. Spawning was induced in the morning when the lights turn on. One hour later eggs were collected, rinsed and reserved for 24h until the exposure experiment. Viable eggs were then randomly distributed into glass petri dishes. Each air sample extract was diluted 500 times in order to make a final percentage of 0.2% DMSO. Each sample was tested with 10 embryos per well, with 6 replicates, plus a negative control (0.2% DMSO), and a positive control (3.7 mg/L 3,4-Dichloroaniline, Sigma–Aldrich Chemical, Germany). The exposure was maintained from 24hpf until 120 hpf renewing the medium every 24h. Anatomical development of embryos was followed daily as described (Kimmel et al., 1995) under a stereomicroscope Nikon SMZ 1500 equipped with a Nikon digital sight DS-Ri1 camera. Lethal and sub-lethal endpoints were determined taking into account the endpoints already described (Brannen et al., 2010; Hermsen et al., 2011).

2.2.3. Statistical analysis

All statistical calculations, including linear and non-linear regression methods and principal component analyses (PCA), were performed using the SPSS v. 19 package (SPSS Inc., Chicago, Ill.) PCA analyses were performed using toxicological data from this work and previously reported chemical analyses for the same samples (Reche et al., 2012). Source apportionment analyses, inferring the putative origins of the different air samples, were performed as described (Reche et al., 2012). Unless otherwise noted, significance levels were set at $p<0.05$.

2.3. Results

2.3.1. Biological response to PM10 samples

AhR ligand activity was detected by the yeast-based AhR-RYA assay in all samples (Figure 1A). Activity values ranged between 0.1 and 2.9 ng.m^{-3} BaPeq, with maximal values corresponding to November and December in both 2008 and 2009. Minimal activity values were recorded in March-May 2009 and September-October from both years (Figure 1A). This trend roughly coincided with the variations of total PAH contents of these samples, which showed differences as high as 10-fold during the analysed period, with values ranging from 0.4 to 4.3 ng.m^{-3} (Figure 1B). The concentrations of BaP ranged from 0.02 to 0.44 ng.m^{-3}.

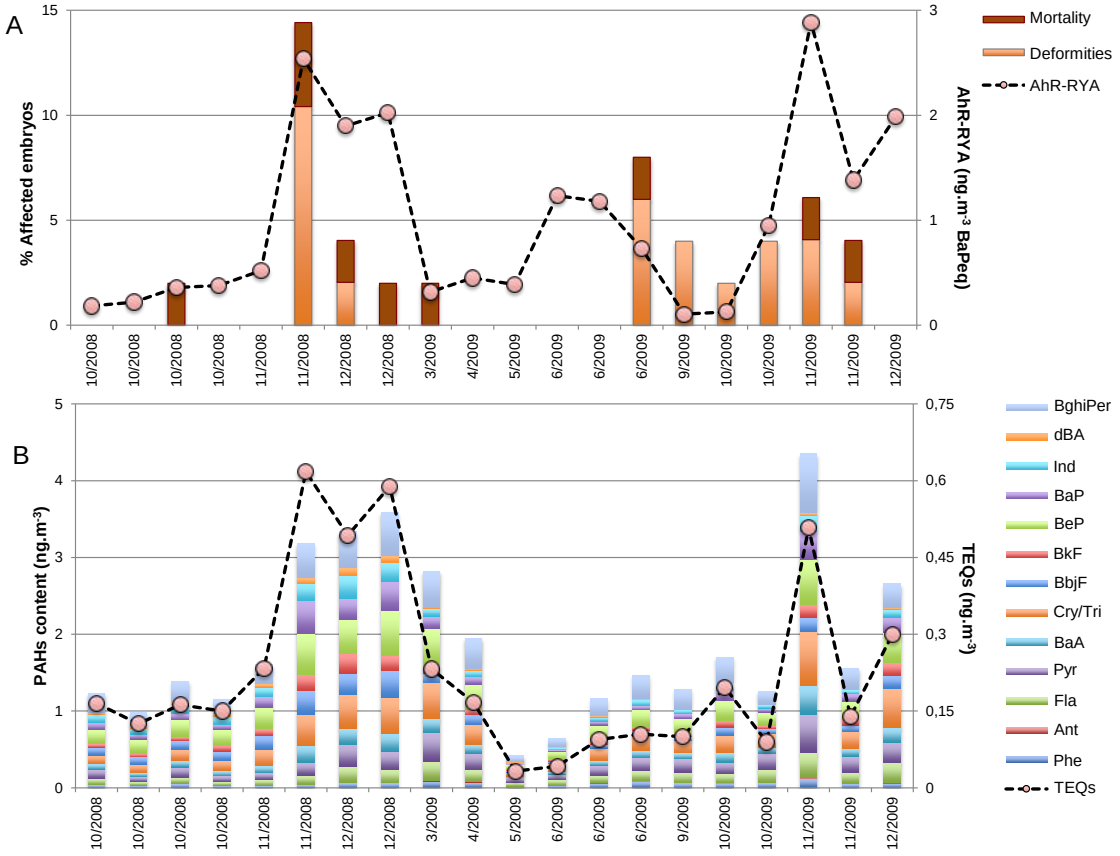

Figure 1 - Biological activity (A) and chemical parameters (B) of PM10 air samples (particulate matter of 10 µm cut-off). Discontinuous line on A represent Benzo[a]Pyrene equivalents (BaPeq) from the Aryl hydrocarbon receptor - recombinant yeast assay (AhR-RYA, in ng.m^{-3}, right axis), and on B represent the calculated toxicity equivalents (TEQs). Bars on A represent the results from the zebrafish embryotoxicity test (ZET, left axis), in percentage of mortality (dark section) or deformations (light section). Collection dates are represented along the X-axis of both graphs. PAHs, polycyclic aromatic hydrocarbons, BghiPer, Benzo[ghi]perylene, dBA, dibenzo[a,h]anthracene, Ind, indeno[123-cd]pyrene, BaP, benzo[a]pyrene, BeP, benzo[e]pyrene, BkF, benzo[k]fluoranthene, BbjF, benzo[b+j]fluoranthene, Cry/Tri, Chrysene + triphenylene, BaA, benzo[a]anthracene, Pyr, pyrene, Fla, Fluoranthene, Ant, Anthracene, Phe, phenanthrene.

A good correlation was observed between biological activity and TEQ values calculated from the concentration of dioxin-like PAH congeners (r= 0.78, Table 1). AhR-RYA data correlated with the concentration of the heavier AhR binding PAH congeners, like BaP, BghiPer, and BkF, but not with more volatile ones (i.e. Phe or Ant, Table 1). More than 50% of the variability of the observed AhR ligand activity in the yeast assay can be explained by the estimated TEQ values of samples (Table 1).

Table 1. Pearson's correlation coefficients for bivariate correlations between biological, chemical and source apportionment data[a] ($p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Abbreviations used as in Figure 1.

		ZET	AhR-RYA	Sea salt	Heavy oil combustion	Industrial - metallurgy	Road dust	Secondary nitrate	Mineral	Secondary sulfate	Vehicular exhaust
Biological effects	ZET	1									
	AhR-RYA	0.471*	1								
Sources	Sea salt	-0.199	-0.453*	1							
	Heavy oil combustion	-0.156	-0.105	0.103	1						
	Industrial - metallurgy	0.378	0.546*	-0.268	-0.259	1					
	Road dust	0.046	0.282	-0.061	0.03	0.557*	1				
	Secondary nitrate	0.187	0.296	-0.102	-0.038	0.033	0.276	1			
	Mineral	0.012	-0.333	0.357	0.354	-0.253	-0.283	-0.103	1		
	Secondary sulfate	0.056	-0.329	0.133	0.029	-0.267	0.028	-0.095	0.195	1	
	Vehicular Exhaust	-0.035	0.335	-0.129	-0.335	0.181	0.581**	0.548*	-0.237	-0.127	1
Chemical parameters	PM	0.157	-0.217	0.344	0.309	-0.051	0.063	0.183	0.831***	0.307	0.011
	TEQs	0.464*	0.767***	-0.403	-0.368	0.414	0.117	0.149	-0.342	-0.436	0.418
	Phe	0.374	0.383	-0.092	0.026	0.681***	0.752***	0.187	-0.102	-0.111	0.293
	Ant	0.397	0.322	0.083	0.168	0.599**	0.804***	0.21	-0.107	-0.02	0.271
	Fla	0.288	0.599**	-0.174	-0.243	0.624**	0.76***	0.429	-0.322	-0.183	0.615**
	Pyr	0.348	0.571**	-0.131	-0.211	0.65**	0.69***	0.335	-0.216	-0.193	0.536*
	BaA	0.46*	0.776***	-0.276	-0.339	0.692***	0.466*	0.215	-0.311	-0.388	0.514*
	Cry/Tri	0.355	0.761***	-0.307	-0.294	0.683***	0.572**	0.336	-0.35	-0.385	0.601**
	BbjF	0.305	0.634**	-0.449*	-0.377	0.256	-0.003	0.14	-0.352	-0.463*	0.383
	BkF	0.419	0.758***	-0.421	-0.355	0.374	0.153	0.158	-0.407	-0.46*	0.428
	BeP	0.395	0.695***	-0.36	-0.275	0.559**	0.396	0.267	-0.332	-0.399	0.536*
	BaP	0.495*	0.791***	-0.367	-0.359	0.452*	0.177	0.196	-0.346	-0.403	0.461*
	Ind	0.391	0.65**	-0.435	-0.375	0.328	-0.021	-0.015	-0.279	-0.456*	0.253
	dBA	0.282	0.519*	-0.467*	-0.32	0.086	-0.276	-0.092	-0.237	-0.462*	0.12
	BghiPer	0.43	0.635**	-0.244	-0.273	0.673***	0.468*	0.084	-0.256	-0.333	0.411

[a] Source apportionment data from Reche et al. (2012).

The ZET assay showed significant *in vivo* toxicity in November 2008 and 2009 PM_{10} samples (Figure 1A, bars), plus a secondary peak of toxicity in June 2009. Deformations observed on exposed zebrafish embryos were spinal deformations, yolk-sac and cardiac edemas, malformation of the swim bladder and balance disruption. Lethality never exceeded 5% of the total exposed embryos, and the total of affected ones (mortality plus deformities) was always below 15% (Figure 1A). Therefore, the data shows a weak, but consistent embryonic toxicity of PM samples, which was detected for the first time in urban air PM samples using the applied methodology. This toxic effect correlated with high TEQ values of samples (r= 0.46), related to the concentrations of BaP and BaA (Table 1, see also Figure 1). No significant correlation was found with the concentration of other PAHs (Table 1).

Actual concentrations of PAHs in ZET exposure water were analysed by Liquid chromatography–mass spectrometry (LC-MS). The results showed a relative standard deviation (RSD %) from the nominal concentrations of less than 20%, with the exception of the most volatile compounds, Phe and Ant (Table 2). Nominal exposure concentrations of total PAHs ranged from 71.0 to 745.2 $ng.L^{-1}$ and the BaP concentrations ranged from 2.9 to 74.0 $ng.L^{-1}$, whereas actual concentrations for the highly toxic November 2008 sample were 545.2 ng total $PAH.L^{-1}$ and 73.9 ng $BaP.L^{-1}$.

Table 2 - Relative standard deviation (RSD %) of the nominal and real concentration of polycyclic aromatic hydrocarbons (PAHs) in the water samples from the ZET assay. Abbreviations as in Figure 1.

PAH	RSD %
Phe	-270.6
Ant	-193.7
Fla	17.2
Pyr	21.7
BaA	20.1
Chy	6.4
BkF	15.6
BaP	9.7
Ind	15.7
BghiP	3.4

2.3.2. Source apportionment and biological activity of PM10 samples

In order to relate potential sources of PM to the observed biological activity and zebrafish toxicity, a principal component analysis was performed combining both chemical and

biological data with an already published dataset of inorganic, organic carbon (OC) and elemental carbon (EC) data on these same PM samples (Reche et al., 2012). About 60% of the total variability observed in the 69 parameters analysed (AhR ligand activity, Fish mortality and deformities, calculated TEQs, PM10 mass, EC, OC, 13 PAHs, 32 inorganic compounds/elements and 8 putative air sources, Table 1) could be described by two principal components (PC, Figure 2, Table 3).

Table 3. Results from principal components (PC) analysis (normalized parameters, rotated solution).

Total Variance Explained						
Component	Initial Eigenvalues			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
PC1	18.840	31.933	31.933	18.547	31.435	31.435
PC2	15.504	26.278	58.211	15.798	26.776	58.211

The analysis clearly separates two groups of parameters: the first one (group A), linked to PC1 and including most of inorganics and cations, and the second one (group B), with higher contribution from PC2 and including all PAHs and some heavy metals, plus elemental carbon and organic carbon (EC and OC respectively, Figure 2). AhR ligand activity and zebrafish toxicity were associated with this second group B, whereas PM10 mass appeared more related to the mineral component of the particles (Figure 2, group A). AhR ligand activity appeared closely linked to the theoretical dioxin-like activity of the samples (calculated TEQ), being both related to the concentrations of dioxin-like PAHs. Zebrafish toxicity, combining lethality and severe deformity data, showed a less strong correlation with the PAH content and dioxin-like activity of PM samples (Figure 2).

Source apportionment data (Reche et al., 2012) was included in the PCA to estimate the putative sources of the effects observed in this study (green labels in Figure 2). Vehicle- and fuel burning-related sources (Vehicular exhaust, Road dust and Industrial/metallurgy) were associated to the parameters included in group B, whereas PM10 mass and many of the inorganic compounds clustered in group A. A third small cluster of putative sources (Secondary sulfate, Sea salt and Heavy oil combustion) appeared separated from both groups (Figure 2).

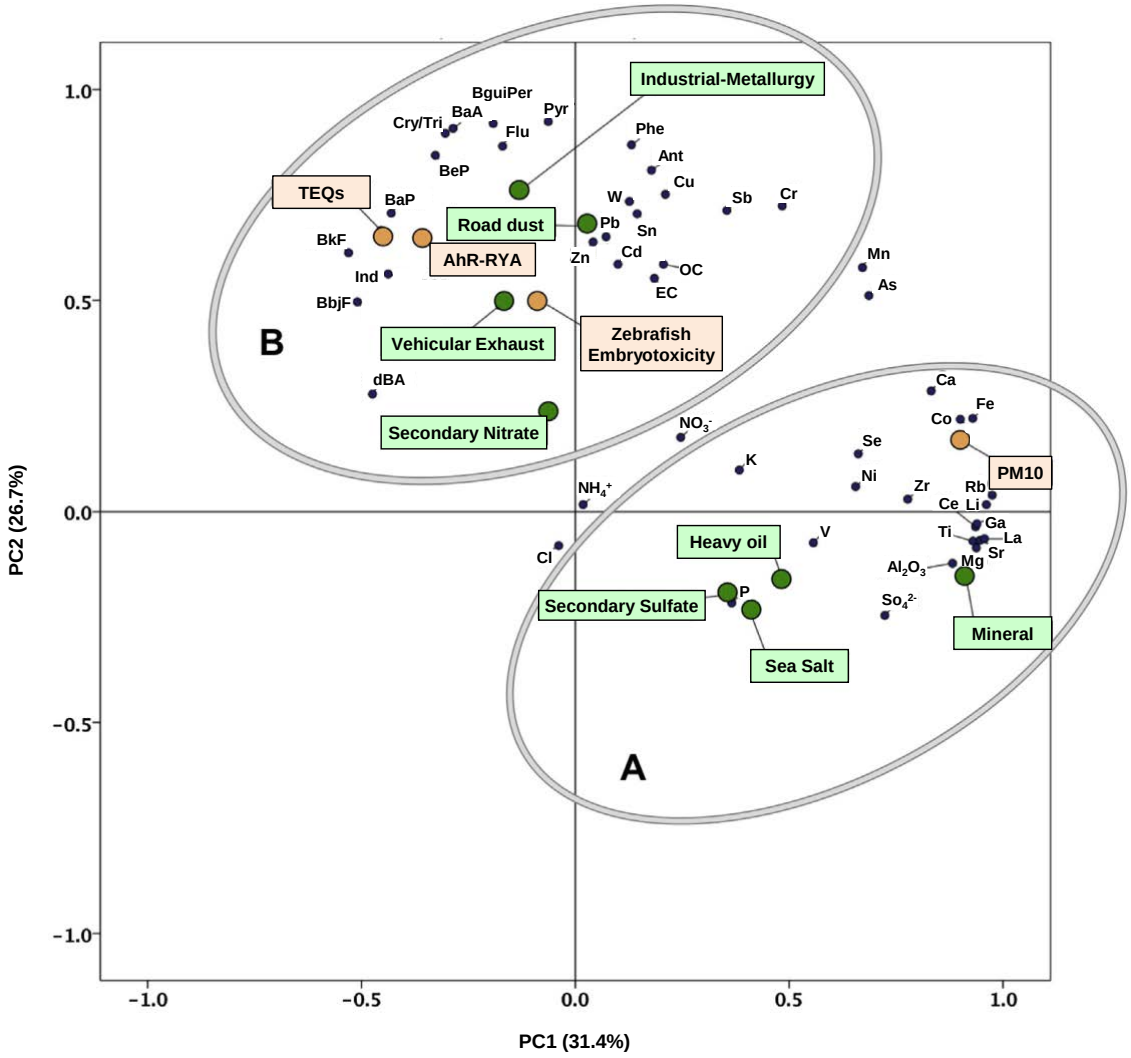

Figure 2 - Loading plots for the different parameters included in the PCA analysis. Orange labels correspond to toxicity/air quality parameters, green ones to estimated emission sources. Ovals indicate the two proposed clusters of parameters. PAHs are indicated as in Figure 1, Zebrafish Embryotoxicity (ZET) represents the sum of dead and deformed animals, and AhR-RYA indicates the measured dioxin-like activity. The rest of parameters and sources correspond to previously reported data (Reche et al., 2012). Cu, copper, Sb, antimony, Cr, chromium, W, tungsten, Sn, tin, Pb, lead, Zn, zinc, Cd, cadmium, Mn, manganese, As, arsenic, NO_3^-, nitrate, NH_{4+}, ammonium, Cl, chlorine, K, potassium, P, phosphorus, V, vanadium, Se, selenium, Ni, nitrogen, Ca, calcium, Co, copper, Fe, iron, Zr, zirconium, Rb, rubidium, Ce, cerium, Li, lithium, Ga, gallium, La, lanthanum, Sr, strontium, Ti, titanium, Al_2O_3, abramant, SO_4^{2-}, sulphate, Mg, magnesium, OC, organic carbon, EC, elemental carbon.

2.3.3. Distribution of biological activity among different size particles

Comparison of the biological activities of PM10, PM2.5 and PM1 fractions showed no differences for AhR-RYA or ZET assays. Table 4 compares the biological activity and PAH contents in PM2.5 and PM1 cut-off fractions with the values obtained for the PM10 fraction. PM2.5 and PM1 fractions showed 112% and 84% of the total AhR ligand activity and 72% and 109% of the embryotoxicity observed for the PM10 fraction, respectively

(Table 4). Similar percentages were found for all analysed PAHs, as well as for the calculated TEQ values (Table 4). As the PM fractions correspond to cut-off sizes, the data indicates that essentially all biological activity and PAH loads corresponded to the smallest PM fraction (PM1) and that the contribution of larger fractions (1<PM<10 μm) was not relevant. On the contrary, total PM mass smaller in the PM1 and PM2.5 cut-off fractions relatively to the PM10 (32% and 58%, respectively), indicating that more than two thirds of the total mass corresponded to particles larger than 1 μm (Table 4).

Table 4 - Comparison of biological activity and PAHs content in PM10, PM2.5 and PM1 fractions of air particles. Abbreviations as in Figure 1.

	% in PM2.5 [a)]	% in PM 1 [a)]	Correlation PM2.5 [b)]	Correlation PM 1 [b)]
ZET	71.7	108.9	0.914	0.973
AhR-RYA	111.9	84.2	0.864	0.832
PM	58.0	32.2	0.912	0.491
TEQs	101.3	93.0	0.990	0.992
Phe	108.6	92.7	0.811	0.905
Ant	102.0	82.0	0.930	0.856
Fla	100.2	80.4	0.946	0.990
Pyr	97.9	78.7	0.952	0.975
BaA	102.6	88.3	0.993	0.994
Cry/Tri	104.7	85.4	0.996	0.993
BbjF	101.5	90.3	0.980	0.985
BkF	99.5	85.4	0.976	0.955
BeP	97.6	92.5	0.964	0.943
BaP	98.8	91.8	0.977	0.976
Ind	113.4	100.4	0.975	0.905
dBA	106.5	98.8	0.990	0.927
BghiPer	101.0	98.2	0.957	0.935
PAHs Sum	101.8	90.7	0.992	0.990

a) Percentage relative to the PM10 value
b) Pearson's correlation between the given fraction and the PM10 fraction for each sample

Therefore, the data suggests a clear separation between the total mass of the PM (which increases with increasing PM size), and the load in PAHs and the biological activity, which concentrated in the sub-micron fraction of PM. This decoupling between PM mass and biological activity and PAH content is further illustrated by the correlation analyses shown in Table 4. Indeed, the biological and chemical parameters measured in PM10, PM2.5 and PM1 fractions showed close mutual correlations, with r-values higher than 0.8 in almost all cases (Table 4). PM mass showed a rather weak correlation between PM10 and PM1 (r = 0.49). This further indicates that PM mass (a standard

measurement for air quality), is a weak predictor of the potential biological activity and toxicity observed in the present study.

2.4. Discussion

The PAH concentrations reported in this study for the Barcelona samples were similar to those observed in previous studies performed at background sites in Barcelona (van Drooge et al., 2012) or in other cities in Spain (Callén et al., 2011). The temporal pattern of PAHs observed during the time period of this study is in agreement with those observed in other studies (Boström et al., 2002; van Drooge and Ballesta, 2009). In no case BaP levels surpassed the 1 $ng.m^{-3}$ legal limit value set by the EU (EC, 2001). Higher PAH atmospheric concentrations are common in winter months as a result of higher PAH emissions, lower planetary boundary layer and less photo-degradation, in comparison to the summer months.

AhR ligand activity strongly correlated with the particle-phase PAH concentrations and TEQs, which is in agreement with previous studies (Olivares et al., 2011; 2013). This relative simple and low-cost assay proved once more to be very sensitive to PAHs, even at low concentration levels. The adverse effects induced by PAHs are commonly mediated through the AhR receptor activation and subsequent increase of the detoxifying response, namely by the induction of genes of the cytochrome P450 superfamily (e.g. CYP1A1). Indeed, the increased expression of CYP1A1 gene has been described for human RAW264.7 macrophages exposed to PM organic fraction (Cavanagh et al., 2009). Therefore, the AhR-RYA can be consider has an early-warning tool for routine screening and control of particle-PAH emissions.

The ZET assay is developing as a valid alternative method for the toxicological use of mammals, particularly in such a sensitive life-stage as the early embryonic development. It is clear that the results cannot be directly extrapolated to possible effects on human health, but they constitute nevertheless an additional tool to study the toxic burden of urban aerosols. It is important to remark that the assay was sensitive enough to be carried out with PM samples corresponding to relatively low volumes of air (90 m^3, in this case), allowing the simultaneous analysis of inorganic and organic pollutants and of their biological activity using a single standard filter. Observed deformations and mortalities in zebrafish embryos partly correlated with PAH content of samples, providing information about the systemic effects of the exposure of a whole living organism to urban PM organic fraction. Further research will be essential to identify the contribution

of different PAH species and of their derivatives to the toxic effects, as well as to determine the role of detoxification mechanisms. This may be especially relevant for PAHs, given their ability to be activated metabolically into reactive, highly toxic diol epoxide intermediates (Jerina et al., 1980). This aspect is often neglected in the cell line assays, since *in vitro* grown cells often lack a substantial set of detoxifying enzymes and essential physiological cascades.

Fully understanding of the PM induced toxicity is very challenging, mainly because PM composition varies greatly and it is influenced by many variables (i.e. climate, season, space and source). The work developed here shows that the organic fraction of urban PM contributes to its toxicity, playing PAHs a substantial role. Even so, it should be clear that the adverse effects observed in this study result only from the PM compounds extractable with the organic solvents used. Although the dichloromethane: methanol mixture covers a large gradient of polarity, there is no control on the extraction efficiency of vast majority of the organic and inorganic constituents of PM, and therefore, there is lack of control on large part of the potential overall PM toxicity. In a previous study using the aliquots of same filter samples as used in the present study, water-extractable components induced oxidative stress. This was detected by both the *in vitro* plasmid scission assay and the dichlorodihydrofluorescin assay (Reche et al., 2012). Therefore, toxicity of both organic and inorganic pollutants needs to be evaluated to fully characterize the toxicity of air particles (de Kok et al., 2005; Valavanidis et al., 2008; Bonetta et al., 2009; Cavanagh et al., 2009). These combined results suggest that PM mass concentration, a normative parameter of air quality regulations used in the last decades, may not be sufficient to ensure quality of urban air samples (Cavanagh et al., 2009; Olivares et al., 2011; Olivares et al., 2013). Recent regulations include BaP concentrations as an air quality parameter, although other PAH species should probably be also included, and the corresponding guideline values defined.

The embryotoxic effects observed on zebrafish gave new insights on the effects of the exposure of an aquatic organism to airborne PM organic fraction. Until date several studies have assessed the adverse effects of individual or mixtures of PAHs on aquatic vertebrates, but since the atmospheric deposition of particulate PAHs is one of the major sources of contamination of the aquatic systems by these and other hydrophobic pollutants (Franz et al., 1998; Jurado et al., 2004; Del Vento and Dachs, 2007; Castro-Jiménez et al., 2012), analysing the effects of the whole PM organic fraction on aquatic species provides relevant information about its environmental toxicity. Ambient PM has been demonstrated to affect the green algae *Selenastrum capricornutum* and the crustacean *Ceriodaphnia dubia* (Sheesley et al., 2004; 2005), related to the PAH content of PM and other environmental parameters. As PAH levels reported for contaminated

surface and coastal waters can easily reach the $\mu g.L^{-1}$ level (WHO/IPCS, 1998), the exposure concentrations used here in the ZET assay (under the $\mu g.L^{-1}$ for total PAH content, and in the $ng.L^{-1}$ for individual PAHs) can be considered as environmentally relevant.

The correlation between biological activity and source apportionment revealed some insights on the relevance of air pollution sources as primary vectors of biologically active PAHs. Vehicle and industry emissions appeared as the major drivers of the observed toxicity of the samples, whereas mineral emissions (essentially, dust, either local or from long-range transport) appear less relevant. Although industrial facilities are spread between the two river basins in the North and South of the Barcelona metropolitan area, mainly along the Llobregat Valley, air masses from these valleys can be transported into the city by land breeze, especially at nighttime (Pérez et al., 2006; Jorba et al., 2013). In addition, stagnant atmospheric conditions may increase their influence on the Barcelona air quality (Amato et al., 2009). The source apportionment analysis indicated that the toxic effects observed in this work were essentially linked to dioxin-like PAHs and traffic exhaust.

Sources with marine origin (including “Heavy oil combustion” in the Barcelona area, Amato et al., 2009) appeared to be negatively correlated with the biological activity observed, despite the contribution of heavy oil combustion sources (basically, cargo ships). This suggests that that seafaring and harbour activities do not contribute significantly to the toxicity of organic PM from this urban site in Barcelona. This can partly be explained by the relationship between the influence of these marine/harbour air masses and the strength of the sea breeze. The same wind that transports air pollution also dilutes it with its speed, resulting in lower ambient air concentrations of many pollutants, including those from local emission sources. It is therefore not surprising that the overall toxicity decreases under active sea breeze conditions. A recent epidemiological study in Barcelona also links high daily mortality rates with traffic emissions and a negative correlation with sea salt contents in air particles (Ostro et al., 2011), consistently with the findings of the present study on the relative toxic potential of these PM sources.

The present results suggest that PAHs and other putative toxic compounds are concentrated in the PM sub-micron fraction, as this fraction presented essentially the same biological activity (and PAH loadings) as PM10 particles collected in parallel at the same time. The association of PM toxicity with the finest fraction of particles has relevant implications for public health. While coarse particles deposit in the respiratory tract, smaller particles can penetrate further in the lungs into the alveolar region, ultimately reaching the bloodstream (Squadrito et al., 2001). Consequently, epidemiological

studies have suggested that the small-size PM is associated with larger risk of cardiovascular toxicity (Pope et al., 2009). Therefore, a more restrict control of the sub-micron fraction of particles is required to improve environmental air quality.

2.5. Conclusions

This study describes for the first time an adverse effect of urban PM samples in the ZET assay, linked to the sub-micron fraction of particles, which might have implications for public and environmental health. The best predictor for ZET toxicity was the dioxin-like activity of samples (measured by the AhR-RYA), closely followed by the predicted TEQ values. This result is consistent with previous findings and underscores the necessity of implementing bioassays to evaluate the toxicity of air samples. The evidence of potential toxic burden of particulate PAHs to an aquatic vertebrate species emphasizes the need of further research on this specific subject. The most used parameter for air quality assessment, the PM mass, had a low predictive capacity for any of the biological activities shown in this study.

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CHAPTER 3

Differential embryotoxicity of the organic pollutants in rural and urban air particles

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3.1. Introduction

Outdoor air pollution, and particularly atmospheric particulate matter (PM), has been recognized as a major human and environmental hazard, triggering the development of legislation and measures for air quality improvement (EC., 2001; Vineis and Husgafvel-Pursiainen, 2005; WHO, 2013). Nonetheless, in 2010 air pollution was still on the top three of the leading risk factors for the global burden of disease (Lim et al., 2012). The adverse outcomes that have been associated to particulate air pollution include oxidative damage, inflammatory reactions, chronic diseases, cancer, cardiopulmonary mortality and also cataracts, diabetes, poor birth outcomes and health effects on neonates after prenatal exposure (Peters and Pope, 2002; Wegener et al., 2002; Jedrychowski et al., 2004; Miller et al., 2004; Hertz-Picciotto et al., 2005; Jedrychowski et al., 2005; Calderón-Garcidueñas et al., 2008; MohanKumar et al., 2008; Cesaroni et al., 2013; Balti et al., 2014; Eze et al., 2014; Fleischer et al., 2014).

Ambient air particles vary in their aerodynamic diameters. Coarse particles (between 2.5 µm and 10 µm) typically originate primarily from mechanical shearing and dust, while fine particles (aerodynamic diameter <2.5 µm) originate from fossil fuel and biomass combustion. Combustion processes tend to originate PM with carbonaceous cores that adsorb different organic toxicants, like polycyclic aromatic hydrocarbons (PAHs), contributing to the strong toxic potential of submicron-sized particles (de Kok et al., 2005; Zhou et al., 2005; Mesquita et al., 2014). Moreover, atmospheric organic compounds (both in gas and particulate phase) generate oxidized derivatives by photo-chemical oxidation processes (Hallquist et al., 2009), mainly found in the finest particle sizes.

In addition to PM size, the origin of particles also influences their organic composition and toxic potential (van Drooge and Ballesta, 2009; Olivares et al., 2011; Dergham et al., 2012). While in urban areas, traffic exhaust is one of the major sources of primary organic aerosols, in rural areas, especially in colder periods, biomass burning is a more important primary source. With this, there is a need to clarify the risk associated with different PM sources and sizes, as well as the inherent toxic mode of action of PM constituents to better understand their actual environmental risk.

Analysis of the systemic toxic effects of PM traditionally rely on rodent intratracheal instillation or similar *in vivo* assays using live mammals (Zhang et al., 2011a). There is a growing social and legal tendency to substitute traditional animal testing by replacement methods (EC, 2010). The zebrafish (*Danio rerio*) embryotoxicity test (ZET, ISO, 2007) and the aryl hydrocarbon receptor-recombinant yeast assay (AhR-

RYA), have been proved useful in previous studies to assess PM toxic potential (Misaki et al., 2007; Olivares et al., 2011; 2013; Mesquita et al., 2014). The zebrafish has become an important vertebrate model in toxicology (Scholz et al. 2008), and it has been used to study the effects of PM in early embryonic development (Mesquita et al. 2014; Olivares et al. 2013b). The AhR-RYA monitors the presence of ligands for the aryl hydrocarbon receptor (AhR), an activity (also known as dioxin-like activity) that substantially contributes to the proliferative, oxidative, and carcinogenic effects of PAHs (Van der Oost et al., 2003).

Transcriptomic analysis have been increasingly used to analyse the adverse effects of pollutants (Rotchell and Ostrander, 2003; Piña and Barata, 2011). Microarray studies allow to predict the possible mechanisms of toxicity of chemical compounds, and the categorization of genes that share functional pathways so that they can be related to specific biological responses leading to relevant toxic modes of action (Oliveira et al. 2013; Raldua and Pina, 2014).

The objectives of the present work were to study the toxicity of the organic fraction of atmospheric PM samples, in different particle sizes collected in urban and rural areas, using both yeast- and zebrafish-based assays (AhR-RYA and ZET). Transcriptomic analyses were used for detecting changes of messenger ribonucleic acid (mRNA) abundance at the whole-genome scale, providing information on the gene-mediated adverse outcome pathways affected upon exposure to PM organic constituents. The obtained data was also used to identify a set of biomarkers that facilitate the assessment of the different toxic potential of particulate air samples. The results were correlated with the chemical composition of samples.

3.2. Methods

3.2.1. Sampling and chemical analysis

Atmospheric particles of different sizes (PM>7.2 µm, PM7.2-3µm, PM3-1.5 µm, PM1.5-1 µm, PM1-0.5 µm and PM<0.5 µm) were collected in an urban background site in Barcelona and a rural site in La Pobla de Lillet in the Pyrenees, Catalonia, Spain (41º23.232'N; 2º6.943'E; 77m a.s.l. and 42º14.731'N; 1º58.488'E; 870m a.s.l., respectively). Four sampling campaigns took place in 2012/2013 at the urban and rural sites (labelled U1-U4 and R1-R4, respectively), and collected samples were classified as "warm period" and "cold period" accordingly with local meteorological conditions

(further details in van Drooge and Grimalt, 2015). Particles were collected on glass-fiber filters for 72h at a sampling rate of 20 $m^3.h^{-1}$. A half of PM filter samples was used for chemical analysis, and the other half reserved for the bioassays.

Chemical analysis was performed for PAHs, oxygenated PAHs (i.e. quinones), hopanes, n-alkanes, nicotine (Nic), levoglucosan (L), galactosan (G), mannosan (M), alcohol saccharides, carboxylic and dicarboxylic acids, malic acid, phthalic acid, cis-pinonic acid, 3-hydroxyglutaric acid, 3-methyl-1,2,3-butanetricarboxylic acid, 2-methylglyceric acid, in a total of 72 compounds. Detailed information on the methodology used for chemical analysis, as well as the relationship of the chemical compounds with primary emissions sources and transformation processes can be found in van Drooge and Grimalt (2015).

Toxicity equivalents (TEQs) meant to reflect the total (human) toxicity of PAHs, were calculated using toxicity equivalence factors for thirteen parental PAHs, as described previously (Nisbet and LaGoy, 1992).

3.2.2. Biological and toxicity assays

For RYA and ZET bioassays, half of each PM filter sample was extracted by sonication with a mixture of dichloromethane:methanol (Merck, Germany, 2:1 v/v, 3x10 mL, 15 min), and the extracts were filtered, evaporated to dryness and dissolved in 1 mL of methanol. Then, 0.5 mL of the extract was reserved for AhR-RYA and the rest exchanged into 0.5 mL of dimethyl sulfoxide (DMSO, Sigma Aldrich Chemical, Germany) for zebrafish assays.

Dioxin-like activity of each size-fraction from the different samples was evaluated by the AhR-RYA, as described in previous studies (Noguerol et al., 2006; Mesquita et al., 2014). AhR-ligand activity of samples is represented as benzo[a]pyrene equivalents (BaPeq, Noguerol et al., 2006; Misaki et al., 2007).

Wildtype zebrafish maintenance conditions and spawning procedure was performed according to the existing regulations (EC, 2010; Mesquita et al., 2014). ZET exposure experiments followed the International standard 15088 (ISO, 2007) for zebrafish eggs. Each sample was tested with 10 embryos per dish (at 0.2% DMSO), with 6 replicates, plus a negative control (0.2% DMSO), and a positive control (3.7 $mg.L^{-1}$ 3,4-dichloroaniline, Sigma–Aldrich Chemical, Germany). The exposure was maintained from 24 hours post-fertilization (hpf) until 120 hpf, renewing medium every 24h. Developmental progression of embryos was observed daily under a stereomicroscope. Lethal and sub-lethal endpoints were determined accordingly to the endpoints already described (Hermsen et al., 2011). Exposure to the canonical dioxin-like PAH congener

BaP, was performed at 0.1 mg.L^{-1} on the ZET assay, under the same experimental conditions. BaP-exposed embryos did not show any phenotypical alteration. Zebrafish embryos were frozen after exposure at -80^{o}C for posterior transcriptomic determinations.

3.2.3. RNA extraction and microarray procedure

Total RNA was isolated from whole embryos (pools of 20 individuals), using the Trizol reagent protocol (Invitrogen Life Technologies, Carlsberg, CA). RNA extraction and processing were performed as described elsewhere (Pelayo et al., 2012). The microarray study was performed using the Agilent Two-colour *D. rerio* Oligo Microarray v3 platform, following the Agilent Microarray – Based Gene Expression Analysis protocol. PM samples analysed were selected from the results obtained from the AhR-RYA and ZET assays; embryos exposed to BaP (0.1 mg.L^{-1}) were also included in the analysis. Three biological replicates were included for each PM sample. Biological replicates, a technical replicate and a self-to-self, were simultaneously amplified and labelled using Cyanine 3 (Cy3) and Cyanine 5 (Cy5) dye. After complement RNA (cRNA) purification (RNeasy Kit, Quiagen GmbH, Hilden, Germany), the cRNA concentration and labelling were quantified in the NanoDrop spectrophotometer, obtaining incorporation rates in the range of 15-20 pmol of cyanide dye/µl cRNA and yield values > 0.825 µg, as recommended. cRNA was fragmented and hybridized in 4x44K arrays, for 17h at 65^{o}C. After hybridization the array slides were washed, and immediately scanned using Microarray Scanner Agilent G2505C system. Data was extracted using Agilent Feature Extraction Software v10.5.1.1, and the quality of microarray data was evaluated using the Quality Control report provided by Agilent Software.

3.2.4. Microarray data analysis

Data from the three biological replicates of each PM extract and BaP exposure were independently normalized for each array using the loess algorithm from the R limma package (Smyth, 2005). Further analyses were performed using R (R Core Team, 2014). Medians for the different probes corresponding to each zebrafish gene in the Agilent arrays were used to construct a 22x12976 matrix containing treated-to-control dual logarithm ratios for each treatment. Genes were selected based in three criteria: different responses to any of the treatments (ANOVA, 3 classes, urban, rural and BaP), differences between rural and urban extracts (T-Test, independent samples), and averages from all treatments different from 0 (H0 =treated equal to controls, single-sample T-test). To minimize the effect of multiple tests, significance was set to p<0.001.

The 4603 genes that showed significant effects by any of these three criteria were selected for further analysis by k-means clustering and principal components analysis (PCA). Biological replicates were averaged to obtain a single set of values for each extract. Functional analysis of clusters was performed using the DAVID Bioinformatic Resources 6.7 (Huang da et al., 2009). Results have been deposited at the NCBI's Gene Express Omnibus database, Series record GSE53522 for PM samples; and Series record GSE41334 for BaP exposure (GMS1015031, GMS1015032, GMS1015033).

3.2.5. Microarray validation and quantitation

The extracted total RNA was first treated with DNAseI (Ambion, Austion, TX) to remove genomic DNA, and retro-transcribed into cDNA using TranscriptorFirst Strand cDNA Synthesis Kit (F. Hoffmann- La Roche, Basel Switzerland). To validate the microarray results 22 primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). The house-keeping gene *ppia2* was used as reference gene (Pelayo et al., 2012; Olivares et al., 2013). The specific transcripts were quantified in cDNA samples by Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-qPCR), using Lightcycler 480 Real Time PCR System (F.Hoffmann- La Roche) with SYBR Green Mix (Roche Applied Science, Mannheim, Germany). Polymerase Chain Reaction (PCR) products were sequenced in a 3730 DNA Analyser (Applied Biosystems) and confirmed by comparison with the corresponding reference sequences at NCBI server. PCR efficiency values for reference and target genes were calculated by dilution series, using five serial 1:4 dilutions for each gene. Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, in triplicate). Cp values of target genes (Cp_{tg}) were normalized to the correspondent Cp values of the reference gene, *ppia2* for each sample ($corrCp_{tg}=Cp_{tg} - Cp_{ppia2}$), and changes in mRNA levels of treated samples relatively to controls, were calculated by the $\Delta\Delta$Cp method (Pfaffl, 2001), using corrected Cp values from treated and non-treated samples ($\Delta\Delta Cp_{tg} = corrCp_{tg\_untreated} - corrCp_{tg\_treated}$). Correlations between RT-qPCR and chemical data were analysed by partial least square analysis (PLS) using the plsdepot R package (Y= $\Delta\Delta$Cp data, X = chemical content, ng/L of zebrafish exposure water, R Core Team, 2014).

3.3. Results

3.3.1. Influence of particle size and origin in PM toxicity

Analysis of the dioxin-like activity of the different PM fractions by AhR-RYA revealed that the PM<0.5 µm accumulated more than 50% of the AhR ligand activity in all cases, a proportion that exceeded the 90% for the most active samples (Figure 1A). Maximal activity was observed for rural samples collected during the cold period (samples R1 and R2), whereas the lowest values corresponded to rural samples, but taken during the warm period (samples R3 and R4, Figure 1A). The dioxin-like activity strongly correlated with the calculated TEQ values (p<0.0001, Figure 1B) for both rural and urban samples. The dioxin-like activity of samples R3 and R4 was so low, that they were not considered in all further assays.

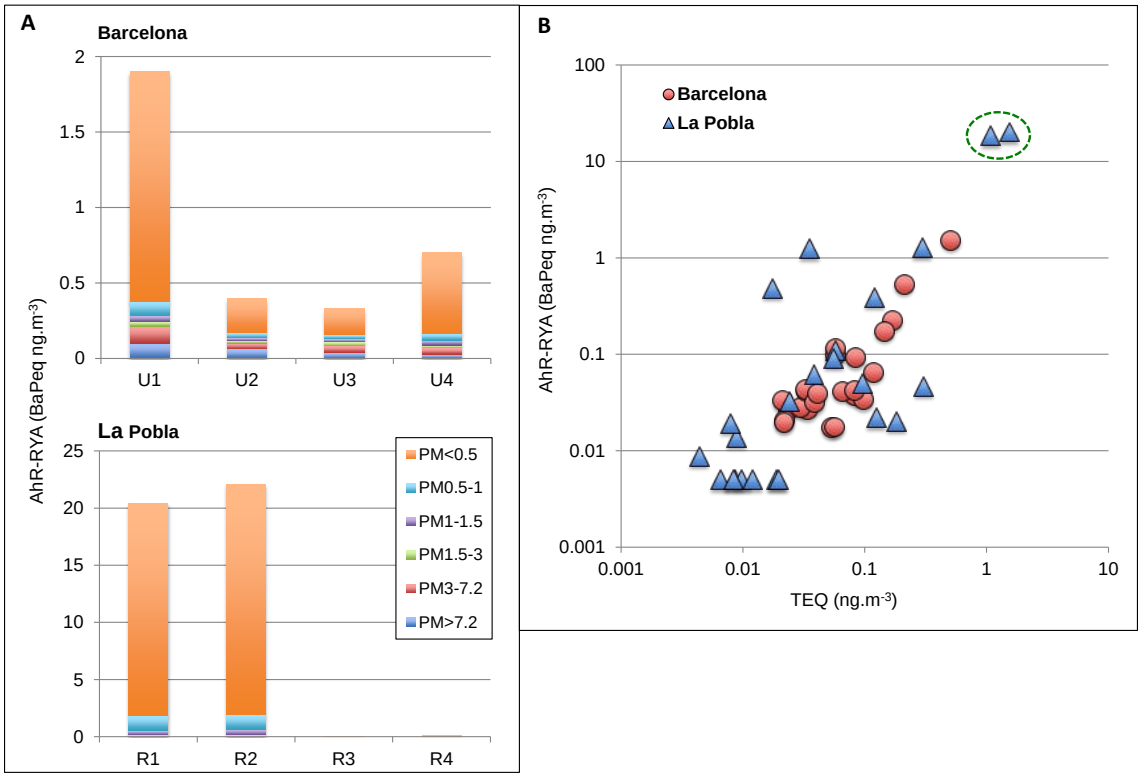

Figure 1 - A) Dioxin-like activity of urban and rural samples (top and bottom, respectively, note the different scale on the Y-axis). The different particulate matter (PM) fractions are represented by coloured sectors. B) Double-log correlation between the expected dioxin-like activity of PM organic extracts based on their content on polycyclic aromatic hydrocarbons (X-axis, toxicity equivalent values, TEQs) and the dioxin-like activity measured on the aryl hydrocarbon receptor-recombinant yeast assay (AhR-RYA, Y axis). Urban and rural samples are indicated by red circles and blue triangles, respectively. Two encircled triangles, corresponding to PM<0.5 µm cold period samples from the rural site.

Only the two extracts from PM<0.5 µm fraction of R1 and R2 samples showed embryotoxic effects on the ZET assay. Toxic effects first appeared at 96 hpf, and included malformed swim bladder, yolk sac and pericardial oedemas and spinal malformations. High mortality ratios were observed at 120 hpf and the surviving embryos were severely deformed, showing abnormal tail, trunk and spinal cord, deformed swim bladder, head oedema with underdeveloped eyes and prominent yolk sac and pericardial oedema with severe heart damage. These effects correlated with the high PAH concentrations and dioxin-like activity of R1 and R2 samples of the smallest size fraction (circled in Figure 1B). Due to their toxicity, these two extracts were diluted 1:12 for all further analysis. This dilution factor ensured that no phenotypical adverse effects were observed on the ZET and that, approximately, the AhR-RYA activity of samples R1 and R2 was similar to the most active urban sample, U1 (Figure 1A). For the transcriptomic analysis, cold period samples were used from the urban and rural sites (U1, U2, R1 and R2), of the smallest size (PM<0.5 µm). Since urban samples of different sizes did not induce any observable phenotypical effects on the ZET assay, the largest fraction of samples U1 and U2 were also used for comparison purposes.

3.3.2. Microarray results

Zebrafish embryos exposed to extracts of the largest and smallest fraction of samples U1 and U2, and to extracts of the smallest fraction of samples R1 and R2, revealed significant changes on mRNA abundance for 4603 genes. Hierarchical clustering indicated differential effects for urban and rural samples, but it showed no differences between urban PM>7.2 µm extracts and their PM<0.5 µm counterparts (Figure 2A). Cluster analysis (k-means) allowed the identification of three main clusters (Figure 2B and Figure 3).

Cluster 1 included 1835 gene transcripts overrepresented in embryos exposed to rural extracts, whereas Cluster 3 included 577 genes whose mRNA abundance was increased in embryos exposed to urban samples and underrepresented in embryos exposed to rural ones. Cluster 2 included 2191 genes showing significant increases in abundance after any of the treatments compared to negative controls (Figure 3). The mutual correlation between the three clusters of genes and the different PM samples can be observed in the PCA analysis in Figure 2B. Note the clear discrimination between rural and urban samples, resulting in the almost perpendicular position of the corresponding PCA loadings (arrows). The analysis also confirms the similarity of the effects induced by urban PM>7.2 µm and PM<0.5 µm extracts. The position for BaP-

treated samples, essentially at the diagonal between the clusters 1 and 3, suggests that the effects induced by the dioxin-like PAH were shared by both types of PM extracts.

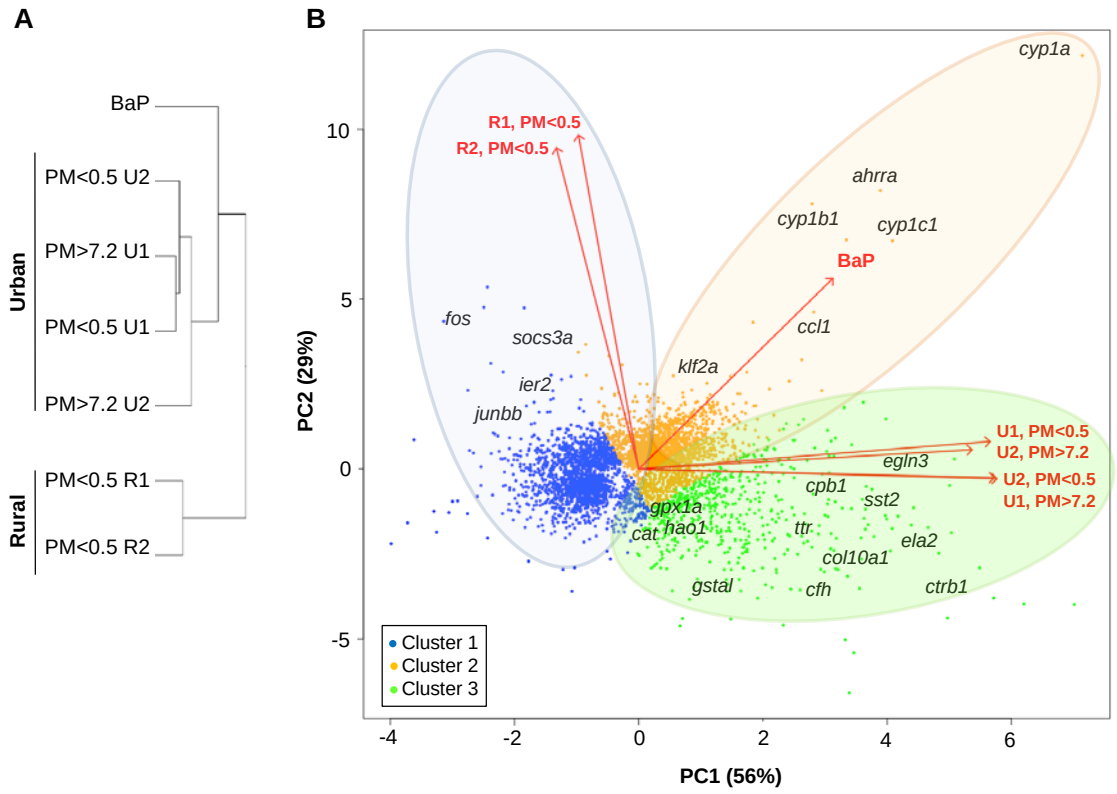

Figure 2 - A) Hierarchical clustering of transcriptomic data from zebrafish embryos exposed to rural PM samples of the cold period (R1, R2) of the smallest size fraction, and to urban PM samples of the cold period (U1, U2) of the largest and smallest size fraction (in µm), and to BaP 0.1 mg.L^{-1}; B) Principal component analysis (PCA) of microarray data from the 4603 genes selected. Dots represent scores for the different genes, coloured according to their adscription to the different k-means clusters (see also, Figure 3). Some representative genes are identified by their official symbol: cytochrome P4501A, P4501B, P4501C (*cyp1a*, *cyp1b*, *cyp1c*, respectively), aryl-hydrocarbon receptor repressor a (*ahrra*), FBJ murine osteosarcoma viral oncogene homolog (*fos*), jun B proto-oncogene b (*junbb*), glutathione S-transferase alpha-like (*gstal*), hydroxyacid oxidase 1 (*hao1*); chymotrypsinogen B1 (*ctrb1*), elastase 2 (*ela2*), immediate early response 2 (*ier2*); catalase (*cat*); kruppel-like factor 2a (*klf2a*); somatostatin 2 (*sst2*); transthyretin (*ttr*), egl-9 family hypoxia-inducible factor 3 (*egln3*), glutathione peroxidase 1a (*gpx1a*), complement factor h (*cfh*), collagen type X alpha 1 (*col10a1*), carboxypeptidase B1 (*cpb1*), chemokine ligand 27a (*ccl1*), suppressor of cytokine signalling 3a (*socs3a*). Arrows represent loadings for the different samples (indicated in red characters). PCA loadings have been scaled to facilitate their representation. Axis legends indicate the corresponding Principal Component (PC) and proportion of variance explained.

Dots in Figure 2B represent scores for the different zebrafish genes, coloured by their classification in the cluster analysis. Clusters 1 and 3 (blue and green in Figure 2B, respectively) showed positive correlations with the PCA loadings corresponding to rural and urban samples, respectively. Genes belonging to cluster 2 (in orange in Figure 2B) appeared correlated to BaP-treated samples, and included genes know to be controlled

by the AhR (cyp*1a, cyp1b1, cyp1c1*, *ahrra,* Figure 2B), configuring the expected dioxin-like response.

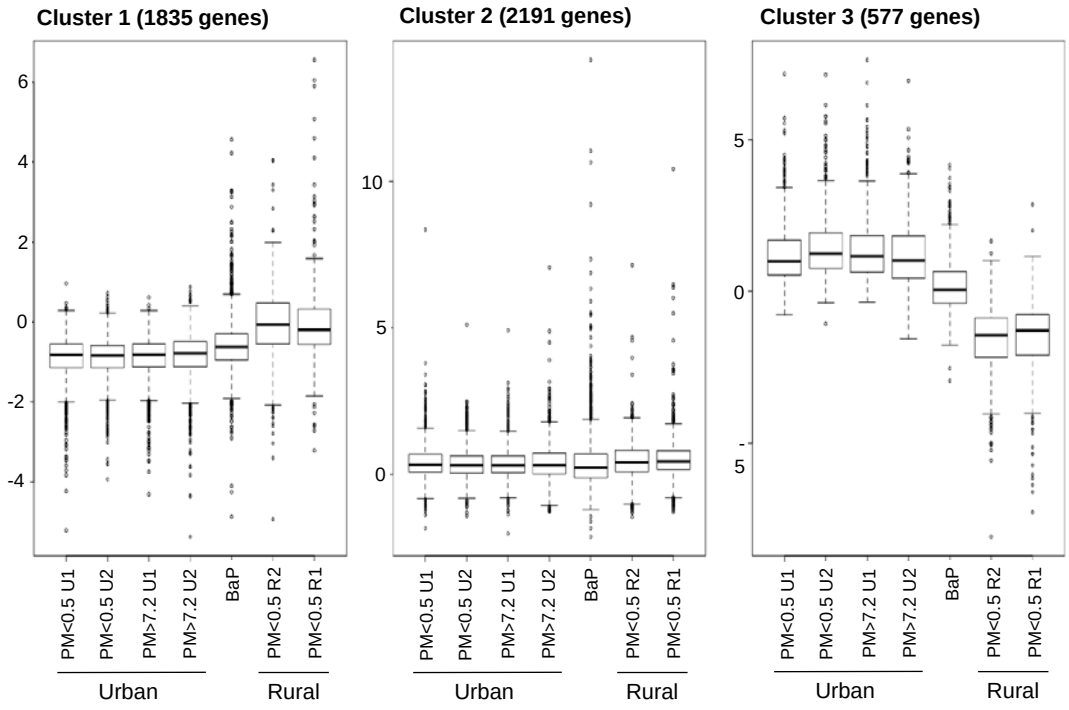

Figure 3 - Box plot representation of 4603 genes showing significant differences between urban and rural particulate matter (PM) samples of the smallest size (in µm, for samples U1, U2 and R1, R2), and the largest size (samples U1, U2), and to BaP 0.1 mg.L^{-1}, grouped by k-means clustering.

The accession number, sequences, amplicon lengths and efficiency of the 22 primers designed to validate the microarray results are described in the Table 1. The observed variation in mRNA abundance of these genes was confirmed by RT-qPCR, whose results showed a very good correlation with the microarray data (Figure 4).

Table 1 - Sequences of primers used in this study.

Gene	Accession number	Primer sequence (5'-3')	Amplicon length (bp)	Efficiency (%)
ahrra	NM_001035265.1	FW: CCGCTGGCATATAACATGAGC	71	100
		RV: AAGACTGTTGACGCTGTGTTCAC		
ccl1	NM_131062.1	FW: AAATGTTTCCAGCTGATGCTTCA	61	90
		RV: GAGCGTAACACACACAGTATATCGC		
cebpb	NM_131884.2	FW: GATGCAACTCATTGTAACAGTATTGGA	81	91
		RV: TAACAGCAGACCAGTTGTACCTTTATC		
cfh	NM_001199190.1	FW: AAGGATGGATGGACTACCGCT	81	90
		RV: TGATTTAATGTCCGACTTCCCG		
col10a1	NM_001083827.1	FW: GTTCTGTACTTCAGCTCAATGAGCA	81	98
		RV: CGGCAGCAAAGACACCAT TAG		
cpb1	NM_001110021.1	FW: TTCATGCCAGAGAATGGATCAC	81	98
		RV: CAGGATCACTGCCATATGTGGA		
ctrb1	NM_212618.1	FW: TTGCCGTTGGAAAGTCCATC	81	93
		RV: TGGCCAGCTTGATCAGCAG		
cyp1a	NM_131879.1	FW: GGTTAAAGTTCACCGGGATGC	101	100
		RV: CTGTGGTGTGACCCGAAGAAG		
egln3	NM_213310.1	FW: AGGGAAACCTTACGTAGCCGA	81	95
		RV: TCATGAGGATTCCTTCGATCTGA		
ela2	NM_001139464.1	FW: ATATCCGCAATGACATCGCC	81	96
		RV: CAGGCAGACAAGCAGGTGAA		
fos	NM_205569.1	FW: TGATTTCTGAACGTGTGAATGTTG	81	93
		RV: CATACTTGGACGTCAGACCATTTAAT		
gstal	NM_213394.1	FW: CCTGCCGAAAACAAAGAGAAA	71	100
		RV: AACACTGGAAGGAAGCGCAC		
hao1	NM_001083542.1	FW: GGCGAATTTCGAGTCTCCAG	81	96
		RV: GTCACATACACCGCCAGCC		
ier2	NM_001142583.1	FW: ACTTGATCTGGTTTGTTAATGGCC	71	97
		RV: GCTTATTTCACGTTGACCTGCAC		
junbb	NM_212750.1	FW: CGCATCTGATATTTTCGTGCAG	81	97
		RV: TTAACATGGCGTCAGGATCAAC		
klf2a	NM_131856.3	FW: GCAATTTGTATGCTGTTAACGTTGA	81	96
		RV: CCAGAACAACTCACATAGCCCTT		
krt4	NM_131509.1	FW: AACTCAACCGCATGATCGC	71	94
		RV: CTCCAAGTTGGCACGCTGT		
ppia	AY391452	FW: GGGTGGTAATGGAGCTGAGA	179	100
		RV: AATGGACTTGCCACCAGTTC		
socs3a	NM_199950.1	FW: CCTGTGTTTTTTTCAGCCTTCTC	81	91
		RV: GAGATGTCTTTACAAAAGGTCTAAATGC		
sst2	NM_131727.1	FW: AAGAGAGGAAGACCGGCTGC	81	97
		RV: GCCAGCATGTTGGTAACTTCG		
rdh1	NM_198069.1	FW:CGCACTTTCTGCACGTCATC	101	99
		RV: TGTCCACTACACACGTGGGC		
ttr	NM_001005598.2	FW: CGGTAAAAATCCTGGATGCTG	81	96
		RV: TCCCACCTTGATCTTGACGAA		

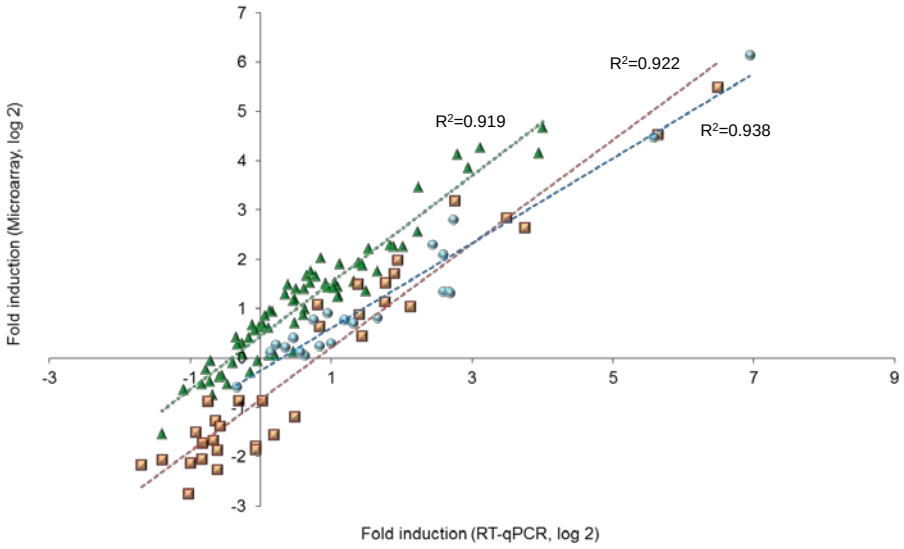

Figure 4 - Correlation between the microarray data and Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-qPCR) data for the genes described in Table 1. Green triangles correspond to urban samples, orange squares to rural samples, and blue circles correspond to BaP 0.1 mg.L^{-1}. Correspondent regression lines and R^2 coefficients are also shown.

3.3.3. Functional analyses

Gene ontology (GO) analysis of microarray results (Table 2) identified functional gene categories affected by the treatments. Cluster 1 presented a significant enrichment of genes involved in protein folding and in RNA-protein interaction, fundamental functions for embryo development. In addition, a subcluster of genes (indicated as Top 100, elevated in rural samples, Table 2), included 10 genes related to the jun/fos signalling pathway, implicated in cellular proliferation. Cluster 2 did not include any functional category above the cut-off level (p<0.05, Bonferroni). As described before, different cytochromes (included the *cyp1a, cyp1b* and *cyp1c* isoforms) appeared as the dominant functional class when only the most highly induced genes were considered (Table 2, indicated as "Top 100", see also their position in the graph in Figure 2B). Finally, Cluster 3 was highly enriched in transcripts related to oxidation-reduction reactions (including Thioredoxin-like enzymes, like glutathione S-transferases), including classical markers of oxidative stress, like catalases and glutathione peroxidases, suggesting an oxidative stress response associated to urban, but not rural, samples. Other functional group of genes included different peptidases of the chymotrypsin group, considered as pancreatic markers (Mudumana et al., 2004). An additional subset of 29 deregulated genes corresponded to beta and gamma crystalline genes (Table 2), which include many minor crystalline components of the eye lens (Greiling et al., 2009).

Table 2 - Functional analysis of transcripts included in the different k-means clusters.

Term	P Value	Genes	Fold Enrichment	Bonferroni
		Cluster 1		
GO:0006457~ protein folding	2.45E-08	DNAJA2L, GRPEL1, FKBP5, FKBP4, FKBP3, PPIL2, ZGC:91922, DNAJC10, CALR, CANX, DNAJB11, DNAJA3B, HSP90A.2, SI:CH211-207K7.4, HSPA9, ZGC:110447, TCP1, CCT6A, CCT7, PPIE, CCT5, PPIH, PFDN1, HSP90B1, PPIG, ZGC:153638, PPIB, LOC554962, PFDN6, PFDN5, ZGC:114162, DNAJB1B, DNAJC5G, FKBP10, ZGC:103752	2.9	3.30E-05
GO:0030529~ ribonucleoprotein complex	1.60E-05	SRP14, MRPS34, RPL36A, RPL19, RPL5A, SNRPD3, RPL5B, SNRPD2, HNRPR, ZGC:171710, ZGC:136591, RPS2, SI:DKEY-265M8.2, RPLP1, ZGC:162401, RPL11, RPL12, PRPF31, ZGC:55701, MRPL9, RPS4X, HNRNPU, HNRPDL, PUF60B, LARP7, SNRPA, RPS13, SNRNP40, RPS11, ZGC:110031, ZGC:73262, ZGC:109888, RPS15A, RPS25, ZGC:123046, RPL30, SNRPFL, RPS29, SNRPD3L, MRPL14, RPL3, RPS20, RPL10A, RSL24D1, HNRNPAB, ZGC:114150, MRPS25, MRPS24, RPS9, RPL27, ELAVL3, HNRNPA0, HNRNPUL1, HUG, POP4, HNRNPH1, RPL28L	1.8	4.22E-03
		Subcluster: Elevated in rural samples (Top10)		
IPR000837 Fos transforming protein	7.67E-09	FOS, ATF3, ZGC:92354, ZGC:92851	748.9	7.67E-08
		Cluster 2		
		Subcluster: High expression in all treatments (Top100)		
IPR017972 Cytochrome P450	1.71E-05	ZGC:63920, CYP1C2, CYP1C1, CYP1A, CYP1B1, CYP24A1L	18.3	2.97E-03
		Cluster 3		
GO:0055114: oxidation reduction	4.75E-19	ACOX1, CYB5R2, CYP2X12, ZGC:65987, RDH1L, ZGC:55856, ZGC:77906, SC4MOL, TDO2A, ZGC:162502, P4HA1, CPOX, CAT, CH25HL1.1, GPX1A, ZGC:86915, ZGC:101673, ZGC:171947, ACADM, ZGC:171945, GPX4A, ZGC:92630, ZGC:192870, RDH1, CDO1, OGFOD1, HAO1, DHRS1, SI:DKEY-91I10.3, CYP2J25, ZGC:91876, CYP2J22, ZGC:158614, HAO2, ZGC:123280, ZGC:103585, HSD11B3, LOC554843, CYP2J28, ME1, ZGC:153647, HSD3B7, SI:DKEY-239I20.2, EGLN3, UGDH, ADH5, ZGC:153269, CYP2J30, ZGC:195056, FMO5, ALDH1A2, ZGC:153597, LOC100148115, DHCR7, ZGC:136371, SDR16C5, SI:DKEY-39N1.2, MPX, GLUD1B, PTGR1, CYP4V2, LOC100006793, CRYZ, ALDH9A1B, GAPDHS, ABP1, ZGC:192851, ZGC:112146, HSDL1, AKR1B1, CP, CYP3C1L2	3.5	1.65E-13
IPR001314: Peptidase S1A, chymotrypsin	4.91E-12	ZGC:92511, ZGC:92590, ZGC:112368, ZGC:112160, CTRB1, ST14B, ZGC:163025, ZGC:66382, F7, LOC562139, ZGC:112302, PROC, ZGC:112285, ZGC:136461, ZGC:101788, ZGC:92041, ELA2L, ZGC:153968, TRY, ELA2, ELA3L, CTRL	7.2	3.72E-09
SM00247: XTALbg - Beta/gamma crystallin	1.59E-12	CRYGM2D2, CRYGM2D3, CRYGM2D4, CRYGM2D6, CRYGM2D7, CRYGM2D8, CRYBA1L, ZGC:173645, CRYBA2B, CRYGM3, CRYBA2A, CRYGM2C, CRYGM2B, CRYGM5, ZGC:162402, CRYGM2D1, CRYGM2D13, CRYBB1L3, ZGC:171792, CRYGM2D10, LOC569000, CRYGM2D11, CRYGN2, ZGC:86723, ZGC:153846, SI:DKEY-57A22.15, ZGC:173493, ZGC:136220, ZGC:173452	11.7	1.95E-10
IPR012335: Thioredoxin fold	2.75E-07	GSTT1B, GLRX3, PDIA2, TMX3, GSTM, ZGC:66350, ZGC:92869, ZGC:136472, ZGC:101897, TFA, GSTM3, ZGC:173994, GSTAL, ZGC:153284, GPX1A, GLRX, SI:DKEY-39N1.2, GPX4A, ZGC:110343, CLIC1, ZGC:112293, ZGC:173961, ZGC:173962, PRDX6, ZGC:92254, CLIC5, ZGC:162356, PDCB, AGR2, CASQ2, GSTP1, ZGC:162938	3.0	4.75E-04

Representatives of the different functional groups are identified by their official gene names in Figure 2B. From these results and from their robust behaviour in the microarray validation assays, 13 genes were selected as potential markers: *cyp1a*; *ahrra*; *fos*, FBJ murine osteosarcoma viral oncogene homolog, *junbb*, jun B proto-oncogene b, *gstal*, glutathione S-transferase alpha-like; *hao1*, hydroxyacid oxidase 1; *ctrb1*, chymotrypsinogen B1; *ela2*, elastase 2; *ier2*, immediate early response 2; *klf2a*, kruppel-like factor 2a; *sst2*, somatostatin 2; *ttr*, transthyretin; *egln3*, egl-9 family hypoxia-inducible factor 3. More information about the selected genes in Table 3.

Table 3– General biological functions for the genes selected as potential molecular markers.

Gene	General biological function	Reference
cyp1a	*cyp1a* activation as an important role in the phase I metabolizing process, participating on the typical dioxin-like response, under AhR regulation	(Van der Oost et al., 2003)
ahrra	Cellular response to xenobiotic stimulus. Participates in the AhR signalling cascade	(Van der Oost et al., 2003)
fos and junbb	Immediate early genes, members of the transcription factor activator protein (AP-1) family. They are leucine zipper transcription factors that play a central role in regulating gene transcription in various biological processes.	(Kovács, 1998)
gstal	*gstal* encodes a cytosolic form of glutathione S-transferase, (GST) alpha-like, involved in defence against oxidative stress. GSTs are phase II metabolizing enzymes that conjugate xenobiotics with reduced glutathione, allowing their excretion from the cell.	(Van der Oost et al., 2003)
hao1	*hao1* encodes a peroxisomal enzyme that oxidizes glycolate to glyoxylate with concomitant production of H_2O_2.	(Recalcati et al., 2003)
ctrb1	*ctrb1* encodes a precursor of the digestive enzyme chymotrypsin, which is a serine endopeptidase.	(Hedstrom, 2002)
ela2	*ela2* belongs to a group of hydrolysis enzymes called serine proteases, which are synthesized in the pancreatic acini and digest insoluble proteins of connective tissues. *ela2*-like genes are considered specific peptidase genes of the exocrine pancreas.	(Mudumana et al., 2004)
ier2	Important gene for the Kupffer's vesicle development, determining embryos left/right symmetry.	(Hong and Dawid, 2009)
klf2a	Immediate-early transcription factor involved in multiple processes, including haematopoiesis and cardiovascular function.	(Kaczynski et al., 2003)
sst2	*sst2* belongs to the somatostatin family, regulatory peptides produced throughout the central nervous system and in most major peripheral organs, with effects on cognitive, locomotor, sensory, inflammatory and hormonal functions.	(Patel, 1999)
ttr	*ttr* encodes a thyroid hormone-binding protein. The up-regulated expression of this gene was observed on zebrafish embryos enduring thyroid endocrine disruption.	(Yu et al., 2013)
egln3	*egln3* is involved in the oxidation-reduction process. *egln3* up-regulation may be a favourable prognosticator for gastric cancer.	(Su et al., 2012)

3.3.4. Marker discovery

Relative mRNA abundance for the 13 selected genes were analysed by RT-qPCR for embryos exposed to PM>7.2 µm and PM<0.5 µm fractions from both rural and urban extracts (samples R1, R2, U1, U2, U3 and U4). Expression data was correlated to the chemical content of the samples (van Drooge and Grimalt, 2015), by PLS analysis (Figure 5). Consistently with the microarray analysis, the PLS analysis grouped the different RT-qPCR probes (purple gene names in Figure 5) into two main clusters, one of them (*egln3, gsta1, ela2, ctrb1, sst2, hao1*, and *ttr*) related to urban extract exposure (U1-U4 in Figure 5) and a second one (*fos, junbb, ier2, ahrra,* and *klf2a*) linked to rural samples (R1, R2 Figure 5).

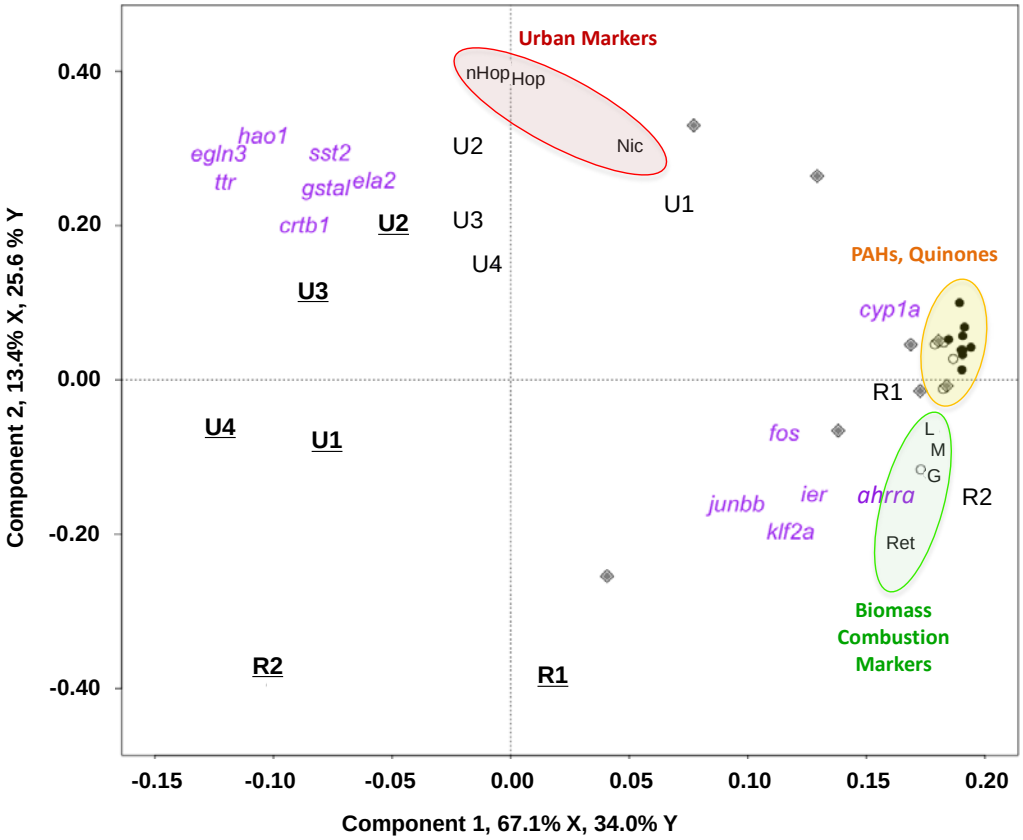

Figure 5 - Mutual correlations betw een gene expression and chemical composition data. The graph shows results from a partial least square, PLS, analysis, plotting component 1 vs. component 2 loadings for mRNA abundance data (gene names as in Figure 2B, here in purple, corresponding to Y-data in the PLS analysis), chemical composition (black or grey symbols and letters, X-data in the PLS analysis, only VIPs are represented), and scores for rural and urban samples (coded as in Figure 1A). Solid and open circles, and grey diamonds correspond to loadings for polycyclic aromatic hydrocarbons, quinones and n-alkanes, respectively. Some key analytes are represented by their abbreviated names: retene (Ret), 17(H)α-21(H)β-29-hopane (Hop), 17(H)α-21(H)β-29-norhopane (nHop), nicotine (Nic), galactosan (G), mannosan (M), and levoglucosan (L). Score values for the different samples are represented by U1-U4 (urban) or R1, R2 (rural) symbols, as in Figure 1A. Bold and underline characters correspond to the PM>7.2 µm fraction, whereas plain ones represent the PM<0.5 µm fraction. Coloured ovals indicate the position of urban (red) and rural (green) chemical markers; the orange oval indicates the position of PAHs (except Ret) and quinones in the graph. Y- and X- axis indicate the fraction of X- and Y- variance explained by each component.

Results from the *cyp1a* probe appeared relatively separated from both clusters. Urban PM>7.2 µm and PM<0.5 µm fractions clustered together, indicating a similar mode of action of PM organic constituents (U1-U4 in bold/underline and plain characters, in Figure 5, respectively), whereas rural PM>7.2 µm appeared clearly separated from their PM<0.5 µm counterparts (R1 and R2 in bold/underline and plain characters, in Figure 5, respectively). This difference can be attributed to the overall low biological activity of the rural PM>7.2 µm fraction. When considering the PLS loadings for chemical compounds (empty, black and grey symbols and text in Figure 5), it becomes clear the correlation of *cyp1a* with PAHs and quinones, consistent with the presence of AhR ligands in the organic fraction of PM (Nisbet and LaGoy, 1992; Mesquita et al., 2014). In addition, urban samples and their associated RT-qPCR probes, positively correlated with the concentration of urban (mostly, traffic-related) markers (hopanes and Nic, Figure. 5, red oval). Similarly, rural samples and their associated RT-qPCR probes, positively correlated with biomass combustion markers (Ret, G, M and L, green oval, Figure 5, assignation of source markers further detailed in van Drooge and Grimalt, 2015).

3.4. Discussion

Assessment of the adverse effects of the different components of ambient PM is of paramount importance for public health. Whereas air quality regulations have been using the PM mass as a normative parameter for air quality (WHO, 2013), an increasing amount of data suggests that the chemical composition of particles and their size are essential factors determining the health risk of ambient air PM (de Kok et al., 2005; Zhou et al., 2005; de Kok et al., 2006; WHO, 2013; Mesquita et al., 2014). The results shown here indicate that both putative toxic components and dioxin-like activity of PM are concentrated in the sub-micron fraction (PM<0.5 µm), in agreement with previous studies (Perez et al., 2009; Zhang et al., 2011b; Mesquita et al., 2014).

Rural samples of the cold period, with high contribution of biomass burning emissions, presented very high levels of PAHs (and quinones), and concurring dioxin-like activity and embryotoxicity. When diluted, the concentration of PAHs and quinones on these samples became similar to the concentrations in urban PM samples. However, the microarray analyses detected at least two physiological responses to PM exposure that appeared different from the common dioxin-like activity, and strongly dependent on the origin (and chemical composition) of PM. Rural PM<0.5 µm samples of the cold period affected general cell functions, like protein folding or ribonucleoprotein complex

components, as well as elements of the jun/fos pathway (related to cell proliferation), indicating a very pleiotropic toxicity pattern. Urban extracts, on the other hand, deregulated genes involved in oxidation-reduction and peptidase reactions, as well as several (up to 29) different eye lens' protein genes. These alterations could be markers for specific toxic effects on the corresponding organs (pancreas, eye lens) of the developing embryo. Thus, the present transcriptomic study points to specific effects in two organs (pancreas and eye lens) that have been reported as targets for air pollution in humans. Epidemiologic studies have linked urban pollution (mostly organic pollutants) and risk of diabetes (Balti et al., 2014; Eze et al., 2014). In addition, exposure to particulate air pollution has been associated to the onset of cataracts and in humans and in model animals (Wegener et al., 2002; Pokhrel et al., 2013), an effect linked to the de-regulation of the oxidoreduction enzymes glutathione-S-transferase and aldose reductase (Shichi, 2004; Terada, 2005). Whereas no direct relation can be drawn at this point, it is worth mentioning that several zebrafish genes encompassing these enzymatic activities (*akr1b1, gstal, gstm, gstm3, gstp1, gstt1b*, ZGC:173961, ZGC:173962, and ZGC:173994) appeared also deregulated upon exposure to urban PM extracts. To the best of my knowledge, this data is the first evidence that emission origins may influence not only the strength, but also the molecular mechanisms implicated in the toxicity of the organic fractions of ambient PM.

Given the complexity of ambient PM samples, it is difficult to individualize compounds, or even families of compounds, that may be considered the triggers for the observed toxic effects. The present data indicates that the dioxin-like response can be almost entirely predicted by the PAH composition of samples, in agreement with previous studies (Olivares et al., 2011; 2013; Mesquita et al., 2014). While there was a strong correlation between mRNA abundance levels of some genes and different chemical markers of biomass burning or urban (mostly, traffic-related fossil fuel combustion) pollution, it is unlike that these compounds could contribute to the observed toxic effects, since they are not biologically active. Nicotine is the only one of the studied source markers with well-known toxic potential, but since it was only present in urban PM<0.5 µm samples, it cannot be linked to the up-regulation of genes common to all tested urban samples. Therefore, other not identified compounds related to these sources may be responsible for the observed effects.

The zebrafish is a widely used vertebrate model and a valid alternative species to the toxicological use of mammals (Raldua and Pina, 2014). Although the use of aquatic models precludes the analysis of lung toxicity, the zebrafish embryo seems promising to evaluate the systemic effects of organic PM and, particularly, adverse effects on the developing human fetus (Goldsmith, 2004; Xu and Zon, 2010). The results

shown in this work suggest that besides molecular composition and particle distribution, air quality assessment requires a third biological component for the early detection of systemic noxious effects on human health.

3.5. Conclusions

This study shows that biologically active, potentially hazardous organic compounds are predominantly present in the ultra-fine PM fraction (PM<0.5 μm). These compounds were present not only in urban but also in rural PM, at particularly high concentrations, especially in the cold period as a consequence of biomass burning. The dioxin-like activity remains as an important part of the overall toxicity of ambient PM, which is consistent with the up-regulation of cytochrome-related genes and the distribution of PAH and quinones. In addition, urban PM induced the expression of pancreatic and eye-lens specific genes, in addition to oxidative stress-related genes. This suggests a specific oxidative stress response associated to urban, but not rural, samples. These results correlated with higher abundances of urban life-style markers such as nicotine, hopane and norhopane, but they most probably reflect the influence of other unknown atmospheric urban pollutants. In contrast, zebrafish exposure to rural samples induced the expression of genes involved in protein folding and RNA-protein interaction, which are fundamental for embryo development. The expression of these genes correlated with specific markers of biomass burning, such as galactosan, mannosan and levoglucosan, suggesting a contribution of specific biomass burning compounds to the observed physiological effects.

3.6. References

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CHAPTER 4

Influence of primary and secondary aerosol constituents on the toxicity of urban particulate matter

Chapter submitted for publication as:

Mesquita, S.R., van Drooge, B.L., Barata, C., Vieira, N., Guimarães, L., Piña, B. Toxic potential of organic constituents of submicron particulate matter (PM1) in an urban road site (Barcelona). Submitted to Environmental Science and Pollution Research. Under review.

4.1. Introduction

Airborne particulate matter (PM) is a highly complex mixture, whose composition varies with PM size, source, weather conditions, space and time. Epidemiological and toxicological studies have described thoroughly the outcomes of exposure to particulate matter, namely inflammatory, respiratory and cardiovascular impairments, preterm birth, cataracts, diabetes, and chronic diseases (Vineis and Husgafvel-Pursiainen, 2005; Kelly and Fussell, 2012; Rohr and Wyzga, 2012; Balti et al., 2014; Fleischer et al., 2014). From a policy perspective, determining the relative importance of different PM constituents is essential for protecting public health (Rohr and Wyzga, 2012).

The major components of atmospheric PM are inorganic salts, siliceous crustal minerals, carbonaceous materials, and water (Seinfeld and Pankow, 2003). The carbonaceous fraction of PM generally accounts for 25-50% of the total particle mass, and is composed by thousands of organic compounds, which strongly contribute to the toxic potential of PM (Rohr and Wyzga, 2012; Mesquita et al., 2014b; 2015). Part of the organic components of particles are originated from direct emissions (constituting primary organic aerosols, POAs), whereas another part is formed in the atmosphere from gaseous precursors, originating secondary organic aerosols (SOAs). POA can enter the atmosphere from combustion processes, such as fossil fuel combustion or biomass burning, and comprises a significant fraction of toxic compounds, such as parental polycyclic aromatic hydrocarbons (PAHs). Nonetheless, road, tire and brake abrasion, plant debris suspension, pollen and dust, also contribute to atmospheric POAs. On the other hand, atmospheric SOAs are formed by oxidation of volatile organic compounds (VOC), from biogenic sources (e.g. trees and plants, such as α-pinene, isoprene, carboxylic and dicarboxylic acids) or anthropogenic sources like traffic emissions or solvent evaporation. Oxidation of these gases produces compounds which have low enough volatilities to form SOAs via either self-nucleation or gas/particle partitioning on pre-existing particles (Hallquist et al., 2009). The relative contribution of primary and secondary sources to ambient PM depends on the nature and strength of local emissions and on the atmosphere meteorological and chemical conditions (Seinfeld and Pankow, 2003).

Ultrafine atmospheric particles have been recognized as especially damaging for exposed organisms since they can penetrate deep into the respiratory system and enter into blood circulation. Once in the body, the bioavailability and potential toxic effects of their constituents highly depend on the physico-chemical properties of the different chemical compounds. Whereas the toxicity of POA organic compounds, such as PAHs,

is relatively well understood (Rohr and Wyzga, 2012; Mesquita et al., 2014b; 2015), there is a major scientific gap concerning the contribution of SOA to the overall adverse effects of PM on public health and the environment. In principle, since SOA partly consists of transformed biogenic compounds from isoprene and terpene oxidation, it is not considered to be a strong contributor to total toxicity of PM. However it has been observed to induce moderate adverse reactions, such as inflammation and cytotoxicity (Gaschen et al., 2010; Rohr and Wyzga, 2012).

Recent studies investigated the toxicity of PM organic fraction, with *in vitro* and *in vivo* assays as alternative to mammalian tests (Mesquita et al., 2014b; 2015); the AhR-RYA (aryl hydrocarbon receptor-recombinant yeast assay) to assess dioxin-like activity of PM samples and the ZET (zebrafish embryotoxicity test, ISO, 2007) to evaluate systemic effects of contaminants in the early embryonic development (Mesquita et al., 2014a; 2014b). Both the AhR-RYA and the ZET have been proved useful to assess PM toxic potential. Transcriptomic analyses have been used to identify adverse outcome pathways resulting from zebrafish embryos exposure to PM constituents (Bui et al., 2012; Mesquita et al., 2015a). PM organic fraction from different origins may induce distinct adverse effects in zebrafish embryos. Embryos exposed to PM organic extracts of an urban background site, showed a specifically de-regulation of genes involved in oxidative stress response and in the exocrine pancreatic function (Mesquita et al., 2015a). In contrast, exposure to PM organic extracts from winter emissions of a rural mountain village with high contributions of fresh biomass burning emissions influenced genes implicated in key cellular signalling pathways and development. In addition, both types of extracts similarly induced the AhR signalling pathway, confirming that the dioxin-like activity remains as an important part of the overall toxicity of ambient PM, correlating with the PM content on PAHs. That study allowed to define a set of gene expression markers to distinguish the different biological activities detected in samples of distinct origins (urban background vs. rural) using high-throughput RT-qPCR techniques (Mesquita et al., 2015a). These gene markers can be used to trace the different origins and toxicological potential of PM samples (Mesquita et al., 2015a).

The present study extends those results by focusing on the potential influence of POA and SOA on the toxicity of PM organic fraction to the zebrafish embryo. Samples of particles with an aerodynamic diameter smaller than 1 µm (PM1) were collected at a road-site in the city center of Barcelona. Traffic density in this city is among the highest in Europe. Moreover, its geographical location favours accumulation of secondary aerosols (Pey et al., 2009; Pérez et al., 2010). Sampling took place continuously over 12h periods (day and night), during one month. Samples were tested for dioxin-like activity (AhR-RYA) and associated to chemical characterization. Embryotoxic effects

(ZET assay) and expression of gene markers of interest were evaluated subsequently. Differential patterns related to human activity cycles (working days vs. Weekends; daytime vs. nighttime) were analysed to provide additional information for risk management purposes.

4.2. Methods

4.2.1. Sampling and chemical analysis

PM filter samples were collected at a road site in the Urgell street (Carrer del Comte d'Urgell, 41º23'18'' N; 02º09''00'' E; 40m a.s.l.), which is located inside a square-grid street network in the neighbourhood of Eixample in Barcelona, Catalonia, Spain. This street is composed by a two-way cycling path and a one-way four-lane vehicle road, with high vehicle intensity (≈ 17 000 vehicles per day passing the street in the study month).

The methodology used for sampling, extraction and chemical analysis has been described elsewhere (Alier et al., 2013). Briefly, PM filter samples were collected using Digitel-DH80 HiVol samplers, continuously for 12h periods between 09.00 and 21.00 (UTC) at a sampling rate of 30 $m^3.h^{-1}$, during one month (September 22 to October 18, 2010). In total, 51 samples were tested, comprising day and night periods, weekdays and weekends, including holidays. Holidays were counted as weekend days. Based on their representativeness of primary organic aerosol emission sources and secondary organic aerosol formation, thirty-two organic compounds have been previously analysed by gas chromatography–mass spectrometry (Alier et al., 2013). The compounds determined were PAHs, namely phenanthrene (Phe), anthracene (Ant), fluoranthrene (Fla), pyrene (Pyr), chrysene (Cry), benzo[a]anthracene (BaA), benzo[bj]fluoranthene (BbjF), benzo[e]pyrene (BeP), benzo[a]pyrene (BaP), benzo[g,h,i]perylene (BghiPer) and coronene (Cor), and hopanes (17(H)α-21(H)β-29-norhopane, nHop and 17(H)α-21(H)β-29-hopane, Hop), nicotine (Nic), levoglucosan (L), galactosan (G) and mannosan (M), hydroxyl and aromatic dicarboxylic acids (DCAs, namely malonic acid, succinic acid, glutaric acid, pimelic acid, suberic acid, azelaic acid, glyceric acid, malic acid, tartaric acid, tricarballytic acid and phthalic acid), cis-pinonic acid (CPA), 3-hydroxyglutaric acid (3HGA) and 3-methyl-1,2,3-butanetricarboxylic acid (MBTCA), 2-methylglyceric acid (2MGA) and polyols, such as C5 alkene triols (C5T) and 2-methyltetrols (2MTs).

Elemental carbon (EC) and organic carbon (OC) were also analysed and PM1 filters were weighted (Dall'Osto et al., 2013). Data from traffic intensity (i.e. averaged

number of vehicles per hour) and traffic intensity corrected by wind speed were also included in the study, along with timetable (nighttime versus daytime) and day of the week (working day versus weekend, including holidays).

Toxicity equivalents (TEQs) were calculated from the TEQ factors described for each individual PAH (BaA, Cyr, BbjF, BeP, BaP, BghiPer, Nisbet and LaGoy, 1992). These values reflect the total (human) toxicity of the different PAHs, irrespectively to their ability to bind to the AhR.

4.2.2. Bioassays

For AhR-RYA and ZET assays PM filter samples were extracted by sonication with a mixture of dichloromethane:methanol (Merck, Germany, 2:1 v/v, 3x10 mL, 15 min). The extract was filtered in a syringe with 0.45 mm PFTE membrane, (Puradisc, Whatman, USA), evaporated on Rotovap followed by gentle nitrogen gas stream, and dissolved in 1 mL of methanol. Then, 0.5 mL of extract were reserved at -20ºC for AhR-RYA, and the other 0.5 mL were exchanged into dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical, Germany), and reserved at -20ºC for the ZET assay.

The AhR-RYA detects and quantifies the receptor-binding activity of dioxin-like compounds, such as PAHs, by incubating samples with a modified yeast strain expressing the human AhR and AhR nuclear translocator genes, under the GAL1-10 promoter (Miller; Noguerol et al., 2006). PM1 samples were tested in duplicate, together with a positive (1µM β-Naphthoflavone), negative (5% of vehicle, methanol), and inhibitory (sample extract plus 1µM β-Naftoflavone) controls in 96-well polypropylene microtiter plates (NUNCTM, Roskilde, Denmark) as described in previous studies (Mesquita et al., 2014b). β-galactosidase activity was measured by fluorescence using 4-methylumbelliferone β-D-galactopyranoside (MuGal, Sigma–Aldrich Chemical, Germany) as a fluorogenic substrate, and 355nm and 460nm as excitation and emission wavelengths, respectively. AhR-ligand activity of samples is represented as BaP equivalents (BaPeq, Noguerol et al., 2006; Misaki et al., 2007).

Wildtype zebrafish maintenance conditions and spawning procedure was performed as described previously (Mesquita et al., 2014b). Fishes were always handled humanely and treated with regard for alleviation of suffering. One hour after spawning fertilized eggs were collected, rinsed and reserved for 24h until the exposure experiments. Viable eggs were randomly distributed into glass petri dishes, and exposed to air samples extracts diluted 500 times with embryo water (total volume of 2.5 ml). Exposure experiments followed the International standard 15088 (ISO, 2007) for zebrafish eggs. Each sample was tested with 10 embryos per dish, with 6 replicates,

plus a negative control (0.2% Dimethyl sulfoxide), and a positive control (3.7 mg/L 3,4-Dichloroaniline, Sigma–Aldrich Chemical, Germany (ISO, 2007). The exposure was maintained from 24 hours postfertilization (hpf) until 120hpf, renewing medium every 24h. Developmental progression of embryos was observed daily (Kimmel et al., 1995), under a stereomicroscope Nikon SMZ 1500 equipped with a Nikon digital sight DS-Ri1 digital camera. Lethal and sub-lethal endpoints were determined accordingly to endpoints already described (Hermsen et al., 2011). Real concentrations of PM organic constituents (10 individual PAHs) have been measured previously, assuring an acceptable relative standard deviation of less than 20% from the nominal concentrations (Mesquita et al., 2014b). After exposure, zebrafish embryos were frozen and kept at -80ºC for posterior transcriptomic determinations.

4.2.3. Gene expression analysis

Total RNA was isolated from whole embryos (pools of 20 individuals), using Trizol reagent protocol (Invitrogen Life Technologies, Carlsberg, CA). RNA concentration was measured by spectrophotometry (NanoDrop Technologies, Wilmington, DE). Total RNA extracted was first treated with DNAseI (Ambion, Austion, TX) to remove genomic DNA and retro-transcribed into cDNA using TranscriptorFirst Strand cDNA Synthesis Kit (F. Hoffmann- La Roche, Basel Switzerland), which was stored at -20ºC. Aliquots of 50 ng were used to quantify specific transcripts in Lightcycler® 480 Real Time PCR System (F.Hoffmann- La Roche) using SYBR® Green Mix (Roche Applied Science, Mannheim, Germany). Ten probes were selected to be tested on the present study: cytochrome P4501A (*cyp1a*), FBJ murine osteosarcoma viral oncogene homolog (*fos*), glutathione S-transferase, alpha-like (*gstal*), hydroxyacid oxidase 1 (*hao1*), chymotrypsinogen B1 (*ctrb1*), elastase 2 (*ela2*), immediate early response 2 (*ier2*), kruppel-like factor 2a (*klf2a*), somatostatin 2 (*sst2*), transthyretin (*ttr*). More details on the biological functions of these genes and their adscription to the functional categories identified can be found in Mesquita et al. (2015). *ier2, klf2a* and *fos* were found up-regulated in zebrafish embryos exposed to PM extracts from biomass burning emissions (rural environment), while *gstal, hao1, ctrb1, ela2, sst2* and *ttr* were up-regulated upon exposure to PM from fossil fuel emissions (urban background). Both extracts commonly induced *cyp1a* expression. The house-keeping gene *ppia2* was used as reference gene. Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, in triplicate). Cp values of target genes (Cp_{tg}) were corrected to the correspondent Cp values of the reference gene, *ppia2* for each sample ($corrCp_{tg}=Cp_{tg} - Cp_{ppia2}$), and changes in mRNA levels of treated samples relatively to

controls, were calculated by the ΔΔCp method (Pfaffl, 2001), using corrected Cp values from treated and non-treated samples ($\Delta\Delta Cp_{tg} = corrCp_{tg\_untreated} - corrCp_{tg\_treated}$). The fold-change ratios were derived from those values.

4.2.4. Statistical analysis

All statistical calculations, including linear and non-linear regression methods and principal component analyses (PCA), were performed in R (packages psych, gplots, multcomp, reshape2, ggplot2, and scales, R Development Core Team, 2008). Correlations between dioxin-like activity values and sample's total PAH content, calculated TEQ values and concentrations of PM1 organic constituents were calculated with the Pearson's coefficients. To accomplish parametric requirements, chemical and AhR-RYA data were used as logarithmic transformation. For transcriptomic analysis, genes were considered as differentially expressed using a confidence p value ≤ 0.05, plus fdr (false discovery rate) correction for multiple determinations when indicated. All statistical calculations for RT-qPCR data were performed using ΔΔCp values, as this parameter fulfilled data assumptions of normality and homogeneity (Shapiro-Wilk and Levene's tests). Differences among groups of samples were analysed by Student's t test (2 groups) or analysis of variance (ANOVA) plus Tukey's test (more than 2 groups).

4.3. Results

4.3.1. Dioxin-like activity of PM1 samples

Dioxin-like activity was detected by the yeast-based assay in all samples. Activity values ranged between 0.26 and 1.04 $ng.m^{-3}$ BaPeq, corresponding the highest values to daytime samples taking during working days and the lowest values to nighttime samples from weekends (Figure 1). The observed dioxin-like activity of PM1 samples showed significant correlation with 12 out of the 32 chemical compounds and environmental parameters tested, including PAHs, TEQ values, EC levels, and traffic intensity (with or without wind speed correction; Table 1). No correlations were observed between dioxin-like activity and PM1 mass concentration or OC levels. Some relevant correlations are shown in Figure 2.

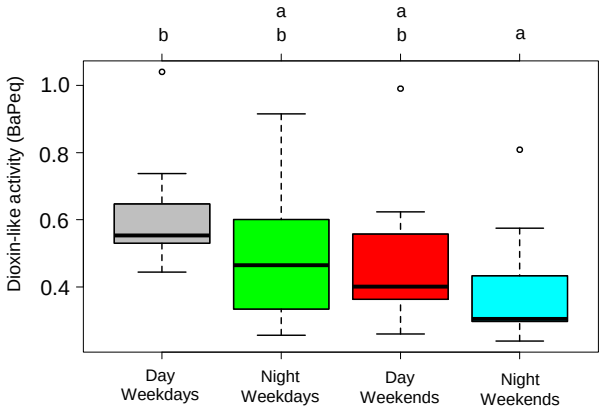

Figure 1 - Dioxin-like activity of PM1 samples as measured by AhR-RYA. Values are represented as ng.m^{-3} BaP eq, and classified according to the collection time (weekdays/weekends and daytime/nighttime). Whiskers represent the 1st and the 4th quartile of the distribution; the box corresponds to the 50% central part of the distribution. The thick bars correspond to the median, circles show outliers. Different letters on top show to statistically different groups of data, as indicated by ANOVA followed by a Tukey's B post-hoc analysis.

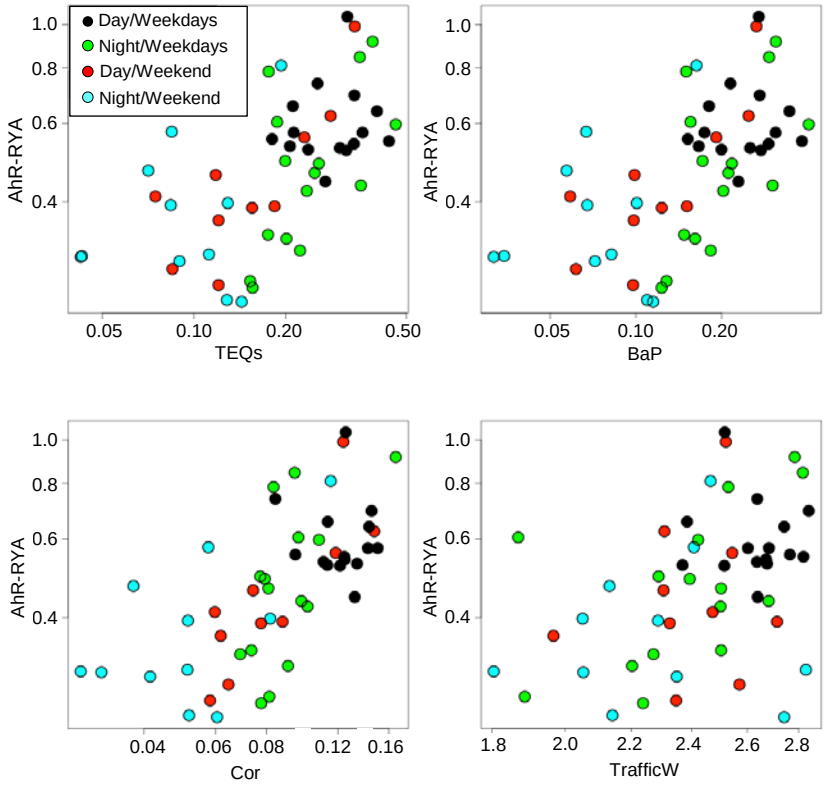

Figure 2 - Correlations between dioxin-like activity in PM1 samples (particles with aerodynamic diameter smaller than 1 µm), concentrations of different constituents of the organic extracts, and traffic intensity with wind correction (TrafficW). Colour codes (as in Figure 1) indicate the time of the day (daytime/nighttime) and differentiate between weekdays and weekend samples. Cor, coronene, TEQs, toxicity equivalent values, BaP, Benzo[a]pyrene.

Table 1 - Pearson correlations between dioxin-like activity (AhR-RYA) and chemical components of organic PM1 extracts and traffic intensity (only significant correlations are shown).

	r	*p*
Cor	0.661	0.00000039
TEQs	0.605	0.0000066
BaA	0.605	0.0000066
BaP	0.605	0.0000066
BghiPer	0.603	0.0000072
∑PAHs	0.586	0.000015
Cry	0.566	0.000035
BeP	0.515	0.00023
BbjF	0.498	0.00041
EC	0.420	0.004
TrafficW	0.412	0.005
Traffic	0.381	0.01

Abbreviations: benzo[a]anthracene (BaA), benzo[bj]fluoranthene (BbjF), benzo[e]pyrene (BeP), benzo[a]pyrene (BaP), benzo[g,h,i]perylene (BghiPer), coronene (Cor), chrysene (Cry), toxicity equivalents (TEQs), sum of polycyclic aromatic hydrocarbons (∑PAHs), elemental carbon (EC), traffic intensity (Traffic), traffic intensity with wind correction (trafficW).

The chemical data and the dioxin-like activity of PM samples were further analysed by PCA. Some chemical compounds were grouped based on their parentage and on preliminary statistical analysis, namely biomass combustion markers (BB markers; sum of G, M, L), hopanes (Hop; sum of nHop and Hop), C3-C5 DCAs (malonic acid, succinic acid and glutaric acid sum), C7-C9 DCAs (pimelic acid, suberic acid and azelaic acid sum), products of α-pinene oxidation (PinOXI; sum of CPA, 3HGA and MBTCA) and products of isoprene oxidation (IsoOXI; sum of 2MGA, C5T, 2MTs). Nic, traffic intensity, and EC were considered individually based on preliminary analysis, and the remaining DCAs (glyceric acid, malic acid, tartaric acid, tricarballytic acid and phthalic acid sum) were grouped as other DCAs. Two components explained 63% of the total variation of samples (Figure 3). PC1, accounting for 47% of the total variation, showed high loadings for all PAHs tested, as well as for TEQ and EC values (Figure 3). It also showed significant, although lower loadings for traffic intensity (both with and without wind correction) and dioxin-like activity, although weaker than EC and TEQ. PC2 (16% of total variation) showed high loadings for different compounds related to biogenic SOA, (i.e. PinOXI, IsoOXI), regional biomass burning (BB markers), and a mixture of short-chain C3-C5 DCAs (Figure 3, which were all related to regional air circulation and accumulation of aged organic aerosols in the urban air shed (Alier et al., 2013; Dall'Osto et al., 2013). Day- to nighttime and working days/weekends differences were only observed for PC1 (see coloured areas in Figure 3). Data suggest that PAHs are major

drivers of the dioxin-like activity (both observed and predicted) in urban PM1 samples, and that their abundance was largely explained by fresh traffic emissions *in situ*. In contrast, PC2 appeared related to the presence of secondary organics and/or regional biomass burning aerosols, with a negative correlation with the urban daytime and traffic-related OA (Figure 3).

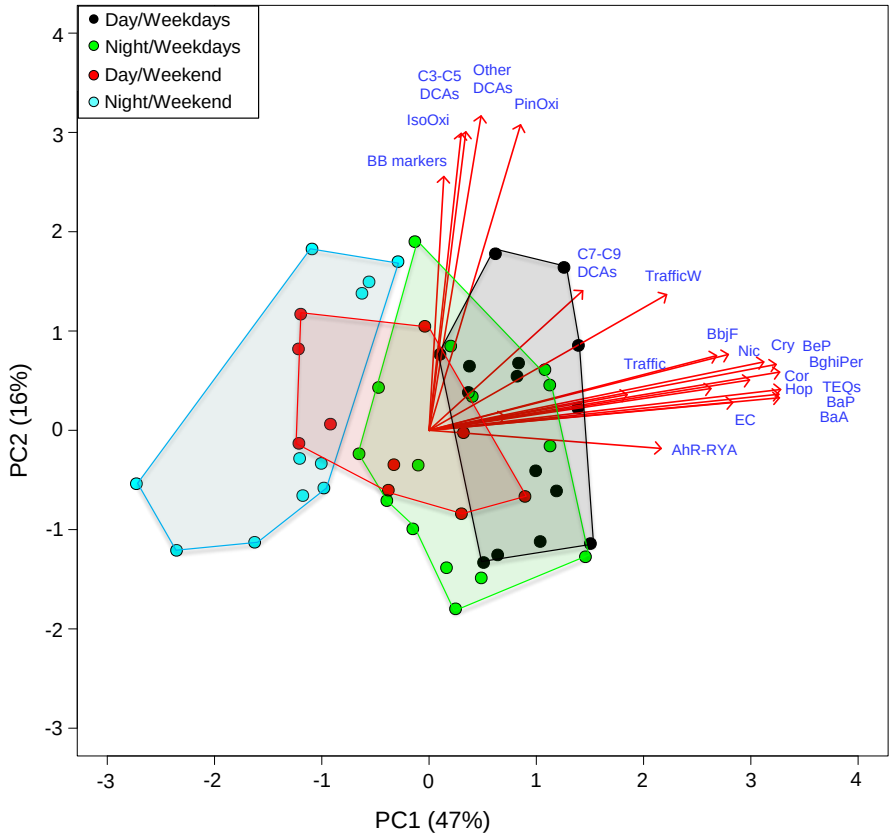

Figure 3 - Principal components analysis (PCA) score- (points) and loading-plots (arrows and blue text) of the aryl hydrocarbon receptor-recombinant yeast assay (AhR-RYA) data, concentrations of different constituents of the organic extracts, and traffic intensity with and without wind correction (TrafficW and Traffic, respectively). Samples are colour-coded as in previous figures. Coloured areas indicate the "territories" for each group of samples: day/weekday (grey), night/w eekday (green), day/weekend (red) and night/w eekend (cyan). The loadings have been scaled to fit in the same graph as scores. The general parameters of the PCA are indicated at the inset. Abbreviations: elemental carbon (EC), phenanthrene (Phe), anthracene (Ant), fluoranthrene (Fla), pyrene (Pyr), chrysene (Cry), benzo[a]anthracene (BaA), benzo[bj]fluoranthene (BbjF), benzo[e]pyrene (BeP), benzo[a]pyrene (BaP), benzo[g,h,i]perylene (BghiPer) and coronene (Cor), hopanes (Hop), nicotine (Nic), biomass combustion markers (BB markers), C3-C5 dicarboxylic acids (DCAs), C7-C9 DCAs, products of α-pinene oxidation (PinOXI), products of isoprene oxidation (IsoOXI), toxicity equivalents (TEQs).

4.3.2 Transcriptional responses elicited by exposure of zebrafish to PM1 organic extracts

Exposure to PM1 organic extracts resulted in moderated toxic effects for a limited number of samples. No mortality was observed and only two samples showed mild morphological defects (mild pericardial edema, malformation of the swim bladder and

balance disruption) in between 10 and 20% of animals. No correlation was observed between these defects and any chemical or environmental parameter). In contrast, transcript level analysis showed changes on mRNA abundance in PM1 extract-treated samples relative to control samples for most of the genes studied. The analysis showed both positive and negative effects, with *cyp1a* and *ela2* showing the most general upregulation effects, whereas *ttr* was downregulated by all tested samples (Figure 4). However, most genes showed increases greater than two-fold over control levels for at least two or three samples (Figure 4). Note that the three genes with the lowest variation from controls, i.e. *fos*, *ier2* and *klf2a*, were previously categorized as gene markers for biomass burning emissions (green boxes in Figure 4), whereas the rest, except *cyp1a*, were related to fossil fuel pollutants (red boxes in Figure 4, Mesquita et al., 2015).

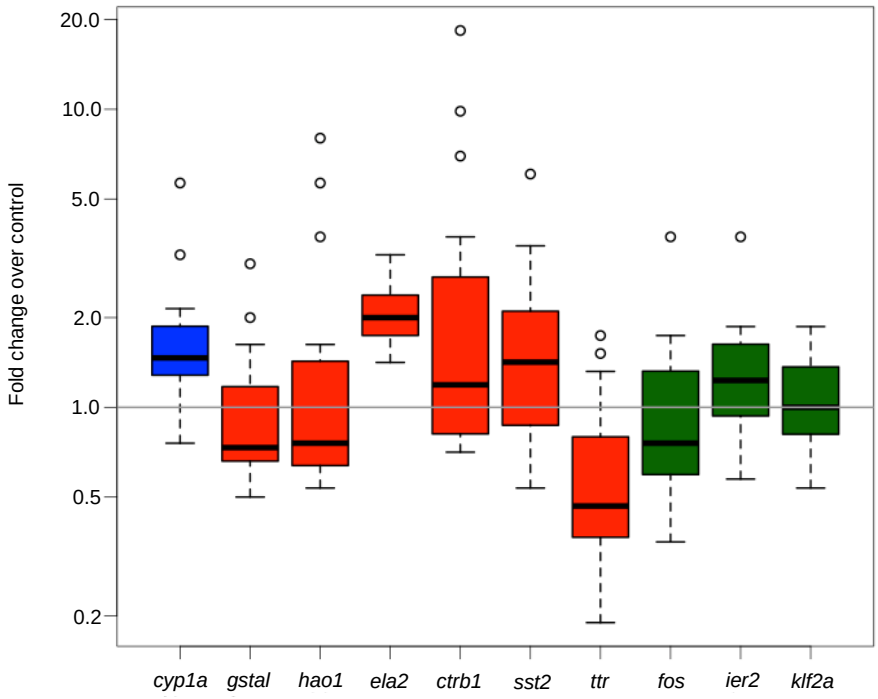

Figure 4 - Distribution of relative mRNA abundances for the tested genes, expressed as fold change over untreated controls. Blue, red and green boxes correspond to *cyp1a*, fossil fuel and biomass burning gene markers, respectively. Note the log y-scale. Control values (fold change=1) is indicated by the grey horizontal line. Cytochrome P4501A (*cyp1a*), FBJ murine osteosarcoma viral oncogene homolog (*fos*), glutathione S-transferase, alpha-like (*gstal*), hydroxyacid oxidase 1 (*hao1*), chymotrypsinogen B1 (*ctrb1*), elastase 2 (*ela2*), immediate early response 2 (*ier2*), kruppel-like factor 2a (*klf2a*), somatostatin 2 (*sst2*), transthyretin (*ttr*).

Changes on gene expression were correlated with pollutant levels in the corresponding PM1 extracts. Clustering analyses defined two groups of gene expression patterns, grossly coinciding with fossil fuel (*gstal, hao1, ctrb1, sst2* and *ttr*) and biomass

burning (*fos, ier2, klf2a*) expression markers, leaving *cyp1a* and *ela2*, in between (Figure 5). *cyp1a* expression showed a significant positive correlation (cyan asterisks and red squares) with the dioxin-like activity of samples, determined by the AhR-RYA (Figure 5). Biomass burning gene markers (*fos, ier2 and klf2a*) positively correlated with EC and PAHs (Figure 5). In contrast, most fossil fuel gene markers (*gstal, hao1, ctrb1, sst2 and ttr*) showed negative (blue squares) rather than positive correlations with several organic PM constituents, particularly with oxidized compounds (IsoOXI, PinOXI) and the presence of aged organic species, as well as with biomass burning-related markers (C3-C5 DCAs, BB markers, Figure 5). No significant differences were found in gene expression data between day- and nighttime or between weekdays and weekends (not shown).

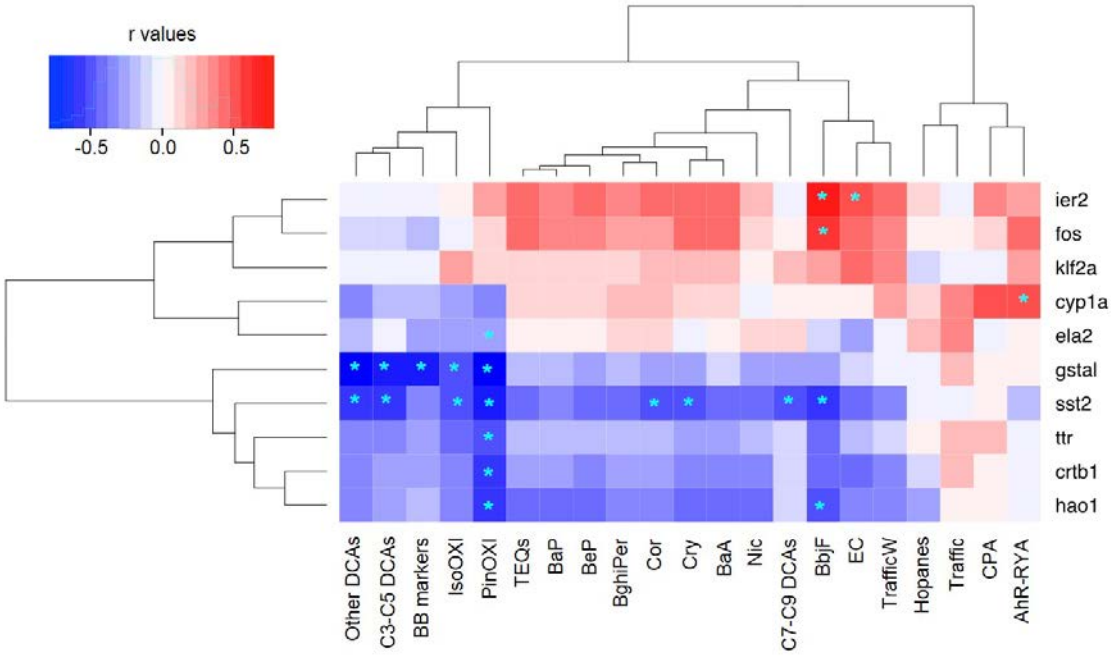

Figure 5 - Correlation analysis of gene expression data against the concentrations of particulate matter constituents and sampling parameters. Red and blue squares denote positive and negative correlations, significant correlations are marked w ith a cyan asterisk. Hierarchical clustering was used to group genes and parameters of similar behaviour. Abbreviations as in Figure 3. Cytochrome P4501A (*cyp1a*), FBJ murine osteosarcoma viral oncogene homolog (*fos*), glutathione S-transferase, alpha-like (*gstal*), hydroxyacid oxidase 1 (*hao1*), chymotrypsinogen B1 (*ctrb1*), elastase 2 (*ela2*), immediate early response 2 (*ier2*), kruppel-like factor 2a (*klf2a*), somatostatin 2 (*sst2*), transthyretin (*ttr*).

4.4. Discussion and conclusions

PM1 samples used in the present study had PAH concentrations in the range of the ones reported in other urban areas of Spain, elsewhere in Europe and in the USA (Ning et al.,

2007; Callén et al., 2011; Křůmal et al., 2013). Particulate BaP concentrations ranged from 0.03 to 0.40 ng.m^{-3}, with a mean concentration of 0.18 ng.m^{-3}, which is very similar to the mean annual BaP concentrations of 0.20 ng.m^{-3} observed in urban traffic sites in Barcelona. BaP concentration did not exceed the annual 1 ng.m^{-3} target value defined by the European Commission (EC, 2015). The dioxin-like activity measured in the present study correlated with TEQ values, as observed previously (Mesquita et al., 2014b; 2015), but also with EC and traffic intensity. EC persists in the particle phase and is generally viewed as only coming from primary emissions, while OC varies in volatility, therefore exchanging between the particle and gas phases. For this, EC is commonly used as a tracer in the urban area for vehicle exhaust and urban-traffic POA (Seinfeld and Pankow, 2003). The dioxin-like activity of PM extracts likely resulted from inputs of fossil fuel combustion (i.e. primary aerosol formation), mainly produced by traffic related activities. PAHs isomeric ratios calculated for several parental PAHs by Allier et al. (2013) using the same PM samples, were consistent with a dominance of traffic emission sources.

However, daily and weekly variations in dioxin-like activity did not simply reflect traffic inputs. Urban traffic has a strong day/night component, and somewhat weaker workday/weekend variation, whereas dioxin-like activity showed greater weekly than daily variation. Correction for the typically quieter night wind conditions (TrafficW) increased the correlation between dioxin-like activity and traffic intensity, although it only explains less than 20% of the observed total dioxin-like variability of samples. The relatively weak day/night variation in dioxin-like activity mirrored the relative minor changes observed for several chemical compounds analysed, whereas the strong weekday/weekend variations correlated with the distribution of PAHs, hopanes and EC, possibly as a result of the overall higher fossil fuel combustion inputs during working days due to congested traffic relatively to weekends. Overall, the results of the present study support the AhR-RYA as an early-warning tool for routine control of particulate PAH emissions, sensitive to daily and weekly fluctuations of these pollutants.

Weak phenotypical adverse effects were observed in zebrafish embryos exposed to PM1 extracts. Nominal exposure concentrations of PAHs in the ZET assay were lower than those that induced embryotoxicity in a previous study with PM samples from a background location in Barcelona (Mesquita et al., 2014b). Nonetheless, exposure to this low concentration of contaminants in the present study lead to relatively mild variations on gene expression. Previous transcriptomic analyses showed different patterns of transcriptional responses for genes involved in biotransformation phase I, oxidative stress and endocrine effects (*ela2*, *ctrb1*, *ttr*, *sst2, gstal,* and *hao1)* and those related to key cellular processes and development (*fos, ier2* and *klf2a,* Mesquita et al., 2015a).

This distribution is also observed in the present work, although one cannot relate the different patterns of response separately to urban fossil fuel combustion or rural biomass burning emissions (Mesquita et al., 2015a). Rather, the second group of gene markers, together with *cyp1a* and *ela2*, appeared associated to PAHs, EC, dioxin-like activity, and traffic-related compounds. In contrast, expression of oxidative stress and hormone-related gene markers appeared negatively correlated to the levels of regional biomass burning and of oxidized compounds. These negative correlations suggest the presence of specific compounds that affect different biological activities, like oxidative stress response or endocrine responses, while being irrelevant for dioxin-like activity. These as yet unknown compounds would be poorly represented in aged regional air masses related to biomass burning and secondary aerosol formation. The present study leads to the conclusion that the contribution of POA and SOA contribution to organic PM toxicity in urban areas with intense human activity and associated vehicle traffic is complex and still poorly understood.

In addition, *cyp1a* activation appeared as strongly correlated with the levels of dioxin-like activity as evaluated by AhR-RYA, but not with TEQ values. This is consistent with previous studies, and suggests the presence in PM1 of dioxin-like compounds aside from the parental PAHs.

Chemical and biological complexity of PM1 organic constituents preclude a definitive evaluation of human health risks associated to emissions from different sources. Moreover, it turns out as unreliable evaluations based purely in quantitative estimations of a limited number of parameters, like the mass of PM1, PM10 (particles smaller than 10 μm) or BaP concentrations. Further investigation should be carried out to deepen knowledge and bring more empirical evidence addressing the complex contribution of POA and SOA to organic PM toxicity in urban areas with intense human activity and associated vehicle traffic. Additional regulatory actions should also be put in place to account for the complexity of PM organic constituents and their potential toxic risks to human and environmental health.

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CHAPTER 5

Toxicity assessment of atmospheric particulate matter in the Mediterranean and Black Seas open waters

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5.1. Introduction

Ambient particulate matter (PM) is considered as one of the most harmful air pollutants to human health by different regulatory agencies (WHO, 2004; EEA, 2012). One of the major contributors to its toxic effects are organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), and their derivatives of photochemical oxidation (Kanakidou et al., 2005; Cavanagh et al., 2009; Mesquita et al., 2014b).

PAHs are formed through the incomplete combustion of organic matter (e.g. biomass burning, fossil fuel combustion, motor vehicle exhaust, waste incineration, home heating) and are considered priority pollutants due to their mutagenic, carcinogenic and teratogenic properties (IARC, 1998; EC, 2000; Zhang and Tao, 2009). Once emitted into the atmosphere, PAHs tend to bind to the soot carbon of air particles due to their semi-volatile character (Dachs and Eisenreich, 2000; Lohmann and Lammel, 2004). When coated onto ambient PM, PAHs have relatively long half-lives, persisting in the environment and travelling long distances before they deposit. Particle-bound PAHs can be removed from the atmosphere through dry deposition (i.e. gravitational settling) or wet deposition (scavenging by rain, snow, fog).

Researchers have highlighted the importance of atmospheric dry deposition as a main vector for the entrance of the most hydrophobic PAHs into coastal aquatic systems and open oceans (Franz et al., 1998; Arzayus et al., 2001; Jurado et al., 2004; Castro-Jiménez et al., 2012; González-Gaya et al., 2014; Kroflič et al., 2015). In parallel, an increasing number of studies have been directed to the assessment of the potential toxic burden of airborne particle-bound PAHs to aquatic biota (Sheesley et al., 2004; 2005; Mesquita et al., 2014a; 2014b). Indeed, an effort has been made to broaden the scope of atmospheric aerosol toxicology to begin to include effects on aquatic organisms (Sheesley et al., 2004; 2005; Mesquita et al., 2014a; 2014b). However, regulatory directives do not contemplate the effects of airborne pollution to aquatic organisms. The latest air quality report of the European Environmental Agency (EEA) addresses the need to protect the environment from the adverse effects of particulate air pollution, but no critical level, target/limit value or long-term objective is defined for PM (neither for airborne PAHs), in order to protect terrestrial or aquatic organisms (EEA, 2014).

The Mediterranean and Black Seas, are semi-enclosed basins surrounded by highly populated countries, with corresponding high levels of atmospheric PM. As a consequence, air masses laden with anthropogenic PM move across the Mediterranean and Black Seas, partially depositing into water. In particular, atmospheric deposition has been referred to as a significant non-point source of PAHs in remote (i.e. non-coastal)

areas of the Mediterranean and Black Seas (Grimalt et al., 1988; Lipiatou et al., 1997; Tsapakis et al., 2003; 2006; Castro-Jiménez et al., 2012; Parinos et al., 2013). The importance of atmospheric input of PAHs to Mediterranean Sea sediments has also been suggested (Lipiatou et al., 1997; Tsapakis et al., 2003).

The organic constituents of atmospheric PM from urban, semi-rural and rural environments have been shown as biologically active and capable of inducing adverse effects on aquatic species, with strong correlations with the PAH content of samples (Sheesley et al., 2004; 2005; Olivares et al., 2011; 2013; Mesquita et al., 2014b; 2015). However, no information is available on the biological activity and effects of PM organic constituents from the atmosphere of open marine environments, such as the Mediterranean and Black Seas. The concentrations of PM organic constituents on such environments, distant from emission sources, are expected to be lower than over continents, however the mixture of chemical compounds bounded to PM should be biologically active, possibly contributing to the toxic burden of marine biota.

A fundamental aspect determining the toxic effects of organic compounds, such as PAHs, is their ability to bind and activate the aryl hydrocarbon receptor (AhR), a key regulator of detoxification cascades (Nebert et al., 1993; Hankinson, 1995). This biological activity is commonly known as dioxin-like activity. This activity can be monitored by the AhR-recombinant yeast assay (AhR-RYA), in which the AhR is challenged with extracts of PM samples to determine their toxic potential (Olivares et al., 2011; Mesquita et al., 2014b).

The zebrafish (*Danio rerio*) embryo is a widely used vertebrate model in toxicology (Westerfield, 2000). The zebrafish embryotoxicity (ZET) test provides a unique opportunity to analyse survival, morphological alterations, and specific gene expression changes that bring insight on the toxic mechanism of action of PM constituents (Olivares et al., 2011; Mesquita et al., 2014b;). The use of transcriptomic tools on zebrafish embryos has proven successful to study and early anticipate potential adverse outcomes of environmental pollutants (Scholz et al., 2008; Piña and Barata, 2011; Raldúa et al., 2012; Mesquita et al., 2015).

Previously, microarray technology was used to identify genes of interest to be used as potential markers for the biological effects of PM organic constituents (Mesquita et al., 2015). These genes included the cytochrome P450 1a (*cyp1a*), a classical AhR-responsive gene, and other genes related to oxidative response, development and pancreatic exocrine function (Mesquita et al., 2015). In the present study the organic extracts of atmospheric PM samples from the Mediterranean and Black Seas were tested, using the AhR-RYA and the ZET assays. The expression of those genes of interest was determined on exposed embryos, by quantitative reverse transcription-

polymerase chain reaction (RT-qPCR). The results were correlated with the PAH composition of PM samples.

The working hypothesis is that the chemical mixture of organic compounds bound to PM from the marine area under study, should elicit measurable biological effects, possibly contributing to the toxic burden of aquatic organisms. To my best knowledge, this is the first study to analyse the biological activity and toxic potential of atmospheric PM organic constituents in open waters from different sub-basins of the Mediterranean and Black Seas.

5.2. Methods

5.2.1. Study area and sampling

The Mediterranean and Black Seas are semi-enclosed environments, with areas of about 2.5×10^6 km^2 and 4.2×10^5 km^2, respectively. The Mediterranean Sea can be divided in Western and Eastern basins, and also in different sub-basins. In the present work, we analysed 29 samples from Western Mediterranean Sea (W Med), Ionian Sea, Aegean Sea, South-East Mediterranean Sea (SE Med), and Black Sea (Figure 1). Air samples were collected on board the R/V Garcia del Cid during two sampling cruises made on June 2006 and May 2007. Both campaigns started and finished in Barcelona (Spain), with Istanbul (Turkey) and Alexandria (Egypt) as intermediate stops (Figure 1).

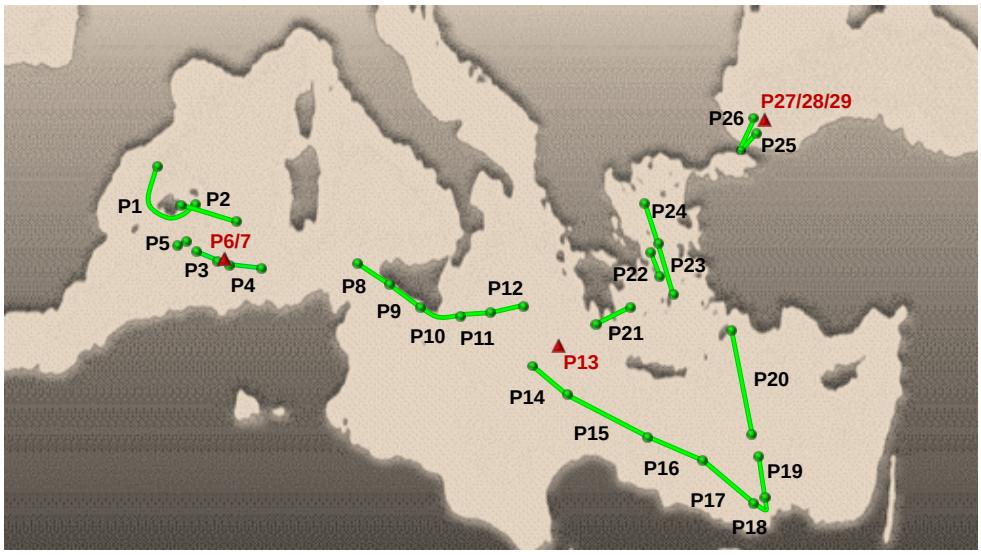

Figure 1 – Particulate matter samples (P1 to P29) collected in the Mediterranean and Black Seas (approximate location, see Table 1 for exact coordinates). Most of the samples were collected while cruising (i.e. transects, green lines delimited by green points), with the exception of samples P6, P7, P13, P27, P28 and P29, which were collected at stations (red triangles).

All further details regarding the sampling procedures and strategy can be found in Castro-Jiménez et al. (2012). Briefly, air samples were collected using a high-volume air sampler (MCV, Barcelona, Spain), located at the upper deck of the boat close to the bow. Samples were collected while cruising (transects), with the exception of samples P6, P7, P13, P27, P28 and P29, which were collected at stations (Figure 1, Table 1). The air was drawn through a precombusted Quartz fiber filter (QM-A; Whatman, 8x10 inches) to collect the atmospheric PM.

Table 1. Particulate matter (PM) sampling details.

		Transect coordinates (degrees)			
		Start		End	
Sample	Period	Latitude (N)	Longitude (E)	Latitude (N)	Longitude (E)
P1	4-5 May 2007	40.59000	2.10000	39.54050	3.86367
P2	4-5 June 2007	39.06133	5.75500	39.50000	3.20000
P3	4 July 2006	37.96517	4.90000	38.25000	3.90000
P4	2 July 2006	37.77750	6.91000	37.85000	5.42000
P5	5 July 2006	38.43767	3.65200	38.41500	3.60767
P6[a]	2 July 2006	37.96517	5.11000	37.96517	5.11000
P7[a]	3-4 July 2006	37.96517	5.11000	37.96517	5.11000
P8	6 June 2006	37.91567	11.33400	37.28817	12.87467
P9	6-7June 2006	37.28817	12.87467	36.70700	14.24633
P10	7 June 2006	36.70700	14.24633	36.45733	16.09550
P11	7-8 June 2006	36.45733	16.09550	36.52967	17.47617
P12	8 June 2006	36.52967	17.47617	36.72867	18.99050
P13[a]	25 June 2006	35.7215	20.7390	35.7215	20.7390
P14	13-14 May 2007	35.08133	19.40067	34.28000	21.02000
P15	14-15 May 2007	34.28000	21.02000	33.11067	24.7265
P16	15-16 May 2007	33.11067	24.72650	32.46000	27.26000
P17	16-17 May 2007	32.46000	27.26000	31.29000	30.01000
P18	17 May 2007	31.29000	30.01000	32.58532	30.23633
P19	21 May 2007	31.44100	29.73600	32.58500	29.43600
P20	21-22 May 2007	33.18620	29.92280	36.08000	28.62000
P21	12 June 2006	36.23470	22.35000	36.67880	23.92000
P22	13-14 June 2006	37.55150	25.28520	38.22480	24.82520
P23	23 June 2006	38.45670	25.22280	37.05650	25.92350
P24	22 June 2006	39.55570	24.62720	38.45670	25.22280
P25	16 June 2006	41.49000	29.71000	41.04000	29.02000
P26	20 June 2006	41.89670	29.60850	41.12770	29.07450
P27[a]	19 June 2006	41.87200	30.07283	41.87200	30.07283
P28[a]	19 June 2006	41.88517	30.03150	41.88517	30.03150
P29[a]	19-20 June 2006	41.90667	29.98267	41.90667	29.98267

[a] Samples taken at station (ship not cruising)

5.2.2. Extraction and chemical analysis

Details on extraction and chemical analysis are reported elsewhere (Castro-Jiménez et al., 2012). Briefly, filters were spiked with PAH labelled standards before Soxhlet extraction (24h). Extract volumes were reduced and fractionated on alumina columns, and PAHs were eluted in the second fraction with hexane/dichloromethane. PAH analysis was performed by high resolution gas chromatography-low resolution mass spectrometry (HRGC-LRMS). The PAHs determined were phenanthrene (Phe), anthracene (Ant), dibenzothiophene (DBT), sum of methyldibenzothiophenes (PMeDBT), sum of methlyl-phenanthrenes (PMePhe), sum of dimethlyl-phenanthrenes (PDimePhe), fluoranthrene (Fla), pyrene (Pyr), chrysene (Cry), benzo(a)anthracene (BaA), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(e)pyrene (BeP), BaP, perylene (Per), benzo(g,h,i)perylene (BghiPer), dibenz(a)anthracene (dBA) and indeno(1,2,3-cd)pyrene (Ind). The concentrations of PAHs in air (gas phase and particulate phase), have been previously described in Castro-Jiménez et al. (2012). Quality control of the chemical analysis was performed and assured (Castro-Jiménez et al., 2012).

Toxicity equivalents (TEQs) were calculated from the content of samples on parent PAHs (BaA, Chy, BbF, BkF, BaP, Ind, BghiPer), by using previously reported toxicity equivalence factors (Murahashi et al., 2007).

For both RYA and ZET assays, particulate filter samples were extracted by sonication with a mixture of dichloromethane: methanol (Merck, Germany, 2:1 v/v, 3 x 10 mL, 15 min). The extract was then filtered in a syringe with 0.45 µm PFTE membrane, Puradisc, Whatman, USA), concentrated on Rotovap to 1 mL, further concentrated to almost dryness with a gentle nitrogen gas stream, and dissolved in 0.5 mL of methanol. Then, 0.25 mL of extract were reserved at -20ºC for AhR-RYA, and the other 0.25 mL were exchanged into dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical, Germany), and reserved at -20ºC for the ZET assay.

5.2.3. Bioassays and gene expression analysis

AhR-RYA

The AhR-RYA allows the detection and quantification of AhR-binding activity of dioxin-like compounds, such as PAHs, by incubating samples with a modified yeast strain expressing the human AhR and AhR nuclear translocator genes, under the GAL1-10 promoter (Miller, 1997; Noguerol et al., 2006). PM samples under study were tested in

duplicate, together with three controls: a positive (1 μM β-Naphthoflavone), a negative (5% of vehicle, methanol), and an inhibitory control (sample extract plus 1 μM β-Naphthoflavone) in 96-well polypropylene microtiter plates (NUNC™, Roskilde, Denmark) as described for previous studies (Olivares et al., 2011; Mesquita et al., 2014b). β-galactosidase activity was measured by fluorescence at 355 nm excitation and 460 nm emission wavelengths. AhR-ligand activity of samples is represented as BaP equivalents (BaPeq, Noguerol et al., 2006; Misaki et al., 2007)

ZET

Wildtype zebrafish (*Danio rerio*) maintenance conditions and spawning procedure was performed as previously described (Mesquita et al., 2014b). One hour after spawning fertilized eggs were collected, rinsed and reserved for 24h until the exposure experiments. Viable eggs were randomly distributed into glass petri dishes, and exposed to the PM samples extracts diluted 500 times with embryo water (volume of 2.5 ml per petri dish). Taking into consideration the dioxin-like activity of PM samples (determined by AhR-RYA), three samples per sub-basin were tested on the ZET assay, together with a negative control (0.2% DMSO) and a positive control (3.7 mg.L-1 3,4-Dichloroaniline, Sigma–Aldrich Chemical, Germany, ISO., 2007). Six biological replicates (of 10 embryos each), were included in the test. The animals were exposed to the different experimental conditions from 24 h postfertilization (hpf) until 120hpf, renewing medium every 24 h. The real concentrations of PM organic constituents (10 individual PAHs) have been measured previously, assuring an acceptable relative standard deviation of less than 20% from nominal concentrations (Mesquita et al., 2014b). Developmental progression of embryos was observed daily (Kimmel et al., 1995), under a stereomicroscope Nikon SMZ 1500 equipped with a Nikon digital sight DS-Ri1 digital camera. Lethal and sub-lethal endpoints were determined accordingly to the endpoints already described (Hermsen et al., 2011). At the end of the experiments the zebrafish embryos were frozen at -80ºC for posterior mRNA quantification.

RNA quantification

Total RNA was isolated from whole embryos (pools of 20 embryos, from 2 biological replicates), using the TRIzol reagent protocol (Invitrogen Life Technologies, Carlsberg, CA). RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDrop ND-8000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The extracted total RNA was first treated with DNAseI (Ambion, Austin, TX) to remove

genomic DNA, and retro-transcribed into cDNA using TranscriptorFirst Strand cDNA Synthesis Kit (F. Hoffmann- La Roche, Basel Switzerland). Primer sequences for target genes, c*yp1a,* FBJ murine osteosarcoma viral oncogene homolog (*fos*), glutathione S-transferase, alpha-like (*gstal*), hydroxyacid oxidase 1 (*hao1*), chymotrypsinogen B1 (*ctrb1*), elastase 2 (*ela2*), immediate early response 2 (*ier2*), kruppel-like factor 2a (*klf2a*), somatostatin 2 (*sst2*), transthyretin (*ttr*) ,and *ppia2* (reference gene, Morais et al., 2007), were designed and validated in Mesquita et al. (2015). The specific transcripts were quantified in cDNA samples using Lightcycler 480 Real Time PCR System (F. Hoffmann-La Roche) with SYBR-Green Mix (Roche Applied Science, Mannheim, Germany). Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, in triplicate). Cp values of target genes (Cp_{tg}) were corrected to the correspondent Cp values of the reference *ppia2* gene, for each sample ($corrCp_{tg}=Cp_{tg} - Cp_{ppia2}$). Changes in mRNA levels of treated samples relatively to controls, were calculated by the ΔΔCp method (Pfaffl, 2001), using corrected Cp values from treated and non-treated samples ($\Delta\Delta Cp_{tg} = corrCp_{tg\_untreated} – corrCp_{tg\_treated}$). Fold-change ratios were derived from those values.

5.2.4. Statistical analysis

Statistical calculations were performed using the SPSS v. 19 package (SPSS Inc., Chicago) and R (packages psych and gplots, R Core Team, 2014). Correlations between dioxin-like activity values and sample's total PAH content, calculated TEQ values and concentrations of different PAH congeners were calculated using the Pearson's correlation coefficient. To accomplish parametric requirements, the logarithmic transformation of the concentration of PAHs, calculated TEQs and AhR-RYA results was used. Heatmap and hierarchical clustering were performed on the Pearson's correlation matrix. Gene clusters were defined attending to mutual correlation coefficients as well as to their positions in the clustering tree. The minimal number of genes required for defining a cluster was set to two.

5.3. Results

5.3.1. Analysis of dioxin-like activity

AhR-ligand activity of organic constituents extracted from PM samples varied by roughly two orders of magnitude (range: 15-1010 pg.m^{-3} BaPeq, bars in Figure 2). Maximal activity was found for the Black Sea (sample P25, Figure 2), whereas low activity samples were more common in the western part of the Mediterranean Sea (Figure 2). Among the analysed samples the total PAH content had differences as high as six-fold, with values ranging from 500 to 3100 pg.m^{-3}. The concentrations of BaP ranged from n.d (not detected) to 110 pg.m^{-3}. In general, PM samples collected in proximity of the coastline (P5, P7, P8, P9, P17, P19, P25) showed higher dioxin-like activity than the ones sampled at open sea (Figure 1 and Figure 2). Indeed, the spatial variability of AhR-RYA activity for the different PM samples reflected the variation of their PAH concentrations (colour areas in Figure 2), particularly for the Aegean and Black Seas.

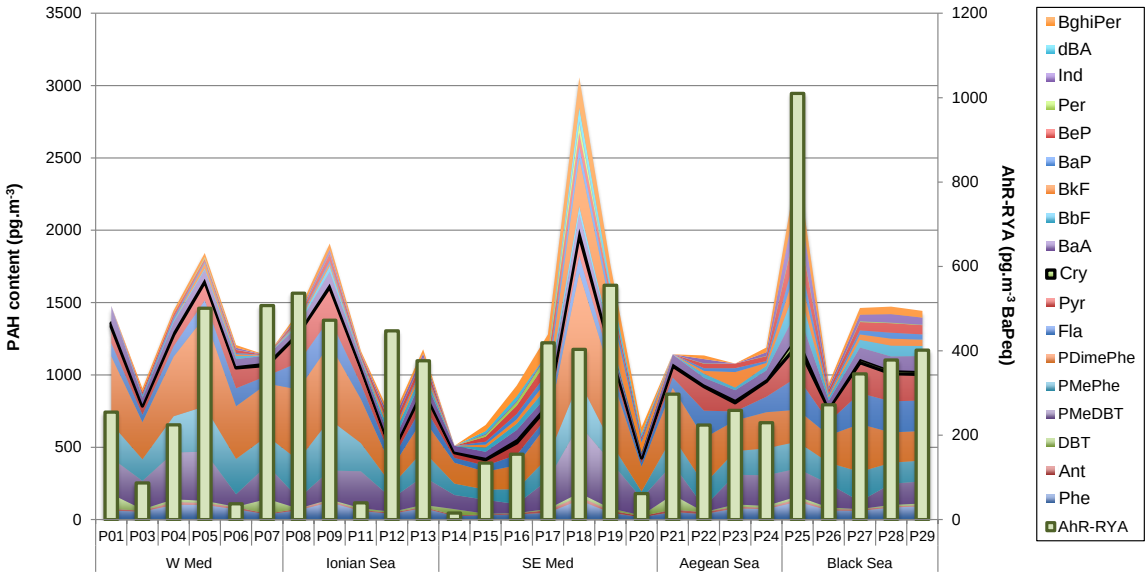

Figure 2 - Dioxin-like activity of atmospheric PM samples from the Mediterranean and Black Seas open waters. Results from the aryl hydrocarbon receptor-recombinant yeast assay (AhR-RYA, pg.m^{-3} BaPeq.) are represented by green bars (right axis), whereas PAH content for each sample (pg.m^{-3}) is represented as coloured areas (left axis). Phenanthrene (Phe), anthracene (Ant), dibenzothiophene (DBT), sum of methyldibenzothiophenes (PMeDBT), sum of methlyl-phenanthrenes (PMePhe), sum of dimethlyl-phenanthrenes (PDimePhe), fluoranthrene (Fla), pyrene (Pyr), chrysene (Cry), benzo(a)anthracene (BaA), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(e)pyrene (BeP), BaP, perylene (Per), indeno[123-cd]pyrene (Ind), dibenzo[a,h]anthracene (dBA), benzo[g,h,i]perylene (BghiPer). PAH congeners are ordered by molecular weight (lighter ones at the bottom); the area corresponding to BaA is marked with a dark outline to distinguish lighter and heavier PAHs.

The dioxin-like activity values showed significant correlation with sample's total PAH content (PAH sum, r= 0.641, p<0.001), with calculated TEQ values (r = 0.675, p<0.001), and with the concentrations of different PAH congeners (Phe, Pyr, BaA, Cry, BbF, BaP, BeP, Per, Ind, and BghiPer, Table 2).

Table 2 - Pearson's correlation coefficients for bivariate correlations between dioxin-like activity (AhR-RYA), PAH content of PM samples and calculated toxicity equivalent values (TEQs, *, p < 0.05; **, p < 0.025; ***, p < 0.01). Abreviations as in Figure 2.

	AhR-RYA
PAHs Sum	0.641 ***
TEQs	0.675 ***
Phe	0.508 **
Ant	0.404 *
DBT	0.054
PMeDBT	0.245
PMePhe	0.323
PDimePhe	0.275
Fla	0.494 *
Pyr	0.516 **
BaA	0.715 ***
Cry	0.634 ***
BbF	0.735 ***
BkF	0.392
BaP	0.642 **
BeP	0.740 ***
Per	0.127
Ind	0.698 ***
dBA	0.764
BghiPer	0.558 **

5.3.2. Zebrafish embryotoxicity and gene transcription analysis

No phenotypical adverse effects were observed on zebrafish embryonic development (ZET assay). The exposure concentrations of organic compounds in the ZET assaywere relatively low, with total PAH content ranging from 90 to 228 ng.L^{-1} (Table 3). Nonetheless, significant changes in gene expression were observed in zebrafish embryos exposed to sample extracts, relatively to non-exposed controls. All samples induced at least two genes by a factor of two or more (Table 3). All tested genes (except for *gstal*) showed two-fold or higher response to at least one of the tested extracts (Table 3). Due to the general low expression of *gstal*, this gene was not included in further analysis.

Table 3 - Gene expression data and summarized pollutant levels for the particulate matter samples tested on the zebrafish embryotoxicity test.

Samples	Fold induction (mRNA levels over controls)										Pollutant content (ng. L^{-1})[a]		
	cyp1a	*hao1*	*crtb1*	*ela2*	*ttr*	*fos*	*klf2a*	*gstal*	*ier2*	*sst2*	PAHs Sum	HMW PAHs	TEQs
P04	2.0	1.0	0.8	1.4	1.0	5.8	0.9	0.8	3.6	4.3	127.5	18.3	41.0
P05	3.5	1.4	2.1	2.8	2.1	1.6	1.1	0.7	0.8	3.7	166.4	18.0	74.6
P07	2.3	0.7	0.4	0.5	1.0	2.7	0.6	0.9	1.0	3.5	108.6	6.6	18.5
P08	3.2	0.9	2.4	2.6	1.1	6.0	1.0	0.8	2.9	11.7	109.4	11.4	43.5
P09	2.4	1.2	12.3	13.8	1.5	7.7	0.8	0.9	2.2	14.4	125.0	19.7	84.0
P13	5.4	3.0	3.8	3.4	3.2	9.8	0.7	1.5	2.1	6.9	131.8	31.3	149.9
P17	5.0	3.4	0.6	1.5	4.5	6.0	1.7	0.6	1.3	9.8	132.5	51.7	200.3
P18	1.9	1.1	1.9	4.8	1.4	1.9	1.3	1.0	0.9	3.0	103.0	37.9	185.8
P19	1.2	1.1	1.1	2.2	1.0	3.1	1.3	0.7	3.0	10.4	130.8	45.0	233.6
P21	5.9	1.0	1.5	1.9	0.8	10.3	2.4	0.5	4.0	8.4	140.8	10.1	24.5
P23	3.3	1.1	13.4	9.7	1.3	6.4	1.0	1.2	2.3	16.7	155.3	39.0	257.8
P24	2.8	1.0	2.3	2.5	1.2	5.0	0.8	0.7	1.5	7.0	90.4	17.7	77.4
P25	5.8	1.3	4.1	8.2	2.1	12.9	2.0	0.6	4.6	16.7	228.3	115.9	536.1
P27	1.6	1.0	2.5	1.1	1.0	0.8	0.8	0.9	0.7	2.7	101.3	26.2	119.7
P28	1.8	1.2	0.8	1.2	0.9	1.0	0.5	0.7	0.9	2.4	108.2	34.1	167.9

[a] Calculated nominal concentrations of the different pollutants in zebrafish test water

Abbreviations: cytochrome P4501A, *cyp1a;* FBJ murine osteosarcoma viral oncogene homolog, *fos;* glutathione S-transferase, alpha-like, *gstal;*, hydroxyacid oxidase 1, *hao1;* chymotrypsinogen B1, *ctrb1;* elastase 2, *ela2;* immediate early response 2, *ier2;* kruppel-like factor 2a, *klf2a;* somatostatin 2, *sst2;* transthyretin, *ttr*; messenger ribonucleic acid, mRNA, polycyclic aromatic hydrocarbons, PAHs; high molecular weight PAHs, HMW PAHs; toxicity equivalent values (TEQs).

Correlation analysis and hierarchical clustering of the responses of the different genes to PM extracts identified three clusters of genes (Figure 3). Cluster A (*fos*, *cyp1a*, *sst2*, *ier2*, and *klf2a*) grouped with the total PAH content, as well as with levels of several individual PAHs, TEQ values and AhR-RYA data, probably reflecting the classical response to dioxin-like compounds. The other two clusters, Clusters B (*ttr*, *hao1*) and C

(*ela2*, *crtb1*) showed significant correlations with a single PAH (Per and Phe, respectively, Figure 3).

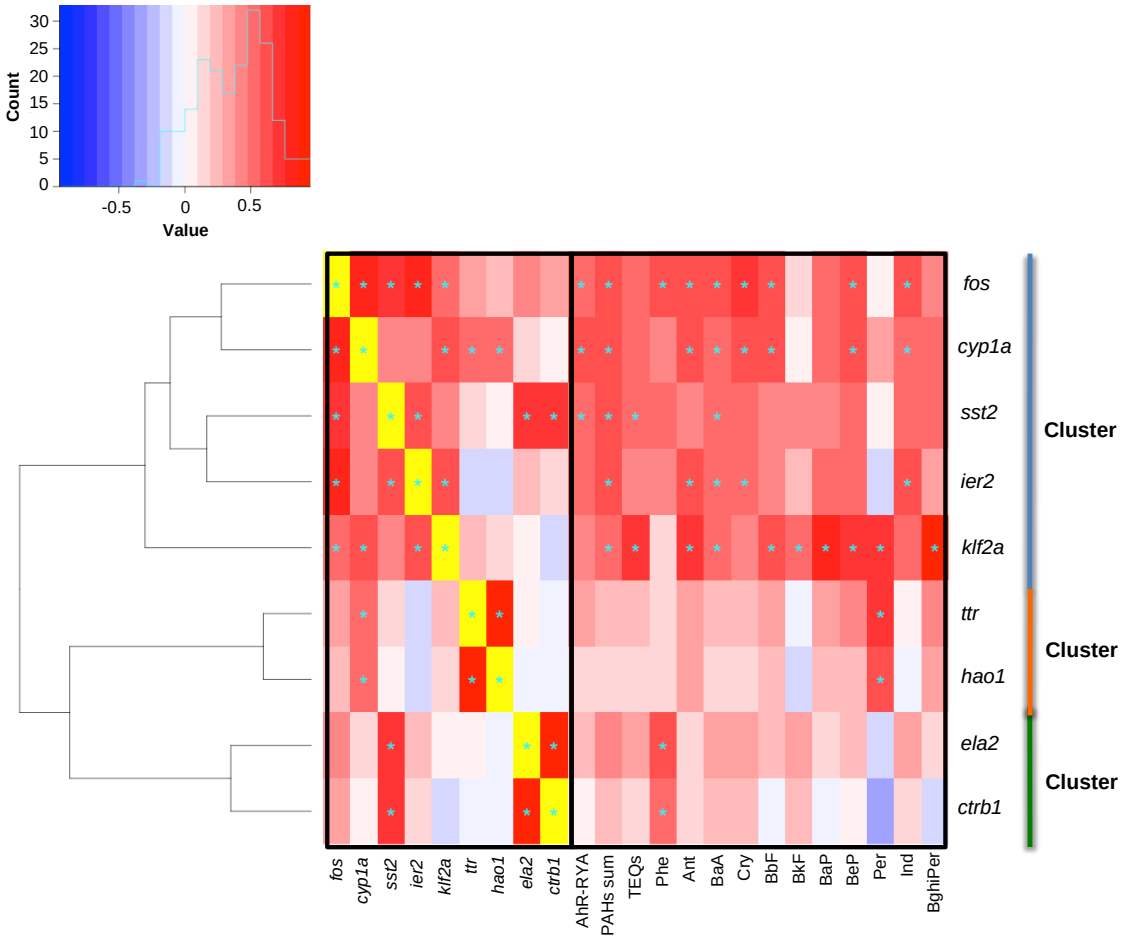

Figure 3 - Correlation heatmap between gene expression (in fold induction over non-treated controls) and PAH concentrations. Positive correlations are marked in red and negative ones in blue (colour scale at the top). Significant correlations (p<0.05) are marked with a blue asterisk. Yellow sectors indicated self-to-self correlations for gene expression data. The three clusters defined by hierarchical clustering are indicated at the right. Abbreviations as in Figure 2 and Table 3.

These clusters corresponded to relatively distinct geographical distributions of the corresponding biological activities (Figure 4). As expected, Cluster A showed a geographical pattern similar to the one followed by PAHs and dioxin-like activity measured by AhR-RYA (Figure 4), whereas the rest of clusters showed peaks that do not correspond to the variation of the total PAH content or dioxin-like response (Figure 4). This discrepancy is particularly notorious for the samples in the central part of the Mediterranean (Samples P8-P17, Figure 4). The graphs in Figure 4 also show that Cluster B includes genes with a limited response to the extracts (lower than 4-fold), while some genes of Clusters A and C responded to some of the extracts with variations of an

order of magnitude or more (Table 3). PM weight showed no correlation with the AhR-RYA or gene expression results (data not shown).

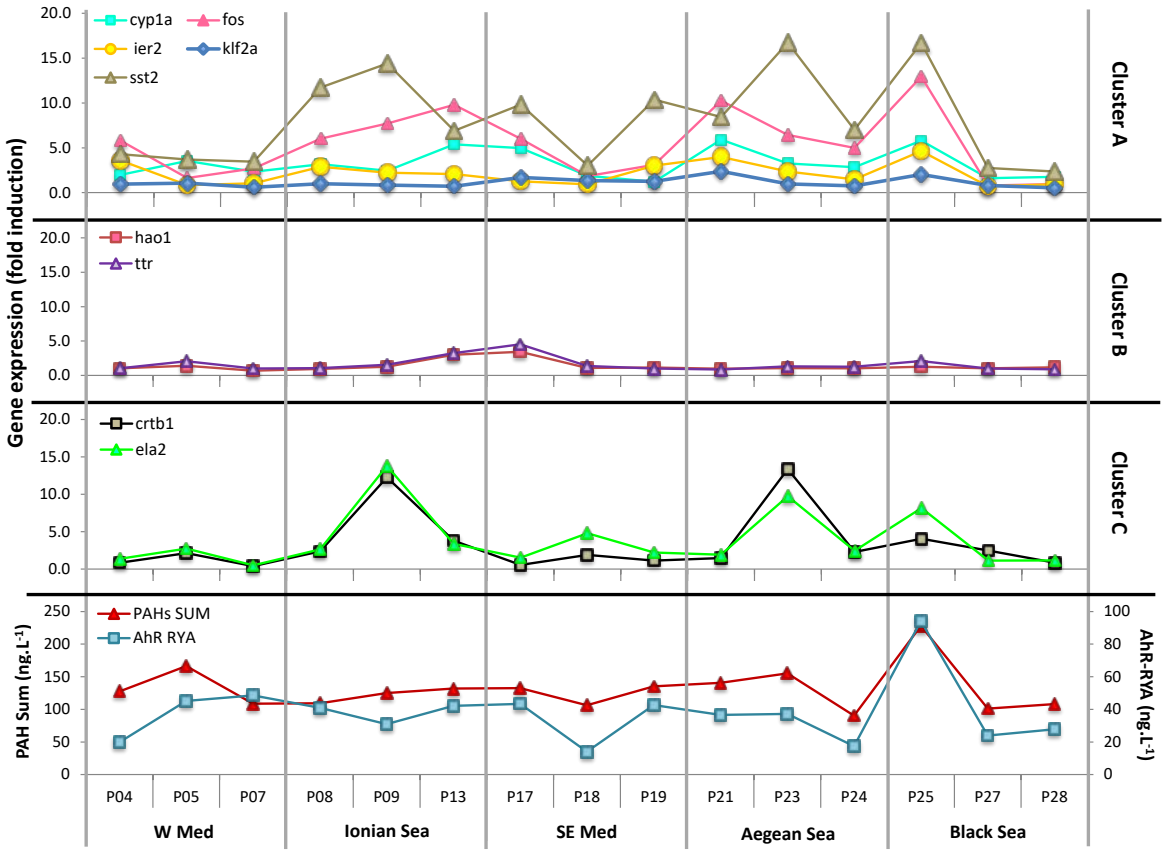

Figure 4 - Gene expression profiles for the three clusters of genes defined in Figure 3. Genes are indicated for each panel, values correspond to fold induction relative to controls. The bottom panel shows the profiles of total PAH content and dioxin-like activity from AhR-RYA, both expressed as ng.L^{-1}. Abbreviations as in Figure 3.

5.4. Discussion

The AhR-RYA has been proved sensitive to the organic content of particulate air samples from urban, rural and semi-rural environments (Olivares et al., 2011; Mesquita et al., 2014b; 2015). In a previous study with urban PM samples from the city of Barcelona (Catalonia, Spain), AhR ligand activity also correlated with particulate PAH concentrations (Mesquita et al., 2014b). The AhR-RYA activity, total PAH content, and BaP concentration of these urban PM samples were 2.9, 1.4 and 4.4 times higher than the correspondent parameters of Mediterranean samples addressed in the present work, respectively. The present study confirms that the dioxin-like activity induced by the low

concentrations of PM organic contaminants found on the Mediterranean and Black seas atmosphere (pg. m^{-3} range) can be also determined by using this bioassay.

Results from the AhR-RYA showed high variability within the different sub-basins, in correlation with particulate PAHs levels. This variability was likely influenced by several environmental factors, such as air-mass trajectory, proximity to coastal sources and losses by deposition (Castro-Jiménez et al., 2012). The correlation between dioxin-like activity and PAH concentrations was essentially driven by the levels of high molecular weight PAHs, which were particularly high in samples from the Aegean and Black seas. However, for the remaining sub-basins other compounds could have a stronger influence. For instance, the concentrations of polychlorinated biphenyls (PCB) congeners are typically higher in the Western Mediterranean Sea, as described in Berrojalbiz et al. (2014) using the same sampling cruises. Organohalogenate compounds can be at least partially responsible for the unexplained dioxin-like activity.

The concentrations of PAHs present in the ZET assay were around 4 times lower than PAH concentrations that induced adverse effects on zebrafish development in a previous study with urban PM samples (Mesquita et al., 2014b). However, the detected gene expression alterations, may indicate a potential risk of induction of several adverse biological effects linked to more polluted PM samples. These effects include spinal deformations, yolk-sac and cardiac edemas, malformation of the swim bladder and balance disruption (Mesquita et al., 2014b; 2015).

Gene expression results revealed at least two distinct robust responses of zebrafish embryos to PM organic extracts. The major response can be related to the presence of PAHs and, particularly, to dioxin-like PAHs, and included the dioxin-like activity (AhR-RYA), the expression of *cyp1a* and of genes related to proliferation and/or development (*fos, sst2, ier2, klf2a*). In addition, the expression of two pancreatic markers (*ela2, ctrb1*) was increased, which has been previously related to exposure to the organic fraction of urban PM (Mesquita et al., 2015). This activity did not correlate with the concentrations of classical dioxin-like PAHs, and it showed a distinct geographic distribution pattern. This further supports that other compounds, in addition to PAHs, are present in the extracted organic fraction of PM, contributing to the altered expression of genes. Organohalogenates are possible candidates, given their presence in ambient PM from the same studied area (Castro-Jiménez et al., 2010; Berrojalbiz et al., 2014).

The results of the present work suggest the involvement of biotransformation, developmental and pancreatic exocrine function related-genes in the response to the organic fraction of PM, in agreement with previous studies. The study of Bui and collaborators (2011) on the effects of combustion-generated PAHs on zebrafish embryos, exposed via water to butadiene soots, also observed up-regulation of *cyp1a*,

fos and *klf2a* expression. These alterations in mRNA levels preceded prominent developmental deformities on exposed zebrafish embryos, such as yolk sac edema and axial malformations (Bui et al., 2012). The genes determined in the present study, in particular *fos, cyp1a, klf2a, ier2, sst2, ctrb1* and *ela2,* may be suitable markers in future research to assess exposure and potential toxic effects of PM organic compounds on native species.

The present work shows relevant changes in gene expression of zebrafish embryos at PAHs exposure concentrations of 100-200 ng. L^{-1}. These concentrations are higher than the concentrations of PAHs measured in water samples (dissolved and particulate phases) collected during the same sampling cruises (Berrojalbiz et al., 2011). However, even higher PAH concentrations have been measured in Mediterranean Sea water, suggesting that the exposure concentrations of the ZET assay may be considered environmentally relevant. Indeed, on the Tyrrhenian Sea (coast of Italy), dissolved and particulate seawater samples collected at sea depth of 43 m and at 0.4 km from the Leghorn coast, had a concentration of PAHs of 398 and 2420 ng.L^{-1}, respectively (Cincinelli et al., 2001). Therefore considerably higher than the ZET exposure concentrations used in the present work. PAH contamination levels on coastal and marine waters can reach the µg.L^{-1} range (WHO/IPCS, 1998; Cincinelli et al., 2001; Maskaoui et al., 2002; Malik et al., 2011), even though such contamination certainly derives from several sources. Further research is needed on the transformation and fate of airborne PAHs in the aquatic environment, in order to better understand and evaluate the real exposure of aquatic organisms.

Nevertheless, it is acknowledged that in the aquatic systems, like in the atmosphere, the fate of PAHs depends on their physicochemical properties. Due to their low aqueous solubility and hydrophobic nature, PAHs bind to suspended particle matter (SPM) or remain dissolved in the water phase (Manoli and Samara, 1999), eventually depositing in the aquatic sediment (Lipiatou et al., 1997; Tsapakis et al., 2003). In general, the concentrations of PAHs in the water-dissolved phase are low, but higher concentrations can be found in SPM and sediments (Manoli and Samara, 1999; Patrolecco et al., 2010). Since fish eggs generally deposit on sediment or on organic matter, and remain nektobenthic in the initial developmental stages, the exposure concentrations and the uptake should be higher than the concentrations considered on the ZET assay. Indeed, several Mediterranean fish species lay their eggs in the bottom sediment and/or organic matter, becoming in close contact with hydrophobic pollutants that concentrate in those matrixes (Castro-Jiménez et al., 2013). The process of uptake of hydrophobic compounds, such as PAHs, is considered passive and controlled by diffusion pressure (fugacity) as a result of the differential between the matrix and tissue

concentration (Meador et al., 1995). PAHs hydrophobic nature allows their direct transfer from particulate matter through embryo membranes without the need for direct particle uptake. The high-lipid content of fish embryos, favours the accumulation of PAHs, particularly on the larvae yolk sac (Bui et al., 2012). With the embryonic development the yolk sac is totally absorbed, and the PAHs metabolized and dispersed through the hatching gland cells, hepatopancreas, gall bladder, gills, eyes and blood vessels, potentiating the body burden of these compounds (Bui et al., 2012). Since early life stages of fish are more sensitive to dioxin-like adverse effects than adults (Peterson et al., 1993), the atmospheric deposition of aerosols on the aquatic environment may constitute a noteworthy risk for the developing embryos.

The latest research studies continue to emphasize the importance of the contribution of combustion-generated aromatic compounds to the aquatic systems budget and consequent hazard to their organisms (González-Gaya et al., 2014; Kroflič et al., 2015). Even though atmospheric deposition is a major source of PAHs for aquatic systems, and even though regulatory directives for air quality recognize the need to protect the environment from the adverse effects of particulate air pollution, no critical level, target/limit value or long-term objective is defined for PM (neither for airborne PAHs), in order to protect aquatic organisms (EEA, 2014). In the same way as other components of air pollution, such as ozone, sulphur oxides, nitrogen oxides have critical levels and long-term objectives defined, for example for the protection of vegetation, the same should be established for PM and airborne PAHs not only for protection of human health, but also the environment.

5.5. Conclusions

This study shows that the organic fraction of atmospheric PM from the Mediterranean and Black Seas is biologically active. The measured dioxin-like activity (AhR-RYA) showed variability within the different sub-basins, correlating with the concentration of particle-bound PAHs. Exposure of zebrafish embryos to the organic fraction of PM up-regulated the expression of biotransformation, developmental and pancreatic related genes. While upregulation of *cyp1a, fos* and different developmental genes followed a pattern similar to that of PAHs and dioxin-like activity, pancreatic markers showed a different geographical distribution. Organic contaminants, other than PAHs, known to be present in PM from the Mediterranean and Black Seas, must be contributing to the observed effects. The genes tested, in particular *fos, cyp1a, ier2, sst2, ctrb1* and *ela2,*

may be suitable markers in future research with autochthonous species to assess exposure and potential toxic effects of atmospheric PM. The organic fraction of airborne PM could increase the toxic burden of aquatic organisms, and therefore efforts should be made to better understand its effects and improve future regulatory guidelines.

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CHAPTER 6

General Discussion

6.1. General Discussion

At the present time the association between exposure to ambient PM and severe health effects is firmly established. The biggest challenge now is to better understand the components and characteristics of particles and their elicited biological effects, in order to develop relevant PM management strategies. The main aims of this thesis were to improve knowledge about the toxicity of ambient PM and investigate a set of sensitive and rapid to use tools to detect toxic effects of PM organic fraction for use in monitoring and risk assessment**.** Given the expected contribution of PAHs to PM toxicity, the base methods selected for this study involved screening of dioxin-like activity and evaluation of embryotoxicity to account for effects possibly resulting from metabolic activation of parental contaminants in the organic fraction into more toxic metabolites.

6.1.1. *In vitro* and *in vivo* assessment of PM organic fraction

AhR ligand activity was detected by the yeast-based AhR-RYA assay in all sets of samples tested. PM samples from a rural area in the Pyrenees (Chapter 3), revealed the highest AhR-RYA activity (Table 1). Among the remaining samples, those collected in a background urban area from a highly populated European city (Barcelona) revealed the highest activity values (i.e. samples investigated in Chapters 2 and 3, Table 1). The AhR-RYA activity measured in all these samples strongly correlated with their content in high MW PAHs and BaP (Table 1). For urban and Mediterranean samples investigated in Chapters 4 and 5, respectively, the correlation of AhR-RYA activity with the concentration of high MW PAHs and BaP, was still significant, though weaker (Table 1). Overall, in spite of the intrinsic chemical variability of PM samples, the AhR-RYA consistently detected their dioxin-like activity, even when atmospheric contamination levels were in the $pg.m^{-3}$ to $ng.m^{-3}$ range.

Table 1 – Minimum and maximum dioxin-like activity values (ng $BaPeq.m^{-3}$, measured in the AhR-RYA) and corresponding correlation with the sum of high molecular weight PAHs (HMW PAHs) and with benzo[a]pyrene (BaP), for each set of samples tested. Pearson correlations coefficients (r), ** p<0.01, *** p<0.001.

	Min	Max	HMW PAHs	BaP
Urban site (Chapter 2)	0.11	3.53	0.766***	0.791***
Urban site (Chapter 3)	0.02	1.52	0.971***	0.977***
Rural site (Chapter 3)	0.00	20.18	0.961***	0.946***
Urban site (Chapter 4)	0.24	1.04	0.606***	0.605***
Mediterranean Sea (Chapter 5)	0.00	1.01	0.679***	0.498**

Over the last decades the ZET assay has been developing as a valid alternative method to the toxicological use of mammals, particularly in such sensitive life stage as the early embryonic development. The work presented on this Thesis showed that this assay is also a useful tool to study the toxic burden of PM. Phenotypical malformations were observed on zebrafish embryos exposed to PM samples of urban (Chapter 2) and rural (Chapter 3) origin. In addition to malformations, high mortality rates were also observed for zebrafish embryos exposed to rural samples (Chapter 3). The malformations observed included spinal deformations, yolk-sac and pericardial oedema with occasional haemorrhages, malformation of the swim bladder and balance disruption. The phenotypical effects described were consistent with literature findings for zebrafish embryos exposed to PAHs (Diamond et al., 2000; Bui et al., 2012).

The ZET assay was sensitive enough to be carried out using only a fraction of each PM filter sample (corresponding to volume of air as low as 45 m^3, Chapter 4), allowing for analysis of AhR-RYA activity in the same samples. The ZET assay provided information about the systemic effects of the exposure of a whole living organism to organic PM and helped elucidating further the mode of action of PM organic constituents. Transcriptomic analysis of zebrafish embryos exposed to the organic PM fraction provided knowledge on its potential modes of action and revealed gene expression patterns whose alterations may lead to the toxicological malformations described.

Both the *in vitro* and *in vivo* assays employed, allied to gene expression analysis, showed characteristics of efficient and rapid early-warning tools for routine screening and monitoring of PM emissions.

6.1.2. Transcriptomic data and molecular markers

Functional analysis performed on microarray data of zebrafish embryos exposed to PM samples of urban and rural origin (Chapter 3) showed a main significant enrichment of genes involved in: i) protein folding and RNA-protein interaction, fundamental functions for embryo development; ii) the *jun*/*fos* signalling pathway; iii) the dioxin-like and phase I biotransformation response (cytochrome superfamily); and iv) oxidation-reduction reactions. Additionally, two other functional groups were related with peptidases of the chymotrypsin group and crystalline genes. Bui and collaborators (2012), studied microarray transcriptomic alterations of combustion-generated PAHs on zebrafish embryos exposed via water to butadiene soots (BDS). The authors observed the up-regulation of biotransformation and oxidative stress gene cascades (Bui et al., 2012). Other research works, using mammalian cell cultures exposed to PAH-rich BDS, or

mouse inhalation of BDS, also described enhanced expression of the same groups of genes (Murphy et al., 2008; Rouse et al., 2008).

Comparing the expression alterations described in Bui et al. (2011) with the microarray data obtained in Chapter 3, similar responses can be observed for some genes. In Bui and collaborators (2011), one of the up-regulated gene cascades was biotransformation, comprising several cytochromes (such as *cyp1a, cyp1b1, cyp1c1, ahrr*). In this work, this cascade was also found to be up-regulated in zebrafish embryos exposed to urban and rural PM samples and to BaP 0.1 $mg.l^{-1}$ (exposure from 24 hpf-120 hpf, data in Table 2), characterising the classical detoxification response to PAH exposure. Even though Bui et al. (2011) exposed embryos only for 48h (24 hpf -72 hpf), the RT-qPCR results obtained were very similar for embryos and young zebrafish adults. These authors suggested that by 72 hpf, biotransformation and oxidative stress transcriptional responses of embryos are fully developed. Biotransformation and oxidative stress transcriptional responses of zebrafish embryos to BDS were also found to be similar, qualitatively and quantitatively, to the responses detected in the lungs of BDS-exposed adult mice (Bui et al., 2012), probably due to high gene homology between species. These oxidative stress transcriptional responses were characterised by altered expression of genes such as heme oxygenase 1 (*hmox1)*, NAD(P)H dehydrogenase, quinone 1 (*nqo1)*, peroxiredoxin 1 (*prdx1)*, glutathione peroxidase (*gpx)*, glutathione S-transferase (*gst)*, thioredoxin reductase 1 (*txnrd1)* and sulfotransferase 6b1 (*sultb6b1)*, which belong to the *Nrf2*-associated oxidative stress response pathway (Bui et al., 2012). In the present Thesis, it was found that these genes were in general up-regulated in zebrafish embryos exposed to urban PM extracts and to BaP 0.1 $mg.L^{-1}$, but were down-regulated by exposure to rural PM extracts (Table 2).

Nrf2 is a major regulator of cellular resistance to oxidants in vertebrates (Timme-Laragy et al., 2012; Ma, 2013). It controls the basal and induced expression of an array of antioxidant response element (ARE)-dependent genes to regulate physiological and pathophysiological outcomes of oxidant exposure (Ma, 2013). Nrf2 positively regulates ARE, which is a cis-acting regulatory element or enhancer sequence, found in promoter regions of genes encoding phase II detoxification enzymes and antioxidant proteins (Lee and Johnson, 2004). Zebrafish embryos in which Nrf2 expression had been knocked-down were more sensitive to toxic compounds, including PAHs, showing significant upregulation of antioxidant genes and exacerbated toxicant-induced malformations (Timme-Laragy et al., 2009; 2012).

In the work described in Chapter 3, *Nrf2* was up-regulated in embryos exposed to urban and rural PM, and BaP 0.1 $mg.L^{-1}$. Those results suggest that activation of the Nrf2-ARE signalling pathway may precede the up-regulation of oxidative stress-related

genes found for urban PM and BaP-treated embryos. However, for rural PM, even though *Nrf2* was over-expressed in exposed embryos, expression of Nrf2-related genes was down-regulated (Table 2). One possible explanation for this may be related to the fact that *fos* negatively regulates the expression of ARE-related genes, by interfering with the binding of *Nrf2* to the ARE binding site (Venugopal and Jaiswal, 1996). Additional evidence suggests that high concentrations of *jun* repress the expression of anti-oxidant genes, such as glutathione genes, presumably due to generation of large amounts of *fos/jun* complex, which interfere with binding of *Nrf2* to ARE (Jeyapaul and Jaiswal, 2000). In the work developed in Chapter 3, *junbb* and *fos* were strongly up-regulated in zebrafish embryos exposed to rural PM, suggesting that in this case the expression of ARE-related genes could be repressed. *fos* is known to dimerize with members of the *jun* family forming a transcription factor referred to as activator protein-1 (AP-1). Components of the AP-1 signalling pathway may act as transcriptional activators or repressors depending on the target gene and on the cellular context. Indeed, AP-1 factors are involved in distinct biological processes, such as cellular differentiation, proliferation, and apoptosis, development and organogenesis, as well as in multistep tumorigenesis, inflammation and oxidative stress (Jochum et al., 2001; Tormos et al., 2004).

Taking this information into account, one may hypothesize that when embryos were exposed to PM organic constituents the AhR was activated, up-regulating phase I biotransformation-related genes, namely of the CYP superfamily. This response was common to all treatments. With the metabolic activation of PM organic compounds, some level of reactive oxygen species (ROS) should have been generated, altering the cellular redox state and eliciting oxidative stress. The induction of oxidative stress by constituents of urban PM has been recognised has an important mechanism of action of urban particulate air pollution (Risom et al., 2005). In the urban environment vehicle emissions are a major source of ambient PM. It has been shown that diesel exhaust particles, via their organic components, modify the cellular redox state, inducing biotransformation phase I and phase II gene expression (Baulig et al., 2003). If the organism is able to eliminate the generated ROS, oxidative injury may be avoided. Therefore, activation of biotransformation phase I and phase II transcriptomic pathways in zebrafish embryos exposed to urban PM extracts and BaP 0.1 $mg.L^{-1}$ probably helped coping with ROS excess. On the other hand, exposure of embryos to rural PM samples (with lower concentrations of PAHs and PAH-related compounds comparatively to urban samples), has led to stronger overexpression of detoxification phase I CYP genes, relatively to urban PM samples, but with no oxidative stress response (even though the *nrf2* gene was over-expressed). High levels of ROS production are known to induce the expression

of genes of the *fos* and *jun* families (Karin and Shaulian, 2001; Tormos et al., 2004). Therefore, in zebrafish embryos exposed to rural PM, the Nrf2-ARE signalling pathway may have been repressed, compromising the expression of phase II biotransformation genes and the protection against oxidative injury.

Table 2 – Summary of data for biotransformation and antioxidant genes obtained from the quantitative microarray data determined in zebrafish embryos exposed to samples of particulate matter (PM) of urban and rural origin, and to benzo[a]pyrene 0.1 mg.L^{-1} (Chapter 3). Mean fold change values obtained for each treatment.

	BaP 0.1 mg.L^{-1}	Urban PM	Rural PM
Biotransformation phase I			
cyp1a	6.14	3.29	5.01
cyp1b1	4.24	0.86	2.55
cyp1c1	3.96	1.65	2.26
ahrra	4.49	1.27	2.71
Nrf2-associated oxidative stress response			
Nrf2[1]	0.54	0.27	0.60
hmox1	-0.97	0.37	-0.69
nqo1	0.57	0.82	-0.86
prdx1	0.82	0.57	-0.47
gstal	0.13	1.37	-2.06
gpx1a	0.14	0.82	-0.64
txnrd1	0.36	0.66	-0.32
sult6b1	1.57	1.59	-0.12
Phenotypic malformations			
emp2	0.07	0.00	-0.57

Cytochrome P4501a, P4501b1, P4501c1 (*cyp1a, cyp1b1, cyp1c1)*; aryl-hydrocarbon receptor repressor a (*ahrra)*; nuclear factor, erythroid 2, (*nrf2)*; heme oxygenase 1 (*hmox1*); NAD(P)H dehydrogenase, quinone 1 (*nqo1*); peroxiredoxin 1 (*prdx1*); glutathione S-transferase, alpha-like (*gstal*); glutathione peroxidase 1a (*gpx1a*); thioredoxin reductase 1 (*txnrd1*); sulfotransferase 6b1 (*sultb6b1*); epithelial membrane protein 2 (*emp2)*.

[1]*Nrf2* is also called *nfe2l2a*

Expression of epithelial membrane protein 2 (*emp2*) was also down-regulated in zebrafish embryos exposed to rural PM, but its expression was unaltered upon exposure to urban PM or BaP 0.1 mg.L^{-1} (Table 2). Previous studies, have shown that morpholino knockdown of e*mp2* in zebrafish embryos leads to severe yolk sac and pericardial edema (Ebarasi et al., 2009; Gee et al., 2014). Microarray data of this study therefore suggests that organic components of rural PM samples may bear a stronger toxic burden than urban samples. This is also supported by the observation of severer toxic effects in zebrafish embryos exposed to non-diluted rural PM samples, relatively to urban PM and BaP 0.1 mg.L^{-1} exposures.

Exposure of zebrafish embryos to urban and rural PM also elicited more specific gene expression responses. Urban PM extracts induced crystalline genes and genes related with peptidases of the chymotrypsin group, such as markers of exocrine pancreatic function. These alterations could be early indications of specific toxic effects on the corresponding organs (pancreas, eye lens) of the developing embryo. Rural samples up-regulated *ier2* and *klf2* genes. *ier2* is an important gene for the Kupffer's vesicle development, determining embryos left/right symmetry (Hong and Dawid, 2009), and *klf2a* is an immediate-early transcription factor involved in multiple processes, including haematopoiesis and cardiovascular function (Kaczynski et al., 2003). Overexpression of *ier2* may cause shortening of the anterior-posterior axis due to inhibition of cell movements in the embryo (Hong and Dawid, 2009). Over-expression of *klf2a* may enhance angiogenesis signalling, leading to cardiovascular defects (Renz et al., 2015).

Overall, from the transcriptomic study performed in the present thesis, it was possible to select a set of probes as molecular markers of toxic potential of particulate air samples, namely *cyp1a*, *fos, ctrb1, ela2, gstal, hao1, ier2, klf2a, sst2* and *ttr*. *cyp1a* characterises phase I biotransformation and the classical dioxin-like response (up-regulated by urban, rural PM and BaP). *fos* belongs to the AP-1 signalling pathway (upregulated by rural PM, together with *ier2* and *klf2a*). The remaining genes were upregulated by urban PM and could be involved in the Nrf2-ARE signalling pathway of antioxidant response, among other processes. The probes selected were tested using additional sets of PM samples, including an urban set from a road site with high vehicle intensity (Chapter 4) and a set originated from a marine atmosphere (Mediterranean and Black Seas open waters, Chapter 5). Altogether they proved to be useful to diagnose exposure to PM organic fraction and brought further insight into the differential toxicity of PM samples.

6.1.3. Characteristics of particles

Atmospheric PM is a heterogeneous mixture that changes in time and space. PM toxic potential is influenced by its chemical components and physical characteristics. PM components have multiple sources, and each source originates multiple components. Hence, identifying and quantifying the influence of PM individual components or source-related mixtures, on measurable biological parameters is one of the greatest challenges of environmental health research. Particularly because PM constituents interact with other atmospheric co-pollutants. Nonetheless, the results presented herein show a

degree of differential toxicity. The work developed identified seasonal, weekly, daily, and PM source and size-dependent patterns of biological effects.

Seasonal differences in the toxic potential of urban PM samples were identified in Chapter 2. AhR-RYA activity and, in general, the adverse effects observed in the ZET, which correlated with particulate PAH concentrations, were stronger in winter months. This was most probably related to higher PM emissions, lower planetary boundary layer and less photo-degradation in winter, in comparison to summer months. Strong seasonal differences were observed also for PM samples of rural origin (Chapter 3). Sampling campaigns for rural PM carried out during winter surpassed the BaP target value of 1 $ng.m^{-3}$, corroborating the extremely high dioxin-like activity and *in vivo* toxicity of these samples. Conversely, chemical loadings and biological activity of rural PM samples collected during summer months were almost un-measurable.

Particulate air pollution sources and origins were addressed on Chapter 2 and Chapter 3. In Chapter 2, vehicle and industry emissions appeared as the major drivers of the observed toxicity of samples, whereas, mineral emissions (essentially, dust, either local or from long-range transport), did not appear to contribute to the toxicity of the organic fraction of PM. Sources of marine origin (including heavy oil combustion) appeared to be negatively correlated with the biological activity observed, despite the contribution of heavy oil combustion sources (i.e. cargo ships). Overall, source apportionment indicated that the toxic effects described in the present Thesis for the organic fraction of PM samples of urban origin, are essentially linked to dioxin-like PAHs and traffic exhaust. This comes in support of the general awareness that the major air pollution source throughout the world is the combustion of fossil fuels (particularly from traffic exhaust), which is consistently associated with the most serious health outcomes (Cassee et al., 2013). In Chapter 3, biomass burning was found to be the dominant source of PM samples of rural origin. High toxicity and concentration of organic compounds found in rural PM samples of the cold period raises concern about the health impact of particles originated from biomass burning. The use of household wood and other biomass combustion for heating is growing in some countries, as a consequence of several factors, namely government incentives/subsidies, the rising costs of other energy sources, or increased public perception that this would be a “green” option (EEA, 2014). Also, a reversion to heating with solid fuels has been happening in households as a consequence of economic hardship (EEA, 2014). However, the burning of biomass either from indoor or outdoor sources (e.g. household combustion, wildfires) has been shown to produce high levels of PM (associated with severe effects on human health, Koenig et al., 1993; Jiang and Bell, 2008; do Carmo et al., 2013; Sarigiannis et al., 2015), comparatively to non-biomass-burning energy sources, such as electricity and natural

gas (Jiang and Bell, 2008). Studies support that biomass burning conditions are dominant factors that determine the hazard of the combustion-derived particles, causing the toxicity of emitted PM to vary significantly (Cassee et al., 2013). But, because incomplete combustion of biomass can originate highly hazardous substances (possibly contingent on the organic fraction), exposure needs to be reduced to a minimum.

Regarding the size of particles, in Chapter 2 urban PM cut-offs (PM10, PM2.5 and PM1) did not differ significantly in dioxin-like activity and toxicity. Since the largest fraction of PM contained the smaller ones, the results suggested that the sub-micron particle fraction (PM1) contained essentially all the activity, with limited contribution of larger particles. The results of Chapter 3 confirmed this finding, showing that for the urban and rural sites under study, the finest PM (PM0.5) induced stronger AhR-RYA activity relative to samples of larger PM size, and that rural PM0.5 induced much stronger toxic effects than larger particles, in agreement with their content on organic compounds. The ultra-fine PM fraction was consistently found as the most biologically active and toxic. Moreover, the organic constituents of PM were mainly contained in the smallest PM fraction, probably due to their affinity to the PM carbonaceous core.

Investigation of weekly and daily effect patterns of PM1 from urban environment (Chapter 4) showed higher dioxin-like activity during daytime and working days, relative to nighttime and weekends, in agreement with particulate concentrations of PAHs, elemental carbon and traffic intensity. Moreover, some of the genes evaluated showed higher expression levels in air conditions in which PAHs and traffic-related compounds predominate than in those with predominance of secondary aerosol formation.

Air quality regulations have been using mostly the PM mass as a normative parameter for air quality (WHO, 2013). However, an increasing amount of data, including the results from Chapter 2 and 4, show that the PM mass is a weak predictor of the potential biological activity and potential toxicity of PM organic fraction. Adverse effects observed in the present Thesis resulted from PM constituents extractable with a dichloromethane:methanol mixture. Therefore, the contribution of inorganic constituents to PM mass was not considered. Other research studies also support that PM mass is frequently insufficient to estimate the toxic potential of PM, failing to represent causal pollutant components (Kelly and Fussell, 2012; Cassee et al., 2013). This is particularly evident for certain characteristics of particles identified as critical for toxicity, such as the PM size. Indeed, the numerical majority of ultrafine particles comprised in a PM sample represents an irrelevant portion of its total PM mass. The present results point, therefore, to the need to revise current standards, guidelines and strategies aimed at reducing PM burden to human health, and define protective limits for biological effects elicited by ambient ultrafine particles.

6.1.4. Aquatic Environment

PAHs of high molecular weight have higher toxic potential than lighter relatives and a greater tendency to bind to aerosols, persisting further on the environment. They reach open waters mainly through dry deposition, concentrating in the aquatic suspended particle matter (SPM), sediments and biota, as a result of their hydrophobic nature (Manoli and Samara, 1999; Arzayus et al., 2001; Nizzetto et al., 2008; Qiao et al., 2008; Patrolecco et al., 2010). Bioavailability and the organism physiology are two of the most important aspects, dictating body burden of organic contaminants in the aquatic environment (Meador et al., 1995). Bioavailability is affected by environmental variables, such as the organic carbon present in SPM and sediments. On the other hand, physiological factors such as lipid content and rates of uptake, metabolism, and excretion influence PAHs body burden.

Assessing effects of organic PM on an aquatic organism provided some information on its potential aquatic toxicity. In Chapter 2, developmental effects of urban PM organic extracts (namely, spinal deformations, pericardial and cardiac edemas, malformation of the swim bladder and balance disruption) occurred at PAH exposure concentrations in the range of those found in the aquatic environment ($ng.L^{-1}$, Table 3). PAHs contamination of surface, coastal and marine waters can reach the $\mu g.L^{-1}$ range (WHO/IPCS, 1998; Cincinelli et al., 2001; Maskaoui et al., 2002; Malik et al., 2011). Particularly for HMW PAHs, which have higher Log Kow values (Table 3), because of their hydrophobicity direct transfer (fugacity) from the particles through embryo membranes may occur. Thus, particles can serve as a delivery system for toxic chemicals. In Chapter 5 zebrafish embryos were exposed to PM organic extracts from the atmosphere of the Mediterranean Sea and Black Sea open waters. At least half of the tested PM samples induced gene expression alterations in zebrafish embryos. These alterations occurred at PAHs exposure concentrations of 100-200 $ng.L^{-1}$

Previous investigation tried to broaden the scope of toxicity studies of atmospheric PM to include potential adverse effects on aquatic organisms, namely using freshwater algae, crustaceans and also zebrafish embryos (Diamond et al., 2000; Sheesley et al., 2004; Sheesley et al., 2005; Bui et al., 2012). Even though studies developed so far show that atmospheric PM has the potential to induce adverse effects on aquatic organisms, further research is needed in order to quantify the actual burden resulting from this contamination source, and to develop critical levels or target values for PM emissions that take into consideration the impact on aquatic life.

Table 3 - Environmental concentrations of polycyclic aromatic hydrocarbons (PAHs) detected in different aquatic systems around the world. Chemical structure, number of aromatic rings, octanol-water partition coefficient (Log Kow), and genotoxicity accordingly to WHO/IPCS (1998) are included. Zebrafish embryotoxicity test (ZET) results obtained in Chapter 2 are also presented as Maximum effect concentration (ZET Emax) and lowest observed effect concentration (ZET LOEC).

							Maximum environmental concentrations for several aquatic systems (ng/L)					
	Chemical structure	N. of rings	Log Kow	WHO/ IPCS[a] [b]	ZET Emax (ng/L)	ZET LOEC (ng/L)	Gomti River, India. [c]	Jiulong River Estuary, China [d]	Estuary of River Thames, UK [e]	New York-New Jersey estuary, USA [f]	Sea surface, Tyrrhenian Sea, Italy [g]	Antarctic Ocean [h]
Phe		3	4.6	+/-	6.9	8.9	1080.0	280.0	288.0	3.3	1840.0	65.0
Ant		3	4.5	-	0.8	1.2	860.0	1000.0	107.0	2.3	8300.0	25.0
Fla		4	5.2	+	20.0	24.7	3190.0	1740.0	940.0	6.2	6730.0	30.0
Pyr		4	5.2	+/-	29.1	35.8	420.0	880.0	1090.0	7.6	9190.0	27.6
BaA		4	5.6	+	37.8	17.3	5760.0	320.0	609.0	4.8	2830.0	11.5
Cry/Tri		4	5.9	+	70.0	38.0		n.d.	726.0	5.7	8480.0	13.8
Per		4	5.5	+			n.d.	n.d.	n.d.		n.d.	n.d.
BkF		5	6.8	+	37.4	12.5	2220.0	690.0	250.0	11.0	4480.0	11.0
BbF		5	6.1	+	51.6	13.1	530.0	1000.0	621.0		10200.0	5.0
BeP		5	6.4	+	89.7	41.5	n.d.	n.d.	207.0	5.2	n.d.	n.d.
BaP		5	6.5	+	74.0	16.4	3410.0	2600.0	909.0	5.5	2980.0	1.0
dBA		5	6.5	+	13.8	1.2	4700.0	970.0	126.0	2.2	4140.0	5.0
Ind		6	6.6	+	38.2	9.6	1004.0	2180.0	n.d.	9.3	n.d.	n.d.
BguiPer		6	7.1	+	76.0	46.8		630.0	627.0	4.4	5860.0	5.0

[a] +, positive; +/-, inconsistent; - negative for genotoxicity; [b] WHO/IPCS, 1998; [c] Malik et al., 2011; [d] Maskaoui et al., 2002; [e] Law et al., 1997; [f] Gigliotti et al., 2002; [g] Cincinelli et al., 2001; [h] Cripps, 1992

n.d. - no data

Phenanthrene (Phe), anthracene (Ant), fluoranthene (Fla), pyrene (Pyr), benzo[*a*]anthracene (BaA), chrysene + triphenylene (Cry/Tri), peryllene (Per), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), benzo[e]Pyrene (BeP), benzo[a]pyrene BaP, dibenzo[*a,h*]anthracene (dBA), indeno[1,2,3-*cd*]pyrene (Ind) and benzo[*ghi*]perylene (BghiPer)

6.1.5. Ambient air quality regulatory framework

In order to reduce exposure and consequent adverse effects of particulate air pollution, current directives define standards for air quality (such as limit and target concentration values for ambient PM). The European Ambient Air Quality Directive (2008/50/EC) establishes legal limits for concentrations in outdoor air of major air pollutants that impact public health such as particulate matter (PM10 and PM2.5) and nitrogen dioxide (NO_2). Set targets include certain toxic metals and BaP. However, continuous pressure to re-evaluate ambient air quality standards has found other PM-related parameters (e.g. organic carbon, elemental carbon) and chemical constituents (other PAHs, transition metals, sulphates) as relevant for evaluating health risks of PM (Cassee et al., 2013).

Nevertheless, due to PM intrinsic complexity it has been concluded there is not sufficient evidence to differentiate additional air quality metrics more closely related to specific health outcomes (Cassee et al., 2013).

Therefore, there is a clear need for parallel efforts within both the scientific and regulatory communities to advance methods to better evaluate and manage the effects of ambient PM. PM contains thousands of chemical compounds and only a fraction of them can be analysed and routinely screened. Even though chemical analysis are fundamental in the assessment of air quality, they are not able to account for effects of mixture interactions eventually intensifying the toxicity of PM to human and environmental health. In addition to determining the standard PM parameters or chemical constituents, it would be valuable to establish effects-based tools (e.g. bioassays and biomarkers) that could be used in the context of different monitoring programmes (surveillance, operational and investigative). Their incorporation in the regulatory framework would bring major air quality advantages safeguarding human health and the environment. Effects-based tools are currently covered in the context of the Marine Strategy Framework Directive (MSFD, 2008/56/EC), and are under consideration in the Water Framework Directive (Wernersson et al., 2015) because of the unique contribution they can give to assess and protect environmental health..

The work developed in the present Thesis suggests that the AhR-RYA and the ZET may be valuable tools for effects-based assessment and quality criteria of ambient PM. An *in vitro* assay (such as the AhR-RYA), sensitively detecting chemicals with similar biological targets or modes of action, would be a valid option for the primary screening of PM toxicity, allowing to easily and rapidly test a high number of samples. The extract thereof should be tested with an *in vivo* bioassay of choice (as the ZET assay), using pre-selected toxicological endpoints, depending on the objective of the study. When addressing PM organic fraction, the determination of dioxin-like activity followed by assays with zebrafish embryos, has been proved useful, also accounting for effects derived from the metabolism of organic contaminants and fulfilling ethical animal welfare requirements.

The work developed also found potential molecular markers of exposure to PM organic constituents, which may act as early signals to predict higher-level effects. The transcriptomic data and the markers identified, provided information about the mode of action of PM organic fraction. Further, this may help to reduce the uncertainties involved in PM environmental risk assessment by providing, for example, tools to elucidate links between exposure and effects in investigative monitoring or a basis for detection and understanding effects across species.

6.2. References

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CHAPTER 7

Conclusions and Future Perspectives

7.1. Conclusions

The specific conclusions of the work developed may be summarised as follows:

- The AhR-RYA proved to be a simple and low-cost tool for measuring the dioxin-like activity of PM samples, distinguishing toxic potentials among them. Its properties are suitable for incorporation in routine screening and control of PM emissions;

- The ZET assay was found to be a valid method to assess systemic toxic effects of PM constituents *in vivo* and to study the mode of action of PM organic constituents in such a sensitive life-stage as early embryonic development;

- The organic fraction was found to contribute to PM toxicity, with PAHs playing substantial role in the induction of the effects observed;

- The ultra-fine PM fraction was consistently found as the most biologically active and toxic. This fraction showed significant correlations with the distribution of biologically active organic compounds, such as PAHs. PM urban samples collected during day-time and working-days showed higher dioxin-like activity than PM samples collected during nighttime and weekends.

- The most used parameter for air quality assessment, PM mass, was found to be a weak predictor of the biological activity and toxicity of PM organic fraction.

- Seasonal differences in PM toxic potential were found for both urban and rural environments, with PM samples collected during winter months inducing stronger toxic effects than samples collected during warmer periods of the year. For the urban environment studied, the effects observed for the organic fraction of PM were essentially linked to traffic emissions. Rural PM collected during the cold period induced strong embryotoxicity and had particularly high concentrations of organic compounds as a consequence of biomass burning;

- Dioxin-like activity represented an important part of the overall toxicity of ambient PM. It was associated with up-regulation of biotransformation phase I transcriptomic pathway (i.e. cytochrome-related genes) and distribution of PAHs and quinones;

- Extracts from rural and urban samples elicited both common and specific transcriptome responses, suggesting different potentially adverse outcomes depending on PM source and composition. Phase I biotransformation was common to both urban and rural PM sources, while phase II biotransformation (possibly under control of the Nrf2-ARE pathway) was specifically activated by exposure to urban PM samples. Exposure to rural extracts, also altered genes implicated in basic cellular functions and AP-1 signalling pathway.

- *Cyp1a, fos, ctrb1, ela2, gstal, hao1, ier2, klf2a, sst2* and *ttr* may be suitable molecular markers of the toxic potential of particulate air samples;

- Incorporation of the approach followed herein in the regulatory framework for ambient air quality would allow rapid site specific characterisation of the potential toxicity that may be elicited by the complex composition in organic compounds of PM, improving protection of human and environmental health.

7.2. Future Perspectives

The studies presented in this Thesis raised several questions and opened perspectives for future research. Transcriptomic data showed that the organic compounds comprised in PM from different environments influence not only the strength, but also the molecular mechanisms implicated in the toxicity of ambient PM. A major future achievement would be to clarify individual compounds, or specific mixtures of compounds characterising different PM samples. For that a broader chemical characterization of the PM samples used is needed possibly including not only products of PAH-photochemical transformation, but also other dioxin-like compounds, such as PCBs or dioxins. Potentially toxic compounds and their mixtures should be tested on zebrafish embryonic development in controlled laboratorial bioassays, with further determination of the expression of the selected probes. This will improve understanting on the chemicals driving the effects observed, allowing to improve future regulatory guidelines. In addition to the organic fraction, PM comprises other components that certainly contribute to its whole toxicity. The inorganic fraction of PM, which includes toxic compounds such as metals, and the presence of bacterial endotoxins on the particle surface, have to be equally addressed in order to fully characterise PM toxic potential. In addition, the work developed raised concern about the potential toxic burden of PM, and particle-bound

PAHs to aquatic species. The fate and transformations of airborne PM in the aquatic environment needs further research, to better understand and evaluate the real exposure of aquatic organisms and the associated risk.

Finally, if it would be possible to actively reduce PM emissions, not only the global burden of disease (0.8 million premature deaths and 6.4 million years of life lost, annually worldwide) would be reduced, but also the morbidity and the massive economic costs associated to PM exposure. This may be accomplished by increasing scientific literacy on air pollution and its detrimental effects, creating social awareness to favour the use of non-fossil transport options and take informed decisions about air quality matters.