Universidade de Aveiro Departamento de Biologia
2013

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Toxicogenómica de efetores naturais e artificiais em eleuteroembriões

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Amadeu Mortágua Velho da Maia Soares, Professor catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação do Doutor Carlos Barata Martí, Investigador Titular do Consejo Superior de Investigaciones Cientificas, Barcelona.

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Dedico aos meus pais e tia Graça.

o júri

presidente

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palavras-chave

Peixe-zebra, microarrays, transcrição, qRT-PCR, disrução endócrina, recetores nucleares, nano particulas

resumo

A introdução de compostos químicos no meio ambiente por atividades antropogénicas pode representar um sério risco para a saúde humana e ambiental. A avaliação de risco ambiental requer o uso de ferramentas eficientes e sensíveis para a determinação do impacto dos contaminantes nos ecossistemas. A utilização do peixe-zebra para a avaliação da toxicidade de produtos farmacêuticos, drogas e poluentes é bem aceite, devido às suas características únicas que permitem fazer o rastreio de poluentes e avaliar o seu risco. O principal objetivo deste trabalho consiste na aplicação de metodologias tóxico-genómicas para identificação de novos biomarcadores, e para a compreensão do modo de ação de contaminantes clássicos e emergentes, capazes de desregular o sistema endócrino (nomeadamente através do recetor do ácido retinóico, do recetor X retinoide e do recetor de hidrocarboneto de arilo). O estudo centraliza-se em diferentes recetores proteicos, nucleares, citosólicos e outros fatores de transcrição (ativados por ligandos ou por stressores) que estão intimamente envolvidos na regulação de genes do “defensoma” e de mecanismos de toxicidade química. Os efeitos transcriptómicos de compostos orgânicos, compostos endógenos e nano particulas foram analisados durante vários estadios de desenvolvimento embrionário do peixe-zebra. Recorrendo à utilização de microarrays, os perfiles de expressão génica de organismos expostos e não expostos aos contaminantes foram estudados, o que permitiu a identificação de marcadores genéticos específicos, assim como o estabelecimento de um “código de genes” para os compostos estudados. As alterações na expressão génica foram observadas a concentrações que não causaram efeitos morfológicos. Inclusive a baixas concentrações, as alterações observadas no nível do transcriptoma, foram bastante robustas para alguns dos genes alvo. Para os genes selecionados os resultados dos microarrays foram posteriormente confirmados através da quantificação da reação em cadeia da polimerase em tempo real (qRT-PCR). A combinação de ferramentas de bio-informática, de análises transcripcionais dos perfiles de expressão génica, e das respostas fenotípicas e bioquímicas para os diferentes tratamentos permitiram a identificação de potenciais mecanismos de ação. Além disto, este trabalho fornece um conjunto de ferramentas integradas que poderão auxiliar nas tomadas de decisão dos organismos de gestão, através da melhoria da capacidade preditiva para a determinação do stress ambiental induzido pelos contaminantes nos ecossistemas dulçaquícolas. Este estudo ilustra igualmente o potencial da utilização de embriões de peixe-zebra, para a análise sistemática e em larga escala dos efeitos de compostos químicos no desenvolvimento de vertebrados.

keywords Zebrafish, microarrays, transcription, qRT-PCR, endocrine disruption, nuclear receptors, nanoparticles

abstract The introduction of chemicals into the environment by human activities may represent a serious risk to environmental and human health. Environmental risk assessment requires the use of efficient and sensitive tools to determine the impact of contaminants on the ecosystems. The use of zebrafish for the toxicity assessment of pharmaceuticals, drugs, and pollutants, is becoming well accepted due to zebrafish unique advantages for the screening of compounds for hazard identification. The aim of the present work is to apply toxicogenomic approaches to identify novel biomarkers and uncovered potential modes of action of classic and emergent contaminants able to disrupt endocrine systems, such as the Retinoic Acid Receptor, Retinoid X Receptor and the Aryl Hydrocarbon Receptor. This study relies on different nuclear and cytosolic protein receptors and other conditional (ligand- or stress- activated) transcriptional factors that are intimately involved in the regulation of defensome genes and in mechanisms of chemical toxicity. The transcriptomic effects of organic compounds, endogenous compounds, and nanoparticles were analysed during the early stages of zebrafish development. Studying the gene expression profiles of exposed and unexposed organisms to pollutants using microarrays allowed the identification of specific gene markers and to establish a "genetic code" for the tested compounds. Changes in gene expression were observed at toxicant concentrations that did not cause morphological effects. Even at low toxicant concentrations, the observed changes in transcript levels were robust for some target genes. Microarray responses of selected genes were further complemented by the real time quantitative polymerase chain reaction (*q*RT-PCR) methodology. The combination of bio-informatic, toxicological analyses of differential gene expression profiles, and biochemical and phenotypic responses across the treatments allowed the identification of uncovered potential mechanisms of action. In addition, this work provides an integrated set of tools that can be used to aid management-decision making by improving the predictive capability to measure environmental stress of contaminants in freshwater ecosystems. This study also illustrates the potential of zebrafish embryos for the systematic, large-scale analysis of chemical effects on developing vertebrates.

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1 - General introduction and conceptual framework of the study

1.1 Conceptual evolution of toxicology

Toxicology can be defined as a branch of science that deals with toxicants, defined as substances that cause harmful effects when administered, either by accident or design, to a living organism. A variety of parameters such as body weight, survival, reproduction, enzyme activity level, histological features of tissue samples or chemical biomarkers may be evaluated to assess the adverse effects of chemicals compounds to organisms. Screenings on the accumulation and effects of several compounds have been performed with relative ease using model organisms. Convenient assays have been extensively described and standardized for different aquatic animal models, like the water flea (*Daphnia magna*) or the zebrafish (*Danio rerio*) (Lele and Krone, 1996; OECD Guideline 211, 1998; OECD Guideline 212, 1998), to perform the risk assessment of relevant ecologically compounds. Nonetheless those methods and endpoints are appropriate for a traditional approach, focused in macroscopic toxic effects, but they do not cover the molecular mechanisms, which constitute the toxic mode of action, nor they provide information for low-level toxicity effects. So, it is of great interest to develop available methodologies to determine, understand and predict the effects of toxicity and disease at molecular level.

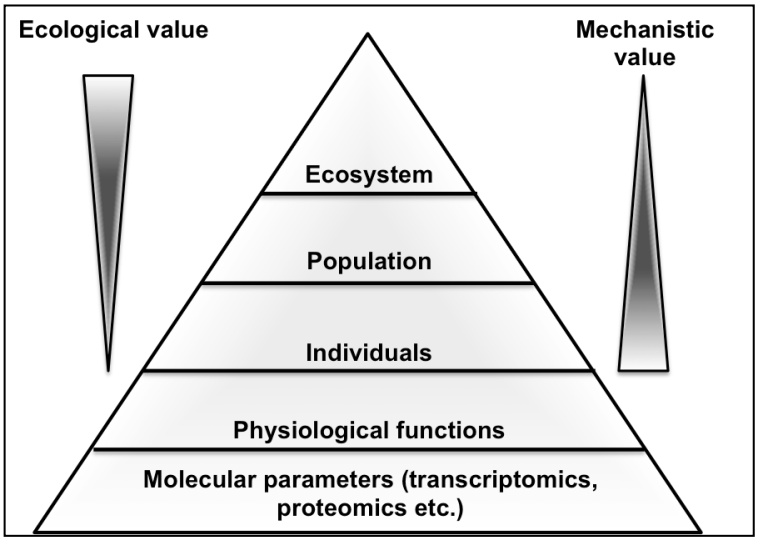


Figure 1.1 Conceptual framework for ecotoxicogenomics (adapted from (Snape et al., 2004).

Hence a new area of study has been developed, ecotoxicogenomics (Figure 1.1). According to Snape et al., 2004, ecotoxicogenomics describes the integration of genomic-based science into ecotoxicology. This area of interest combines the fields of molecular biology and ecotoxicology with the study of chemical and physical interactions and their effects when released into the environment, using DNA- and RNA-based technologies, as well as proteomic and metabolomic analyses.

Ecotoxicogenomics offers a different approach to the Environmental Risk Assessment (ERA), and its application as a standardized procedure for risk assessment is under continuously discussion, weighting its advantages and disadvantages (Table 1.1). It provides the characterization and identification of genomic signatures of environmental toxicants as gene and protein expression profiles.

Table 1.1 Advantages and disadvantages of ecotoxicogenomics in ERA -table adapted from (Robbens et al., 2007).

Advantages	**Disadvantages**
ERA is based on mechanistic information and mode of action (broader knowledge base)	Genomic information for several ecotoxicological model organism is scarce
Possibility of reducing uncertainty, and deducing a more realistic assessment factor for ERA	Confounding factors make it difficult to interpret genomic data
Enables reduction in animal testing	High expertise is required for analysis, and there is currently a lack of experienced genomics experts in regulatory agencies
Short time for exposure, compared with classical tests	High investment and costly equipment required
Possibility of assessing effects from bioavailability fraction	High need for data-mining tool that can also be used in ecotoxicology
Possibility of "learning lessons" in ecotoxicology from the experience with human toxicology	Lack of standardization in terminology
Early-warning tools	Validation of cross-platform is insufficient

Ecotoxicogenomics research allows to achieve a better understanding of the mechanisms of toxicity and to identify gene expression patterns that accurately reflect and predict specific and measurable ecotoxicological endpoints. Exposure to chemical compounds may affect a cascade of genes and gene interactions rather than a single gene (Aardema and MacGregor, 2002). Since gene expression can be altered either directly or indirectly as a result of the exposure to toxicants, it is necessary to determine the changes at gene expression level to completely understand their mode of action. This knowledge can be used to develop new molecular biomarkers and, in many cases, to discover new target genes. Also, the extrapolation from animal models to human and *in vitro* to *in vivo* represents a main challenge for a better understanding of molecular mechanisms.

One of the important aspects of ecotoxicogenomics is the development of bioinformatics tools and databases that facilitate the analysis, mining, visualization, and sharing of the vast amounts of biological data that can be generated (Coverdale et al., 2004). Moreover, some answers need to be addressed due to the lack of studies in some key ecological species, the controversy between the doses and times of exposures, and the need to improve experimental designs to perform a correct interpretation of the obtained results.

The assessment of how a stressor affects gene regulation in several metabolic pathways and/or what changes are produced in the transcription level of a particular gene is an important tool to assess the ecological status of an ecosystem. There are

several techniques to measure mRNA levels: Northern blot and reverse-transcription quantitative polymerase chain reaction (qRT-PCR) when few genes are studied; DNA microarrays and, more recently, quantitative massive sequencing to analyse the transcription levels of numerous genes at once (expression profile). DNA microarray and qRT-PCR are the two major techniques used in this work, and are described below.

1.1.1 Microarray technologies

Transcriptional profiling by DNA chip (microarrays) methodologies are highly informative and are among the most promising novel techniques for ERA (Figure 1.2). Information retrieved from those chips enables the identification of new biomarker genes, hence is becoming a major tool in Life Sciences.

The advances in biotechnology have made possible the monitoring of thousands of nucleic acid sequences simultaneously – either specific expressed RNAs or sequence variants (polymorphisms) in DNA (Brown and Botstein, 1999). Microarray analysis of the expressed mRNAs probes can be compared to the execution of thousands of Northern blot analysis, and offers the opportunity to monitor the expression of individual genes across the entire genome (i. e. global gene expression analysis, or transcriptomics). Thus, microarray technology provides a methodology of studying multiple pathways and mechanisms at the same time. Such a global analysis of gene expression has the potential to provide a much more comprehensive view of ecotoxicity than it has been possible until now, since toxicity generally involves change not only in a single or few genes but rather is a cascade of gene interactions (Aardema and MacGregor, 2002).

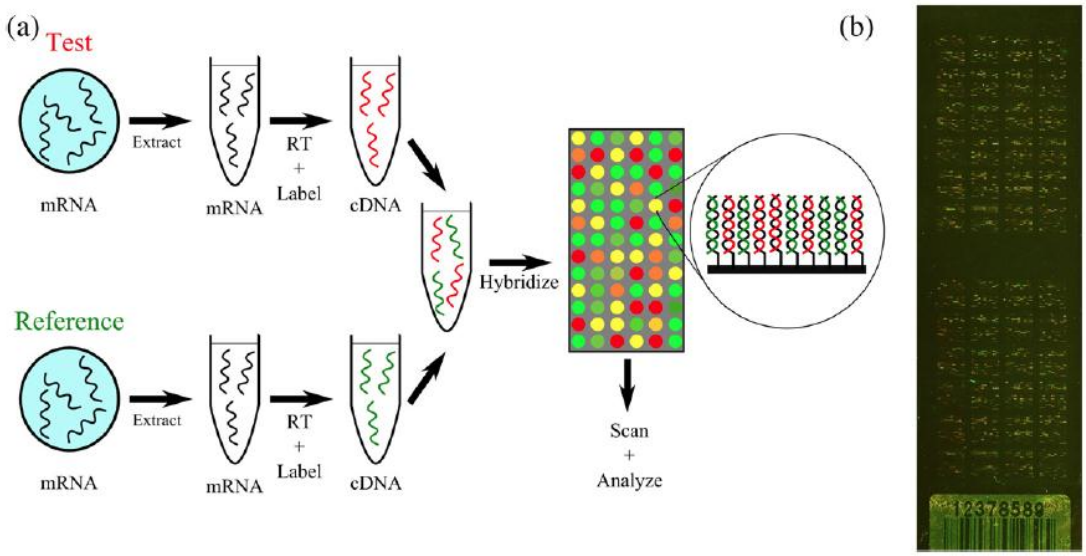


Figure 1.2 a) General outline of a microarray experiment for ERA in different species b) Scanned image of a cDNA microarray, adapted from (Karakach et al., 2010).

Microarrays consist in a small rigid support made of glass or silica, that hosts thousands of cDNAs or oligonucleotides spotted sequences called probes, which are printed or attached in known and fixed locations. Hybridization between these probes and complementary target sequences labelled with a fluorescent dye is the principle behind microarrays technique. Total RNA from control and treated samples is extracted from cells or tissues of the desired organ(s)/organism for which the array was designed. Target sequences are obtained by reverse transcription of this material, using nucleotide derivatives that are either fluorescent or that can be attached to fluorescent molecules (for example: Cy3-dUTP and Cy5-dUTP) or that can be subsequently attached to fluorescent molecules. The conditions in which hybridization and subsequent washings are carried out, promote the formation of only strongly paired strands, that is, those with a high number of complementary base pairs, avoiding non-specific interactions. In this context, the total fluorescent signal emitted by each spot depends upon the amount of target sequence binding to the probes present on that spot. Scanning and analysis of intensity data, allow the estimation of the relative steady-state mRNA levels for all genes present in the microarray. Comparison of these gene expression patterns allows the identification of genes whose expression change due to the condition under study. Although microarray technology has a very high throughput interrogating thousands of genes simultaneously, the process includes numerous sources of variability being necessary to apply statistical tools in experimental design and in data analysis. This is essential to obtain high quality results from microarray experiments.

There are a large number of microarray platforms that differ in the type of probe and manufacturing technology. There are essentially two protocols for designing and analysing microarray experiments, differing in the use of a single-colour versus two-colours for labelling the probes (Figure 1.3). In one-color experiments, all samples are labelled with the same fluorescent dye and hybridized to separate microarrays. In two-colour experiments, samples are differentially labelled, using two different fluorescent dyes, and are directly compared by mixing and hybridizing the two samples on a single microarray. The one-colour approach requires very consistent manufacturing to minimize array-to-array variation. The two-colour approach benefits from the direct comparison between samples on a single array. However, comparing more than two samples becomes complicated, necessitating either the use of a common reference sample or a hybridization strategy that combines multiple different pairs of samples. Both single- and dual-channel platforms are frequently used and yield comparable results.

Microarray experiments performed in this study are based on the Agilent two-colour labelling approach, in which each sample and condition was hybridized versus a common reference sample. In fact, when a control sample can be defined, the two-colour platform approach is normally preferable to the single-colour strategy.

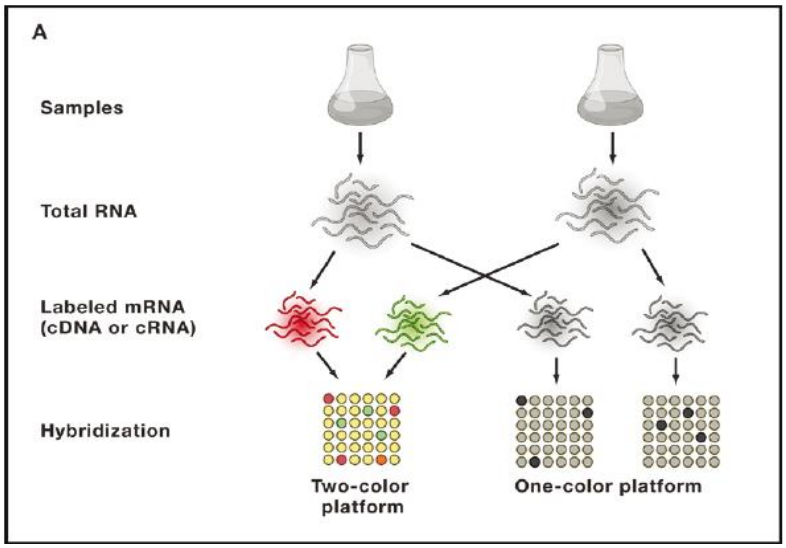


Figure 1.3 Comparison between single- and double-colour labeling strategies.

Those advantages will be described here:

- In two-color designs, the hybridization of two samples to the same array allows a direct comparison, minimizing variability due to processing multiple microarrays per assay. This reduced variability theoretically results in increased sensitivity and accuracy in determining levels of differential expression between sample pairs. More complex hybridization schemes are also an option when using two-color approach, including hybridization with common reference samples or the use of loop designs.
- Dual-colour derived ratios are more robust against moderate hybridization artefacts and thus less prone to false-positive results. This is because the most frequently occurring hybridization artefacts tend to affect both colour channels similarly. Therefore, the resulting detraction is often minimized/equalized after calculating ratios of intensities of the two channels;
- In some cases, the dual-colour approach is more reliable in detecting small intensity differences;
- Depending on the underlying biological study design and the objective, the dual-colour approach might be advantageous regarding the overall costs, especially if the study should primarily serve as a pilot or screening approach.

This technology could be very suitable for environmental toxicology studies, but in order to obtain accurate information it must be carefully planned and interpreted. In addition, inherently to the methodology, intrinsic sources of variability in the gene expression profiles may occur due to the physiological state of the animals, their age, season of analysis, sex and genetic polymorphism. Temperature and day length are known signals that start the process of reproduction in fish, thus innately changing gene expression. Being aware of these sources of variability is of major importance to

conduct experiments with sufficient statistical power to obtain useful data. Moreover, it is necessary to be aware of inaccuracies that are present in microarray analyses. They do not provide a truly quantitative assessment of gene expression, but instead show changes in biochemical pathways and processes that are linked together. Transcriptomes are highly redundant, and many genes are only expressed at very low levels, in very specific stages of the development and/or under very specific conditions.

There are some limitations to the technology due to the incomplete knowledge of the entire genome of many existing species for proper microarray design. Although a large number of complete genomes are being released in public databases (http://www.genomesonline.org/cgi-bin/GOLD/index.cgi), the functional annotation for many of these genomes is still incipient. Another limitation of this technique is that the DNA sequence does not provide (yet) directs information about the portion of the genes (the coding sequences) that become actually transcribed and ultimately translated into proteins. In addition, the transcriptomic profiles may vary depending on the concentrations used to stress the organism. At low concentrations only the genes related to specific targets of the chemical compound would be affected by its presence. On the other hand, an exposure to higher concentrations would induce a generalized response of proper stress genes masking the specific target changes.

New functional data, novel developments in Bioinformatics, development and completion of sequencing projects will be required for an in-depth analysis of environmental impacts and to overcome the limitations of functional and transcriptomic analyses. Ideally, transcriptomic tools may allow both the identification of mechanisms of toxicity of new substances and the direct identification of the source of pollution by specific changes in the transcriptome.

1.1.2 Real time PCR and molecular targets

Specific RNA detection has become one of the most robust tools of molecular biology, especially since the introduction of the polymerase chain reaction (PCR). There are also particular limitations associated to this technique, but in general it allows the detection and quantification of even small amounts of mRNA molecules. The application of devices able to monitor the PCR process in real time (quantitative Real-Time PCR, or qRT-PCR) makes the detection and quantification of an amplified sequence more accurate, reproducible and reliable. The high sensibility of the qRT-PCR technique allows the development of a new family of biomarkers based on the analysis of gene-expression patterns for environmental monitoring (Piña et al., 2007) with suitable application for small organisms (*Danio rerio*) or even for some structures of the

body, i.e. scales (Tanguay et al., 2000; Quiros et al., 2007a; Quiros et al., 2007b; Pelayo et al., 2011).

Most of the mRNA-quantification methods rely on the knowledge of the exact sequence of at least a portion of the genes to be monitored. Genomes of several species are getting sequenced at unprecedented speeds (Figure 1.4), although many phyla and classes of great environmental interest (e.g. molluscs, annelids, and among vertebrates, birds, reptiles and amphibians) are still severely underrepresented in gene databases.

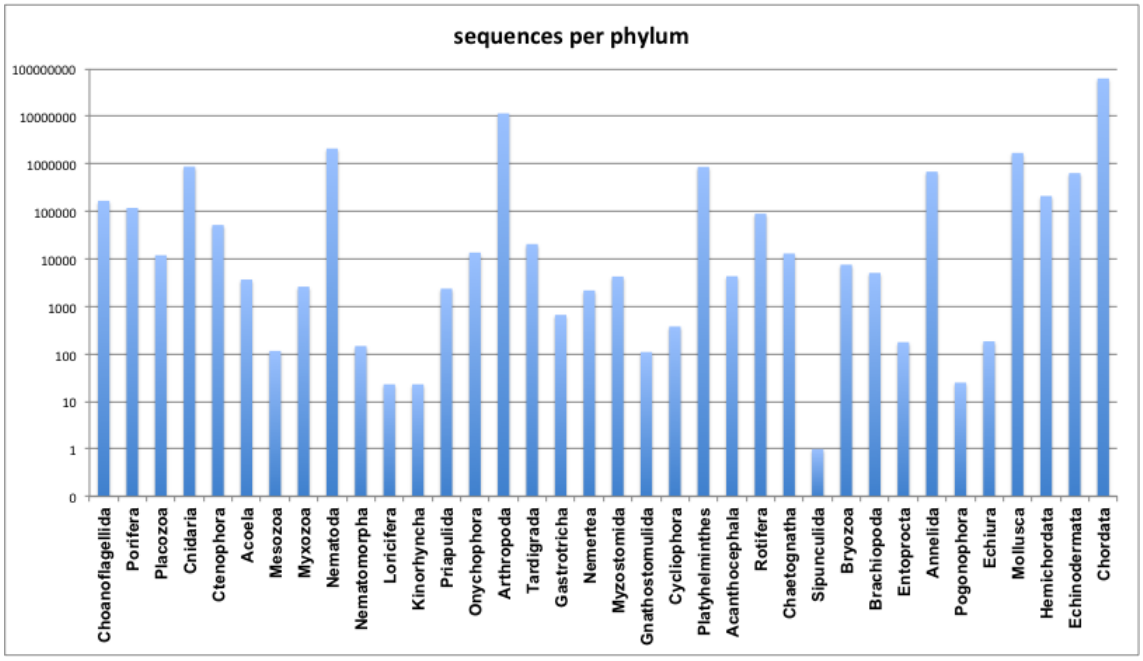


Figure 1.4 The total number of DNA sequences published per phylum (values corrected from http://www.ebi.ac.uk/ena/ at 7-5-2013).

Polymerase Chain Reaction (PCR, developed by Kary Mullis and his co-workers) is a method that allows exponential amplification of short DNA sequences within a longer double stranded DNA molecule. PCR is based on the specificity of complementary sequences, the activity of a thermo-stable polymerase enzyme and the dependence of both with temperature. A pair of oligonucleotides complementary to both, sense and anti-sense, strands of the DNA sequence of interest is required. Once they are hybridized to the target DNA, those primers are extended by the DNA polymerase and synthetize a copy of the designated sequence. Since the separation of the double stranded DNA molecule needs higher temperature than oligonucleotide hybridization and polymerase extension, the reaction relies on a cyclic process of temperature changes. In each cycle, new copies of target sequence are synthesized leading to exponential amplification of the desired fragment.

In the standard PCR, reaction product is detected at the end of the entire process, but in qRT-PCR the amplified DNA is detected as the reaction progresses in real time. The detection is carried out by monitoring the signal of a fluorescent molecule added to

the reaction mixture, whose fluorescence is directly related to the amount of DNA molecules accumulated in each cycle (Higuchi et al., 1992). Common methods for detection of products are DNA sequence-non specific fluorescent dyes that intercalate with any double-stranded DNA, like SYBR Green, and sequence-specific DNA probes consisting of oligonucleotides labelled with a fluorescent reporter.

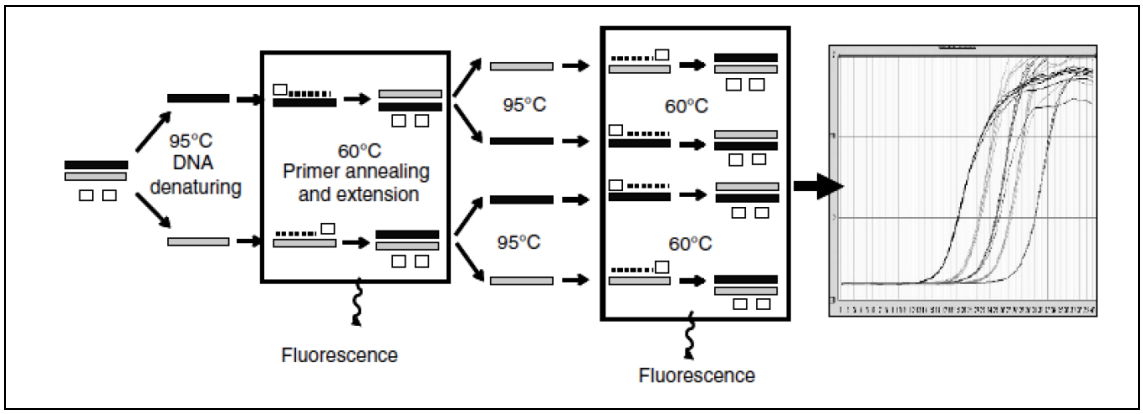


Figure 1.5 Process of mRNA quantification by qPCR and monitored in real-time by fluorescence methods (Piña et al., 2007). The scheme applies to non-specific, DNA intercalating dyes.

Quantification of the starting amount of DNA must be carried out in the exponential phase of the PCR amplification, since it is only here where the amount of amplified target is directly proportional to the input amount of transcript. The parameter used for quantification is the quantification cycle, Cq, which is defined as the cycle number at which the reporter dye emission intensity rises above background noise (Figure 1.5). Cq value is inversely proportional to the logarithm of the starting number of target DNA molecules.

In comparison to others common techniques of mRNA quantification such as Northern blot, qRT-PCR can quantify smaller samples and it is sensitive enough to quantify the RNA from a single cell (Roth, 2002). The technique is suitable for microarrays validation since provides accurate and detailed results of the expression of specific genes.

Environmental toxicology studies can benefit from qRT-PCR since they could provide early warning information associating pollutants to specific molecular responses, either activatory or inhibitory. So far, a large part of the work on gene-expression biomarkers uses counterparts of well-established biochemical biomarkers, (e.g., cytochromes P450 in liver for organic pollution, metallothionein for metals in liver and kidney and egg-forming proteins, as vitellogenin for endocrine disruption in fish liver).

In summary, environmental genomics provide specific information's about the response of organisms to environmental stress conditions at genome level. A great advantage of using a standard model organism in environmental studies, such as zebrafish, is the possibility that the obtained data could be relevant to other aquatic vertebrate and also to humans. The wide number of characteristics and resources of the zebrafish make it an excellent model for environmental genomic studies. To fulfil the entire potential of ecotoxicogenomics, collaborative efforts are necessary to establish protocols that integrate data from different model organisms, including microorganisms (*Saccharomyces cerevisiae*, algae, etc.), aquatic species (*Danio rerio, Daphnia magna*, *Xenopus laevis*) and terrestrial species (*Enchytraeus albidus, Eisenia foetida*). With those tools, we can obtain the complete assessment of the functional activity of biochemical pathways, and structural genetic (sequence) differences among individuals and species, that were previously unreachable.

1.2 Environmental Risk Assessment

Natural and anthropogenic impacts on ecosystem and human health constitute pressing international issues. Environmental Risk Assessment (ERA) is defined as 'the procedure by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies' (Anderson et al., 1994).

Since environmental scientists as well as the general public have learned that chemicals, which are not toxic to human, may have deleterious effects on natural resources, ERA has become increasingly important. Risk is not an inherent property of a chemical toxicant but rather the product of its toxicity times the exposure received by a given organism.

Risk assessment can be divided into two different components: risk analysis and risk management. The scientifically oriented *risk analysis* is a process, which comprises some or all of the following elements: hazard identification, effect assessment, exposure assessment and risk characterization, (Walker et al., 2000). On the other hand, *risk management* is more politically oriented, dealing with regulatory measures based on risk assessment (Walker et al., 2000). Those two components are intrinsically related one each other, the former determines the risk of a certain stressor, and the later examines the possible solutions to the problem.

Environmental Risk Assessment includes a set of guidelines allowing determination of acute and chronic toxicity parameters. REACH is the European Community Regulation on chemicals and their safe use (EC 1907/2006, 2007). It deals with the Registration, Evaluation, Authorization and Restriction of Chemical

substances. The law entered into force on June 1st, 2007. The aim of REACH is to improve the protection of human health and the environment through the better and earlier identification of the intrinsic properties of chemical substances.

Moreover, environmental risk assessment can also be viewed in a more scientific context. Besides acute and lethal effects, there is the need to evaluate sublethal effects, both in the field and in the laboratory. This kind of environmental risk assessment allows for the evaluation of sublethal, more subtle parameters.

The ability of various pollutants (and their derivatives) to mutually affect their toxic actions complicates risk assessment, usually based on environmental levels of single chemicals (Calabrese, 1991). Deleterious effects on populations are often difficult to detect in feral organisms since many sublethal effects are only measurable after long periods of time. When the effect finally becomes clear, the damage may have gone beyond the point at which it may be reversed by remedial actions of risk reduction (Hylland, 2006). Discrimination between survival (lethal) and sublethal (i.e. resource acquisition) effects of toxic substances is important in ecotoxicology because their combination may have unexpected and severe consequences at the community and ecosystem level.

1.2.1 Aquatic toxicology

Aquatic toxicology can be defined as the study of the effects of potentially toxic chemicals on aquatic organisms, with special emphasis on the harmful effects. The environment is continuously loaded with foreign organic chemicals (xenobiotics) released by urban communities and industries. A wide variety of chemical compounds, including Polychlorinated Biphenyls (PCBs), Organochlorine Pesticides (OCPs), Polycyclic Aromatic Hydrocarbons (PAHs), Polychlorinated Dibenzofurans (PCDFs) and Dibenzo-*p*-dioxins (PCDDs), have been continuously released into the environment. The ultimate sink for many of these contaminants is the aquatic environment, either due to direct discharges or to hydrologic and atmospheric natural processes (Heid et al., 2000). Historically, this discipline has used toxicity tests to identify putatively harmful effects. Standardized tests evaluate dose-response relationships (toxicity at different concentrations) and mechanisms of action in a variety of organisms that are representative of the different ecosystem niches. Those tests aim to evaluate the response of individuals or populations to varying concentrations of the chemical.

The occurrence of a xenobiotic compound in the aquatic environment does not imply deleterious effects by itself. It is of great importance to establish connections between the external levels of exposure, internal levels of tissue contamination and

early adverse effects. Thus the identification and the assessment of the impact of several hydrophobic organic compounds and their associated metabolites is very important due to the lack of information and the increasing number of this type of substances. Hence, the evaluation of the fate, exposure and effects of those chemicals in aquatic organisms has been extensively studied by environmental toxicologists.

It's important to recognize that toxicity data will not always be available for all potential species in a particular environment. Given this limitation, the overall objective of test organism selection is to choose alternatives that are representative of the major ecosystem components. Aquatic algae and plants are representative of photosynthetic organisms, also called primary producers. Invertebrate species such as scuds and water fleas, feed on algae and decaying plant materials and bacteria, and are in turn important sources of food for a variety of larger fish, birds and mammals. Fish species have attracted great interest in the assessment of biological and biochemical responses to environmental contaminants (Powers, 1989). They represent the largest and most diverse group of vertebrates, and they play a major ecological role in the aquatic food webs because of their function as energy carriers from lower to higher trophic levels (van der Oost et al., 2003). Therefore, the understanding of toxicant uptake, behavior and responses in fish has high ecological relevance.

1.2.2 Fish and environmental monitoring

Actinopterygii, a class of bony fish, is the most diverse class of vertebrates. It includes approximately 48% of the known species of the subphylum Vertebrata. Their geographical and environmental diversity makes them important experimental models in environmental toxicology, since they could live in a wide range of aquatic habitats: from fresh to salt water; from the cold polar seas to warm tropical reefs and from shallow surface waters to the ocean depths. Certain species also possess many specific characteristics that support their use in environmental toxicology. Examples of these favourable attributes are listed below:

- Fish live in intimate contact with the aquatic environment;
- Many hormonal and regulatory functions common with mammals and other terrestrial vertebrates;
- Many species possess specialized functions of higher vertebrates; some species with relatively short life-cycles and with high production of eggs;
- Several fish species are suitable for field and laboratory experiments (e.g. cage monitoring studies), facilitating extrapolations between lab-based studies and field observations.

Finally, fish are fundamentally important to the function of most aquatic ecosystems and have a high economic value, Therefore, there is a need to protect fish populations against the potential adverse effects of chemical discharges.

Consequently to these characteristics, fish have been largely used for ecotoxicity tests to assess the environmental impact of individual chemicals or their mixtures, (e.g. effluents), and to establish criteria for monitoring water quality in rivers and estuaries. The scientific community, regulatory bodies and the chemical industry as a whole, have long considered fish as crucial representatives for studies on aquatic environment.

1.2.3 Ecotoxicological standard tests

Classical ecotoxicological strategies encompass a variety of fish species and are considered indispensable components of integrated toxicity testing approaches for the aquatic environment. In particular, the fish acute toxicity assay plays an important role for environmental risk assessment and hazard classification, allowing the evaluation of the relative toxicity of different chemicals also in several species (Wedekind et al., 2007).

The test species that are recommended by OECD guidelines are rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), bluegill (*Lepomis macrochirus*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), guppy (*Poecilia reticulata*) and common carp (*Cyprinus carpio*). This recommendation helps to reach comparable results and thus saves experiments and resources (i.e. they increase feasibility). However, it may be discussed that none of the recommended species is native to Western Europe. Current guidelines at the OECD level are good example of this with fish-targeted guidelines covering acute toxicity (OECD Guideline 203, 1992), early life-stage toxicity (OECD Guideline 210, 1992), short-term toxicity test on embryos and sac-fry stages (OECD Guideline 212, 1998) and juvenile growth test (OECD Guideline 215, 2000). Those guidelines have become widely required for the aquatic hazard assessment of new and existing synthetic substances as well as for the use in evaluation of complex mixtures (Hutchinson et al., 2003). Thus, single species LC_{50} data are questionable with respect to accuracy and, in more general terms, to toxicological relevance (Braunbeck et al., 2005), and it seems largely accepted that there is no a single susceptible species that could be used in an unified conservative testing approach. All manufacturers and importers of chemical substances in the EU, in quantities exceeding one tonne per year will be required to submit a registration dossier to the new European Chemicals Agency (ECHA). The registration dossier will contain

detailed information on the substance, including: the acute toxicity for freshwater fish (96h LC_{50}), the acute toxicity for daphnids 48h EC_{50} (OECD Guideline 202, 2004) and the growth inhibition test on freshwater algae (growth rate 72h ErC_{50} and/or biomass 72h EbC_{50} and/or biomass: (OECD Guideline 201, 2006).

Due to the consideration of the animal welfare consortium, fish tests have been increasingly questioned, and alternatives or refinements to that type of assays are being considered i.e. the usage of cell fish cultures or early developmental stages of fish. Another possibility of reduce the number of fish animals used for toxicological determinations is the use of Quantitative Structure-Activity Relationships (QSARs) which attempt to correlate chemical structure with activity using statistical approaches for the prediction of activities of unutested chemicals.

1.2.4 The need for alternative methods to environmental risk assessment

The scientific relevance, reliability and ethical acceptability of acute toxicity testing with mammals and other vertebrates have been under intense scrutiny for several years. In a critical paper, (Fentem and Balls, 1993) concluded that "fish are used as an ecologically "representative" species and, as such, the toxicity data obtained only provides an approximate guidance for environmental risk assessment purposes", and that the relevance of the results did not justify the amount of animals used. This line of reasoning points, account for the need of alternative approaches to chemical testing to be developed.

Since the implementation of the Animal Welfare Guideline 86/609/EC in 1986 (EC, 1986), the development and validation of alternatives to animal testing is strongly promoted by EU institutions (Lilienblum et al., 2008). Since 2004, on the basis of the 7^{th} amendment to the Cosmetic Directive (EC, 2003), animal testing has been banned for finished cosmetic products, and this ban was extended to ingredients of cosmetic products in March 2009 (EC, 2008). Likewise, REACH explicitly promotes non-animal testing and gives preference to alternative methods for animal testing whenever possible, (EC, 2001; Lilienblum et al., 2008).

Alternative test methods are generally regarded as technologies that incorporate the principles of **Replacement, Reduction, or Refinement** (**3Rs**) of animal use (Stokes and Marafante, 1998). In the frame of safety testing and safety assessment, the definition of alternative methods includes "testing methods" (e.g. *in vitro, ex vivo* or reduced/refined methods *in vivo*) as well as "non-testing methods" such as the use of expert systems. Under REACH, non-testing methods also include the adequate use of existing data of a substance or other considerations such as the chemical category or

chemical analogue approach that may contribute to reduce or avoid testing *in vivo* (Lilienblum et al., 2008).

Russell and Burch (Russell, 1959) developed the first significant step towards such alternative approaches in their book *The Principles of Humane Experimental Techniques*. In this book, the authors indicated that all experiments using animals should be designed to diminish or avoid inhumane practices and should follow the 3Rs. The principles of the 3Rs are described as follows:

Reduction refers to the number of animals used to obtain information of a given amount and precision;

Replacement means the substitution for conscious living higher animals of insentient material;

Refinement means any decrease in the incidence or severity of inhumane procedures applied to those animals, in the cases animal testing is still to be used.

Regulatory authorities, such as the EURL ECVAM (European Union reference laboratory for alternative to animal testing), works continually in order to promote the scientific and regulatory acceptance of non-animal tests which are of importance to biomedical sciences, through research, test development and validation and the establishment of a specialised database service. The EURL ECVAM provides technical support to the following Commission services: European Chemicals Agency (ECHA), European Partnership for Alternative Approaches to Animal Testing (EPAA), Organisation for Economic Co-operation and Development (OECD) in relation to the Guidelines for the Testing of Chemicals. The European Union (EU) put forward the "white paper" strategy for future chemicals policy in the community with the overriding goal of *sustainable development,* a policy that became integrated into the REACH guidelines (EC, 2001). All this legislation states the requirement for a maximization of non-vertebrate animal test methods, encourages the development of new non-animal test methods, and calls for the minimization of animal test programmes (COM, 2001). It is important to note that the White Paper calls for the collection of information about chemicals, rather than for testing.

1.2.5 Zebrafish in environmental toxicity testing (model organism)

The zebrafish *Danio rerio* (formerly also named *Brachydanio rerio*) is a small benthopelagic cyprinid fish (Figure 1.6), living in rivers of South Asia, chiefly northern India, northern Pakistan, Bhutan and Nepal (Dahm and Geisler, 2006).

Figure 1.6 On the left adult female and on the right representation of an adult male Zebrafish. The man adult length varies between 3 and 5cm. Males can easily be distinguished from females by their more slender body shape and an orange to reddish tint in the silvery bands along the body. During spawn maturity, due to the large number of eggs produced, females can be recognized by their swollen bellies.

Table 1.2 Taxonomic hierarchy of *Danio rerio*

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Superclass	Osteichthyes
Class	Actinopterygii
Subclass	Neopterygii
Infraclass	Teleostei
Superorder	Ostariophysi
Order	Cypriniformes
Superfamily	Cyprinoidea
Family	Cyprinidae
Genus	Danio
Species	*Danio rerio* (Hamilton, 1822)

Zebrafish is a common and useful model organism for studies of genetics, developmental biology, neurophysiology and biomedicine (Sumanas and Lin, 2004; Alestrom et al., 2006; Dahm and Geisler, 2006), which has been recently incorporated into ecotoxicological research.

Zebrafish presents several features that make it an ideal vertebrate model. Maintenance under laboratory conditions is easy during all year; and yields a large number of non-adherent, fully transparent eggs (Laale, 1971). Their short generation, typically 3-4 months, makes them suitable for multi-generational experiments. Fertilization and development are external, and single adult female spawns hundreds of eggs. Embryos are transparent, and they can be monitored through all developmental stages under a dissecting microscope. The development is fast, completing organogenesis at the fifth day post fertilization (dpf), coinciding with the complete yolk consumption and the start of external feeding. Zebrafish reaches maturity within three months after fertilization (all time points apply to fish reared at 28ºC, (Scholz et al., 2008)).

The zebrafish was first identified as a genetically tractable organism in the 1980s, and the subsequent advances in gene mapping; phenotype analysis and extensive EST databases make it a convenient model organism for many biological fields. The zebrafish genome is virtually complete and available in the zv9 assembly on the

Ensembl website (http://www.ensembl.org/Danio_rerio/Info/Index). It encompasses orthologues for approximately 70% of human genes, confirming its status of excellent vertebrate model with relevant applicability to human health issues, including pollution effects (Howe et al., 2013).

1.2.6 Embryonic development of zebrafish

Zebrafish shows a set of particularities that makes them suitable for ecotoxicological studies. The transparency of embryos and their notable characterization during early stages of development gives unique properties to perform whole-mount *in situ* hybridization (Fjose et al., 1992), antibody staining (Wilson et al., 1990) and also the embryo can be subjected to the microinjection of nucleic acids and expression of specific proteins can be suppressed throughout the developing embryo by antisense agents. Zebrafish embryonic development has been extensively described in detail in several studies (Kimmel et al., 1995; Nagel, 2002). The zebrafish egg is telolecithal, cleavage is meroblastic and discoidal. Kimmel et al 1995 describe seven broad periods of embryogenesis; the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching period (table 1.3). The main developmental stages that will be presented in the following chapters are shown in detail in Figure 1.7.

Table 1.3 Stages of embryonic development of zebrafish (*Danio rerio*) at 26±1ºC (Nagel, 2002).

Time (h)	Stage	Characterization (after Kimmel et al., 1995)
0	Fertilization	zygote
0	Zygote period	cytoplasm accumulates at the animal pole, one-cell stage
0.75	Cleavage period	discoidal partial cleavage
		1. median vertical division: two-cell-stage
1		2. vertical division: four- cell-stage
1.25		3. vertical and parallel to the plane of the first: eight-cell-stage
1.5		4. vertical and parallel to the second plane of division: 16-cell-stage
2	Blastula period	start of blastula stage
3		late cleavage; blastodisc contains approximately 256 blastomers
4		flat interface between blastoderm and yolk
5.25	Gastrula period	50% of epibolic movements; blastoderms thins and interface between periblast and
		blastoderm become curved
8		75% of epibolic movement
10		epibolic movement ends, blastopore is nearly closed
10.5	Segmentation period	first somite furrow
12		somites are developed, undifferentiated mesodermal component of the early trunk,
		tail segment or metamere
20		muscular twiches; sacculus; tail well extended
22		side to side flexures; otoliths
24	Pharyngula period	phylotypic stage, spontaneous movement, tail is detached from the yolk; early pigmentation
30		reduced spontaneous movement; retina pigmented, cellular degeneration of the tail end;
		circulation in the aortic arch 1
36		tail pigmentation; strong circulation; single aortic arch pair, early motility; heart beating starts
72-96	Hatching period	heart-beat regularly; yolk extension beginning to taper; dorsal and ventral stripes meets at tail;
		segmental blood vessels: thickened sacculus with two chambers; foregut developmental; neuromasts

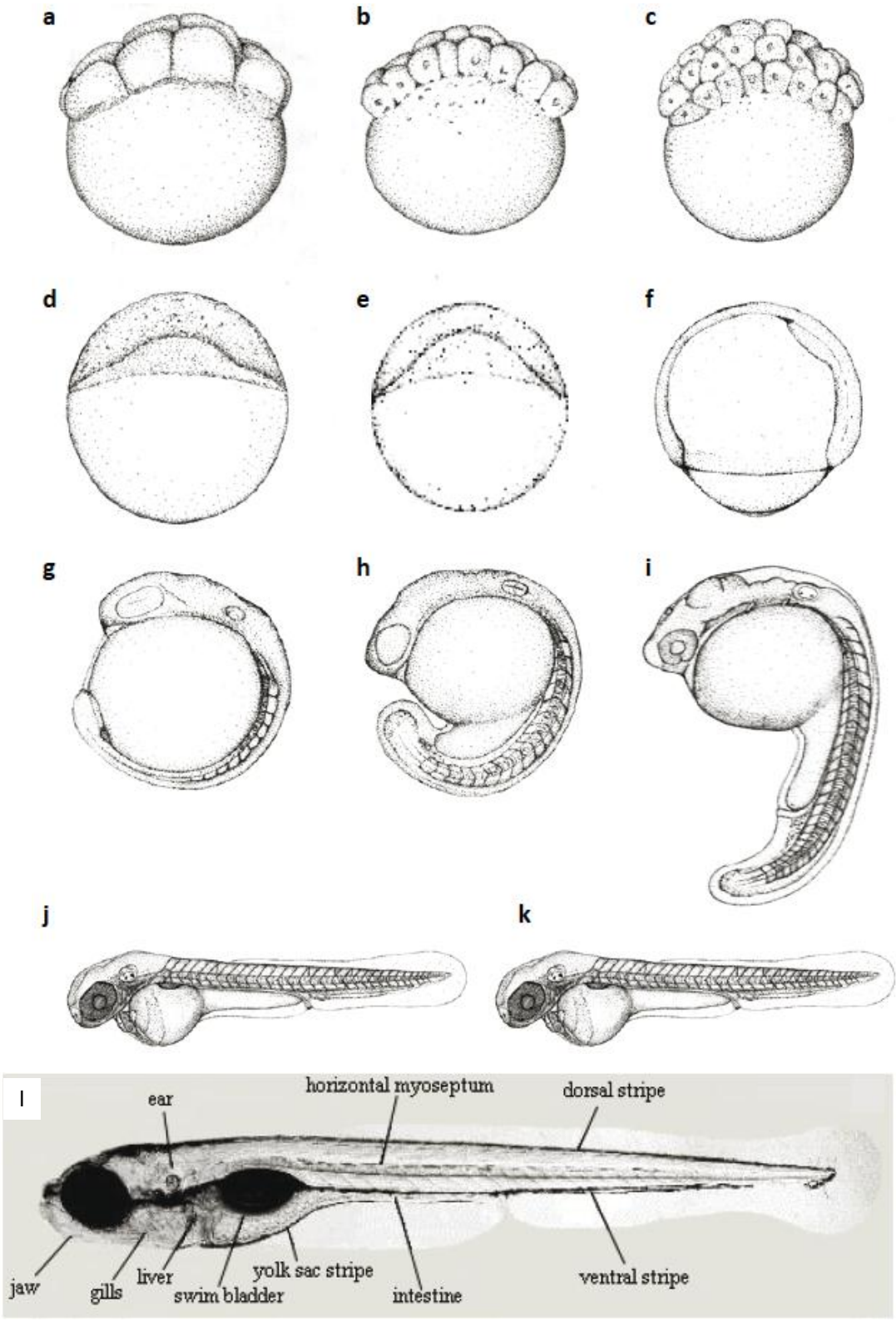


Figure 1.7 Selected stages of the embryonic development of *Danio rerio* –illustrations without chorion (adapted from Kimmel 1995):

a 8-cell stage (1.25h)
b 16-cell stage (1.5h)
c 32-cell stage (1.75h)
d beginning epiboly (4.3h)
e 30% epiboly stage (6h)
f 75% epiboly stage
g embryo at an age 14h
h embryo at an age of 18h
i embryo at an age 22h
j eleutheroembryo at an age 48h
k eleutheroembryo at an age of 72h
l larvae at an age 120h

The embryonic stage of development in fish refers to the autotrophic stages, in which the embryo develops without external feeding and relying on the yolk sac reserves. This period includes both the pre-hatching developmental period and the first two or three days post hatching, at which the development continues and the fish is still feeding on its own reserves. This post hatch/sac fry stage of development is referred as the eleutheroembryo stage, whereas the term embryo is usually restricted to pre-hatching stages of development. This distinction is important because the capacity of self-feeding, not hatching, is a key legislatory time point to apply animal experimental rules (EC, 2010).

1.2.7 The use of fish embryos in ecotoxicological assays

The application of fish embryos to predict environmental toxicity has been recognized has a tool in order to develop an approach to the animal testing (Nagel, 2002; Scholz et al., 2008; Thienpont et al., 2011; Pelayo et al., 2012; Olivares et al., 2013). Fish embryos are used to predict both acute and chronic toxicity (Nagel, 2002; Braunbeck et al., 2005), and have been proposed as an alternative to the regular mammalian endpoints in the earlier development of new drugs (Van Leeuwen et al., 1990; Teraoka et al., 2003; Ton et al., 2006). In particular, the acute fish test with mortality as an exclusive endpoint should be replaced by alternative methods, since there is an increasing evidence that some form of pain perception, similar to what is present in mammals, may be present in bony fish (teleost), and that adult fish exposed to acute toxic concentrations of chemicals may at least be suspected to suffer severe distress and pain (Nagel, 2002; Sneddon et al., 2003; Chandroo et al., 2004; Braunbeck et al., 2005; Huntingford et al., 2006). This undue suffering is not compatible with the current animal welfare legislation in Europe (COM, 2012). The switch to alternative methods is particularly obvious with the model organism *Danio rerio*, as there are an increasing number of papers that rely on the usage of embryos instead of adult organisms (Figure 1.8), in accordance with the change of the research methodology orientation.

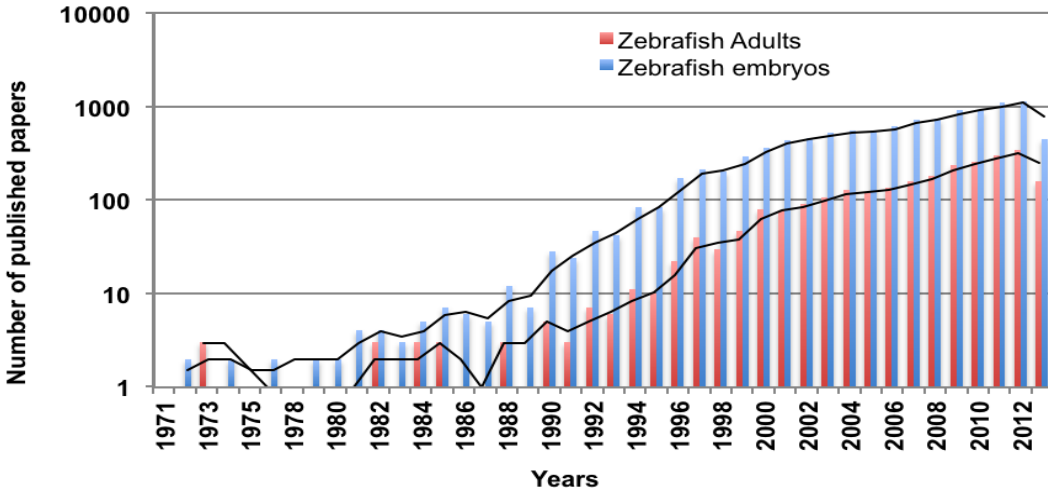


Figure 1.8 Differences between the numbers of published papers in which the words zebrafish adults and zebrafish embryos appear in the tittle. This search was conduct on the web site www.scopus.com at the day 27/05/2013. The search was performed with the following key-words, zebrafish adults and zebrafish embryos.

There are many potential benefits to the use of fish embryos for acute toxicity assessments. The embryo-larval and the early juvenile stages appeared as the most or among the most sensitive stages during the fish life cycle in a large analysis including 56 life-cycle tests completed during a decade with 34 organic and inorganic chemicals and four fish species (McKim, 1977). Therefore, toxicity tests with these early life stages of fish should be useful in establishing water-quality criteria and in screening large number of chemicals, as the prediction is that long-term toxicity could be predicted by other studies with early-stages in about 80% of the cases. In this context, the embryo toxicity test (Embry et al., 2010), was developed as a good tool for the replacement of the acute fish testing (Nagel, 2002; Braunbeck et al., 2005). According to the UK Animals (Scientific procedures) (Act, 1986) and the EU (ECETOC, 2005; EFSA, 2005) fish are classified as legally protected once they reach the capacity of independent feeding. The potential benefits of using Fish Embryos Toxicity Test (FET) compared with adult test include:

- Under laboratory conditions thousands of eggs can be easily daily produced, and they may be used in parallel experimental treatments (Scholz et al., 2008), which allows simultaneous toxicity assessments of a larger number of substances than adult tests, that typically require one or several aquaria for each substance (plus the controls).
- As fish embryo tests are conducted in 24-well microtier plates, the volume of medium required to perform the assay is really small, limiting the required volume of the tested substance or sample.

- The test duration is also short, limited to two or three days, compared to the costumer 96h for acute toxicity and 28 days for chronic toxicity in adult fish experiments.
- The transparency of fish embryos enables the possibility of evaluation of several sublethal endpoints. Thus, the effect of chemicals on fish embryos can be evaluated, not only in terms of overall lethality, typically characterized by coagulation of the egg, lack of movements and absence of heartbeat, but also a series of other end-points relating to diverse physiological functions that may be indicators of chronic endpoints.
- FET test can be applied not only to zebrafish embryos, but also to the early embryonic stages of other OECD species as the fathead minnow (*Pimephales promelas)* and the Japanese medaka (*Oryzias latipes*).
- Fish embryos can be used as a tool for determining the genetic effects of substance exposure, since approximately 90% of the genome is active during embryogenesis, whilst only approximately 10% is functional during adult life.

In the process of validation/acceptance of the FET test, the correlation between the alternative and the conventional tests was determined (Figure 1.9 a,b) for a vast majority of substances, with certain exceptions for which embryo tests were significantly more or less sensitive (Braunbeck et al., 2005). The Endpoints suggested to determine the toxicity of the chemical compounds in zebrafish embryos are listed below:

- Non-completion of gastrulation (12 hours),
- No somites (16hours),
- No heartbeat (48hours),
- No movement (48hours),
- Coagulated egg.

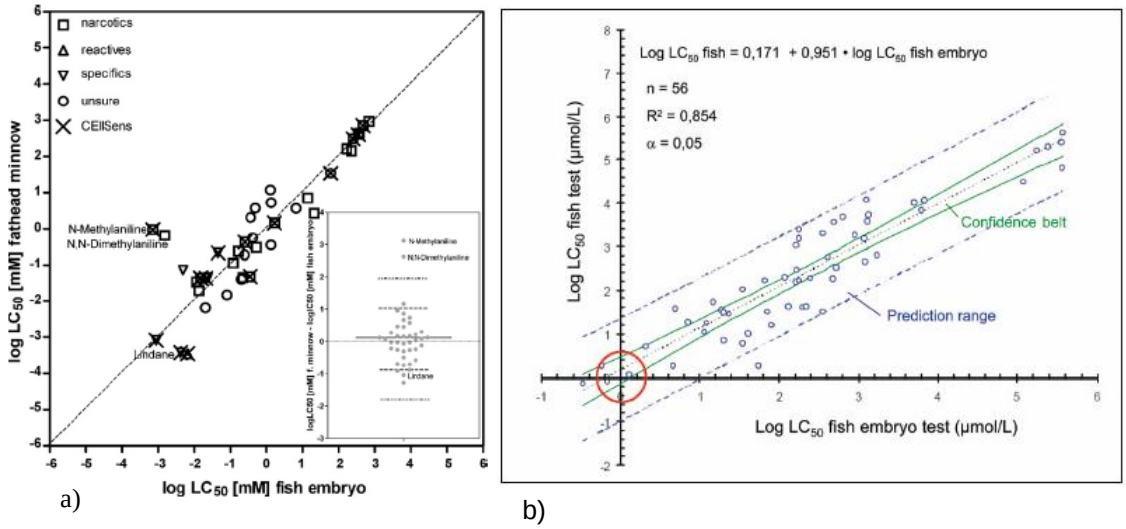


Figure 1.9 a) Plot of U.S. EPA fathead minnow data vs. UBA zebrafish embryo toxicity data. All 40 chemicals for which both log LC50 (*Fathead minnow*) and log LC50 (zebrafish embryo) were available were sorted according to their modes of action and correlated adapted from (Schirmer et al., 2008). B) Correlation between the zebrafish (*Danio rerio*) embryo test and the 48 h acute fish LC50 test (various species; n = 56). The slope of the regression is close to 1, and that the regression line almost perfectly crosses the origin of the axes, indicating that even lowest toxicities can be recorded adapted from (Braunbeck et al., 2005).

Although there are many benefits to the use of embryos and a significant amount of research has been conducted on their use as an alternative approach, there are indications that this embryonic approach may be considered less sensitive than adult tests. The metabolic capability of fish embryos is not well developed, making them less sensitive to toxins that require metabolic activation (e.g. Benzo(a)pyrene). In addition, fish embryos metabolites are not all synthesized *in ovo*. Finally, the chorion could be acting as a barrier to certain chemicals and reduce their toxic effect (Lange et al., 1995; Westerfield, 2000).

Whereas zebrafish embryos proved to be a good model for toxicological assays using typical toxicological endpoints, it gives relatively little information about the mode of action of the toxicants. To cover this aspect, genomic tools are extremely useful for the study of several chemical compounds acting as endocrine disrupting compounds (EDC) or for other compounds that are considered new and for which we do not have information on their mode of action.

1.3 Nuclear Hormone Receptors - Endocrine system

The development of a complex endocrine system constitutes a particular feature of the bilateral animal evolution, allowing organisms to coordinate their reaction to the

environment, to regulate their development, and to maintain homeostasis. In vertebrates, different types of hormone systems could principally be targets of environmental chemicals. . Natural hormones, such as reproductive hormones such as estrogens and androgens, thyroidal hormones, corticosteroids, growth hormone and their associated hypothalamus/ pituitary releasing and stimulating hormones, are responsible for the regulation of the expression of genes, through the binding to proteins called Nuclear Hormone Receptors (NRs). Those proteins are clustered into a large superfamily of intracellular protein receptors, which are believed to be evolutionary derived from a common ancestor (Figure 1.11). They act as ligand-activated transcription factors, providing a direct link between signalling molecules that control processes and transcriptional responses.

Ligands for some of these receptors have been recently identified: hydrophobic molecules such as steroid hormones (estrogens, glucocorticoids, progesterone, mineralocorticoids, androgens, vitamin D, ecdysone, oxysterols, bile acids…), retinoic acids (all-trans and 9-cis isoforms), thyroid hormones, fatty acids, leukotrienes and protaglandins (see Table 1.4). On the other hand, there are NRs for which ligands either do not exist or still have to be identified, and they are referred to as orphan receptors.

Table 1.4 Subfamilies of zebrafish nuclear receptors.

Receptor	Natural ligand	Contaminants	Effects
Estrogen receptor	Estradiol	Genistein, Nonylphenol, BAP	Feminization
Androgen receptor	Testosterone	PCBs	Differentiation of the male sexual phenotype
Dioxin-like receptor (AhR)	?	PAHs, TCDD, Dioxin like PCBs	Oxidative stress
Thyroid hormone receptor	Thyroid hormones	PBDEs? BPA?	Regulation of basal metabolism
Peroxisome proliferator activated receptor	Fatty acids, Fibrates	Pharmaceuticals	Lipid metabolism
Retinoic acid receptor	Retinoic Acid	Pharmaceuticals?	Embryonic development, and tissue patterning
Retinoid X receptor	9-cis Retinoic Acid	TBT, TPT	Cellular growth, differentiation and development

Evolutionary analysis of the receptors has led to a subdivision in six different subfamilies (Figure 1.10). One large family is formed by thyroid hormone receptors (TRs), retinoic acid receptors (RARs), vitamin D receptors (VDRs) and peroxisome proliferator-activated receptors (PPARs) as well as different orphan receptors. The second subfamily contains the retinoid X receptors (RXRs) together with chicken ovalbumin upstream stimulators (COUPs), hepatocyte nuclear factor 4 (HNF4), testis receptors (TR2) and receptors involved in eye development (TLX and PNR). The third family is formed by the steroid receptors and the hihly related estrogen-related receptors

(ERRs). The fourth, fifth and sixth subfamilies contain the orphan receptors NGFI-B, FTZ-1/SF-1, and GCNF, respectively. In addition, the Aryl hydrocarbon Receptor (AhR) belongs to a different family of transcriptional regulatory proteins, as evidenced in numerous studies, whose members play key roles in development, circadian rhythmicity, and environmental homeostasis, mediating the dioxin toxicity.

The NR pathway comprises the production of the signal, its transport to peripheral organs, binding of the ligand to the receptor, and transcriptional activation of the receptor. . NRs share a common structural organization composed of several functional domains, each domain has a particularly function, they are named from A-F domains, with a central, well-conserved DNA-binding domain (DBD, also termed C domain) is responsible for the recognition of specific DNA sequences, a variable NH2-terminal region (A/B domain) a variable linker D non-conserved hinge (D domain) connects the DBD to the moderately conserved E/F region that contains the ligand-binding domain (LBD) within the COO-terminal portion of the LBD (Figure 1.10).

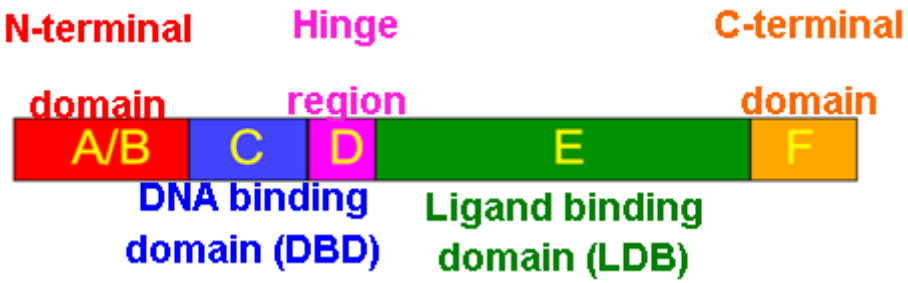


Figure 1.10 Schematic representation of a nuclear receptor. A typical nuclear receptor is composed of several functional domains.

NRs are promising pharmacological targets as they bind small molecules, which can be easily modified by drug design, and control functions associated with major pathologies (cancer, osteoporosis, diabetes, etc). Moreover some environmental chemicals may act as ligands of these NRs and thus, affect different types of hormone systems, effect known as endocrine disruption.

In the following sections the Nuclear Receptors studied in this work, will be described in detail:

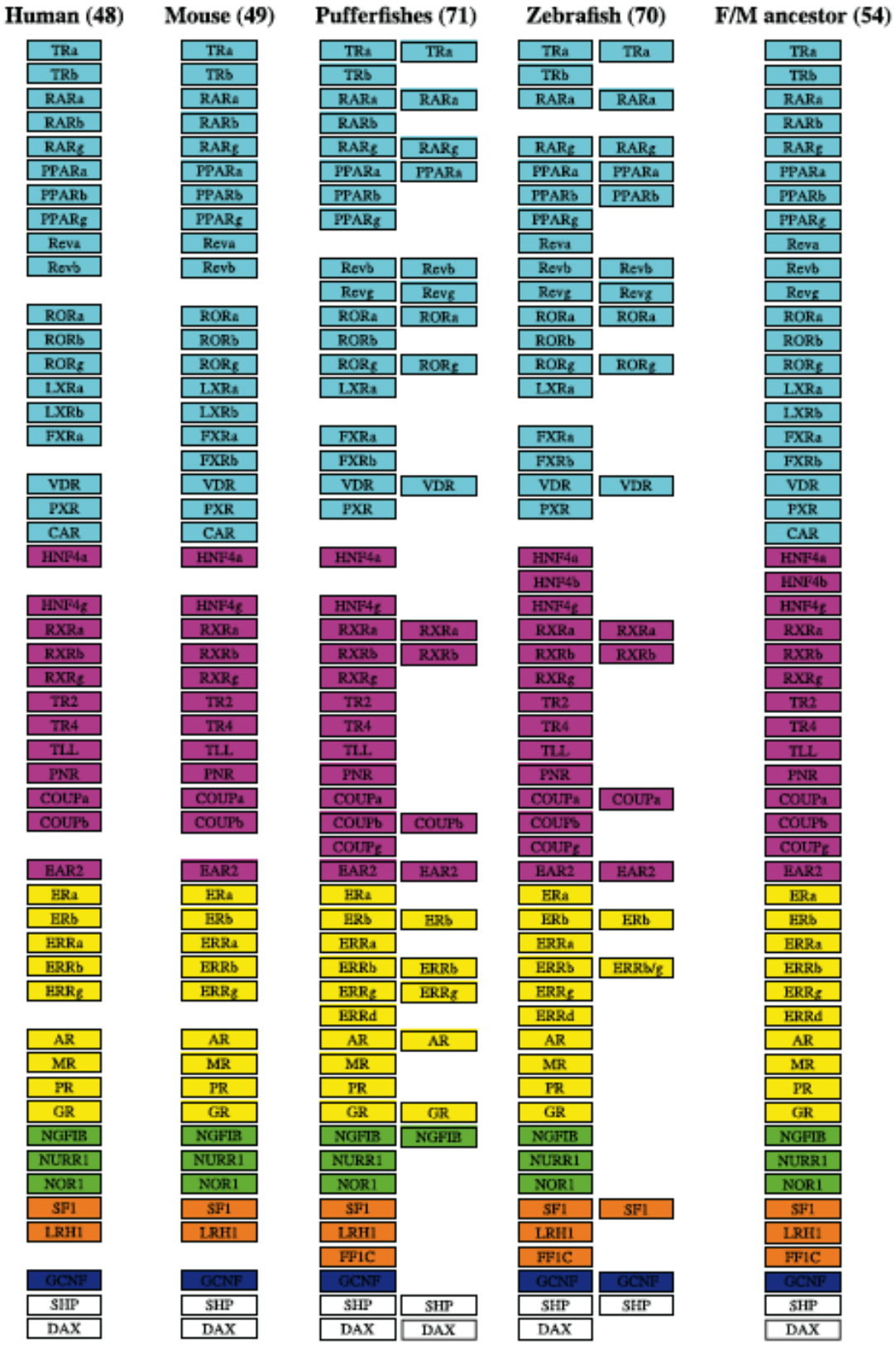


Figure 1.11 NR Complement in Human, Mouse, Zebrafish, Pufferfish, and the Inferred Complement in the Common Ancestor of Actinopterygian Fish and Mammals (Indicated by ‘‘F/M Ancestor’’) Each colour corresponds to a specific NR subfamily: light blue, purple, yellow, orange, dark blue, and white for subfamilies 1, 2, 3, 4, 5, 6, and 0, respectively. doi:10.1371/journal.pgen.0030188.g001 - Adapted from (Bertrand et al., 2007).

1.3.1 Retinoic Acid Receptors

Natural retinoids- vitamin A and its metabolites - are mediators of various important processes in eukaryotic organisms. Retinoic acid (RA) signalling is important for multiple aspects of embryonic development, tissue homeostasis and patterning of both the anterioposterior and dorsoventral axes of the CNS with primary sites of action in the hindbrain and anterior spinal cord (Maden, 1999). Heterodimers of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) transduce RA signalling. The RARs can bind all-trans RA and a 9-cis RA (Bastien and Rochette-Egly, 2004; Mark et al., 2006), whereas RXRs only bind 9-cis RA. However, ligand binding seems to be not always essential for RXR activity (Jones et al., 1995). In addition, RXRs can interact with and function in the transcriptional activation pathway of other nuclear hormone receptors (Aranda and Pascual, 2001), including thyroid-hormone receptors (TRs), peroxisome proliferator-activated receptors (PPARs), vitamin D receptor (VDR), and liver X receptor (LXR) (Aranda and Pascual, 2001), indicating that RXRs mediate expression of a large variety of hormone-responsive genes.

RA signalling operates essentially through RAR/RXR heterodimers. Upon ligand binding, the RAR/RXR heterodimers undergo conformational changes and modulates (either by activation or repression) the transcription of RA signalling target genes (Bastien and Rochette-Egly, 2004). In mammals there are three members of the retinoid X receptor, RXRα, RXRβ and RXRγ, while zebrafish possesses six RXR genes, r*xraa*, *rxrab*, *rxrba*, *rxrbb*, *rxrga*, *rxrgb*) (Jones et al., 1995; Tallafuss et al., 2006; Waxman and Yelon, 2007). Due to the capacity of RXR to form heterodimers with many known and orphan nuclear receptors, therefore affecting the subsequent regulation of their target genes, RXRs are considered to be involved in the regulation of most drug metabolizing enzymes and transporters. However, the ultimate role of RXR heterodimer complex appears to be multifaceted and yet uncertain (Rushmore and Kong, 2002; Wang and LeCluyse, 2003).

RA receptors (RARs) also constitute a nuclear hormone family of receptors, of which there are 3 paralogs (alpha, beta and gamma) in mammals (Aranda and Pascual, 2001; Bastien and Rochette-Egly, 2004). Zebrafish genome encompasses four RARs (*raraa*, *rarab*, *rarga*, and *rargb*), probably as a consequence of a whole genome duplication and a subsequent deletion of two genes (Joore et al., 1994; Hale et al., 2006; Waxman and Yelon, 2007)

Evidence for redundancy has also come from treating embryos with pharmacological RAR antagonists, who have graded effects on A-P patterning of the hindbrain depending on antagonist concentration, and also disrupts neurogenesis at larger stages (Dupé and Lumsden, 2001; Linville et al., 2004). A key step in hindbrain segmentation is the specification of posterior rhombomeres by RA. RA is thought to act

as a morphogen during gastrulation, forming a gradient that directly regulates homeotic (Hox) gene or the *cyp26a1* (Figure 1.12) expression and specifies the segmental identities of rhombomeres in a concentration-dependent manner (Conlon, 1995; Begemann and Meyer, 2001).

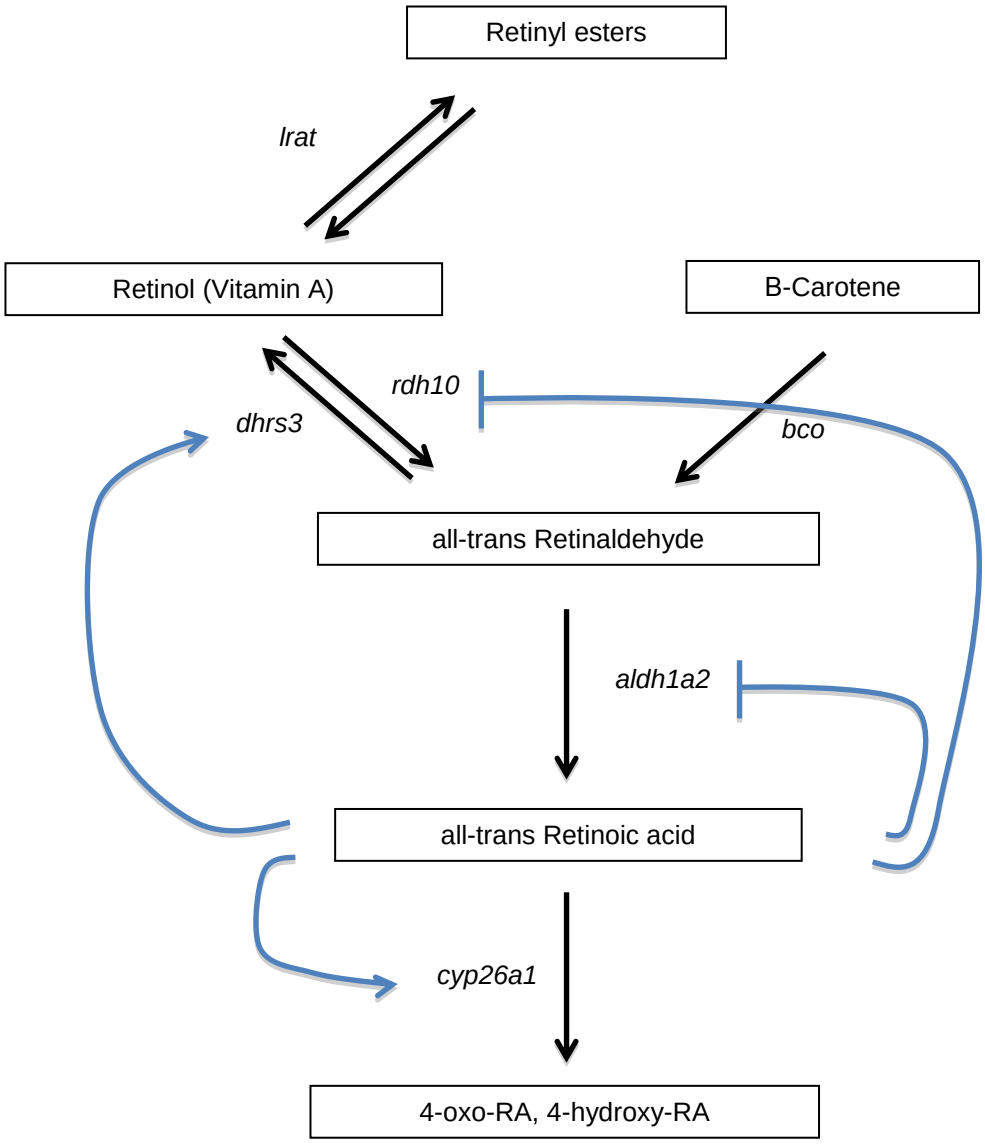


Figure 1.12 Retinoid pathways for the synthesis and oxidation of retinoic acid. Retinoic acid comes from either vitamin A or beta-carotene that is converted to retinal via retinol dehydrogenases. Retinoic acid is produced by the action of one of several retinal dehydrogenases, the major early embryonic enzyme being retinaldehyde dehydrogenase 2. Blue arrows indicate levels of feedback control.

1.3.2 Aryl hydrocarbon receptor (AhR)

The aryl hydrocarbon receptor, also known as a dioxin receptor (Figure 1.13), is a ligand-activated member of the basic helix–loop helix (bHLH) Per-ARNT-Sim (PAS) superfamily of nuclear receptors (Billiard et al., 2006). AhR is known to recognize a

range of chemical structures, including halogenated aromatic hydrocarbons, such as 2,3,7,8-tetra- chlorodibenzo-p-dioxin (TCDD), and polycyclic aromatic hydrocarbons. AhR binds to DNA as a heteromeric complex with the ARNT cofactor (Schmidt and Bradfield, 1996). Both the bHLH and PAS domains are required for DNA-binding, and thus presumably for dimerization with the Ah receptor nuclear translocator (Arnt).

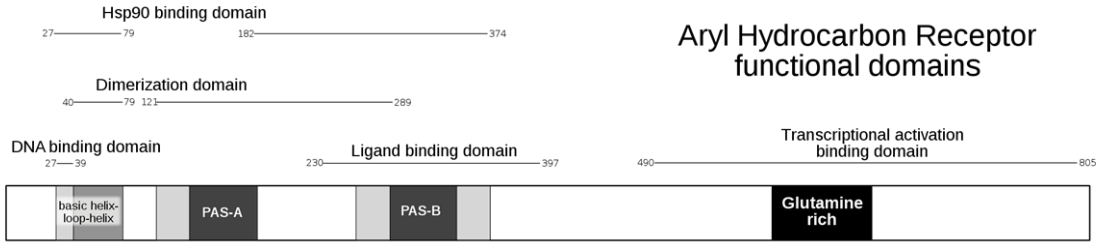


Figure 1.13 Structure of Aryl Hydrocarbon Receptor.

The AhR and Arnt proteins have a single transactive domain (TAD) in their C-terminals, comprising amino acids 521-640 in the AhR and amino acids 582-774 in Arnt (Schmidt and Bradfield, 1996).

Ligand-free AhR is found almost exclusively in the cytoplasm of the cell, and binding to the ligand causes the migration of the complex into the nucleus. During the process, it dimerizes with the ARNT and binds to specific DNA sequences called DREs or dioxin-responsive elements (Billiard et al., 2006). Activated AhR regulates expression of a number of genes of the AhR battery, including the phase I enzymes *cyp1a*, *cyp1a2*, and *cyp1b1*. Two divergent AhRs, *ahr1* and *ahr2* have been identified and characterized in zebrafish (Evans et al., 2005). Cytochrome P450 1A (*cyp1a*) induction is the most sensitive known response to AhR activation and may contribute to dioxin toxicity.

Inherent to the scope of this work, the next section briefly describes the main chemical compounds used in this work.

1.4 Natural and anthropogenic effectors

1.4.1 Natural effectors of RAR and RXR receptors

The family of retinoids comprises an enormous number of compounds related to vitamin A. RXR can be activated by 9-cis Retinoic Acid (9cRA), an isomer of all-trans Retinoic Acid (atRA), (Heyman et al., 1992). The occurrence of this molecule *in vivo* has been questioned, presumably, it is generated from atRA trought specific

isomerization, lends support for its relevance in the whole organism (Romert et al., 1998). However, discussions if 9cRA is a natural ligand for RXR persist.

Many of the retinoids (Figure 1.14) compounds are naturally occurring substances generated during the biological functioning of retinoids: the conversion of dietary sources of retinoids (β-carotenes, retinyl esters) to those that can be taken up by the absorptive epithelium of the gut (retinol); the conversion of absorbed forms to stored forms in the liver (retinyl esters); the conversion of stored forms to active forms as mediators of vision (retinals), skin differentiation (retinoic acids), and general cell differentiation and proliferation (retinoic acids). In addition, there are different synthetic ligands, like the retinobenzoic acids, developed as more potent and less teratogenic retinoids for pharmaceutical use. The following two chapters will be related with atRA and 9cRA, zebrafish transcriptomic responses in an embryological context.

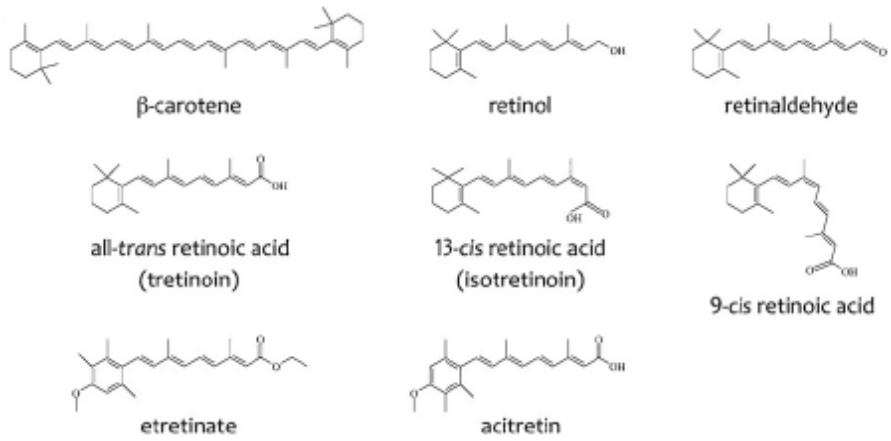


Figure 1.14 Structures of retinoic acid (RA) isomers.

atRA, a vitamin A metabolite, has proven to be necessary for the proper development of the vertebrate embryo. Embryos deficient in vitamin A (vitamin A deficiency syndrome or VAD) fail to complete development and demonstrate patterning defects in the CNS, circulatory system, hematopoietic system, limbs, and trunk reviewed by, (Maden, 2000; Ross et al., 2000; Zile, 2001). In contrast, when RA is available to embryos in the wrong places or at the wrong times it is a potent teratogen (Shenefelt, 1972). According to (Costaridis et al., 1996), atRA is present and its level does not change significantly during zebrafish embryogenesis, they fail the detection of other retinoids like 9cRA and 4-oxo-RA. In any case, and given the potential for developmental defects when RA is either absent or in excess, a mechanism must be in place to create the appropriate pattern of RA activity in the vertebrate embryo.

1.4.2 The nature of anthropogenic effectors

1.4.2.1 Nanoparticles

Nanomaterials are categorized as materials whose size ranges from 1-100nm (Figure 1.15). Nanoscience and nanotechonology deal with materials science and its application at, or around, the nanometer scale. Within the diverse definitions provided for nanotechnology, the one that was made by the National Nanotechnology Initiative (NNI; http://www.nano.gov), a U.S. Federal research, reads "as the study and application of extremely small things and can be used across all the other science fields, such as chemistry, biology, physics, materials science, and engineering, like a collection of multidisciplinary technology with broad ranges of applications across many fields".

Many chemical processes have been using nanomaterials for many decades, such as the production of polymers, or large molecules made up of tiny nanoscale subunits. However, it is only in recent years that sophisticated tools have been developed to investigate and manipulate matter at the nanoscale levels. In addition, the development of proper analytical and imaging technologies allows better understanding of the behaviour and properties of the matter at the nanoscale.

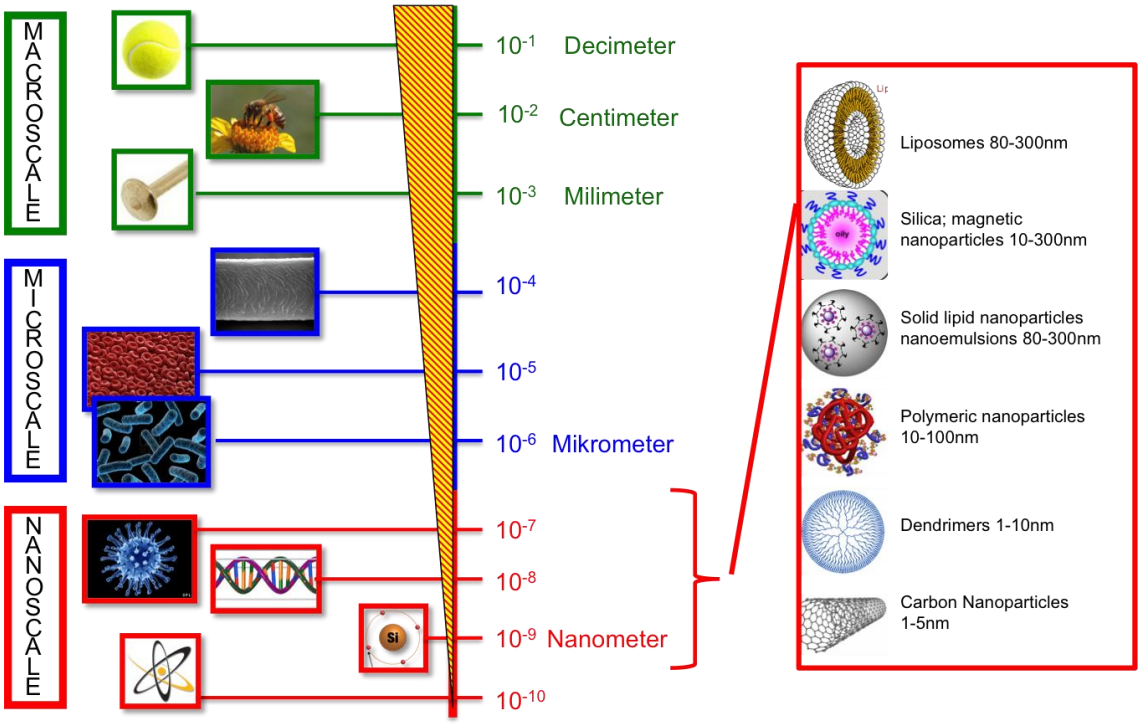


Figure 1.15 Schematic comparison between the diverse substances and materials and their corresponding size.

Nanomaterials are divided into different classes, depending on their natural, incidental or engineered origin. Within the engineered nanomaterials, this category includes metal oxides, quantum dots and carbon nano particles (Figure 1.15). Engineered nanomaterials will create new opportunities for novel applications, and also will enhance the performance of the traditional products, bringing us some innovation.

In this work a chapter is focused in the investigation on a specific group of engineered nanomaterials, the Polyamidoamine PAMAM dendrimers.

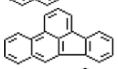
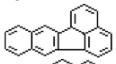
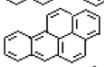
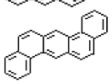
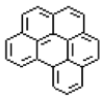
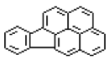
1.4.2.2 PAMAM Dendrimers

Traditionally polymer technologies were focused mainly on linear polymers. Currently highly branched polymers have been found whose properties of branched macromolecules are quite different from conventional polymers. The unique structural features, of these dendritic and hyperbranched macromolecules, rely on the number of chains whose ends combined with a high degree of branching which leads to a variety of new physical properties when compared with traditional linear polymers (Nanjwade et al., 2009). Dendrimers have well-defined nanoscale architecture and potential novel applications in the biomedical field (Svenson and Tomalia, 2012). Polyamidoamine (PAMAM) dendrimers contain a 2-carbon ethylenediamine core and primary amino groups on the surface. The systematically variable structural architecture and the large internal free volume make these dendrimers an attractive option for drug delivery and other biomedical applications, such as the cancer therapy (Rosen and Abribat, 2005; Tomalia, 2006; Tomalia et al., 2007). It is possible to passively target PAMAM dendrimers to a tumour because of the increased permeability of tumour vasculature to macromolecules and of its limited lymphatic drainage. Amino terminated PAMAM dendrimers result in enhanced antiovalbumin immunoglobulin-G and immonuglobulin-M levels and have also been used as adjuvants in vaccine delivery systems. PAMAM dendrimers have also been proposed for intracellular drug, protein and vaccine delivery systems. Because of the potential widespread use of these systems, a complete evaluation of their toxicity to human is required. Furthermore, although they are not in widespread use yet, future uses may result in significant environmental and/or accidental exposures, notably in fresh water via industrial effluent and domestic waste. This results in the requirement of a comprehensive ecotoxicological study in a fresh water ecosystem model. For the purpose of this work, PAMAM dendrimers generation 3 and 4 will be assessed for toxicogenomics response, which may serve to develop a fundamental understanding of their interaction and as guidelines for the future prediction of responses into the aquatic environment.

1.4.2.3 PAHs and dioxin like compounds

Polycyclic Aromatic Hydrocarbons (PAHs) are a variable group of compounds, made up of two to six fused aromatic rings composed only by carbon and hydrogen. The number and disposition of these atoms determine their physicochemical properties. PAHs (Table 1.5) are ubiquitous environmental contaminants of both natural and anthropogenic sources, derived from the incomplete combustion of carbon. The main sources of pollution come from the processing and combustion products of fossil fuels (coal and oil), other industrial processes, burning wood, and tobacco smoke. Although they are not considering proper persistent pollutants, their environmental levels are increasing, rather than decreasing, at the global scale, largely due to the burning of fossil fuels (Van Metre and Mahler, 2005). The lighter PAH (2-3 rings), which are generally not carcinogenic, are mostly found in the gas phase while the heavier ones are mainly associated with airborne particles. Heavier PAH (with more than three rings) are rapidly attached to existing particles, usually soot particles, by adsorption or combustion upon cooling of fuel gas (Kamens et al., 1995). While there is an extensive literature describing the effects of Polycyclic Aromatic Hydrocarbons (PAHs) on adult or juvenile animals, few studies have addressed the effects of PAHs on embryonic and early larval development in fish. These early life history stages may be particularly susceptible to PAHs exposure, especially for species that spawn or rear near human settlements.

Table 1.5 Main physical-chemical characteristics of 16 Polycyclic Aromatic Hidrocarbons (PAH) defined as "priority toxic pollutants" by the US Environmental Protection Agency. [a]Octanol-water partition coefficients, relative molecular mass, genotoxicity, carcinogenicity: data from Env. Health Criteria 202 (IPCS, 1998). [b](Sverdrup et al., 2003) (+) positive; -, negative; ?, questionable; parentheses, result from small database.

PAHs	Molecular formula	N. rings	Log Kow[a]	Relative molecular mass[a]	Genotoxicity[a]	Carcinogenicity[a]	Aqueous solubility (mg/L)
Naphthalene		2	3.4	128.2	-	(?)	31
Acenaphthylene		3	4.1	152.2	(?)	No studies	16
Acenaphtene		3	3.9	154.2	(?)	(?)	3.8
Fluorene		3	4.2	166.2	-	-	1.9
Phenanthrene		3	4.6	178.2	(?)	(?)	1.1
Anthracene		3	4.5	178.2	-	-	0.045
Fluoranthene		4	5.2	202.3	+	(+)	0.26
Pyrene		4	5.2	202.3	(?)	(?)	0.13
Benzo(a)anthracene		4	5.6	228.3	+	+	0.011
Chrysene		4	5.9	228.3	+	+	0.006
Benzo(b)fluoranthene		5	6.1	252.3	+	+	0.0015
Benzo(k)fluoranthene		5	6.8	252.3	+	+	0.0008
Benzo(a)pyrene		5	6.5	252.3	+	+	0.0038
Dibenzo(ah)anthracene		5	6.5	278.4	+	+	0.0006
Benzo(ghi)perylene		6	7.1	276.3	+	-	0.0003
Indeno(1.2.3-cd)pyrene		6	6.6	276.3	+	+	0.0002

AhR mediates many of the biological and toxic effects of 2,3,7,8-tetrachlorodibenzo- p-dioxin (TCDD), PAHs, and others. In addition to TCDD, many types of planar ligands with the size below 14Å×12Å×5Å are considered to bind in to the ligand binding pocket of AhR. Most bioassays for the determination of dioxin toxic equivalents are based on the assumption that all dioxin related compounds act through the AhR signal transduction pathway. The current understanding of the system is that a dioxin-like compound: (1) binds to the AhR, (2) the complex is translocated to the nucleus of the cell, (3) where it induces the transcription of a number of genes, and subsequently, (4) the production of proteins including cytochrome P-450 (CYP) 1A, an enzyme involved in oxidation, reduction, and hydroxylation reactions, also called mixed function oxidases (Behnisch et al., 2001).

In aquatic systems, atmospheric deposition and urban runoff pose significant threats to early life stages of fishes, which are sensitive to toxicity of PAHs (Brinkworth et al., 2003; Incardona et al., 2004; Wassenberg and Di Giulio, 2004; Incardona et al., 2006). Resulting deformities include pericardial edema and craniofacial malformations. Other environmental contaminants, such as chlorinated dioxins and polychlorinated biphenyls (PCBs), cause similar toxicity in developing fish, and this toxicity has been shown to be mediate by the aryl hydrocarbon receptor (Carney et al., 2004; Billiard et al., 2006).

The study of those compounds is of great importance due to the fact that some of these compounds are carcinogenic, mutagenic or teratogenic and are characterized by their ability joining the AhR and activate genes related to the metabolism of xenobiotics. Therefore, is known that some PAHs or their metabolites have the ability to form adducts with DNA resulting in gene mutations and can lead to tumours and cancer (Dipple, 1995; Denison and Heath-Pagliuso, 1998).

1.5 Objectives and thesis structure

Although several studies have focus on the developmental and reproductive toxicity of chemical compounds in zebrafish embryos, the main goal of this thesis is to use a set of toxicogenomic tools in order to study the differential gene expression of zebrafish eleutheroembryos when exposed to different types of chemical compounds. Some of them act through the binding to Nuclear Hormone Receptors and the main challenge is to investigate their mode of action. This thesis is divided into 6 different chapters that will be described here.

1st chapter- In this chapter an overview of Ecotoxicogenomic field is presented, focusing their application to Environmental Risk Assessment and also a description of alternative approaches for non-mammals tests, describing how fish embryos fit into the harmonized approach in terms of the three “Rs” of animal testing. Essentially, this first chapter will focus on the use of zebrafish embryos in ecotoxicological testing, how fish embryos relate to alternative approaches and the potential research needs associated with fish embryos.

2nd chapter- The characterization of the expression of two different nuclear receptors is performed, by the assessment of the expression of the mRNA of the different isoforms of the RAR/RXR receptors during the early embryo and larvae stage of zebrafish development. In addition, the expression of several target genes associated

within the function and action of the nuclear receptors will be evaluated - *Retinoic acid receptors' expression and function during zebrafish early development*.

3rd chapter- In this section, a comparison between two isoforms of retinoic acid will be made in terms of gene expression. Zebrafish fish embryos will be exposed to 9-cis Retinoic Acid and to all-trans Retinoic Acid during different periods of time. Toxicogenomics tools will be applied in order to conduct the molecular interpretation of the differential action of those two isoforms of RA, during the early development of zebrafish - *All-trans Retinoic acid and 9-cis Retinoic Acid transcriptomic differences in Danio rerio early stages of development*.

4th chapter- Assessment of the transcriptomic changes of zebrafish embryo when exposed to dioxin-like compounds. The dioxin like compounds studied were: Benzo(a)pyrene, Benzo(k)Fluoranthene and b-Naphtoflane - *Transcriptomic effects of dioxin-like compounds in Danio rerio embryos*.

5th chapter- In this section, molecular techniques will be applied to novel compounds, the PAMAM dendrimers. Their recently application as drugs delivery systems could be a potential source of nocive effects, not only for humans but also for aquatic organisms, as the aquatic environment is the ultimate sink for toxicants - *Transcriptomic response of zebrafish embryos to PAMAM dendrimers*.

6th chapter- Final remarks, thesis overview, concerns and future research needs. General conclusions are drawn and future research needs are formulated.

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2 - Retinoic acid receptors' expression and function during zebrafish early development

Retinoic acid receptors' expression and function during zebrafish early development

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2.1 Abstract

Retinoic acid (RA) regulates many developmental processes through its binding to two types of nuclear receptors, the retinoic acid receptor (RAR), and the retinoid-X receptor (RXR), which preferentially binds to the 9-cis isomer. Here we analyzed the RAR/RXR regulatory system during the first five days of development of zebrafish. Analysis of the relative transcript abundances for the four RAR and the six RXR zebrafish genes present in the zebrafish genome indicates a transition from maternal to embryonic transcripts during the first 24 hours post fertilization. These changes did not affect the response to exogenous RA of the known RARresponsive genes *cyp26a1*, *dhrs3a*, *hoxb1b*, *hoxb5a*, and *hoxb5b*. At the transcriptomic level, RA treatment elicited a negative feedback of genes involved in the endogenous RA synthesis and reduced levels of transcripts related to organ and anatomic development. These effects occurred at concentrations at which no morphological changes were observed. Data analysis suggests that exposure to exogenous RA results in an advance of the developing program, activating genes that should remain silent until later developmental stages and inhibiting expression of development-related genes. We conclude that zebrafish embryos are particularly sensitive to potential disruptors of the RAR/RXR regulatory system.

Keywords: endocrine disruption, microarrays, retinoids, hormone receptors, embryo, Danio rerio.

2.2 Introduction

The development of many chordate-and vertebrate-specific characters is under control of a retinoic acid (RA), a member of the retinoid family of lipids. RA signaling participates in the formation of the body axes and in the development of a number of organ systems, including retina, brain, heart, the urogenital system, and lungs [1, 2]. Excess or deficiency in RA during early development is teratogenic, mainly due to anteroposterior patterning defects linked to the anomalous expression of homeobox genes [3-8]. There are at least two active forms of RA, all-trans RA (atRA) and 9-cis RA, both synthesized from Vitamin A and other dietary sources. Retinoic acid acts by binding to two types of nuclear receptors: The retinoic acid receptor (RAR), which recognizes both forms, and the retinoid-X receptor (RXR), which binds preferentially 9-cis RA [9, 10]. RAR/RXR heterodimers bind to specific DNA sequences (retinoic acid response elements or RAREs) in target gene promoters and modulate their transcription rates [11, 12]. RXR is known to participate in many independent regulatory pathways, involving other nuclear receptors, like the thyroid receptors, the Vitamin D receptor, the Peroxisome Proliferator-Activated Receptor (PPAR) family, and others [5, 9, 10]. In general, vertebrate genomes encode three RAR and three RXR genes, typically referred to as α, β, and γ subtypes. They derived from ancestral single RAR and RXR genes by two successive whole genome duplications of the vertebrate genome [13-17]. The teleostei genome suffered an extra duplication [13], which resulted in the presence of additional RAR and RXR paralogs. As zebrafish apparently lost the RARβ subtype during evolution, its genome encompasses four RAR genes (*raraa, rarab, rarga,* and *rargb*) and six RXR genes (*rxraa, rxrab, rxrba, rxrbb, rxrga,* and *rxrgb*) [17, 18]. The role of these different subtypes and isoforms during zebrafish development is unclear, but it is known that their relative abundance (either as protein or transcript) changes significantly during development and from one tissue to another [15-17, 19, 20]. Here we provide a survey on the changes on mRNA for all RAR and RXR subtypes and isoforms in the developing zebrafish embryo from 0.5 dpf to 5 dpf, combined with an analysis of the transcriptional response to RA, to investigate the putative functional meaning of these changes in receptor variant composition. Many genes are regulated by RA, including several regulators of vertebrate development, like homeobox genes and co-factors and different nuclear receptors [16, 21-27]. RA activates the transcription of the enzymes related to its own degradation to limit its effective concentration within the cells, including different types of the cytochrome P450 *cyp26* or the short-chain dehydrogenase/reductase *dhrs3a* [1, 28, 29]. This metabolic feedback is essential

for zebrafish development, with particular importance in the brain [22, 30]. We analyze here the transcriptomic changes induced by ectopic exposure to RA of zebrafish eleutheroembryos to elucidate the role of RA in development and the possible consequences of exposure to RA-mimicking or counteracting substances during development for exposed organisms, including humans.

2.3 Materials and methods

2.3.1 Zebrafish embryos and eleutheroembryo maintenance

Zebrafish (*Danio rerio*) embryos (from fertilization to hatching) and eleutheroembryos (from hatching to the self-feeding larval stage) were obtained by natural mating and raised at 28,5ºC [31] with a 12L:12D photoperiod in 90ug/ml of Instant Ocean (Aquarium Systems, Sarrebourg, France) and 0.58mM $CaSO_4.2H_2O$, dissolved in reverse osmosis purified water ("embryo water", http://zfin.org/). Animal stages were recorded as days or hours post-fertilizarion (dpf or hpf, respectively).

2.3.2 Chemicals and embryo and eleutheroembyo treatment

DMSO and all-*trans*-Retinoic acid (atRA) were purchased from Sigma Aldrich (St. Louis, MO). All dilutions are reported as nominal concentrations. Stock solutions were prepared in DMSO on the day of the experiment. Experimental solutions with the same final concentration of DMSO (0.1%) were directly prepared in embryo water. Zebrafish embryos were exposed to 1 μM atRA in three windows of exposure (6.5 to 11 hpf, 48 to 72 hpf and 96 to 120 hpf), designed to cover different developmental stages: segmentation stage (0.5 dpf), pharyngula stage (1 and 2 dpf), and post-hatching stage (3 to 5 dpf, [32]). Untreated embryos were collected at 0.5, 1, 2, 3, 4, 5 dpf to calculate the relative abundances of the RAR and RXR transcripts. Parallel analyses determined 100 nM as the non-adverse effect concentration for morphological alterations in 2-5 dpf eleutherioembryos (Supplementary Figure 2.1, [33, 34]). This concentration was therefore used for microarrays experiments.

2.3.3 RNA extraction and quantitation by qRT-PCR

Total RNA was isolated from 20 embryos or eleutheroembryos per replicate (at least three biological replicates per datapoint) using the Trizol reagent protocol

(Invitrogen Life Technologies, Carls), and purified using standard methods following the manufacturer's protocol (RNeasy Kit; Qiagen). RNA concentration was measured by spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality checked by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was treated with DNAseI (Ambion, Austin, TX) to remove genomic DNA contamination, and retro-transcribed to cDNA using First Strand cDNA Synthesis Kit (F. Hoffmann-La Roche, Basel Switzerland). Appropriate primers for 15 genes, -*cyp26a1*, *dhrs3a*, *hoxb1b*, *hoxb5a*, *hoxb5b*, *raraa*, *rarab*, *rarga*, *rargb*, *rxraa*, *rxrab*, *rxrba*, *rxrbb*, *rxrga* and *rxrgb*, (zfin.org, [35])- were designed using Primer Express 2.0 software (Applied Biosystems) and the Primer-Blast server (http://www.ncbi.nlm.nih.gov/tools/primerblast/ index.cgi?LINK_LOC=BlastHome). Primer sequences and amplicon lengths are shown in the Supplementary Table 2.1). House-keeping genes *ef1a* and *ppia2* were selected as reference genes [36, 37]. PCR products (amplicons) were sequenced in a 3730 DNA Analyzer (Applied Biosystems), and compared to the corresponding reference sequences at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Supplementary Table 2.2 shows the actual sequences obtained from the amplification and their match to the corresponding sequences deposited in GeneBank. Aliquots of 50 ng of total RNA 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). Amplification efficiencies were calculated as better than 90% for all tested genes as described [38]. Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated by triplicates). To minimize errors on RNA quantification among different samples, Cp values for stress-related target genes (Cp_{tg}) were normalized to the average Cp values for *ppia2*, used as reference gene, following Eq 1.

$$\Delta Cp_{tg} = Cp_{ppia2} - Cp_{tg} \quad \text{Eq1.}$$

Changes in mRNA abundance in samples from different treatments were calculated by the ΔΔ Cp method, [39], using corrected Cp values from treated and non-treated samples (eq 2.)

$$\Delta\Delta Cp_{tg} = \Delta Cp_{tg\_untreated} - \Delta Cp_{tg\_treated} \quad \text{Eq2.}$$

To facilitate the comprehension of the data, mRNA abundance values are represented as mRNA copies of target gene per 1000 copies of the reference gene *ppia2* mRNA (‰ of reference gene, $1000x2^{(\Delta Cptg)}$). Fold-change ratios were derived from those values.

2.3.4 RNA extraction and microarray analysis

Zebrafish 96 hpf eleutheroembryos were exposed for 24h to 100 nM atRA in100- ml glass beakers, with 50 eleutheroembryos in each. Total RNA was isolated, purified and analyzed as described above. Only samples with RIN (RNA integrity) values ranging between 9.5 and 10 were further processed. Microarray studies were performed using the Agilent *D. rerio* (Zebrafish) Oligo Microarray v3 platform, using two-color labelling. The study included three biological replicates (independent eleutheroembryo pools either untreated or treated with 100nM atRA), labeled and hybridized at the same time (results are deposited at GEO, reference: GSM1015046, GSM1015047 and GSM1015048). No statistical differences were observed between the biological replicates. Microarray data were analyzed using Robin [40] and MultiExperiment viewer MeV4 [41] software. Gene ontology analyses were performed using the AmiGO! webpage (http://www.geneontology.org, [42]); metabolic pathway information was obtained from KEGG (http://www.genome.jp/kegg/kegg2.html, [43]). Data on expression of zebrafish genes during normal development was obtained from http://www.ebi.ac.uk/microarrays/aer/result?queryFor=Experiment&eAccession =E-TABM-33.

2.3.5 Statistical analyses

Statistics tests were performed using the SPSS 19 (SPSS Inc., Chicago, Ill) package. All statistical calculations were performed using ΔΔCp values, as this parameter followed normal distributions (Levene's test). Differences among control and treated groups were analyzed by Student’s t-test (2 groups) or ANOVA plus Tukey's tests (more than 2 groups).

2.4 Results

2.4.1 Analysis of mRNA levels of RXR and RAR genes during zebrafish early development.

Sequence analysis of amplification products demonstrated the specificity of the primers pairs used in this work. As shown in the supplementary Table 2.2, targeted sequences for all the four RAR and the six RXR genes present in the zebrafish genome were unequivocally amplified by their intended primer pairs. Quantitative analysis of mRNA levels of these ten genes during the first 5 dpf revealed a large decrease of overall receptor mRNA levels (50-fold on average) during the first 24 hpf and a relative stabilization from 1-2 dpf on (Figure 2.1). This decrease was less pronounced for RAR than for RXR transcripts, with the result that the fraction of RAR subtype mRNAs represents the 24% of the total at 11 hpf and about 40% at 120 hpf (not shown). The observed decrease was specific for RAR and RXR mRNAs, as it was not observed for the constitutive gene *ef1a* (not shown).

The relative proportion of the different receptor subtypes' mRNAs changed during development (Figure 2.1, columns). Transcripts from *raraa*, *rarab* and *rarga* were similarly represented (25%, 25% and 40%, respectively) at 11 hpf, whereas *rarga* predominated at later stages (60 to 70%). Already a minor component at 11 hpf, *rargb* became residual after 24 hpf (Figure 2.1A).

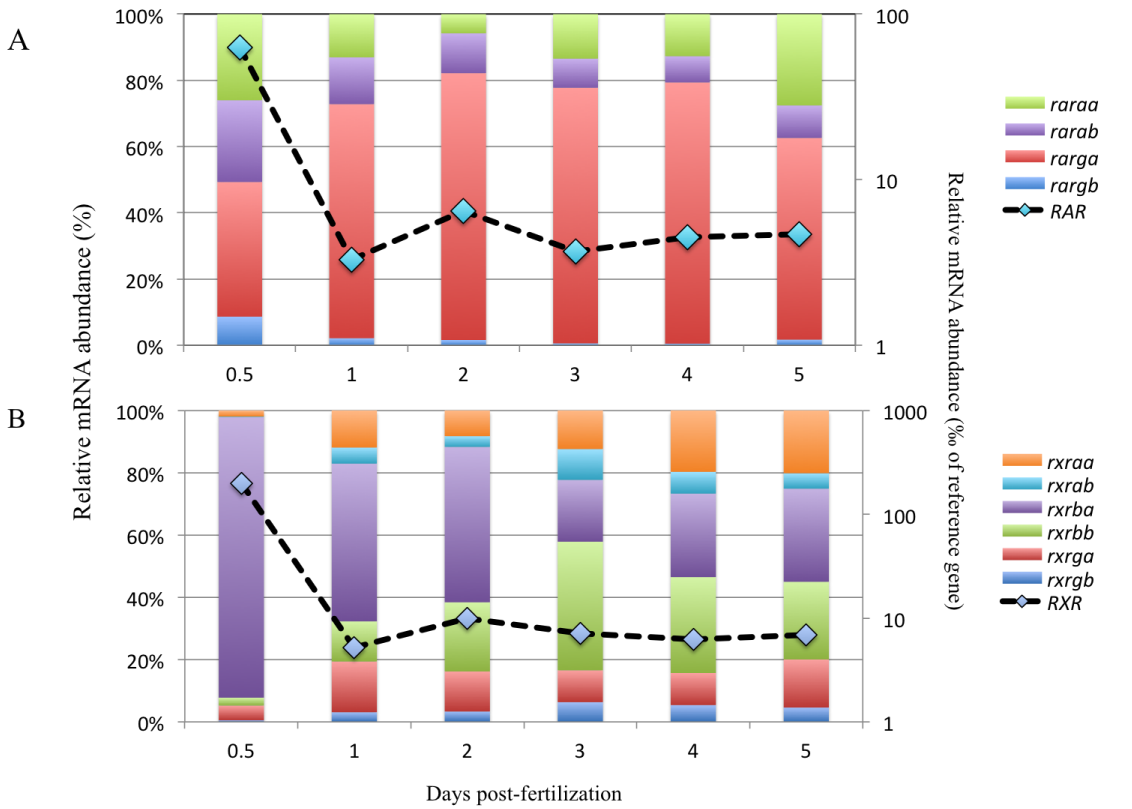

Figure 2.1. Changes in mRNA levels of RAR and RXR genes. Sectored columns indicate relative abundances of RAR (A) and RXR transcripts (B) during the first 5 days of embryo development (left Y-axis scale). Discontinuous lines represent aggregated results for all RAR (A) and RXR (B) transcripts, expressed as mRNA copies per 1000 copies of ppia2 mRNA (right Y-axis scale).

Analysis of mRNA abundance of RXR genes showed a progressive substitution of *rxrba* transcripts by the rest of genes during zebrafish development (Figure 2.1B). *rxrba* accounted for 90% of the total RXR mRNA present in the 11 hpf embryo, but only 30% of the total RXR at 3-5 dpf (Figure 2.1B). At 4-5 dpf, mRNA levels of *rxraa*, *rxrba*, *rxrbb* and *rxrga* were similar, and *rxrab* and *rxrgb* transcripts appeared as minor contributors to the overall RXR transcript composition (Figure 2.1B, columns). Whereas RAR mRNA subtype composition appeared to stabilize at 24 hpf, coinciding with the overall reduction on total RAR/RXR mRNA, RXR mRNA subtype composition showed a continuous evolution until at least 72-96 hpf, already after the hatching period (Figure 2.1B).

2.4.2 Analysis of transcriptional response of RA-responsive genes at different stages of embryo development

Exposure to a saturating (1 μM) concentration of atRA resulted in a significant increment on mRNA levels for all five RAR-responsive genes analyzed (*cyp26a1*, *dhrs3a*, *hoxb1b*, *hoxb5a*, and *hoxb5b* [29, 44, 45]), both in the early embryo (6.5 to 11

hpf exposure period), during the hatching period (48 to 72 hpf), and in the late eleutheroembryo stage (96 to 120 hpf, Table 2.1). In the absence of external atRA, *cyp26a1* and *dhrs3a* mRNAs were 5 to 10 times more abundant in early embryo than in the eleutheroembryo or hatching embryos. These levels increased notably (more than ten fold in all cases) upon addition of atRA. Maximal activation responses (140-150 fold for *cyp26a1* and around 35 fold for *dhrs3a*) were observed in hatchlings and eleutheroembryos, probably due to the higher basal mRNA levels shown for these transcripts at earlier stages (Table 2.1). The homeotic *hoxb1b* gene showed a similar pattern, with a 30-fold decrease on its basal mRNA levels between early embryos and late eleutheroembryos, although in this case the activation response was essentially the same in all three stages (3-4 fold, Table 2.1). Finally, *hoxb5a* and *hoxb5b* genes showed only a moderate decrease in mRNA levels during the examined period (about two-fold), as well as only moderate changes in their activation response. These data demonstrate that RAR/RXR signaling pathway is active all through the zebrafish embryonic stages, independently of the regulation of their basal expression levels.

Table 2.1 mRNA abundance of RA-responsive genes in control and atRA treated zebrafish embryos at different early developmental stages

	Control[a)]	1 µM *at*RA	Fold induction	*p*-value
6.5-11h				
cyp26a1	76.5 ± 43.0	2534.4 ± 1110.6	33.1 ± 14.5	2.9E-07
dhrs3a	40.0 ± 28.2	588.5 ± 489.9	14.7 ± 12.2	1.3E-04
hoxb1b	311.0 ± 201.0	930.9 ± 593.3	3.0 ± 1.9	0.014
hoxb5a	59.0 ± 38.3	410.8 ± 279.3	7.0 ± 4.7	4.9E-04
hoxb5b	52.9 ± 19.5	310.6 ± 129.4	5.9 ± 2.4	1.5E-05
48-72h				
cyp26a1	6.8 ± 1.9	1021.6 ± 258.5	149.3 ± 37.8	1.5E-11
dhrs3a	8.3 ± 2.5	304.1 ± 104.5	36.4 ± 12.5	3.0E-09
hoxb1b	24.7 ± 7.0	91.9 ± 38.4	3.7 ± 1.6	8.1E-05
hoxb5a	45.2 ± 13.3	145.8 ± 67.9	3.2 ± 1.5	3.9E-04
hoxb5b	33.3 ± 11.1	187.8 ± 77.3	5.6 ± 2.3	1.2E-05
96-120h				
cyp26a1	5.4 ± 2.4	757.9 ± 91.6	140.6 ± 17.0	2.1E-06
dhrs3a	8.4 ± 7.0	296.3 ± 60.8	35.1 ± 7.2	3.2E-06
hoxb1b	9.1 ± 5.8	31.2 ± 9.0	3.4 ± 1.0	0.014
hoxb5a	37.4 ± 25.4	80.1 ± 17.0	2.1 ± 0.5	0.026
hoxb5b	20.5 ± 8.5	121.0 ± 35.0	5.9 ± 1.7	0.001

a) mRNA copies (‰ of reference gene)

2.4.3 Transcriptomic response of zebrafish embryos to atRA

Transcriptomic analysis was performed in 5 dpf eleutheroembryos treated for 24h with 100 nM atRA, equivalent to an EC_{30} for *cyp26a1* and *dhrs3a* induction (not shown). At the selected atRA concentration no phenotypic effects were detected even

after a 3-day exposure (2 to 5 dpf, Supplementary Figure 2.1). In contrast, exposure to 1 μM atRA for 24h (4 to 5 dpf) induced pericardial edema, craniofacial malformation and impairment of the yolk consumption (Supplementary Figure 2.1), whereas a 3-day exposure (dpf 2 to 5) induced a similar, but more severe, phenotype, including not only pericardial but also yolk sac edema (Supplementary Figure 2.1).

Exposure to 100 nM atRA exposure induced changes in less than 15% of the probes (5596 probes out of 43803) present in the zebrafish array, corresponding to 4495 unique sequences (Table 2.2). Comparison of fold induction values for RAresponsive genes showed a good correlation between microarray and qRT-PCR data (Figure 2.2). Functional analysis of the 2200 transcripts that increased their relative abundance upon atRA exposure showed a moderate enrichment of protease-regulating genes ("negative regulation of molecular function", with serpins as the most remarkable group), and of heme-binding proteins, including several monooxigenases of the P450 family (Table 2.2). On the contrary, transcripts whose concentrations decreased upon atRA treatment (2295 unique sequences in total) showed a significant enrichment in development-related genes (Figure 2.3). Table 2.2 shows atRA-reduced transcripts belonging to three subsets (child GO terms) of the developmental process function, affecting nervous, circulatory, and skeletal system development. Table 2.2 also shows the relatively high number of regulatory genes whose transcripts decreased upon atRA treatment ("DNA binding", including many hox and hox-related genes). This suggests that at least part of the observed effects were related to the disruption of different signalling pathways.

Table 2.2 Functional analysis of zebrafish genes whose transcripts appeared as over- or under- represented in atRA-exposed embryos (partial results)

Observed change	Probes	Unique sequences	GO Term		P-value	Genes
Overrepresented	2772	2200	GO:0044092 negative regulation of molecular function	P	2.89E-06	serpine3 setd6 serpina7 bcl2l1 serpina1l cst3 camk2n2 agt serpina1 mcl1a pkig serpind1 pik3ip1 serpinb1l1 mcl1b il17rd dusp1 serpina10a serpinc1 dusp5 serpinf1 pkib usp44
			GO:0020037 heme binding	F	2.97E-03	sdhda tbxas1 ptgis cyp2v1 ngb cyp3c1 cyp2p6 hbbe3 cyp51 cyp24a1 cyp1c1 cyp2ad3 cyp4v7 cyp2ad2 mpx cyp1a cyp26b1 cat hbbe2 cyp2x7 cyp2aa12 cyp26a1 cyp27a7 tdo2b cyp2aa2 cyp26c1 cyp2n13
Underrepresented	2824	2295	GO:0007399 nervous system development	P	2.72E-11	celsr2 cdh2 top2b sfrp2 szl ctnnb1 ednrb1a lepr nlk2 pitx2 tfap2a pard3 sncb kcnc3a smo msi1 gli2b id3 nf1b lamc1 vangl2 aldh1a2 robo1 agtpbp1 igf1rb slit2 shha sox9a fzd6 nrp2a dpf3 fgf17 jag1b plxna3 nadl1.1 fzd3a epha4a cxcl12b mycbp2 ntn1a nadl1.2 pcdh19 mapk3 fam57ba nfasca hdac1 mvp vegfaa nrp1a pafah1b1b slit1a synj1 neurog1 mib pitpnb sncgb notch3 leo1 efna5b gli2a pbx4 phox2bb rab8a bcor cox5ab dvl2 six1a atp2b1a ewsr1b ptn tgif1 spna2 appb dlc tcf7l2 ralgapa1 pafah1b1a col4a5 sox3 sfpq emx3 pou4f2 stmn2a fzd3b glra4a robo2 foxa3 dpysl2b fezf2 dpysl3 tnc notch1a ctnna2 nrp2b hnf1ba lamb1a ntn1b arnt2 sema3ab disc1 ache atg5 dicer1 dbx1b notch1b stat3 mkrn2 plp1a fmr1
			GO:0072359 circulatory system development	P	1.82E-08	cdh2 sfrp2 szl notch2 ctnnb1 mtp nrarpb pitx2 twsg1a pabpc1a bmpr2a smo wdr43 nf1b fbln1 robo1 bbs7 nxt2 igf1rb slit2 shha fzd6 csrp1a nrp2a ctsz dpf3 cav1 ppp1cab kras fzd3a cxcl12b slc8a4a thsd7a ntn1a kdrl elmo1 pak2a taz mef2cb vegfaa nipblb nrp1a ptpn11a nrxn3a mib fgfr1a nrarpa csnk1a1 hoxc9a nr2f2 leo1 bai3 meis1 cobl dvl2 lmo7b gipc1 fermt2 plxnb2a mbnl2 tp53 plekhh1 ctr9 npnt apln pkd2 fzd3b unc5b ptenb nrxn3b rtf1 rbm24a nrp2b fgfr2 arnt2 sema3ab gsk3b tbx2a skia notch1b ptena
			GO:0001501 skeletal system development	P	2.05E-06	cdh2 notch2 ctnnb1 pitx2 sec24d tfap2a smo dnmt1 mbtps2 gfpt1 fras1 aldh1a2 rerea shha sox9a jag1b kras plxna3 and1 wwtr1 ucmab nrp1a kat6a nog1 and2 col27a1b leo1 col11a2 pbx4 bmp3 furina sec13 whsc1 disc1 runx2b skia sec23b fmr1
			GO:0003677 DNA binding	F	6.92E-04	top2b tfap2d taf3 upf2 pitx2 emx1 hmbox1b dr1 pparab tfdp1a hdgfrp2 ilf2 foxf1 ilf3b pbx3b rab11fip4a dnmt1 otud7b hoxc6b mybbp1a rabgef1 onecut1 nfya arid4a ncor1 hmgn2 rerea cwc22 arid1aa polr1a fbxo21 zbed4 prdm5 pparaa roraa mybl2 zbtb4 smarcc1a vdrb hmga1b fzr1 foxn2b h3f3b.1 elf2b ahr2 hmga1a pax5 irx3a nkx2.1a fosl2 mef2cb atf4b2 hells ssrp1b fam60al taf1 hoxa11b etv5a mll kat6a polb neurog1 iqgap2 pbx3a dbpb upf1 nfia nr5a1b lhx6 trim33 xpc fos hoxc9a brip1 nr2f2 ttna fosb pbx4 phox2bb meis1 kdm2ba mafba arid2 hoxc13a six1a cxxc1l xbp1 shox pola1 rybpa ctcf erfl3 tgif1 mcm5 sox21b tp53 tcf7l2 pbrm1l crsp7 atrx irf2 sox3 emx3 pou4f2 baz1a akap8l hic2 foxp4 foxa3 ssbp3b cbfb znf292a esrrd setdb1b sim1b cbx2 smarcc1b nfe2l3 rtf1 zfand5b fezf2 hoxb13a dnajc1 mxi1 srf mta2 phf21ab her13 hnf1ba mll4b arnt2 hoxc8a lcor cdx1b ebf3 dbpa uhrf1 runx2b hlx1 tbx2a dbx1b ybx1 hmga2

A metabolic pathway interpretation of the variations observed in the microarray analysis show a coordinated effect on the retinoic acid metabolic pathway (Figure 2.3). The data shows a general increase of transcripts encompassing enzymes that inactivate atRA, either by reduction of its direct precursor (all-*trans*-Retinal) to vitamin A (*adh5*, *dhrs3b*) or by oxidizing it to inactive compounds (*cyp26a1*, *cyp26b1*, *cyp26c1*, *cyp1a*). At the same time, transcripts for *aldh1a2* show a significant reduction in their concentration, therefore limiting the production of atRA from its precursor (Figure 2.3). We interpret these data as reflecting the known negative feedback mechanism induced by the presence of atRA [29].

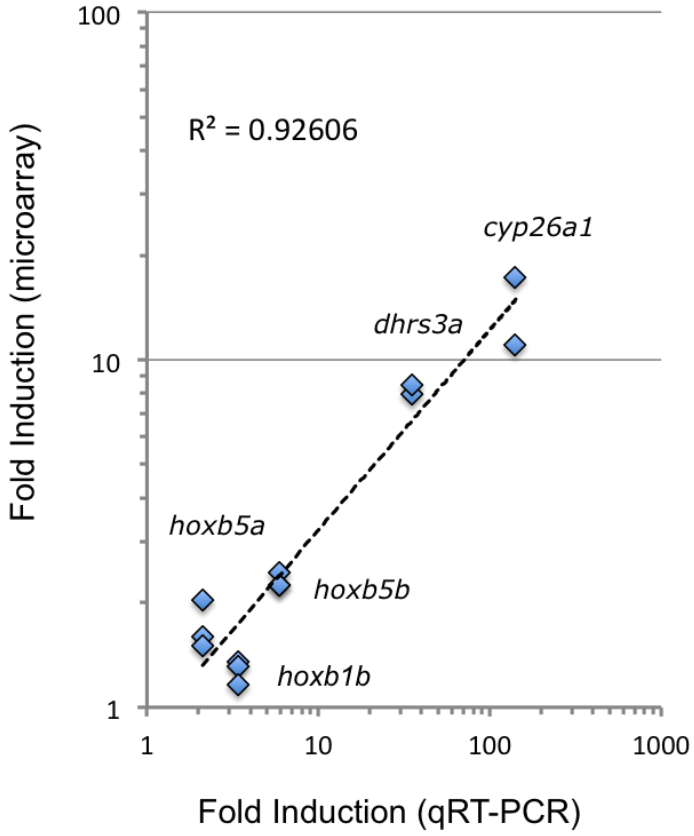

Figure 2.2 Correlation between microarray and qRT-PCR results. Fold induction values of cyp26a1, dhrs3a, hoxb1b, hoxb5a, and hoxb5b transcripts upon atRA exposure are plot as logarithmic values. The X-axis, corresponds to qRT-PCR data from Table 2.1 (96–120 h), whereas the Y axis represents microarray data. Note that each gene is represented by more than a single probe in the microarray. Gene identification, regression line and the corresponding R^2 coefficient are also represented.

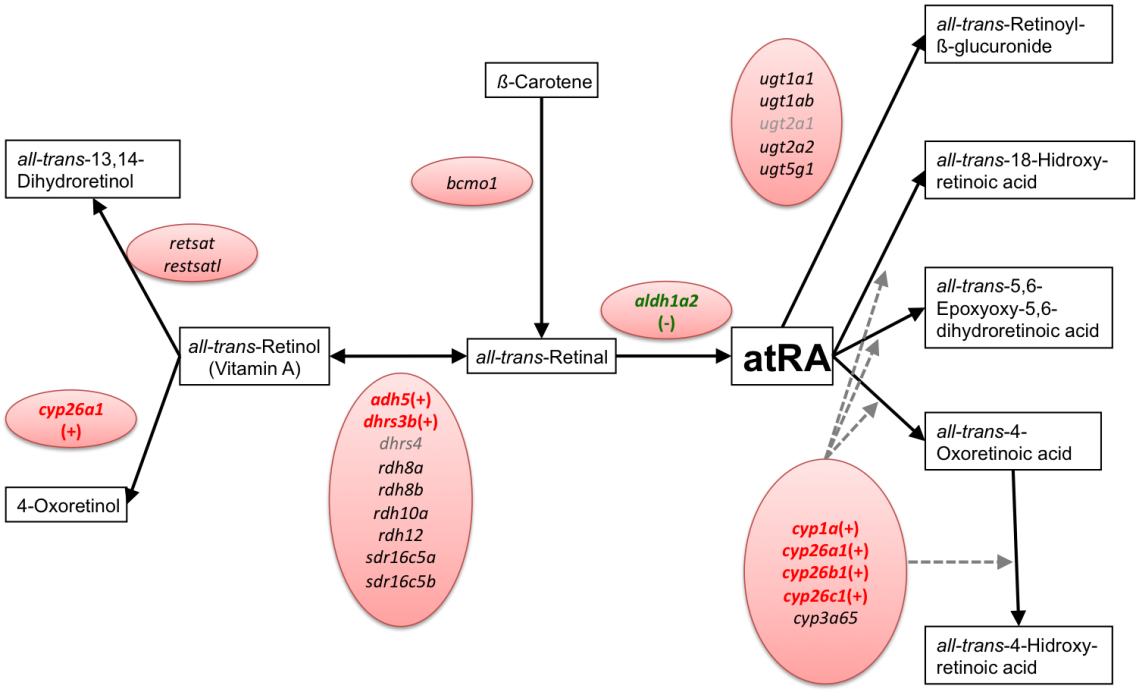

Figure 2.3 Graphic representation of the metabolic pathways for atRA synthesis and degradation. Relevant metabolites are indicated in squares, implicated enzymes are indicated by their respective gene names in colored ovals. Genes whose transcripts were significantly enhanced (red) or reduced (green) upon atRA treatment are indicated in bold and with "+" and "–" signs, respectively. Information about gene function, pathways and relevant metabolites are presented as indicated by KEGG consortium (Section 2.3.4); genes included in the KEGG database that are not present in the microarray are indicated in gray.

The close relationship between retinoic metabolism and the developmental processes occurring in the eleutheroembryo is summarized in the Venn diagram on Figure 2.4. Transcripts related to nervous, circulatory and skeletal system development show a considerable degree of overlap. In all three categories, about 30% of transcripts showed significant changes in concentration upon atRA exposure, with underrepresented transcripts roughly doubling the number of overrepresented ones (Figure 2.4). This unequal distribution of under- and overrepresented transcripts in atRA treated samples can be observed in the volcano plot on Figure 2.5. On the contrary, 5 out of the 14 genes related to the retinoic acid metabolism incremented their mRNA levels upon atRA treatment, whereas only two showed a significant decrease (Figures 2.4 and 2.5). The Venn diagram reveals that all retinoic metabolism-related transcripts that became overrepresented in atRA-treated samples are also functionally related to developmental processes, presumably through signaling pathways (Figure 2.4). Therefore, the data suggests a relationship between the ectopic presence of atRA, the negative feedback of retinoic metabolism, and the general decrease on the expression of development-related genes.

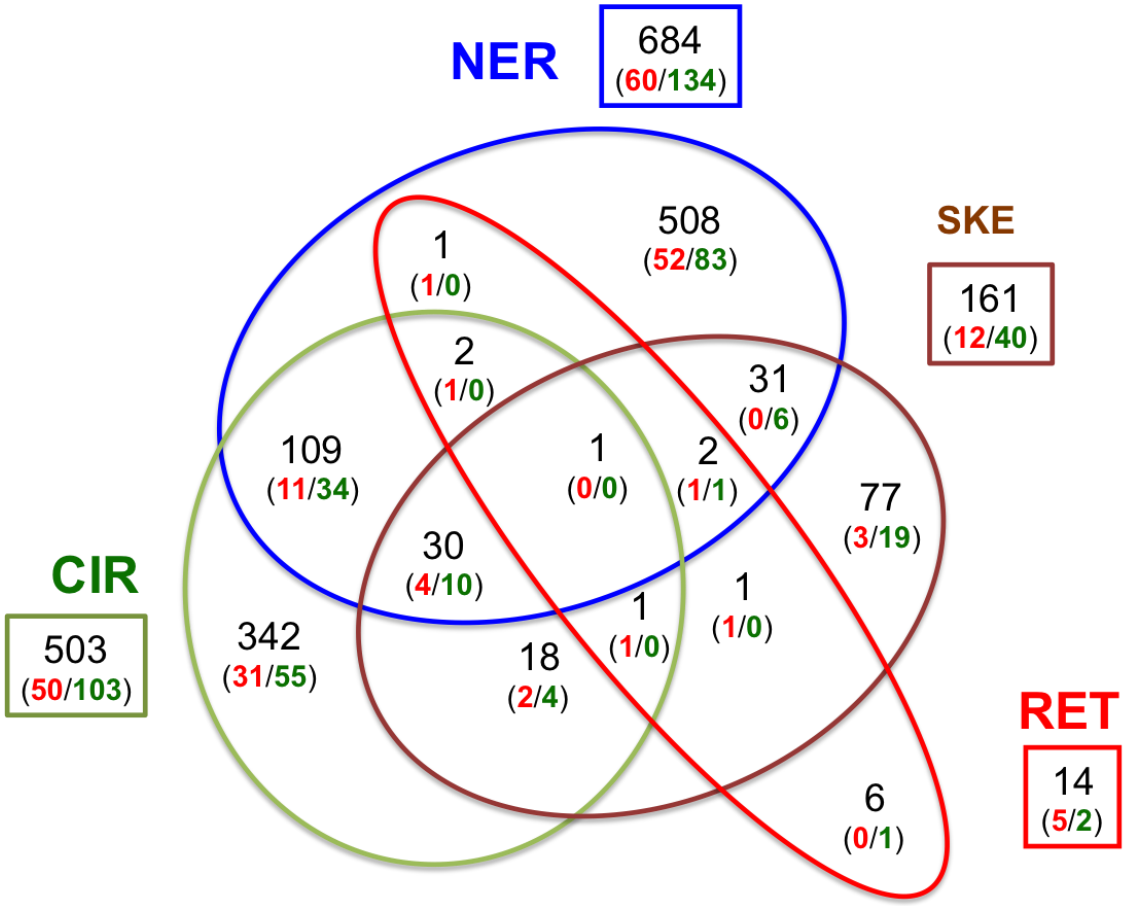

Figure 2.4 Venn diagram with microarray results. The graph includes genes included in GO categories of nervous system development (NER, GO:0007399, blue oval), circulatory system development (CIR, GO:0072359, green oval), skeletal system development (SKE, GO: 0001501, brown oval), and retinoid metabolic process (RET, GO:0001523, red oval). Square boxes indicate total numbers of genes ascribed to each category (black figures on top) and the number of those whose transcripts became enhanced (red figures, bottom left) or reduced (green figures, bottom right). Corresponding values are represented (with the same codes) in the corresponding sector of the Venn diagram.

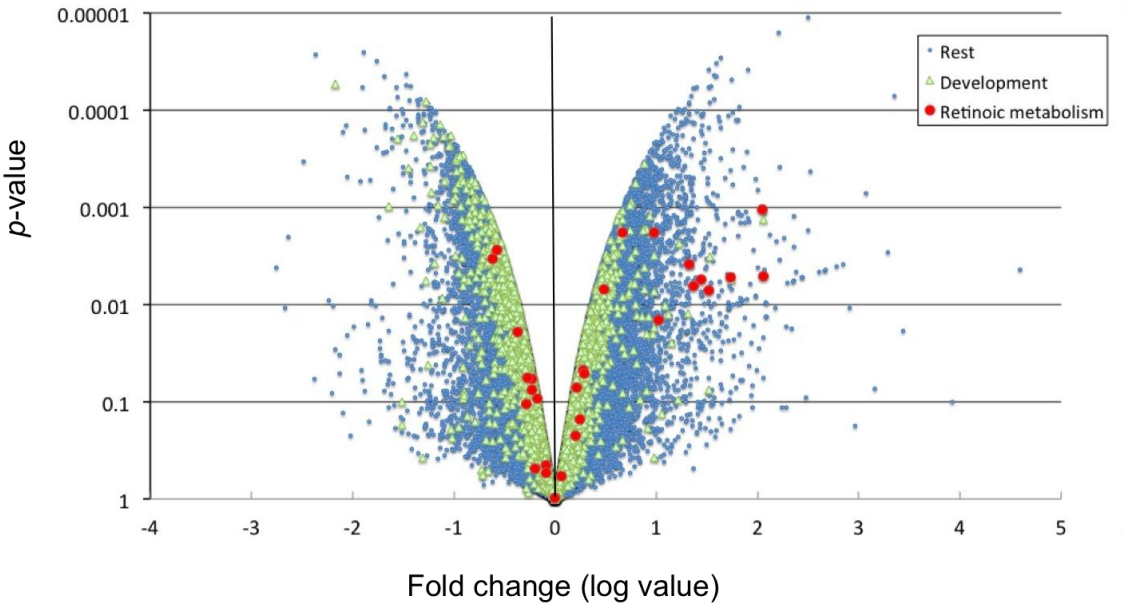

Figure 2.5 Volcano plot. Transcriptomic changes in zebrafish embryos upon atRA treatment are represented in a volcano plot (inverse of p-value versus fold change, log values). Each symbol represents a probe in the microarray, and therefore a single gene may be represented by more than one spot. Green triangles indicate transcripts included in the nervous, circulatory, or skeletal system development GO functional terms; red dots represent transcripts included in the retinoid metabolic process GO term (see also Fig. 2.4). Remaining transcripts are represented by small blue dots.

2.4.4 Temporal pattern of expression of RA-responsive genes during zebrafish development.

The observed changes in the relative abundance of mRNA upon atRA exposure were compared with the normal evolution of zebrafish transcriptome during development up to the pre-adult stage (90 days, data from http://www.ebi.ac.uk/arrayexpress/experiments/E-TABM-33; last visit, January 2013). At least 75% of zebrafish transcripts that increased their abundance upon atRA treatment correspond to genes whose mRNA levels naturally increase at the post-hatching stage (3-5 dpf, vertical dotted line in Figure 2.6), whereas most transcripts that become reduced upon atRA treatment are also less abundant in the post-hatching phase than in the earlier embryonic stages (Figure 2.6). These trends are even more evident when data from the initial 16 hpf are not taken into account, covering the period at which maternal transcripts likely represent a relevant contribution to the total mRNA abundance (grey shadowed areas in Figure 2.6). A heatmap showing the evolution of atRA enhanced or reduced transcripts along zebrafish development is shown in Supplementary Figure 2.2. These results agree with the functional analysis of the changes on the transcriptome upon atRA exposure (Table 2.2), showing a general decrease of transcripts involved in organ- and anatomic structure development processes, which are particularly active during the first hours of development.

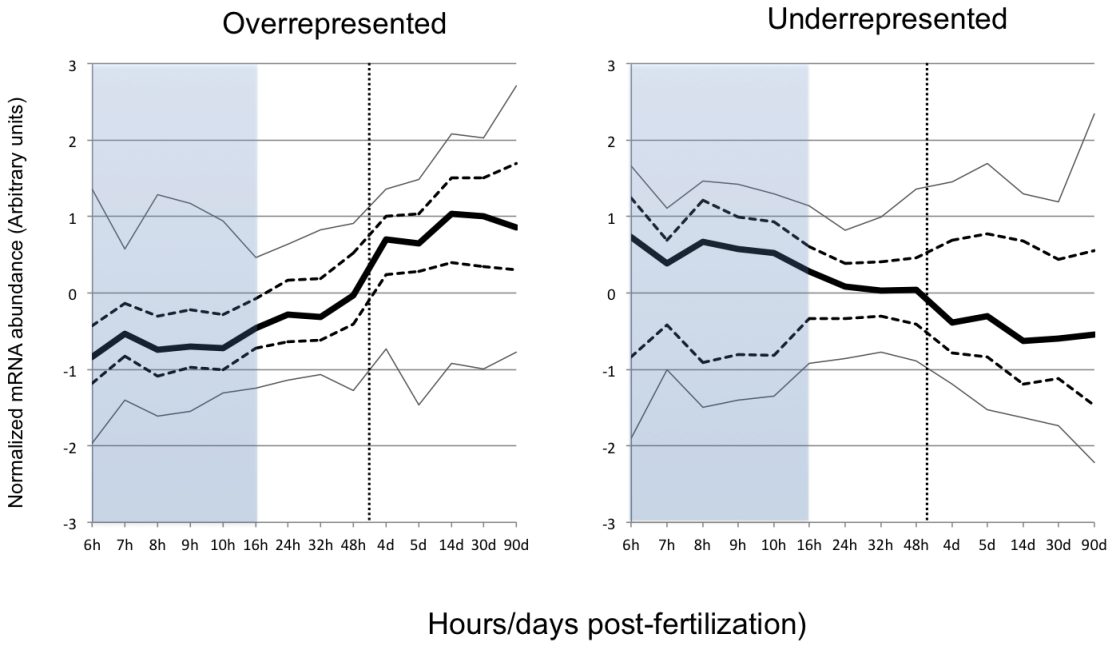

Figure 2.6 Relative abundance changes of RA-responsive transcripts during the first 90 days of zebrafish embryo development. The graphs are based on published data on mRNA levels of transcripts during the first 90 days of development (Section 2.3.4). Only genes showing significant changes, either overrepresented (left) or underrepresented (right) upon atRA exposure were included. In each panel, the thick line corresponds to the median values of the expression of the analyzed genes at each time point; discontinuous lines correspond to the first and third quartiles, and hairlines indicate the 5 and 95 percentiles. The heatmap corresponding to this analysis is shown in Supplementary Fig. 2.2. The shadowed area represents the period at which maternal transcripts are assumed to be relevant; the vertical dotted line indicate the approximate period of hatching.

2.5 Discussion

Zebrafish is becoming a preferred model for the analysis of sublethal effects of toxicants in vertebrates [46, 47]. The excellent knowledge of its genome and the availability of many genetic and molecular biology tools allow the study of toxicity mechanisms at the molecular level. Unlike purely *in vitro* (or in culture) models, zebrafish embryos open the possibility to analyze systemic modes of action, an essential aspect for studying endocrine disruption. Under the current EU directive, animal welfare rules only apply to fish larvae capable of independent feeding [48]. Assays performed before this stage accord with the 3Rs principles (relative replacement of animal tests), a target for many international regulatory bodies [49]. We therefore consider that the development of molecular markers for analyzing RAR/RXR function in zebrafish eleutheroembryos represents a significant contribution to the establishment of alternative methods for the analysis of toxicants affecting the retinoic system.

Relative proportions of RAR and RXR subtypes' mRNA showed a dramatic change during the first 24 hpf, coinciding with the transition from maternal mRNAs to

zygotic ones [31, 50]. Zygotic genes remain silenced for the first 10 divisions and become fully activated at division 13th [50, 51], which corresponds to the 6 hpf [31]. Maternal mRNAs become largely degraded after this period, although there is evidence for maternal contribution to the embryonic development until at least 24 hpf [50, 51]. Therefore, our data indicate that the RAR/RXR subtype complement in the maternally inherited mRNA is very different from the composition in the developing embryo, likely driven by the specific regulation of the different genes and cell types. These results agree with previous reports [15-17] showing a significant contribution of maternal mRNA transcripts in the early stages of zebrafish development. These combined results suggest that the 48-96 hpf period marks the transition from early to late embryonic mRNA levels for the different RAR/RXR genes' mRNAs. The functional meaning of the presence of different RXR/RAR subtypes and of the changes in their relative proportions during development is unclear. Our data is consistent with a model in which a single RXR gene (*rxrba*) accounts for essentially all maternal RXR mRNA, being gradually replaced by other RXR transcripts as the zygote genome becomes activated. Conversely, maternal mRNA likely contains roughly equals amounts of messengers from three out of the four RAR genes (*raraa*, *rarab* and *rarga*), whereas in late stages *rarga* transcripts predominate without completely substituting the rest of RAR transcripts. Whether or not these changes are related to the organogenesis occurring during the studied period is presently unknown. Despite the changes in subtype composition, our data show that RXR/RAR regulatory system is fully functional at all embryo/eleutheroembryo zebrafish stages from the somite-formation to the free-feeding larval stages. Not only all RAR/RXR genes are expressed, but their known regulated genes are responsive to the addition of external ligands with similar fold induction values for the three developmental stages investigated: just after the activation of the zygotic genome (6.5-11 hpf), during the hatching period (48-72 hpf) and in the late eleutheroembryonic period (96-120 hpf). Whereas induction factors varied among the different genes, the overall pattern was very similar across all the examined period, reinforcing the hypothesis that the different composition in RAR/RXR subtypes has a limited influence on the regulation of responsive genes. Therefore, the easiest hypothesis is that promoters, rather than protein functions, are the main factor determining the relative abundance of RAR/RXR subtype mRNAs. Treating late zebrafish eleuteroembryos (4 dpf) with subsaturating concentrations of atRA (100 nM) resulted in the ectopic activation of adult genes and a decrease of typically embryonic (i.e., development-related) ones. The selected concentration, roughly equivalent to EC_{30} for *cyp26a1* activation, did not induce any gross morphological effect, even after long-term (3 to 4 days) exposure. However, a 10 fold higher concentration did induce clear phenotypic effects on the head, potentially involving both skeletal and nervous system, as well as on the circulatory system (pericardial

edema). These effects increased in severity when exposure started at 48 hpf. Interestingly, atRA should activate preferentially RARs and we have found that during the exposure window (4-5 dpf) the predominant RAR subtype mRNA is *rarga*. This subtype of RAR is specifically expressed in nervous system (hindbrain and anterior spinal cord) and skeletal system (branchial arches and head mesenchyme) in 48 hpf embryos [52], which its perfectly with the identified developmental process functions altered after exposure to 100 nM atRA (Table 2.2) and with the phenotypic effects found at higher concentrations. The analysis also showed a transcriptional negative feedback mechanism by which genes implicated in RA degradation are activated and at least one gene involved in its synthesis becomes inhibited [29]. The temporal analysis of the data suggests that RAR-regulated genes become active only after the first 16 hpf, and that the evolution to the juvenile/adult pattern is not complete at least until the 30/90 dpf. Retinoic Acid is present at very low concentration (at the 10 nM range) during the early stages of zebrafish embryo development, and these levels increase steadily during the first four months of development and growth [53]. As our results show that developing zebrafish embryos respond to atRA already during the first 12 hpf, we concluded that the low intracellular concentration of RA is responsible for the nearly basal expression of these genes during the first 16-24 hpf. Consequently, their progressive activation during the subsequent embryonic stages can be attributed to intracellular increases of the corresponding ligands (Figure 2.6). A clear implication of this model is that any external alteration of RA concentrations, either by direct addition, changes on RA metabolism, or the presence of RA-like chemicals, may represent a significant hazard for the developing zebrafish embryos, even at concentrations at which no macroscopic developmental effects could be observed. Given the similitudes between fish and mammalian early development, it would be sensible to extend these concerns to developing humans.

2.6 Conclusions

Major changes occur in RAR and RXR variant composition during the first days of zebrafish embryo development, likely linked to the transition from maternal to embryonic messengers. As addition of exogenous atRA results in strong transcriptional response at all developmental stages analyzed, we concluded that the RAR/RXR signaling system is operational since the very early embryonic stages, and that the receptor variant composition has minor to no effects on the responsiveness of the RA-regulated genes. Our data shows that even subsaturating levels of ectopic atRA induced a negative feedback on RA metabolism and an advance of the developmental program. Therefore, any exogenous substance able to interfere with this response should be

regarded as a potential hazard for the early embryonic development of zebrafish and other vertebrates, even if their concentrations are insufficient to induce morphological alterations.

2.7 Acknowledgements

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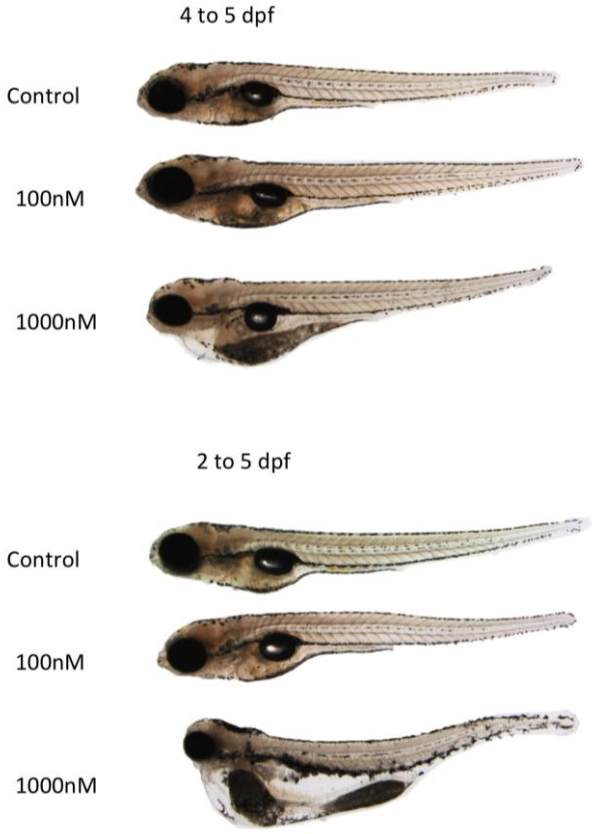
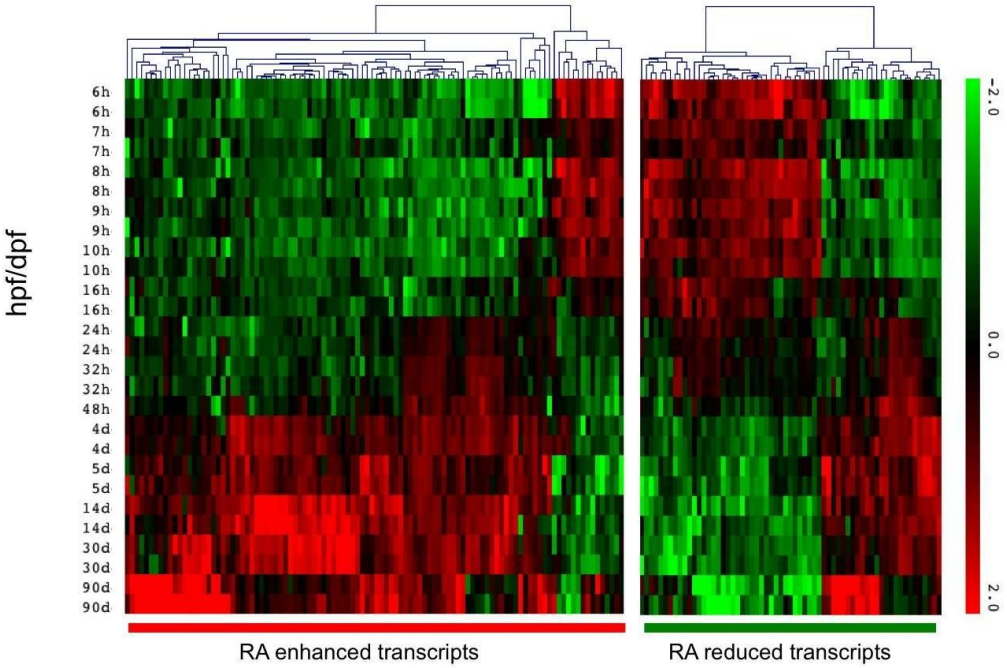
2.8 Supplementary information



Supplementary Fig 2.1. Five-day old zebrafish eleutheroembryos treated with atRA for at the specified concentrations and times of exposure. Top, embryos exposed for 24 h (4–5 dpf). Bottom, eleutheroembryos exposed for 3 days (2–5 dpf).



Supplementary Fig 2.2 Heatmap corresponding to the relative abundance changes of RA-responsive transcripts during the first 90 days of natural (i.e., untreated) zebrafish embryo development (Section 2.4). Only transcripts showing significant changes upon atRA exposure are shown. The two parts of the graph represent transcripts enhanced (left) or reduced (right) by atRA treatment.

Supplementary Table 2.1 Sequences of primers used in this study

Gene	Accession Number	Primer Sequence (5'-3')		Amplicon length (bp)
		Forward	Reverse	
ef1a	X77689	cgtctgccacttcaggatgtg	acttgcaggcgatgtgagcag	376
ppia2	AY391452	gggtggtaatggagctgaga	aatggacttgccaccagttc	179
cyp26a1	NM_131146.2	aactacatccccttcggagga	ttgcaatgctgcgttaactca	101
dhrs3a	NM_001003477	gggatgagcgaatcgagatg	tgtgcagtccagtatagggcaat	101
hoxb5a	NM_131101.2	cccaagacctgctacgtggt	aaggtgccgcgatgtacagta	101
hoxb5b	NM_131537.2	actcgctaccagacgctcga	tctgagaggcagagagcgtg	101
hoxb1b	NM_131142.2	tccatggccagcagaacata	gcgcgctctggtcaagtatt	101
raraa	NM_131406.2	atactgccggctgcagaagt	tgcattcgggctttttctctt	101
rarab	NM_131399.1	ccagagagcagaattccggta	aggctcggcctgtgtgtaac	101
rarga	BC098519.1	aagagccagagcgtgtcgat	gcgagggaacatgtgaggttt	101
rargb	NM_001083310.1	cgctgaagatctacgctcgc	tcctttagtgctgatgcccc	101
rxraa	NM_001161551.1	gaagacatgcccgtggagaa	ggtgaatttgatggcatggg	101
rxrab	NM_131153.1	tttcctcatggagatgctgga	tggacagtatttagcgcattgc	101
rxrba	NM_131275.1	attggagacaccccaatcga	caagccggattgaactggat	101
rxrbb	NM_131238.1	agtgcagaggtgggagcctt	gcacgcagacagccaagtt	101
rxrga	NM_131217.2	gcagatggacaagacggagc	cccttaacgcctccacctct	101
rxrgb	NM_001002345.1	gagaaggtttacgcctcgctg	gagcgaagcgcaggaagac	101

Supplementary Table 2.2 Blast analysis of RAR and RXR amplicons obtained in this work

Amplicon[a]	Hits	Position	Sequence	*gene*[c]
raraa	Query[b]	1	GCCTGGAAGTGGGGATGTCCAAAGAATCGGTGAGGAATGACAGAAATAAGAAGAAGAAAG	
	S74155	613	.....	*raraa*
	NM_131406	1042	.....	*raraa*
	L03398	524	.....	*raraa*
	NM_001083310	379	..G..C..A.....G.....C....C.....G.....	*rargb*
	NM_131399	446	.....C.....T.....T..T.....C...C.G..C..A.G.....	*rarab*
	L03399	615	.....C.....T.....T..T.....C...C.G..C..A.G.....	*rarab*
	BC098519	684	..G..T..C.....G...G.T...C.C..C..TC.....	*rarga*
	NM_131339	676	..G..T..C.....G...G.T...C.C..C..TC.....	*rarga*
	L03400	415	..G..T..C.....G...G.T...C.C..C..TC.....	*rarga*
rarab	Query	1	GACTTTGACCCGGACTGAACAGTAAATCTGGCCAGCTTTCATTCGCTGAAGTCCAGTAGA	
	AC146500	38002	.....	*rarab*
	CR392003	149575	.....	*rarab*
	BX649211	37503	.....	*rarab*
	NM_131399	1491	.....	*rarab*
	L03399	1655	.....-.....C.....	*rarab*
	CU915566	63947	...A.....TC......A...	*nsun3*
	CR762421	96204	...A.....TC......A...	*nsun3*
	BX890568	83340	....C....A...GG.....	*gtf2a1*
rarga	Query	1	CGTCTGCAGGAGCCCTTGCTGGAAGCTCTGAAGATTTATGCACGACGCCGCCGACCCAAC	
	BC098519	1287	.....T.....	*rarga*
	BX465864	95478	.....T.....	*rarga*
	L03400	1018	.....C.....	*rarga*
	NM_131339	1279	.....C..A.....	*rarga*
	NM_001083310	985	.....GC.C.....G..G.....C..C..T..C.....T.....	*rargb*
	L03399	1223	..T.....G..C.....C....	*rarab*
	CR392003	149061	..T.....G..C.....C..	*rarab*
	BX649211	36989	..T.....G..C.....C..	*rarab*
	NM_131399	1054	..T.....G..C.....C..	*rarab*
rargb	Query	1	GCAGGTCGGTGACTTTCATCAGCATTCGGGGGAACATGTGAGGTTTGTTGGGACGGCGGCG	
	NM_001083310	1087	.....	*rargb*
	BC098519	1390	..A..A....TC.....G..A.....T.....	*rarga*
	BX465864	95375	..A..A....TC.....G..A.....T.....	*rarga*
	AL935205	97627	..A..A....TC.....G..A.....T.....	*rarga*
	NM_131339	1382	..A..A....TC.....G..A.....T.....T..	*rarga*
	L03400	1095	.....G.....G.....	*rarga*
	L03399	1324	.....T....TC.....CTT.....T.....	*rarab*
	BX649211	37090	.....T....TC.....CTTA.....T.....	*rarab*
	NM_131399	1155	.....T....TC.....CTTA.....T.....	*rarab*
	CR392003	149162	.....T....TC.....CTTA..A.....T.....	*rarab*
rxraa	Query	1	TACATTAGTCTCAATGTAAGTCTCCGTCTTGGGCTCCACAGCAAGCTCTGCTTCCAGTATC	
	NM_001161551	750	.....T.....	*rxraa*
	CU062650	62847	.....T.....	*rxraa*
	EF028132	753	.....T.....	*rxraa*
	DQ017629	471	.....T.....	*rxraa*
	NM_131217	767	...C.....CT..T.....T..C.....A..G.....G..	*rxrga*
	U29940	718	...C.....CT..T.....T..C.....A..A.....G..	*rxrga*
	EF028133	789	....TG....C.....A.....T..G..T.AC..T.....A.....G..	*rxrgb*
	NM_001002345	789	....TG....C.....A.....T..G..T.AC..T.....A.....G..	*rxrgb*
	AF495876	300	....TG....C.....A.....T..G..T.AC..T.....A.....G..	*rxrgb*
	BX296562	65576	....TG....C.....A.....T..G..T.AC..T.....A.....G..	*rxrgb*
rxrab	Query	1	GGCGCCGCATCAAATCACATAAACTCCTCATGTACGAAACACACGGCAGGAACATTAC	
	FP016250	47304	.....	*rxrab*
	BX950178	98941	.....	*rxrab*
	AL929057	113819	.....	*rxrab*
	NM_131153	1179	.....	*rxrab*
	CR354540	129075	.....G...T...A.....T...	
	BX649515	28494	.....TA.....C..-.....	
	BX088579	188335	...C.....C.C.....	
	AL954130	162078	...C.....C.C.....	
	CU929413	53396	.....T....C....G...T....	
rxrba	Query	1	CGAGACTGGTCCAGGTTAGGTCAGCTGATGAGGCGCTTCAAGCATTTCCATTAAGAAGGTG	
	FP101875	17607	.....	*rxrba*
	BX890617	176672	.....	*rxrba*
	AL672176	4842	.....	*rxrba*
	NM_131275	1719	.....	*rxrba*
	BC054649	1544	...T.....	*rxrba*
	EF028133	1406	.....T.....C.....C.....G.G...A..	*rxrgb*
	NM_001002345	1406	.....T.....C.....C.....G.G...A..	*rxrgb*
	BX296562	57479	.....T.....C.....C.....G.G...A..	*rxrgb*
	NM_001161551	1377	...T....TT..G..T..A.....T.....C.....G.G.....	*rxraa*
	CU062650	34630	...T....TT..G..T..A.....T.....C.....G.G.....	*rxraa*
rxrbb	Query	1	ATTCGACAGGGTTCTTACTGAGCTGGTCTGCAAGATGCGTGACATGCAAATGGGCAAAACTG	
	BC162302	1014	.....A.....	*rxrbb*
	BC162301	1014	.....A.....	*rxrbb*
	NM_131238	1020	.....A.....	*rxrbb*
	FP067425	66193	.....A.....	*rxrbb*
	CR848674	44607	.....A.....	*rxrbb*
	BC054649	1175	.....G..G..C.....GA....A...A.G.....G....AT.....	*rxrba*
	NM_131275	1350	.....G..G..C.....GA....A...A.G.....G....AT.....	*rxrba*
	NM_131153	847	..T.....A..G..G..A.....T..G.CA.....A.A.....A...G..	*rxrab*
	NM_131217	1040	...T.....AT.A..A.....G.CT.....A.G.....G....A...G..	*rxrga*
	U29940	991	...T.....AT.A..A.....G.CT.....A.G.....G....A...G..	*rxrga*
rxrga	Query	1	GATGGGTTTGATAGTCCTTTTGCATCTGGGTTGAAGAGGACGATTGCTCTTAAACAGCCCA	
	NM_131217	1165	.....	*rxrga*
	U29940	1116	.....	*rxrga*
	BC054649	1298	....C....C.A...C.....T....T...G.....G.GG..T....	*rxrba*
	NM_131275	1473	....C....C.A...C.....T....T...G.....G.GG..T....	*rxrba*
	EF028133	1184	..A.....C.AG.....G..G..A.....G..C..A.GG.....	*rxrgb*
	NM_001002345	1184	..A.....C.AG.....G..G..A.....G..C..A.GG.....	*rxrgb*
	NM_131153	971	.....A..A..C.A.....A.....A.....C..A..A..G..	*rxrab*
	NM_001161551	1145	.....C..G.....G.AG.....A..T..T..A..G....	*rxraa*
	DQ017629	866	.....C..G.....G.AG.....A..T..T..A..G....	*rxraa*
	CU929314	86037	.....	
rxrgb	Query	1	GAGACCTACACTAAACAGAAATACCCCGACCAGCCTGGCAGGTTTGCTAAGCTGCTGCTGC	
	EF028133	1227	.....	*rxrgb*
	NM_001002345	1227	.....	*rxrgb*
	NM_131153	1012	...G.....TG.....C.....T.....C.....	*rxrab*
	BX296562	61022	.....	*rxrgb*
	NM_131217	1211	.....C.....C..C.....G..T.....A.....C.....A..C....	*rxrga*
	U29940	1162	.....C.....C..C.....G..T.....A.....C.....A..C....	*rxrga*
	NM_001161551	1201	.....T..G.....T..A..A.....A.....T.....	*rxraa*
	DQ017629	922	.....T..G.....T..A..A.....A.....T.....	*rxraa*

a) Predicted sequence from primer design
b) Matching zebrafish gene from ZFIN
c) Actual amplicon sequenced (primers excluded)

3 - All-trans Retinoic acid and 9-cis Retinoic Acid transcriptomic differences in Danio rerio early stages of development

All-trans Retinoic acid and 9-cis Retinoic Acid transcriptomic differences in *Danio rerio* early stages of development.

Eva Oliveira, Amadeu Soares, Carlos Barata and Benjamin Piña

In prep.

3.1 Abstract

The normal vertebrate development requires appropriate amounts of Retinoic acid (RA). All-trans retinoic acid (atRA) and 9-cis retinoic acid (9cRA) are potent metabolites of Vitamin A and operate by affecting gene expression through the binding of atRA or 9cRA to retinoic acid receptors (RARs) and the binding of 9-cis RA to retinoid X receptors (RXRs). In zebrafish, exogenous application of the retinoid isomers is known to have deleterious effects of varying magnitudes on development. In this study, we report the phenotypic and transcriptomic effects of exposing zebrafish larvae to concentrations of retinoids for 24h and 72h. Whereas major phenotypic effects were similar for both compounds and stronger at 1000nM for longer than for shorter exposures, transcriptomic data showed a differential regulation of 4587 spots, corresponding to some 2920 genes. The strongest transcriptional signal corresponded to the long atRA exposure, and affected (either positively or negatively) key genes related to development and/or metabolism. In contrast, the weakest response corresponded to the long 9cRA treatment, suggesting that the cellular action of this isomer was transient. Chemical stability was similar for both compounds in treatment conditions, as well as their ability to activate *cyp26a1*, a known marker gene for retinoic activity. We concluded that the ectopic presence of both retinoic acid isomers affect zebrafish embryonic development.

Key words: all-trans Retinoic Acid, 9-cis Retinoic acid, development, microarrays

3.2 Introduction

The developing zebrafish embryo is becoming a standard model for environmental risk assessment of chemicals (Scholz and Mayer, 2008; Stegeman et al., 2010). The research link between molecular biology and toxicology is producing new insights into how toxicity of chemical compounds directly regulates gene expression. Retinoids, naturally occurring vitamin A derivatives, regulate metabolism by activating specific nuclear receptors and have pleiotropic effects in all tissues of vertebrate organisms involving cellular development, proliferation, differentiation, metabolism and apoptosis (Nielsen et al., 2008; Delacroix et al., 2010). The widespread nature of the bioactivities of retinoids is due, in large part, to their ability to bind retinoic acid receptors (RARs and RXRs, see below), which are key regulators of the expression of different target genes. In a previous work, we analysed the RXR/RAR regulatory system during the first 5 days of development of zebrafish (Oliveira et al., 2013). This analysis has led to a proliferation of information on the contribution of each nuclear hormone receptor related to the metabolism of retinoic acid (RA) during the early stages of zebrafish development and to evaluate the expression of RA target genes during these stages.

In this work, we analyse the effects of two bioactive isomers of retinoic acid (RA), the all-trans RA and 9-cis RA (atRA and 9cRA, respectively). The general course of retinoid metabolism, transport and nuclear action is diagrammed in Figure 3.1. In vertebrates, there are two classes of retinoid receptors, the retinoic acid receptors (RARs) and the 9 cis retinoid acid receptor (RXRs). Both the RARs and RXRs bind the naturally occurring metabolites of Vitamin A with a similar preference order (all-tans RA> retinal> retinyl acetate > retinol), but RXRs require from 10 to 40 fold more ligand than RARs for comparable activation (Mangelsdorf et al., 1995). Conversely, RXRs bind with very high affinity to the 9cRA isomer, a downstream metabolite of atRA (Heyman et al., 1992).

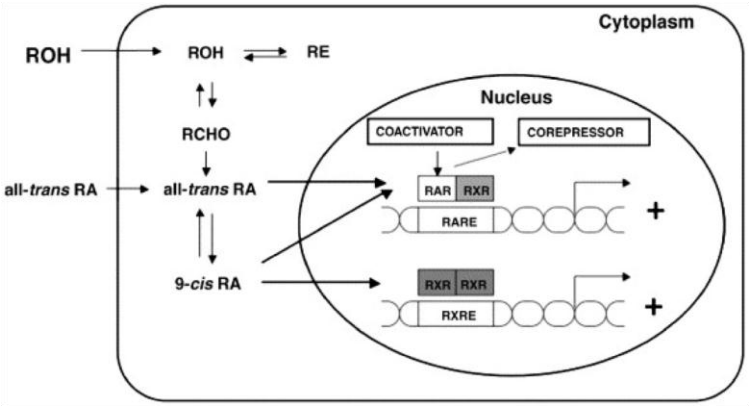


Figure 3.1 Intracellular retinoid metabolism, transport and nuclear action.

A description of the multiple RAR and RXR subtype for zebrafish was provided by (Hale et al., 2006; Tallafuss et al., 2006). Each receptor subtype has different isoforms generated by alternate splicing and differential promoter usage of the individual gene (Chambon, 1996). In addition, the RXRs serve as heterodimeric parteners for RARs, thyroid hormone receptors (TRs), vitamin D receptors (VDRs), the peroxisome proliferator-activated receptor (PPAR), the chicken ovalbumin upstream promoter-transcription factor (COUPTF), farnesoid X- activated receptor, liver-X receptor and other less-well characterized NRs (Aranda and Pascual, 2001). The RXRs can also function as homodimers dependent on the 9cRA ligand (Mangelsdorf et al., 1992). Many of the ligands for the Nuclear Receptors (NRs) that heterodimerize with RXR are products of lipid metabolism, such as fatty acids, leukotrienes, prostaglandin and cholesterol derivatives (Aranda and Pascual, 2001). RARs and RXRs have conserved modular structures, particularly in the DNA binding domains, which define, in part, their membership in the nonsteroisd, thyroid/retinoid/vitamin D superfamily of NRs. Such heterodimeric receptors offers two distinct ligand binding sites with where chemicals (endogenous or exogenous) can interact to modify receptor functions. An extensive number of studies have tested the effects of exogenous retinoids on different vertebrate embryos and suggested possible normal functions of vitamin A (Durston et al., 1989; Ruiz i Altaba and Jessell, 1991). One of RA well-known roles is in determining anterior-posterior (A-P) positional identity during early development, through regulation of HOX gene expression (Oliveira et al., 2013).

The Zebrafish are easy to maintain in laboratory, have a short life cycle, and readily produce relatively large quantities of transparent embryos, the development of which has been extensively studied and can be observed using a variety of optical methods. The small sizes of the embryos allow high throughput approaches, as the use of microtiter plates or robotized control mechanisms. Moreover, and unlike purely in vitro (or in culture) models, the embryos allow the possibility to integrate alternative modes of action. The excellent current knowledge of the zebrafish genome and the availability of many genetic and molecular biology tools allow precise analysis of the observed processes at the molecular level. Whereas the use of the acute embryo toxicity test DarT (Nagel, 2002) is becoming increasingly implemented, many efforts are devoted to use zebrafish embryos as model to understand toxic mechanisms at sublethal levels and to predict possible adverse and long-term effects. This would require the development of suitable molecular markers as indicators of the mode of action and the establishment of links between short-term effects in embryos and long-term toxicity in adults (Scholz et al., 2008; Scholz and Mayer, 2008).

In this study, zebrafish eleutheroembryos were exposed to atRA and 9cRA (100nM) from the 2^{th} to 5^{th} dpf (72h) and from 4^{th} to 5^{th} dpf (24h). These windows of exposure were selected considering that zebrafish embryos are completely developed by

the end of the endotrophic period (typically, at 6-7 dpf), and avoiding the first 48hpf in order to ensure that no major distortion of the embryo was caused by the treatment. The sublethal effect concentration of 100nM was selected based on previous range finding tests for the compounds. The major objective of this study was to determine and analyse the transcriptomic profile of those RAR/RXR agonists in zebrafish early stages of development. We expected to improve the knowledge of atRA and 9cRA metabolic mode of action in zebrafish.

3.3 Material and Methods

3.3.1 Zebrafish embryos and eleutheroembryo maintenance

Zebrafish (*Danio rerio*) embryos (from fertilization to hatching) and eleutheroembryos (from hatching to the self-feeding larval stage) were obtained by natural mating and raised at 28,5ºC (Kimmel et al., 1995) with a 12L:12D photoperiod in 90ug/ml of Instant Ocean (Aquarium Systems, Sarrebourg, France) and 0.58mM CaSO4.2H2O, dissolved in reverse osmosis purified water ("fish water", http://zfin.org/). Animal stages were recorded as days or hours post-fertilizarion (dpf or hpf, respectively).

3.3.2 Chemicals and embryo and eleutheroembyo treatment

DMSO, all-*trans*-Retinoic acid (atRA) and 9-*cis* Retinoic Acid (9cRA) were purchased from Sigma Aldrich (St. Louis, MO). All dilutions are reported as nominal concentrations. Stock solutions were prepared in DMSO on the day of the experiment. Experimental solutions with the same final concentration of DMSO (0.1%) were directly prepared in fish water. Zebrafish embryos were exposed to each compound in two different windows of exposure (48-120hpf and 96-120hpf) covering the post-hatching stage (2 to 5dpf). The tested concentrations for embryo toxicity assessment were: 100nM and 1000nM of each RA isomer. The larvae gross anatomical development for the controls and treatments was observed at the end of each assay, based on the normal developmental stages of zebrafish reported in the literature (Kimmel et al., 1995). The larvae were evaluated and images were taken in order to record phenotypic characteristics during the development using a Nikon digital Sight DS-Ri1 camera and NIS Elements AR Software (version 3.0). The sub-lethal and teratogenic endpoints based on the criteria established (Nagel, 2002; Lammer et al., 2009) were specifically evaluated at the selected time points. Exposures for microarrays

analysis were performed with a 100nM concentration for each compound in a 100ml glass beaker, 6 biological replicates were made per treatment. The selected concentration is considered as a non-adverse effect concentration for these two windows 2-5dpf and 4-5dpf.

3.3.3 RNA extraction and microarray analysis

Total RNA was isolated from the eleutheroembryos using Trizol reagent protocol (Invitrogen Life Technologies, Carls) and purified using standard methods following the manufacturer's protocol (RNeasy Kit; Qiagen). RNA concentration was measured by spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality checked by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples that registered the RIN (RNA Integrity Number) values between 9.5 and 10 were selected to further investigation. Microarray studies were performed using the two-color strategy Agilent *D. rerio* (Zebrafish) Oligo Microarray v3 platform. The study included three biological replicates (independent eleutheroembryo pools either untreated or treated with 100nM atRA or 100nM of 9cRA), labelled and hybridized at the same time (results are deposited at GEO, reference- GSE41335: GSM1015039, GSM1015040, GSM1015041, GSM1015042, GSM1015043, GSM1015044 and GSM1015045 for 9cRA exposures, for atRA the following: GSM1015046, GSM1015047, GSM1015048, GSM1015049, GSM1015050, GSM1015051). The quality of the microarray data was evaluated manually using the quality control report generated by the by the Agilent Feature Extraction Software. No statistical differences were observed between the biological replicates of each treatment. Microarray data were analysed using Robin (Lohse et al., 2010) and MultiExperiment viewer MeV4 (Saeed et al., 2003) software. Gene ontology analyses were performed using the AmiGO! Webpage (http://www.geneontology.org (Carbon et al., 2009)).

3.3.4 Microarray validation (quantitation by qRT-PCR)

Total RNA was treated with DNAseI (Ambion, Austin, TX) to remove genomic DNA contamination, and retro-transcribed to cDNA using First Strand cDNA Synthesis Kit (F. Hoffmann-La Roche, Basel Switzerland). Aliquots of 50ng of total RNA 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). Appropriate primers (Table 3.1) for 9 test genes (*cyp26a, dhrs3a, hoxb1b, hoxb5a, hoxb5b, acox1, acsl1, apoa1, lpl*), were designed using Primer Express 2.0 software (Applied Biosystems) and the Primer-Blast server

(http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Amplification efficiencies were calculated as better than 90% for all tested genes as described (Pfaffl et al., 2002). House-keeping genes *eef1a1l1* and *ppiaa* were both selected as reference genes (Morais et al., 2007; Pelayo et al., 2012), as mRNA levels of neither gene changed upon treatment (Pfaffl et al., 2002). PCR products (amplicons) were sequenced in a 3730 DNA Analyzer (Applied Biosystems), and compared to the corresponding reference sequences at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Table 3.1 shows the actual sequences obtained from the amplification and their match to the corresponding sequences deposited in GeneBank. Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (*Cp*, calculated by triplicates). To minimize errors on RNA quantification among different samples, *Cp* values for stress-related target genes (Cptg) were normalized to the average *Cp* values for both reference genes.

Table 3.1 Sequences of primers used in this study.

Gene	Accession Number	Primer Sequence (5'-3')	Amplicon length (bp)	Amplicon sequence
eef1a1l1	X77689	FW: CGTCTGCCACTTCAGGATGTG RV: ACTTGCAGGCGATGTGAGCAG	376	TACAAAATTGGAGGTATTGGAACTGTACCTGTGGGTCGTGTGGAGACTGGTGTCCTCAAGCCT GGTATGGTTGTGACCTTCGCCCCTGCCAATGTAACCACTGAGGTCAAGTCTGTTGAGATGCAC CACGAGTCTCTGACTGAGGCCACTCCTGGTGACAACGTTGGCTTCAACGTTAAGAACGTGTC TGTCAAGGACATCCGTCGTGGTAATGTGGCTGGAGACAGCAAGAACGACCCACCCATGGAGG CTGCCAACTTCAACGCTCAGGTCATCATCCTGAACCACCCTGGTCAGATCTCTCAGGGTTACG CCCCAGTGCTGGATTGCCACA
ppiaa	AY391452	FW: GGGTGGTAATGGAGCTGAGA RV: AATGGACTTGCCACCAGTTC	179	GCCGACGTTGTCCCCAGAACTGCAGAGAATTTCAGGCAGTTGTGCACGGGTCAGCCTGGCTA TGGCTACAAGGGATCCTCTTTCCATCGTGTAATTCCTGGTTTCATGTGCCAGGGAGGTGACTTC ACAAACCACAATG
cyp26a1	NM_131146.2	FW: AACTACATCCCCTTCGGAGGA RV: TTGCAATGCTGCGTTAACTCA	101	ACTAAAAAGATCTTGAGTAACACTTTGGCGAACTCTTTGCCCACACACATCCTGGATCC
dhrs3a	NM_001003477	FW: GGGATGAGCGAATCGAGATG RV: TGTGCAGTCCAGTATAGGGCAAT	101	CAAATCCAAATTCATCCATGTAAGACTTGCAGACATAACAGTTCTTGGGACAACAAGC
acox1	NM_001005933.1	FW: GATAAGGCCCAATGCAGTGG RV: TGTGCTCATAGACGTTGCCATC	101	ATATCGTCCCAGAGAAGAATTAAGCATCTCATCACGGTAGTCAAAAGCATCAACGAGAG
acsl1	NM_001031837.1	FW: TGTCATGCTGTTGGGAGTGAA RV: GGCTGAGGGATGACTACAGCTC	101	CTCCAAGTGGTAGAAAAAAAAACAAAAATAAAAAAAAAAAGAGTCCACTAGTGGTCC
lpl	NM_131127.1	FW: TCCTGTTGACCACGGATGC RV: GGTGTCGGAGTTCCACCAAG	101	AGACATCGGAGAGCTGCTGATGGTTAAACTTCTCTGGGAGAAAGACACGCTCATCAGCTGGC
hoxb1b	NM_131142.2	FW: TCCATGGCCAGCAGAACATA RV: GCGCGCTCTGGTCAAGTATT	101	ATACGCACCAATTTCACCACCAAGCAGCTCACGGAACTTGAGAAGGAGTTTCACTTCAACA
hoxb5a	NM_131101.2	FW: CCCAAGACCTGCTACGTGGT RV: AAGGTGCCGCGATGTACAGTA	101	CCCTGTAGGTAATGTTTTGAGATCTCTTTTTCaTCTCATTTTCCGCGTAATTTTTTGCAC
hoxb5b	NM_131537.2	FW: ACTCGCTACCAGACGCTCGA RV: TCTGAGAGGCAGAGAGCGTG	101	GCTGGAGAAAGAGTTCCATTTCAATAGATATCTGACGCGCAGGAGGGGAATAGAGATAGCT
apoa1	NM_131128.1	FW: ACACCCAGGACCTCCAGACTC RV: TTATGCCTGGATGGCCTTG	101	GCGATGGTCTCATACATCTGTGCGAATGTGGTCCTCACGTTCTCCATGTAGGGCTCCATGC

3.3.5 Retinoids detection by HPLC

The detection and identification of both Retinoic Acid isomers was carried out by HPLC (Alliance 2695) coupled to a Photo Diode Array detector (Diode Array PDA 996). Mixtures of both compounds were separated on a silica column, LiChroCART® 125-2 Purospher® STAR RP-18 endcapped (5µm- Merck) with a pre-column, at a flow rate of 0.3 ml/min. For each analysis a volume of 10ul was injected onto the HPLC

column. Mobile phase consisted of (A) water (LiChrosolv®, Merck) and (B) acetonitrile (LiChrosolv®, Merck), both acidified with 0.1% formic acid, and its composition (relative concentration of A and B) varied according to the gradient indicated in (Figure 3.2). UV detection in the wavelength range of 210-400 nm was used to determine isomers after separation. Each Retinoic Acid was determined at its absorption maximum was 350 nm for 9cRA and 355 nm for atRA. Total acquisition time for each sample was 29 min. Data were acquired with MassLynx v4-1 scn627 software. Column and autosampler were kept at room temperature, the latter protected from light in order to avoid retinoic acids decomposition. Both compounds were stable during the time. A small decrease in peak area around 10% was observed (Figure 3.3). Calibration curves for 9cRA and atRA, establish a linear working range from 19.5 to 2500ppb. The lower LOD (limit of detection) was 39.1ppb according to the obtained calibration curve.

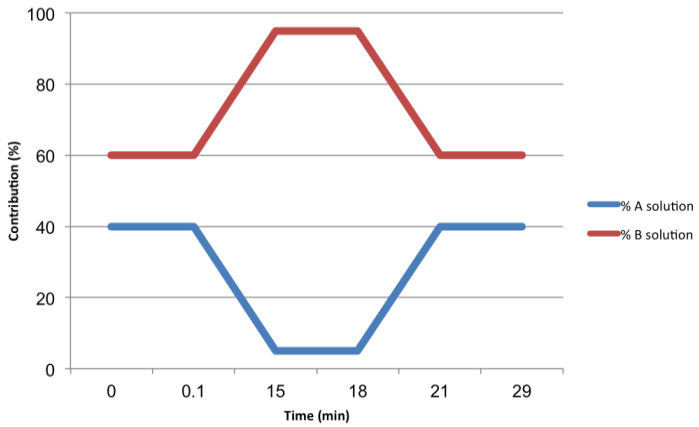


Figure 3.2 Chromatographic gradient. (A: water + 0.1%formic acid; B: acetonitrile + 0.1% formic acid) during the 29 minutes of injection.

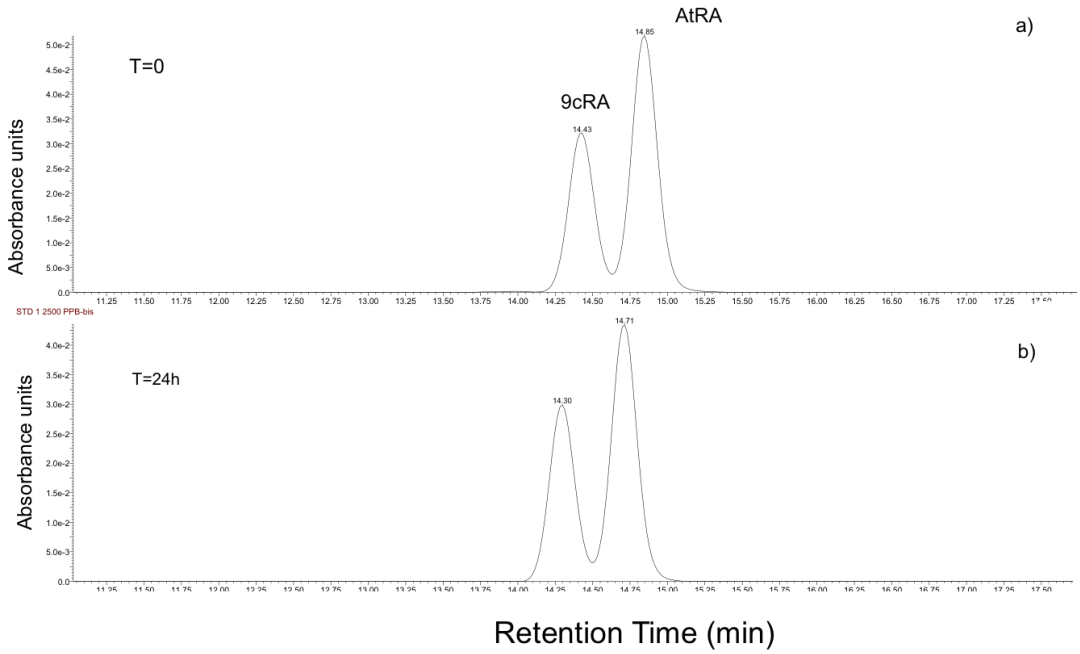


Figure 3.3 Chromatogram showing separation of 9cRA and atRA standards using HPLC system optimized for RA separation. a) states for time 0 and b) after 24h.

3.3.6 Statistical analyses

Statistics tests were performed using the SPSS 19 (SPSS Inc., Chicago, Ill) package. All statistical calculations were performed using ΔΔCp values, as this parameter followed normal distributions (Levene's test). Differences among control and treated groups were analyzed by Student's t-test (2 groups) or ANOVA plus Tukey's tests (more than 2 groups).

3.4 Results

3.4.1 Sublethal morphological effects of atRA and 9cRA

The overall analysis of the toxicological assays performed with zebrafish eleutheroembryos, showed the absence of mortality for the tested concentration during atRA or 9cRA treatments. No significant deformities or mortality were observed in either control group, larvae presented a well-developed tail, head and a normal body structure for the complete set of toxicological assays. In contrast, several malformations were observed after 72h of exposure for both compounds (Figure 3.4). Larvae exposed to 1000nM of atRA or 9cRA showed tail curvature (lordosis), pericardial and yolk sac edema, lack of spontaneous movements, a strong developmental delay. Similar, albeit reduced morphological effects were observed after 24h exposure, being pericardio

edema and impairment of the yolk consumption the most conspicuous effects at the highest tested concentration (Figure 3.4).

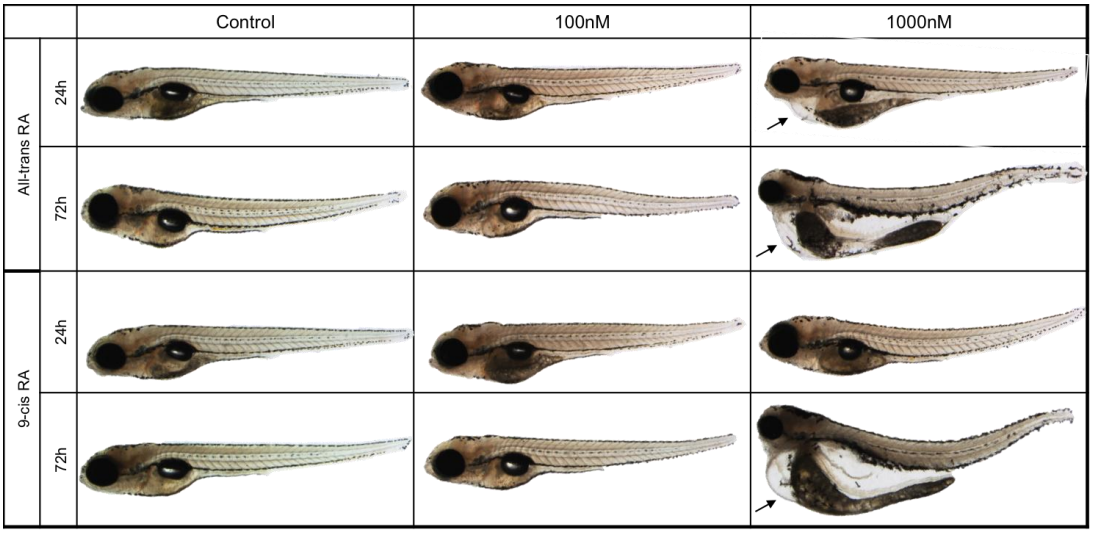


Figure 3.4 Zebrafish eleutheroembryos treated with atRA and 9cRA at the specified times of exposure and concentrations. Three different windows of exposure were selected to determine specific teratogenic effects at the 5dpf were registered after a 24h and 72h exposure periods. The black arrow evidences the common pericardio edema.

3.4.2 Transcriptomic responses to atRA and 9cRA

Treatment of zebrafish eleutheroembryos with 100nM of either atRA or 9cRA for 24h and 72h showed significant changes on the hybridization levels for 4587 probes out of 43,803 present in the zebrafish microarray (One-way Anova $p<0.01$, Figure 3.5). Hierarchical analysis of significant changes shows dissimilar behaviours of the different treatments, both in terms of compound and of time of exposure, indicating different toxic/regulatory mechanisms (Figure 3.5). The most relevant feature revealed by this analysis is the differentiation between 9cRA and atRA-treated samples, which was more evidenced for the 72h treated samples from the rest (Figure 3.5). We consider that these differentially affected genes may serve as indicators of the different modes of action (from both the toxicological and the physiological points of view) of these otherwise closely related ligands.

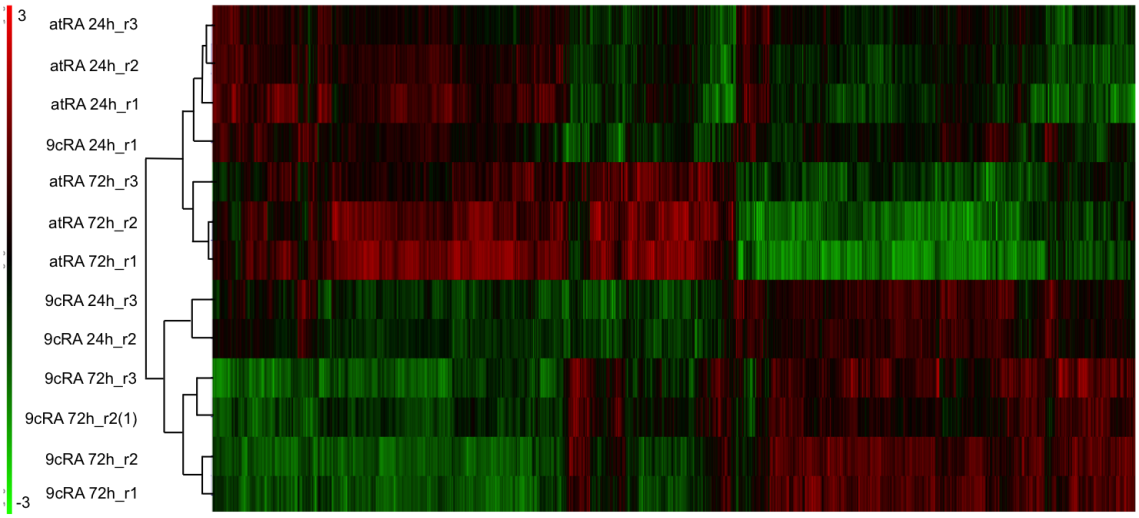


Figure 3.5 Heatmap, corresponding to the relative abundance changes, only significative spots are shown, the results belongs to the 5th dpf upon atRA and 9cRA exposures during 24h and 72h. A visibly separation on the action of both compounds is observed.

3.4.3 Gene clustering by Self-Organizing Map (SOM) analysis.

The multidimensional data set generated by our primary analysis includes 4587 probes that may include hidden or non-obvious relationships between both compounds and times of exposure. We used SOM analysis to project the differential set of probes onto a two-dimensional display (Figure 3.6), to "check" the spatial distribution of the effects on the mRNA abundance of both RA isomers and to provide a quality indicator of the degree of similarities or differences between the samples that generate one or more differential responses. SOM analysis individualized four different responses to atRA and 9cRA exposure, coded as c1-c4 (Figure 3.6). Clusters c1 and c4 (727 probes) include probes that were inhibited by the long (72h) 9cRA treatment, but activated, or at least not affected) by the rest of treatments. Clusters c2 and c3 include probes that were only affected by atRA, either positively (c3, 1844 probes) or negatively (c2, 2016 probes). In both clusters, the long atRA treatment showed a clearly stronger effect than the short one (Figure 3.6). In general, the strongest differences between atRA and 9cRA effects appeared after the long treatments, whereas the short ones appeared as intermediate and similar one to each other. These results show that short-term effects were essentially identical for both isomers, whereas 9cRA effects decreased after time while atRA effects increased.

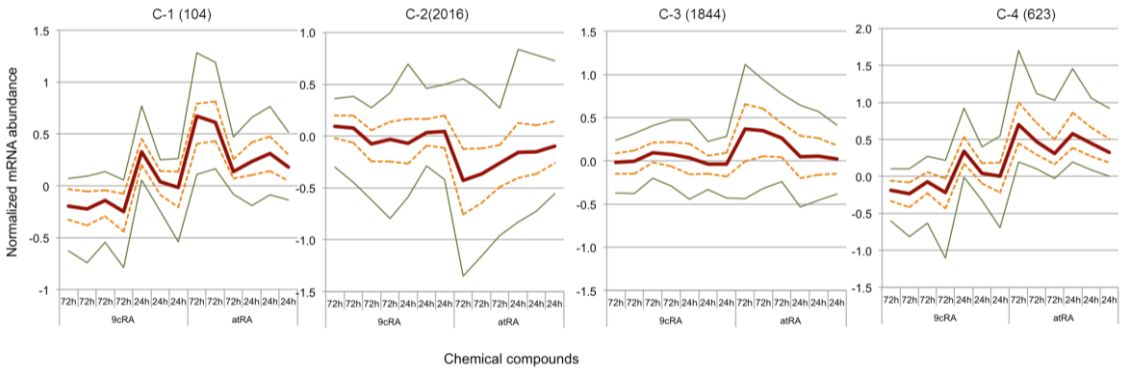


Figure 3.6 Use of SOMs to identify similarity patterns for atRA and 9cRA. In each panel, the thick line corresponds to the median values of the expression of the analyzed spots at each time point; discontinuous lines correspond to the 25 and 75 percentiles, and hairlines indicate the 5 and 95 percentiles.

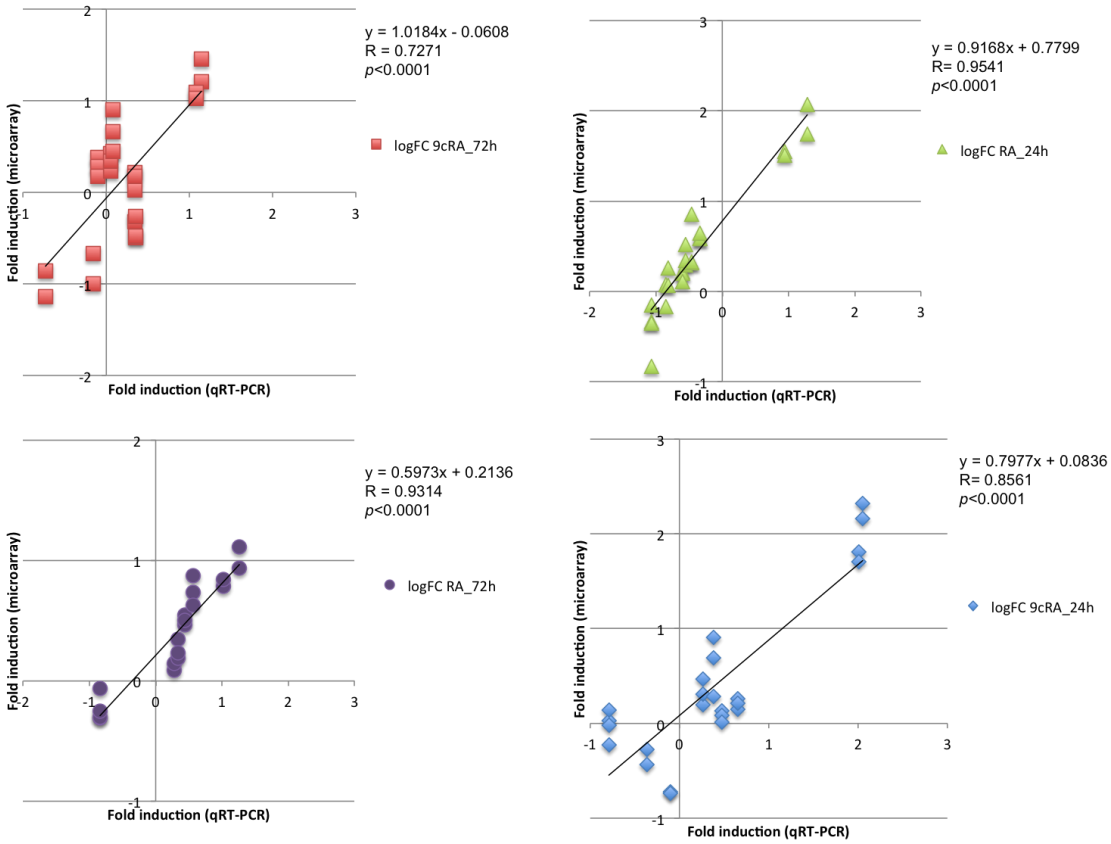


Figure 3.7 Correlation between microarray and qRT-PCR results. Fold induction values for 9 genes (*cyp26a1*, *dhrs3*, *hoxb1b*, *hoxb5a*, *hoxb5b*, *acox1*, *acsl1*, *apoa1* and *lpl*). The x-axis, corresponds to qRT-PCR data for each compound and time of exposure, whereas the y axis represents microarray data. Note that each gene is represented by more than a single probe in the microarray. Gene identification, regression line, the corresponding r coefficient and p-values are represented.

3.4.4 Functional analyses of atRA and 9cRA – induced transcriptomic changes

Analysis of the 308 transcripts included in c1 and c4 clusters showed an enrichment on different GO (gene ontology) functional categories, including translation-related functions and some metabolic processes (Table 3.2) More significantly, many genes from the p450 family were also found in these two SOM clusters. As these genes participate in the general response to RA in embryos (Oliveira et al, 2013), and given the temporal profile of the probes included in these two SOM clusters (inhibited or unaffected only after the long 9cRA exposure), these results are consistent with the weakening, or even loss of the 9cRA response after 72 hours of exposure.

Functional analysis of genes included in clusters c2 and c3 are shown in tables 3.3 and 3.4, respectively. Both clusters, which corresponds to genes repressed or activate after the 72h atRA treatment, respectively, appear enriched in different developmental functional categories, including development of anatomical structures, at the organ, system, whole embryo, and nervous system levels (Table 3.3 and 3.4). Although more difficult to interpret, these results are also consistent with the persistence, and even enhancement, of atRA effects after 72h of treatment. The observed variations in mRNA abundance for some key Retinoic Acid metabolism genes was confirmed by qRT-PCR (Figure 3.7), showing a significant correlation with the microarray data and corroborated the similar effect of the four treatments applied to zebrafish larvae.

Table 3.2 GO analysis of genes included in the cluster 1 and cluster 4, data from the SOM clusters. Functional analysis of zebrafish genes whose transcripts appeared different in atRA when compared to 9cRA.

Go term	Aspect	*P*-value	Sample frequency (n=308)	Brackground frequency (n=18439)	Genes
Biological process					
GO:0006413 translational initiation	P	7.58E-05	10 (3.2%)	59 (0.3%)	*denr eif4a2 eif4a1b eif2b4 eif3jb eif6 eif4ebp3l brf1a eif3s10 eif3m*
GO:0006412 translation	P	1.67E-03	19 (6.2%)	305 (1.7%)	*denr rpl13 piwil1 eif4a2 kars piwil2 rplp0 dars eif4a1b rps5 eif2b4 eif3jb rpl5a eif6 eif4ebp3l brf1a eif3s10 rars eif3m*
GO:0008152 metabolic process	P	7.65E-03	146 (47.4%)	6461 (35.0%)	*fggy ak2 casp8 gata2b tsr2 lpl cyp24a1 ppm1k tdh adam8a cct8 denr pmt rpl13 cyp2aa12 tcp1 gna14 esrp2 cyp4v7 agt piwil1 nr1d1 pter atp1a1a.4 prmt2 eif4a2 prmt3 pla2g7 prkab1a pdgfra fbp1a ctsl1a nr1i2 gnsa wdr45 kars apol1 piwil2 pck1 mycb dhodh glulb cyp26b1 foxa1 cct4 rplp0 tmprss4a dohh atg4b cyp4t8 ptgis aldob mettl5 pnp4b riok2 dars grhprb ddb2 eif4a1b chac1 rps5 ctsl1b rnaset2 mmp30 mdh1a rgs4 tat hsd17b10 fkbp5 meis4.1a cyp1c1 nr1d2b eif2b4 pdk2 pcbd1 npsn gpx1b map4k5 aldh6a1 eif3jb aup1 sgk1 echdc2 pfkfb4l cnot6l mst1 nmnat1 cish gli2a rpl5a socs3b mcl1a c1qb eif6 h1fx dpp7 esr2b tdo2b mlycd atp1a1a.2 gys2 ivd scpep1 akt2l rdh14a gbp nr1d2a eif4ebp3l tdo2a b3gnt5a cda brf1a atp1a1a.3 pfkfb1 pfkfb3 gnl2 wbscr22 ugt5e1 ctsa acat1 arg2 fbxo44 eif3s10 eno3 acadm ctssb.2 gpt2l pnp5a plg gpx4a nr5a5 rars foxo3b rdh12l ctssb.1 spint1a acsl4l cct6a aspg edf1 tbx1 hsd11b2 nkx6.2 cnppd1 cyp3c1 eif3m*
Molecular function					
GO:0003743 translation initiation factor activity	F	4.21E-04	9 (2.9%)	54 (0.3%)	*denr eif4a2 eif4a1b eif2b4 eif3jb eif6 brf1a eif3s10 eif3m*
GO:0020037 heme binding	F	9.49E-04	13 (4.2%)	138 (0.7%)	*cyp24a1 cyp2aa12 cyp4v7 cyp26b1 cyp4t8 ptgis cyp1c1 hbbe3 tdo2b nenf tdo2a hbbe2 cyp3c1*
GO:0046906 tetrapyrrole binding	F	1.55E-03	13 (4.2%)	144 (0.8%)	*cyp24a1 cyp2aa12 cyp4v7 cyp26b1 cyp4t8 ptgis cyp1c1 hbbe3 tdo2b nenf tdo2a hbbe2 cyp3c1*

Table 3.3 GO analysis of genes included in the cluster 2, data from the SOM clusters. Functional analysis of zebrafish genes whose expression is under-regulated in an atRA treatment for 72h.

Go term	P-value	Sample frequency (n=758)	Background frequency (n=18439)	Genes
Biological process				
GO:0007275 multicellular organismal development	4.39E-16	185 (24.4%)	2338 (12.7%)	*ak2 cebp1 pou3f3b pou4f2 neurod4 mapk8b fzd7a tp63 wnt3 rassf8b tagln3b igf2a rps7 csrnp1b top2b c1orf109 thraa appb bbs7 cyp26c1 mbnl2 lhx2b hoxc8a pho smoc2 pbx1a vmhc gata2a glcci1 snrpd1 itga6a frzb tbpl1 fgfr2 arrb1 ctnna2 ptrfb cyp26b1 dusp6 fstl1a hoxb9a sestd1 dnmt1 hdc myhz1.1 six1a nrp2b nipblb maea arhgef11 stmn2a tfap2c col2a1b melk bbs4 synj1 cdc42 igf2b ephb3a ascl1b col17a1a slc40a1 hoxc13a gli2a ddx46 sfrp5 cdh2 lama1 her8a atg5 axin1 ocrl cyt1 mstnb efna5b ccnd1 jag2 pou3f3a ccdc85cb lmo4a tgif1 foxg1a mta3 dpysl2b smad2 shrprbck1r neurog1 tspan12 rab8a larp6 crkl fn1b prkci smg5 irx3a tcf7 scinla plxnb2a ndrg4 atp6v0b cdh6 ihhb hoxd9a tcf7l1a slit2 dctn1a ccdc40 top2a tal1 csk socs3a otx1a tagln itgb4 mych dctn2 ryk col27a1a eef2b runx1 kdm6al trim33 hoxb2a ptprub rbpjb robo3 sart3 bmp4 usp5 rbp4l dusp4 tfap2a mcl1b plp1a stk11 nusap1 otx2 wnt11r foxo3a ppdpfa col2a1a fstl1b rab11a notch3 gli2b cdc25 dpysl3 sox1a neurod6a atp2b2 mtor hoxd3a nrarpb aggf1 ahcyl1 egfl7 glra4a dazl nr6a1a klf2a neurod2 cryaa a2ml olig2 cdkn1c nrg2b smarca5 znf503 ier2 clptm1 adcy8 tnc rbmx egr2b srgap2a tspo copb2 whsc1 fzd10 upf1 notch1a crb2b zeb2a tmem2 hoxa11b*
GO:0032502 developmental process	1.93E-15	192 (25.3%)	2500 (13.6%)	*ak2 cebp1 pou3f3b smyhc1 pou4f2 neurod4 mapk8b fzd7a tp63 wnt3 rassf8b tagln3b igf2a rps7 csrnp1b top2b c1orf109 thraa appb bbs7 cyp26c1 mbnl2 lhx2b hoxc8a pho smoc2 tnnt3b pbx1a vmhc gata2a glcci1 snrpd1 itga6a frzb tbpl1 fgfr2 arrb1 ctnna2 ptrfb cyp26b1 dusp6 fstl1a hoxb9a sestd1 dnmt1 hdc myhz1.1 six1a nrp2b nipblb maea arhgef11 stmn2a tfap2c col2a1b melk bbs4 synj1 cdc42 igf2b nkd3 ephb3a stau2 ascl1b col17a1a slc40a1 hoxc13a gli2a tmem231 ddx46 sfrp5 cdh2 lama1 her8a atg5 axin1 ocrl cyt1 mstnb efna5b ccnd1 jag2 pou3f3a ccdc85cb lmo4a tgif1 foxg1a mta3 dpysl2b smad2 shrprbck1r neurog1 tspan12 rab8a larp6 crkl fn1b prkci smg5 irx3a tcf7 scinla plxnb2a ndrg4 atp6v0b cdh6 ihhb hoxd9a tcf7l1a slit2 dctn1a ccdc40 top2a tal1 csk socs3a otx1a tagln itgb4 mych dctn2 ryk col27a1a eef2b runx1 kdm6al trim33 hoxb2a ptprub rbpjb robo3 sart3 bmp4 usp5 rbp4l dusp4 tfap2a mcl1b plp1a stk11 nusap1 rnf41 otx2 wnt11r foxo3a ppdpfa col2a1a fstl1b rab11a notch3 gli2b cdc25 dpysl3 sox1a neurod6a atp2b2 mtor hoxd3a nrarpb aggf1 ahcyl1 egfl7 glra4a dazl nr6a1a klf2a neurod2 cryaa a2ml olig2 cdkn1c nrg2b smarca5 znf503 ier2 clptm1 adcy8 tnc rbmx egr2b srgap2a tspo copb2 whsc1 fzd10 upf1 notch1a crb2b zeb2a crebl2 tmem2 hoxa11b*
GO:0048856 anatomical structure development	2.88E-13	172 (22.7%)	2243 (12.2%)	*ak2 cebp1 smyhc1 pou4f2 neurod4 fzd7a tp63 wnt3 rassf8b tagln3b igf2a rps7 csrnp1b top2b appb bbs7 cyp26c1 mbnl2 lhx2b hoxc8a pho smoc2 tnnt3b pbx1a vmhc gata2a glcci1 snrpd1 itga6a frzb tbpl1 fgfr2 arrb1 ctnna2 ptrfb cyp26b1 dusp6 fstl1a sestd1 dnmt1 hdc myhz1.1 six1a nrp2b nipblb maea arhgef11 stmn2a tfap2c col2a1b melk bbs4 synj1 cdc42 igf2b nkd3 ephb3a stau2 ascl1b col17a1a slc40a1 hoxc13a gli2a tmem231 ddx46 sfrp5 cdh2 lama1 her8a atg5 axin1 ocrl cyt1 mstnb efna5b ccnd1 jag2 lmo4a tgif1 foxg1a mta3 dpysl2b smad2 shrprbck1r neurog1 tspan12 rab8a larp6 crkl fn1b prkci smg5 irx3a tcf7 scinla plxnb2a ndrg4 atp6v0b cdh6 ihhb tcf7l1a slit2 dctn1a ccdc40 top2a tal1 csk socs3a otx1a tagln mych dctn2 ryk col27a1a eef2b runx1 trim33 ptprub rbpjb robo3 sart3 bmp4 usp5 rbp4l dusp4 tfap2a mcl1b plp1a stk11 nusap1 rnf41 otx2 wnt11r foxo3a ppdpfa col2a1a fstl1b rab11a notch3 gli2b cdc25 dpysl3 neurod6a atp2b2 mtor nrarpb aggf1 egfl7 glra4a nr6a1a klf2a neurod2 cryaa a2ml olig2 cdkn1c nrg2b smarca5 znf503 ier2 adcy8 tnc egr2b srgap2a tspo copb2 whsc1 fzd10 upf1 notch1a crb2b zeb2a tmem2*
GO:0032501 multicellular organismal process	8.47E-13	199 (26.3%)	2781 (15.1%)	*ak2 cebp1 pou3f3b smyhc1 pou4f2 neurod4 mapk8b fzd7a tp63 wnt3 rassf8b tagln3b igf2a rps7 csrnp1b top2b c1orf109 thraa appb bbs7 rlbp1a cyp26c1 mbnl2 lhx2b hoxc8a pho smoc2 pbx1a vmhc gata2a glcci1 snrpd1 itga6a frzb tbpl1 fgfr2 arrb1 ctnna2 ptrfb cyp26b1 dusp6 fstl1a hoxb9a sestd1 dnmt1 hdc myhz1.1 six1a nrp2b nipblb maea arhgef11 stmn2a tfap2c col2a1b melk bbs4 synj1 cdc42 igf2b adra2b ephb3a stau2 ascl1b col17a1a slc40a1 hoxc13a gli2a ddx46 sfrp5 slc1a2b cdh2 lama1 her8a atg5 npvf axin1 ryr1b ocrl ik cyt1 mstnb efna5b ccnd1 jag2 pou3f3a ccdc85cb lmo4a tgif1 foxg1a rgrb mta3 dpysl2b smad2 shrprbck1r neurog1 tspan12 rab8a larp6 crkl fn1b prkci smg5 irx3a tcf7 scinla plxnb2a ndrg4 atp6v0b cdh6 ihhb hoxd9a tcf7l1a slit2 dctn1a ccdc40 top2a tal1 csk socs3a otx1a tagln itgb4 mych dctn2 ryk col27a1a eef2b runx1 kdm6al trim33 hoxb2a ptprub rbpjb robo3 pde6h sart3 bmp4 usp5 rbp4l pmchl dusp4 tfap2a mcl1b plp1a stk11 nusap1 otx2 wnt11r foxo3a ppdpfa col2a1a fstl1b smyhc2 rab11a notch3 gli2b cdc25 dpysl3 sox1a neurod6a atp2b2 mtor hoxd3a nrarpb aggf1 ahcyl1 egfl7 glra4a dazl nr6a1a klf2a neurod2 cryaa a2ml olig2 cdkn1c nrg2b smarca5 znf503 ier2 clptm1 rho adcy8 tnc rbmx egr2b srgap2a tspo copb2 whsc1 fzd10 stx1b upf1 notch1a crb2b zeb2a tmem2 hoxa11b*
GO:0048731 system development	5.04E-11	144 (19.0%)	1848 (10.0%)	*ak2 cebp1 pou4f2 neurod4 tp63 wnt3 rassf8b tagln3b igf2a rps7 csrnp1b top2b appb bbs7 cyp26c1 mbnl2 lhx2b hoxc8a pho smoc2 pbx1a vmhc gata2a glcci1 snrpd1 itga6a frzb fgfr2 arrb1 ctnna2 ptrfb cyp26b1 dusp6 fstl1a sestd1 dnmt1 hdc six1a nrp2b nipblb maea arhgef11 stmn2a tfap2c melk bbs4 synj1 igf2b ephb3a ascl1b col17a1a slc40a1 gli2a ddx46 sfrp5 cdh2 lama1 her8a atg5 axin1 ocrl mstnb efna5b ccnd1 jag2 lmo4a tgif1 foxg1a mta3 dpysl2b shrprbck1r neurog1 tspan12 rab8a crkl prkci irx3a tcf7 scinla plxnb2a ndrg4 atp6v0b cdh6 tcf7l1a slit2 dctn1a ccdc40 top2a tal1 socs3a otx1a tagln mych dctn2 col27a1a runx1 trim33 rbpjb robo3 sart3 bmp4 rbp4l tfap2a plp1a stk11 nusap1 otx2 wnt11r foxo3a ppdpfa col2a1a fstl1b rab11a notch3 gli2b dpysl3 neurod6a atp2b2 mtor nrarpb aggf1 egfl7 glra4a nr6a1a klf2a neurod2 cryaa a2ml olig2 cdkn1c smarca5 znf503 adcy8 tnc egr2b srgap2a tspo copb2 whsc1 fzd10 notch1a crb2b zeb2a tmem2*
GO:0030154 cell differentiation	8.21E-11	91 (12.0%)	949 (5.1%)	*ak2 cebp1 smyhc1 neurod4 wnt3 top2b appb mbnl2 lhx2b pho tnnt3b pbx1a vmhc gata2a glcci1 ctnna2 dusp6 fstl1a hdc six1a nrp2b maea tfap2c melk bbs4 synj1 ephb3a stau2 ascl1b slc40a1 gli2a cdh2 lama1 her8a axin1 ocrl efna5b jag2 foxg1a dpysl2b smad2 neurog1 rab8a crkl prkci ihhb tcf7l1a slit2 dctn1a tal1 mych dctn2 runx1 trim33 ptprub rbpjb robo3 sart3 bmp4 dusp4 tfap2a stk11 nusap1 rnf41 wnt11r ppdpfa fstl1b notch3 gli2b dpysl3 neurod6a atp2b2 aggf1 egfl7 glra4a dazl nr6a1a klf2a neurod2 olig2 cdkn1c clptm1 adcy8 tnc egr2b srgap2a tspo notch1a crb2b zeb2a crebl2*
Molecular function				
GO:0003677 DNA binding	*2.49E-05*	*97 (12.8%)*	*1308 (7.1%)*	*cebp1 pou3f3b pou4f2 polr3k neurod4 arntl1b ddx21 mybl2 tp63 rassf8b top2b thraa lhx2b hoxc8a smarcc1b pbx1a gata2a atf7a tbpl1 smarca2 kdm5ba hoxb9a dnmt1 mycl1b six1a mcm7 ilf3b irf9 sos1 ascl1b hoxc13a rybpa maf zbed4 her8a pou3f3a arid1aa ssh1b tgif1 hmga1a foxg1a mta3 smad2 neurog1 cebpd irx3a hoxd9a tcf7l1a taf1 tfdp2 smad3a top2a tal1 otx1a pds5b znf711 runx1 h3f3b.1 her2 trim33 hoxb2a atrx ar rbpjb atf6 nfya nusap1 pknox2 otx2 aebp2 foxo3a nr2e1 nfe2l3 lcor pole3 sox1a neurod6a hoxd3a gon4l dazl nr6a1a klf2a neurod2 nr1d2a smarca5 pbx3b arid2 rbmx egr2b tmpoa nr5a5 upf1 pc thap11 crebl2 fam60al hoxa11b*
GO:0005515 protein binding	*2.43E-04*	*106 (14.0%)*	*1541 (8.4%)*	*dok6 smyhc1 neurod4 arntl1b ywhae1 coro6 tp63 wnt3 rassf8b kpnb1 kif3a igf2a top2b tbca thraa appb fbxw11b vmhc frs2b frzb fgfr2 arrb1 ctnna2 pth1b dnmt1 ktn1 mapre3b myhz1.1 mycl1b maea arhgef11 rel pfn2l bag2 igf2b epb41l3a sos1 ascl1b polr2c sfrp5 farp2 cdc37 lama1 ldb2b zbed4 her8a dlg1l axin1 calrl2 sst1.1 mstnb mark1 ccnd1 uspl1 jag2 uxt foxg1a*

GO:0097159 organic cyclic compound binding	*1.40E-03*	*261 (34.4%)*	*4882 (26.5%)*	*tmsb shrprbck1r bhlhe22 neurog1 cul5a scinla islr2 tcf7l1a ccnt2a ubr5 smad3a tal1 mych ryk h3f3b.1 her2 robo3 cct4 bmp4 usp5 pmchl nusap1 cfl2 wnt11r foxo3a calrl smyhc2 stc1l tcf12 pole3 gnao1a neurod6a nr6a1a neurod2 brd9 cryaa olig2 nrg2b eif4ebp3 znf503 ier2 gmfg srgap2a fzd10 stx1b ywhae2 ube2nb cul1b appl1 ak2 cebp1 ddx43 pou3f3b cdk8 smyhc1 pou4f2 arl4ab polr3k neurod4 mapk8b arntl1b rbmx2 rab8b denr ddx21 mybl2 tp63 khdrbs1a gnl1 rassf8b csnk1g2b mrps6 kif3a zbtb33 ptgs2b klf3 top2b srpk1a uba2 thraa ssb rbm4.2 ck2a2a nlk1 cyp26c1 eif4e1c lhx2b hoxc8a smarcc1b pbx1a scrt2 vmhc gata2a atf7a snrpd1 acadvl tbpl1 glis3 fgfr2 smarca2 zcchc24 cyp26b1 kdm5ba rbm18 fkbp4 hoxb9a cntn1b dnmt1 myhz1.1 plk3 mycl1b six1a mcm7 rnaset2 hemk1 fabp6 melk rap2b fkbp5 synj1 tlk1a rab3c cdc42 pabpc1b ilf3b rhot1b exosc1 actc1b ephb3a stau2 irf9 sos1 ascl1b hoxc13a pfkfb4l gli2a cyp2ad2 ddx46 fgfr3 egfra tia1 hnrnpl2 rybpa nme2a maf zbed4 fa2h prkacaa her8a ppil4 zbtb12.2 actr1 xgb mark1 insm1b pou3f3a chtf18 drg2 rnd2 arid1aa khsrp fabp10a ssh1b safb tgif1 parn hmga1a znfl1 foxg1a src mta3 sept7b hnrnph1l mak smad2 srsf6a neurog1 cebpd gne rab8a larp6 acin1a prkci irx3a rnd1 skiv2l myef2 sdhda hoxd9a tcf7l1a magoh arfrp1 ubr5 syncrip taf1 tfdp2 csnk1g1 smad3a rab6a top2a tal1 csk atp5a1 otx1a sept4a pds5b nnt znf711 ryk eef2b runx1 h3f3b.1 prkag1 pcca map2k1 her2 trim33 hoxb2a ftcd atrx pck1 ckmt2 eif4ba plagx ar pcbp3 mknk2a tuba2 rbpjb rnd3a pde6h sart3 atf6 nfya cct4 puf60a atp2a1l bcl6a stk11 nusap1 pknox2 otx2 aebp2 foxo3a sucla2 smyhc2 rab11a cyp1c1 pak1 rab7 nr2e1 insm1a gli2b nfe2l3 znf865 ak4 lcor pole3 gnao1a mapk7 larp7 sox1a neurod6a atp2b2 mtor hoxd3a pank4 hnrnpa1 aggf1 gon4l eif6 mapk4 raf1b dazl tceb3 nr6a1a klf2a neurod2 nr1d2a ptk2.1 smarca5 sp4 znf503 pbx3b khdrbs2 sept5b arid2 adcy8 pabpn1 scaper rbmx egr2b nme2b.2 cdk11b arl3l2 tmpoa nr5a5 upf1 klf9 pc thap11 adssl1 zeb2a xrn1 krr1 cyp2ad3 crebl2 fam60al cdkl5 hoxa11b*

Table 3.4 GO analysis of genes included in the cluster 3, data from the SOM clusters. Functional analysis of zebrafish genes whose expression is over-regulated in an atRA treatment for 72h.

Go term	p-value	Sample frequency (n=799)	Background frequency (n=18439)	Genes
Biological process				
GO:0009058 biosynthetic process	6.23E-05	157 (19.6%)	2373 (12.9%)	*eif4bb onecut1 gfm1 taf6 gmppaa hoxc9a dbt hoxa2b mrps7 setd8a nr1d1 rfc1 mthfd2l gata6 eif3ha med7 adcy7 hars agpat9 gabpa atp5l glulb b3gnt5b meox1 pbx2 amd1 foxo1a dlx5a mrpl19 hoxc12b dr1 polr2gl smyd2b meis4.1a prtfdc1 rx2 c1galt1b hif1an eif3ea got2a gtf2a1 rpl5b iars chst10 rpl8 hprt1 got1 phkg1a mocs3 pdhb paf1 rps23 msxa ccnk tp53rk mllt3 max rps2 adkb pigq coq3 rnasekb hmga2 edf1 gtf2f1 eif2s3 pcyt1ab eif3s6ip hif1al nfkb2 elp3 hmga1a foxg1a dbx1b pde8a crsp7 alg11 hoxa9b fos atp5d gne med4 st3gal4 eif2ak1 mrpl14 dnajc2 lias pts ass1 elp4 hmgcra yeats4 gmppb mppe1 cbsb foxf1 dhps eif3eb med20 mrps11 atp1a1b stt3b rpl3 mapk14a cars kars fezf1 adi1 ext1c narfl hey1 foxa1 setdb1b mrpl27 sars hoxb10a cebpg tarbp2 taf6l adsl hoxd13a ciapin1 rps5 gpib mtap alg6 dlat pkd2 nkx2.1b nr2e1 hoxa9a hoxc5a smyd2a eif3d mrrf nrarpb eif2s2 gpr161 hopx eif3s10 dph1 rfc5 trip4 dbx1a tpi1b taf12 elovl4b gmds esrrgb adra2da gch2 traf3 ccbl2 mcm3 hoxa11b tfb2m eef1g*
GO:0008152 metabolic process	1.93E-04	351 (43.9%)	6461 (35.0%)	*tceb1b eif4bb onecut1 cry1b gfm1 hist2h2l grpel1 taf6 prdx6 gmppaa capn1a trub1 hoxc9a atp6v0a1a dbt ppt1 jak2b atp6v1ba spsb4b snrnp27 gtpbpl senp3a hoxa2b mrps7 hsd17b4 setd8a prkar1aa pfkfb4 nr1d1 rfc1 mthfd2l gata6 bzw2 nlk1 ube2v2 gnai1 psmd4a eif3ha med7 adcy7 extl2 fnta hars pdha1a agpat9 hhip gabpa atp5l capn2a cst3 hk2 acy3.1 sdhb capn2l vbp1 fgfr2 glulb tgfbr2 b3gnt5b rdh10a meox1 fkbp14 pbx2 amd1 mettl7a foxo1a hibadhb dusp12 ndufa4 pgam1b agfg1b adam17a dlx5a mrpl19 opn1mw1 hoxc12b ecsit dr1 pin1 polr2gl smyd2b trmt5 rock2b ptprfb prkab1b pgm2 melk npc2 pop7 meis4.1a prtfdc1 tmx3 rx2 me3 c1galt1b asb4 hif1an psma5 rpe klfd pld1a sdhdb cyp51 eif3ea got2a bckdhb gtf2a1 rpl5b iars chst10 aph1b hnrnpl2 rpl8 hprt1 got1 phkg1a ptp4a1 dpysl5a fah mocs3 opn1lw2 pdhb tpst1l ndor1 paf1 phex ptbp1b rps23 gstm msxa ccnk tp53rk mllt3 max calrl2 jak1 rps2 adkb pigq mrpl44 coq3 camk2d2 hsdl2 rnasekb galca ca2 cdk7 sox10 hmga2 edf1 ppil2 gtf2f1 eif2s3 pcyt1ab ndufs2 b4galt4 ddc mmadhc eif3s6ip hif1al nfkb2 elp3 hmga1a foxg1a dbx1b pde8a crsp7 alg11 dpysl2b mtmr8 hoxa9b fos atp5d brca2 gne med4 st3gal4 tm7sf2 tmem68 eif2ak1 nap1l4a mrpl14 dnajc2 lias rnasen fuca1 pts ass1 pla2g6 papss2b ehmt1a araf vrk2 elp4 mmp13a hmgcra yeats4 tcp1 gna14 uba1 gmppb mppe1 cbsb foxf1 ptpn11a pglyrp6 glb1 dhps eif3eb med20 mrps11 dusp11 atp1a1b stt3b kdsr dnajb11 rpl3 trmt2a enpp1 adam9 pak2b cyp1c2 nnt mapk14a npsnl cars prkri kars fezf1 wdr12 wdr32 timm50 adi1 ide shmt1 ext1c bop1 narfl slc27a1b hey1 xab2 il17rd foxa1 sgk3 setdb1b dldh mrpl27 sars hoxb10a uap1 cebpg tfr2 sgk2b tarbp2 ercc1 trmt11 taf6l ctbs adsl hoxd13a tpte wdr4 ciapin1 rps5 gpib hk1 mtap alg6 sulf2l dlat pkd2 bcl2l1 mcat adam10a kras nkx2.1b nr2e1 ldhba msrb3 arl1 hoxa9a hoxc5a dpysl3 cct5 smyd2a eif3d sps2 sgk1 mrrf ppp1r14bb nrarpb eif2s2 socs3b immp2l ahcyl1 mapk4 uqcrh slc27a1a rdh10b gpr161 hpse n6amt2 irg1l prcp ugp2b map3k7 dnajc6 ftsj1 gnat2 map2k2b fem1c apg3l hopx hnmt baxa mut prkacbb rhbdl2 pitrm1 eif3s10 dph1 eno3 rfc5 trip4 d2hgdh htra1b dbx1a pfkmb opn1sw2 cyp27c1 tpi1b aldh5a1 taf12 ralgapa1 elovl4b p4hb ctsba gmds esrrgb adra2da pnp6 gch2 traf3 ccbl2 mcm3 cdkl5 hoxa11b abcb9 tfb2m eef1g pus7l*
GO:0009987 cellular process	7.00E-04	449 (56.2%)	8748 (47.4%)	*gosr1 tceb1b bbs1 xirp1 kazald3 eif4bb onecut1 thbs4b cry1b gfm1 hist2h2l grpel1 taf6 gmppaa trub1 slc39a1 gga1 rhbg hoxc9a atp6v0a1a dbt oxa1l snx5 ppt1 jak2b atp6v1ba coro6 slc25a33 spsb4b snrnp27 wdr43 gtpbpl cdc23 tlr5b hoxa2b mrps7 setd8a prkar1aa sdc2 pfkfb4 antxr2a paqr5a nr1d1 rfc1 mthfd2l gata6 bzw2 nlk1 ube2v2 atp6v1f gnai1 rab20 psmd4a tprb eif3ha med7 adcy7 fnta hars pdha1a taar14g ap1b1 agpat9 tnni2a.4 hhip gabpa atp5l asna1 hk2 sdhb parvb vbp1 or136-1 tomm20b fgfr2 glulb slc12a8 cx43.4 tgfbr2 pwp2h b3gnt5b slc2a15b rdh10a meox1 slc16a3 fkbp14 pbx2 daam1b amd1 foxo1a hibadhb dusp12 apobec2a lrrc39 ndufa4 pgam1b agfg1b adam17a dlx5a mrpl19 opn1mw1 hoxc12b dync1li1 dr1 lgals3bpb pin1 slc25a14 polr2gl smyd2b trmt5 nipblb fgf4 rock2b ptprfb ucp2 prkab1b stmn2a sox9b igf1 melk pop7 meis4.1a prtfdc1 tmx3 rx2 me3 c1galt1b asb4 timm23 hif1an arf3a psma5 rpe klfd gpr56 slc25a12 pld1a sdhdb vcl wisp2 ntn1b eif3ea got2a rab2a gtf2a1 slit1a rpl5b stxbp2 iars aph1b hnrnpl2 rpl8 hprt1 got1 phkg1a ptp4a1 plrg1 dpysl5a fah mocs3 opn1lw2 pdhb tpst1l pitpnb tmem41b ndor1 paf1 cnih ptbp1b sec31a rps23 msxa nitr9 ccnk tp53rk mllt3 arpc5lb max deptor calrl2 jak1 rab32a mstnb rps2 adkb pigq mrpl44 coq3 camk2d2 ewsr1a mfsd8 sept9b pa2g4b rnasekb rnd2 galca ca2 cdk7 sox10 slc37a4b hmga2 selt2 dcbld2 edf1 ppil2 gtf2f1 eif2s3 myd88 asb12b ddc amotl2a flvcr1 mmadhc eif3s6ip cradd hif1al nfkb2 elp3 lgals3bpa hmga1a foxg1a rab24 dbx1b bbs2 pde8a crsp7 atp6v0d1 dpysl2b fbln1 mtmr8 hoxa9b fos atp5d brca2 slc16a1 gne med4 dlb st3gal4 fzd6 eif2ak1 nap1l4a mrpl14 dnajc2 lias rnasen pcdh1a4 pts mul1b ass1 papss2b ehmt1a araf slc12a10.3 vrk2 elp4 mmp13a hmgcra yeats4 tcp1 gna14 uba1 gmppb mppe1 cbsb krit1 foxf1 ptpn11a dhps eif3eb med20 rab1a mrps11 dusp11 atp1a1b stt3b dnajb11 klf4a rpl3 bbs5 trmt2a slc43a1b adam9 pak2b mapk14a arf1l per1b tmx1 cars slc47a1 prkri zw10 kars fezf1 slc25a47b ssr3 mibp2 wdr12 wdr32 plagx timm50 adi1 shmt1 tomm22 ext1c bop1 hey1 xab2 il17rd syn1 foxa1 tusc3 sgk3 setdb1b dldh mrpl27 sars hoxb10a sept5a cebpg disp1 sgk2b tarbp2 ercc1 taf6l adnp2b adsl hoxd13a tpte wdr4 cnn3a cax1 ciapin1 rps5 itgb1b copa gpib hk1 mtap alg6 nup93 dlat pkd2 bcl2l1 tnnt2d adam10a kras nkx2.1b*

GO:1901576 organic substance biosynthetic process	7.42E-04	149 (18.6%)	2309 (12.5%)	*nr2e1 ldhba arl1 napgl hoxa9a hoxc5a dpysl3 ppp2r4 stmn2b cct5 smyd2a eif3d sps2 txndc9 sgk1 sox7 mrrf ppp1r14bb nrarpb eif2s2 socs3b immp2l ahcyl1 mapk4 uqcrh rdh10b glra4a gpr161 slc35b1 capza1a slc25a5 ap3s1 irg1l slc35b3 atp6v1e1b ric8b map3k7 dnajc6 ftsj1 atp6v0cb rgs14a gnat2 map2k2b fem1c tnfsf10l apg3l ntn1a thbs3a nrp1b hopx abcd1 baxa prkacbb opa1 eif3s10 dph1 eno3 rfc5 aktip trip4 slc9a3.2 gpr143 htra1b dbx1a pfkmb opn1sw2 dld sec24d tpi1b aldh5a1 taf12 ralgapa1 arl11 elovl4b p4hb cacnb2b aqp1a.1 ap2m1a gmds vsg1 esrrgb adra2da pnp6 chordc1 erh gch2 traf3 lamc1 ccbl2 cx35.4 mcm3 cdkl5 magt1 hoxa11b rgs19 abcb9 spns1 tfb2m optn eef1g pus7l*
GO:0044237 cellular metabolic process	8.46E-04	277 (34.7%)	4936 (26.8%)	*eif4bb onecut1 gfm1 taf6 gmppaa hoxc9a dbt hoxa2b mrps7 setd8a nr1d1 rfc1 mthfd2l gata6 eif3ha med7 adcy7 hars agpat9 gabpa atp5l glulb b3gnt5b meox1 pbx2 amd1 foxo1a dlx5a mrpl19 hoxc12b dr1 polr2gl smyd2b meis4.1a prtfdc1 rx2 c1galt1b hif1an eif3ea gtf2a1 rpl5b iars chst10 rpl8 hprt1 phkg1a mocs3 pdhb paf1 rps23 msxa ccnk tp53rk mllt3 max rps2 adkb pigq coq3 rnasekb hmga2 edf1 gtf2f1 eif2s3 eif3s6ip hif1al nfkb2 elp3 hmga1a foxg1a dbx1b pde8a crsp7 hoxa9b fos atp5d gne med4 st3gal4 eif2ak1 mrpl14 dnajc2 lias pts ass1 elp4 hmgcra yeats4 gmppb mppe1 cbsb foxf1 dhps eif3eb med20 mrps11 atp1a1b stt3b rpl3 mapk14a cars kars fezf1 adi1 ext1c hey1 foxa1 setdb1b mrpl27 sars hoxb10a cebpg tarbp2 taf6l adsl hoxd13a rps5 gpib mtap alg6 dlat pkd2 nkx2.1b nr2e1 hoxa9a hoxc5a smyd2a eif3d mrrf nrarpb eif2s2 gpr161 hopx eif3s10 rfc5 trip4 dbx1a tpi1b taf12 elovl4b gmds esrrgb adra2da gch2 traf3 mcm3 hoxa11b tfb2m eef1g* *tceb1b eif4bb onecut1 cry1b gfm1 hist2h2l grpel1 taf6 gmppaa trub1 hoxc9a atp6v0a1a dbt ppt1 jak2b atp6v1ba spsb4b snrnp27 gtpbpl hoxa2b mrps7 setd8a prkar1aa pfkfb4 nr1d1 rfc1 mthfd2l gata6 bzw2 nlk1 ube2v2 gnai1 psmd4a eif3ha med7 adcy7 fnta hars pdha1a agpat9 gabpa atp5l hk2 sdhb vbp1 fgfr2 glulb tgfbr2 b3gnt5b rdh10a meox1 fkbp14 pbx2 amd1 foxo1a hibadhb dusp12 ndufa4 pgam1b agfg1b dlx5a mrpl19 opn1mw1 hoxc12b dr1 pin1 polr2gl smyd2b trmt5 rock2b ptprfb prkab1b melk pop7 meis4.1a prtfdc1 rx2 me3 c1galt1b asb4 hif1an psma5 rpe sdhdb eif3ea got2a gtf2a1 rpl5b iars hnrnpl2 rpl8 hprt1 got1 phkg1a ptp4a1 dpysl5a fah mocs3 opn1lw2 pdhb tpst1l paf1 ptbp1b rps23 msxa ccnk tp53rk mllt3 max calrl2 jak1 rps2 adkb pigq mrpl44 coq3 camk2d2 rnasekb galca ca2 cdk7 hmga2 edf1 ppil2 gtf2f1 eif2s3 ddc mmadhc eif3s6ip hif1al nfkb2 elp3 hmga1a foxg1a dbx1b pde8a crsp7 dpysl2b mtmr8 hoxa9b fos atp5d brca2 gne med4 st3gal4 eif2ak1 nap1l4a mrpl14 dnajc2 lias rnasen pts ass1 papss2b ehmt1a araf vrk2 elp4 hmgcra yeats4 tcp1 gna14 uba1 gmppb mppe1 cbsb foxf1 ptpn11a dhps eif3eb med20 mrps11 dusp11 atp1a1b stt3b dnajb11 rpl3 trmt2a pak2b mapk14a cars prkri kars fezf1 wdr12 wdr32 timm50 adi1 shmt1 ext1c bop1 hey1 xab2 il17rd foxa1 sgk3 setdb1b mrpl27 sars hoxb10a cebpg sgk2b tarbp2 ercc1 taf6l adsl hoxd13a tpte wdr4 rps5 gpib hk1 mtap alg6 dlat kras nkx2.1b nr2e1 ldhba arl1 hoxa9a hoxc5a dpysl3 cct5 smyd2a eif3d sps2 sgk1 mrrf ppp1r14bb nrarpb eif2s2 socs3b immp2l ahcyl1 mapk4 uqcrh rdh10b gpr161 irg1l map3k7 dnajc6 ftsj1 gnat2 map2k2b fem1c apg3l hopx prkacbb eif3s10 dph1 eno3 rfc5 trip4 dbx1a pfkmb opn1sw2 tpi1b aldh5a1 taf12 ralgapa1 elovl4b gmds esrrgb adra2da pnp6 gch2 traf3 ccbl2 mcm3 cdkl5 hoxa11b abcb9 tfb2m eef1g pus7l*
GO:0044249 cellular biosynthetic process	1.14E-03	146 (18.3%)	2267 (12.3%)	*eif4bb onecut1 gfm1 taf6 gmppaa hoxc9a dbt hoxa2b mrps7 setd8a nr1d1 rfc1 mthfd2l gata6 eif3ha med7 adcy7 hars agpat9 gabpa atp5l glulb b3gnt5b meox1 pbx2 amd1 foxo1a dlx5a mrpl19 hoxc12b dr1 polr2gl smyd2b meis4.1a prtfdc1 rx2 c1galt1b hif1an eif3ea gtf2a1 rpl5b iars rpl8 hprt1 phkg1a mocs3 pdhb paf1 rps23 msxa ccnk tp53rk mllt3 max rps2 adkb pigq coq3 rnasekb hmga2 edf1 gtf2f1 eif2s3 eif3s6ip hif1al nfkb2 elp3 hmga1a foxg1a dbx1b pde8a crsp7 hoxa9b fos atp5d gne med4 st3gal4 eif2ak1 mrpl14 dnajc2 lias pts ass1 elp4 hmgcra yeats4 gmppb mppe1 cbsb foxf1 dhps eif3eb med20 mrps11 atp1a1b stt3b rpl3 mapk14a cars kars fezf1 adi1 ext1c hey1 foxa1 setdb1b mrpl27 sars hoxb10a cebpg tarbp2 taf6l adsl hoxd13a rps5 mtap alg6 dlat nkx2.1b nr2e1 hoxa9a hoxc5a smyd2a eif3d mrrf nrarpb eif2s2 gpr161 hopx eif3s10 dph1 rfc5 trip4 dbx1a taf12 elovl4b gmds esrrgb adra2da gch2 traf3 mcm3 hoxa11b tfb2m eef1g*
GO:0071840 cellular component organization or biogenesis	7.29E-03	90 (11.3%)	1273 (6.9%)	*xirp1 kazald3 gfm1 hist2h2l atp6v0a1a oxa1l coro6 gtpbpl cdc23 setd8a sdc2 antxr2a atp6v1f rrs1 eif3ha ap1b1 tnni2a.4 kctd5 asna1 fgfr2 pwp2h daam1b lrrc39 smyd2b fgf4 stmn2a psmg2 vcl wisp2 ntn1b eif3ea slit1a pitpnb tmem41b sec31a rpl7a arpc5lb rab32a ewsr1a amotl2a eif3s6ip elp3 foxg1a bbs2 atp6v0d1 dpysl2b fbln1 fzd6 nap1l4a dnajc2 mul1b ehmt1a krit1 eif3eb kctd10 zw10 mibp2 wdr12 timm50 bop1 narfl setdb1b disp1 cnn3a ciapin1 nup93 tnnt2d arl1 dpysl3 ppp2r4 stmn2b smyd2a eif3d immp2l capza1a atp6v1e1b apg3l ntn1a rsl24d1 nrp1b opa1 eif3s10 aktip htra1b dld sec24d chordc1 lamc1 tfb2m optn*
GO:0044281 small molecule metabolic process	9.86E-03	68 (8.5%)	888 (4.8%)	*gmppaa dbt atp6v1ba gtpbpl mthfd2l gnai1 adcy7 hars atp5l glulb amd1 hibadhb agfg1b npc2 prtfdc1 tmx3 me3 rpe got2a iars hprt1 got1 dpysl5a fah pdhb adkb coq3 ca2 ddc mmadhc dpysl2b atp5d gne lias ass1 hmgcra gna14 gmppb cbsb dhps atp1a1b cars kars adi1 shmt1 ext1c sars adsl mtap dlat kras arl1 dpysl3 ahcyl1 gpr161 irg1l gnat2 tpi1b aldh5a1 ralgapa1 elovl4b p4hb gmds adra2da pnp6 gch2 ccbl2 abcb9*
Molecular function				
GO:0003824 catalytic activity	4.08E-03	279 (34.9%)	5065 (27.5%)	*cry1b gfm1 mpped2 prdx6 gmppaa dhx38 capn1a trub1 dbt ddx49 ppt1 jak2b atp6v1ba gtpbpl senp3a hsd17b4 sult2st1 setd8a prkar1aa ctdsp1 pfkfb4 rfc1 mthfd2l nlk1 ube2v2 atp6v1f gnai1 adcy7 extl2 fnta hars gyg2 pdha1a agpat9 hhip capn2a asna1 hk2 acy3.1 vps4a sdhb nsun2 capn2l fgfr2 glulb tgfbr2 b3gnt5b rdh10a fkbp14 amd1 mettl7a hibadhb dusp12 apobec2a ndufa4 pgam1b adam17a dync1li1 ecsit pin1 hccs polr2gl smyd2b rbbp9 trmt5 rock2b ptprfb prkab1b pgm2 melk pop7 prtfdc1 tmx3 me3 c1galt1b myo5b nudt5 hif1an pofut1 psma5 rpe pld1a cyp51 uqcrc1 got2a bckdhb nat8l iars chst10 hprt1 got1 phkg1a ptp4a1 plrg1 dpysl5a fah mocs3 pdhb tpst1l ndor1 phex gstm tp53rk hddc2 jak1 adkb pigq mrpl44 coq3 camk2d2 hsdl2 nudt2 pa2g4b rnasekb galca ca2 abhd3 cdk7 ppil2 gtf2f1 eif2s3 pcyt1ab ndufs2 b4galt4 ddc cpped1 elp3 pde8a fpgt dpysl2b mtmr8 ddx23 atp5d gne st3gal4 tm7sf2 tmem68 eif2ak1 lias rnasen fuca1 pts mul1b ass1 skiv2l pla2g6 papss2b ehmt1a araf vrk2 mmp13a hmgcra gna14 uba1 gmppb mppe1 cbsb hccsa rnaseh1 ptpn11a pglyrp6 glb1 dusp11 atp1a1b stt3b kdsr trmt2a enpp1 adam9 pak2b cyp1c2 nnt mapk14a ppp1cab npsnl cars prkri kars mest neu1 timm50 adi1 ide shmt1 ext1c naa10 slc27a1b syn1 uqcrc2a ptrh2 sgk3 atxn3 setdb1b dldh sars uap1 tfr2 glo1 sgk2b ercc1 trmt11 ctbs adsl tpte wdr4 gpib hk1 mtap alg6 sulf2l dlat mcat adam10a kras ldhba msrb3 arl1 dpysl3 ppp2r4 smyd2a sps2 sgk1 cant1a dcp2 immp2l ahcyl1 mapk4 uqcrh slc27a1a rdh10b exosc4 hpse fhit ppapdc3 n6amt2 irg1l atp6v1e1b prcp ugp2b map3k7 dnajc6 ftsj1 gnat2 map2k2b apg3l ola1 nrp1b hnmt abcd1 neu3.2 mut prkacbb rhbdl2 opa1 pitrm1 ppap2c eno3 rfc5 aktip d2hgdh htra1b pfkmb cyp27c1 tpi1b aldh5a1 atp6v1g1 p4hb ctsba gmds pnp6 gch2 traf3 ccbl2 mcm3 cdkl5 abcb9 tfb2m nsfb pus7l*

3.4.5 Chemical analysis

Given the different temporal patterns of 9cRA and atRA effects, we considered important to check the relatively stability of both atRA and 9cRA at the embryo treatment conditions. HPLC analysis verified that both compounds were stable and detectable in filtered embryo water, at least for 44h, a period of time considered sufficient, as fish water was changed daily. Similar results were obtained in a functional assay, in which mRNA abundance of *cyp26a1* was determined after an exposure to freshly prepared dilutions or aged for 1 or 2 days for both compounds. In this experiment, no significant differences were observed on *cyp26a1* activation between aged or fresh atRA or 9cRA solutions (Figure 3.8).

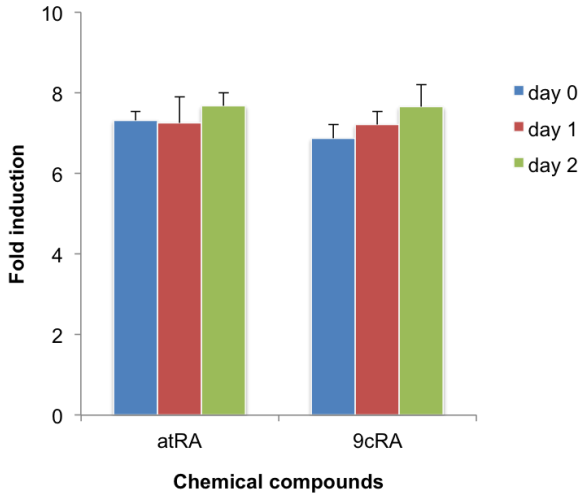


Figure 3.8 *cyp26a1* mRNA abundance at 72hpf after an exposure to 200ppb of 24h of each compound. The solutions prepared were aged for 1 and 2 days under the conditions of the assay.

3.5 Discussion

There are numerous advantages in using zebrafish as a toxicological model (Spitsbergen and Kent, 2003; Teraoka et al., 2003) since there is probably more knowledge about ‘‘what is normal’’ in the zebrafish than in any other fish species, which may help the identification of the adverse effects of chemical compounds. Zebrafish genome is fully sequenced and annotated, and there is precise information about its development, morphology, biochemistry and physiology not only at the embryo stage but also for juveniles and adults of both sexes.

The term "retinoids" refer to those chemicals that are structurally or functionally similar to retinol (Tang and Gudas, 2011), an essential biomolecule for embryonic development and adult body homeostasis. All retinoids have in common the presence of

a polyene hydrophobic tail attached to a cyclic 6-carbon ring. The polyene tail is characterized by the alternating conjugated carbon-carbon double bonds, which makes retinoids light-sensitive. Given their structures, retinoids are highly oil-soluble and able to diffuse across cell membrane. In general, retinoids are involved in regulatory processes related to cellular growth, apoptosis, immune response, and epithelial growth (De Luca, 1991; Smith et al., 1992; Means and Gudas, 1995; Ross et al., 2000) through the interaction with RAR/RXR receptors. The major active form of retinoids during early development, the atRA, regulates germ layer formation, body axis formation, neurogenesis, cardiogenesis, and the development of pancreas, lung and eye. It is also a critical element for visual function (Davies et al., 2010).

The RA metabolism forms a loop that regulates and balances atRA levels in embryos. Such regulation not only maintains the endogenous atRA level within a normal range, but also allows the organisms to respond to exogenous atRA fluctuation. The ectopic exposure to atRA is also teratogenic to the early embryonic stages of zebrafish. In zebrafish, embryos the degradation of endogenous RA works by the regulation of *raldh2* and *cyp26a1* expression and its synthesis is yielded by the *rdh10* expression. Thus the RA signalling down-regulate the expression of enzymes for atRA production, but up-regulated enzymes that can reduce atRA level in embryos (Oliveira et al., 2013).

In the present study we attempt to distinguish the action of Retinoic Acid isomers after an exposure to different concentrations of RA isomers, and determine the teratogenicity effects of each one. The stabilitity of each compound in "embryo water" was studied, and the results showed that for a period of 48h of aging the expression of *cyp26a1* did not change and that the UV absorption of both compounds was stable during the period of exposures. As described in (Herrmann, 1995) with respect to the chemical structure, the order of potency is found to be, atRA>=9cRA, for their scoring system. In our case the toxicity of both compounds was similar. No phenotypic differences were observed for the effects induced by both isomers in each related window of exposure. The longer exposure, during 72h, was the one inducing the most extreme deformations to the larvae, in comparison with the 24h exposures. As first hypothesis, we propose that the action of both compounds was similar and Figure 3.4 confirms that morphologically there are no differences on the effect. The principal morphological effects observed were pericardial and yolk sac edemas, tail malformation and a delay on the development.

To characterize the difference between long and short exposures, a microarray analysis was conducted with a sublethal-concentration for both compounds (100nM). The analysis was conducted in order to determine if the related compounds act through the same mode of action, or not. The period selected to collect the larvae was 5dpf after a 72h and 24h exposures. The transcriptomic analysis revealed a clear differentiation on

the mode of action for both compounds and also individualized the action of atRA in a longer exposure. The functional analysis evidences a set off genes that have clearly differentiated the action of atRA and 9cRA. The GO term enrichment analysis shows that genes related to transcription regulation are the ones that establish the differential behaviour after an exposure to atRA and 9cRA. Those genes are important for the starting machinery of eukaryotic translation. It was evidenced in (Fahrenkrug et al., 1999), that a particular isoform of the eukaryotic translation genes, the *elf4e* is a translational factor whose activity appears to be both ubiquitous and central for the regulation of protein synthesis in all cell-types during vertebrate oogenesis, gastrulation and erythropoiesis because translational control plays an important role during early development (Curtis et al., 1995; Groisman et al., 2001; Sonenberg and Hinnebusch, 2007).

The atRA 72h treatment exhibited stronger effects on the transcriptome, upon which we observed down regulation of genes from the *hox* family. This set of genes modulate the development of organs, physiological mechanisms, cellular processes, thus alterations on the expression of those genes lead to several teratogenic effects on the eleutheroembryo. On the other hand, 9cRA treatments did not show any clear transcriptomic differentiation between the two periods of exposure.

The establishment of the action of both retinoic acid isomers is of great importance, due to their similar properties and phenotypic effects on *Danio rerio*. Whereas both it has been described that atRA is a natural ligand and its presence is essential for the early development of zebrafish, the presence of 9cRA in early zebrafish embryo stages is controversial (Costaridis et al., 1996), and it may play only a minor role in the development. Intracellular concentration of 9cRA may be controled by its isomerization to atRA (Chen and Juchau, 1998), and this may explain the observed decrease of its transcriptional response upon time.

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4 - Transcriptomic effects of dioxin-like compounds in *Danio rerio* embryos

Transcriptomic effects of dioxin-like compounds in Danio rerio embryos

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In prep.

4.1 Abstract

Zebrafish embryos constitute a convenient model for studying effects of environmental stressors on development. Among these stressors, dioxin-like pollutants are especially relevant because of their ubiquity among environmental compartments and their known toxic effects (oxidative stress, genotoxicity, cardiotoxicity, and others) in many animal species, particularly in vertebrates. The study performed allowed to detect the transcriptional responses of *Danio rerio* embryos at sublethal concentrations of three compounds with recognized dioxin-like activity; two PAHs (Benzo[k]fluoranthene and Benzo[a]pyrene) and the natural compound β-Naphtoflavone. A total of 2201 unique genes (out of 15,883) appeared as differentially affected (p<0.001) by at least one of the treatments. Cluster analysis separated data from β-Naphtoflavone treated samples from the PAH treated ones. A defined cluster of genes showed a common pattern of variation for the three treatments, including an enhanced expression of the cytochrome P450 superfamily and other stress- related genes, corresponding to the well-known activation of the regulatory AhR by dioxin-like compounds. Another interesting group of genes was those whose transcripts were only affected upon a (5 days) longer exposure to Benzo[a]pyrene, a known mutagen. This group included many mitochondrial genes and other ones related to apoptosis or to cell death. We consider that these data suggest a novel mechanism of embryonic toxicity for B[a]Pyr, which compromises the cellular energy process and that may be related with other well known effects of B[a]Pyr, like cardiotoxicity or edemas. The analysis performed here may contribute to distinguish between different modes of action for pollutants that may share some targets in the cell (in this case, the aryl hydrocarbon receptor) but that, at the same time, differ in their toxic effects at the systemic level. Zebrafish embryos thus demonstrate their suitability for predicting the potential for developmental toxicity of environmental air/water samples.

Keywords: zebrafish, PAHs, β-Naphtoflavone, gene expression, mitochondria

4.2 Introduction

The determination of the role of the aryl hydrocarbon receptor (AhR) pathway in the toxicity of different Polycyclic Aromatic Hydrocarbons (PAHs) has been a major focus in recent years. There is a general agreement that dioxin-like toxicity is largely mediated through binding of pollutants to the AHR in mammalian models (Fernandez-Salguero et al., 1997), a ligand-activated transcription factor found in vertebrate species from fish to human (Schmidt and Bradfield, 1996). Polychlorinated dibenzo-*p*-dioxins, dibenzofurans, biphenyls and several other classes of chemicals, including some PAHs, bind to AhR and elicit a number of toxic and biochemical responses, such as induction of cytochromes P4501A1 and P4501A2, which are major enzymes of metabolic activation of many promutagens (Safe, 1993). The activation of AhR is considered an important determinant of carcinogenic and/or co-carcinogenic potency of chemicals (Cheung et al., 1993). The AhR-dependent induction of gene expression is widely used as a biomarker of dioxin-like toxicity, based on the assumption that AhR activation is a common initial event in adverse effects of dioxin-like compounds. In general, AhR pathways are similar among vertebrates, including fishes, reptiles, birds, and mammals (Hahn, 1998). Upon activation, the AhR translocates into the nucleus and heterodimerizes with the AhR nuclear translocator. The resulting complex binds to xenobiotic responsive elements (XREs) in the promotor region of AhR-responsive genes, initiating transcription (Hahn, 1998).

Unlike the single form of AHR found in mammals, zebrafish were found to express two forms of AhR, the *ahr1* and *ahr2*. Dioxin-like toxicity in zebrafish embryos is mediated via the aryl hydrocarbon receptor2 (*ahr2*, the functional AhR isoform) (Dong et al., 2004; Antkiewicz et al., 2006), a master regulator of the xenobiotic metabolism (e.g. cytochrome P450 1A) and implicated in the normal development and homeostasis (Fernandez-Salguero et al., 1997; Hahn, 1998; Walisser et al., 2004). Conversely, zebrafish *ahr1* lacks the ability to bind PAHs or planar halogenated aromatic hydrocarbons (pHAHs) *in vitro* and is transcriptionally inactive.

PAHs are a class of pollutants of global concern due to their toxicity, environmental persistence and widespread occurrence (Hafner et al., 2005). A wide range of PAHs have been reported to induce ecotoxicological effects in a diverse group of biota, such as microorganisms, terrestrial plants, aquatic and soil invertebrates (Bispo et al., 1999). PAHs are released due to both natural and anthropogenic activities, like burning of biomass or of fossil fuels, and they are found widespread in the environment. Many PAHs are suspected or known potent mutagens and carcinogens that may pose serious human and environmental health risks (Durant et al., 1996; Delistraty, 1997; Who 202, 1998; Durant et al., 1999). They also cause toxicity to the early life stages of fish species. Symptoms include increased cardiovascular dysfunction, pericardial and

yolk sac edemas, subcutaneous haemorrhages, craniofacial deformities, reduced growth, and increased mortality rates (Wassenberg and Di Giulio, 2004; Incardona et al., 2005; Billiard et al., 2006; Incardona et al., 2006). Benzo[a]Pyrene (B[a]Pyr), a high molecular weight (5-ring) PAH and a potent carcinogen and mutagen (Shaw and Connell, 1994; Banni et al., 2009), shows strong binding affinity for the AhR and is a potent inducer of cytochrome P4501A (Hahn, 1998; Aluru and Vijayan, 2008). This compound was selected as reference compound for this study, since it is generally used as the reference compound for carcinogenic PAHs (Durant et al., 1996; Delistraty, 1997; Who 202, 1998; Durant et al., 1999).

Zebrafish (*Danio rerio*) is recognized as a valuable vertebrate model for studying the effects of exogenous stressors on development, as well as a model for study of human diseases (Barut and Zon, 2000; Langheinrich, 2003; Spitsbergen and Kent, 2003; Hill et al., 2005). It is an oviparous species, with external fertilization and development, which allows for easy detection of morphological alterations and manipulation of the transparent embryos until ≈120 hours post fertilization (hpf). Zebrafish have rapid growth, high egg yield, and short generation time, and their genetics and developmental biology are well characterized. The sequenced zebrafish genome is currently in the final stages of assembly and annotation, and many molecular techniques have been developed to study gene function in this system. While there is extensive literature describing the effects of PAHs on adult or juvenile animals, few studies have addressed the effects of PAHs on embryonic and early larval stages of development. These early stages may be particularly susceptible to PAHs exposure, especially for species that spawn or rear near human settlements. There is evidence that fish embryos are highly sensitive to PAHs dissolved from oil; the biological effects of petrogenic PAHs in developing fish have been described in detail and are consistent across species (Marty et al., 1997; Carls et al., 1999; Heintz et al., 1999; Heintz et al., 2000; Kiparissis et al., 2003; Incardona et al., 2004; Incardona et al., 2005; Rhodes et al., 2005; Farwell et al., 2006). Zebrafish embryos exposed to individual PAHs, develop lordosis (spinal curvature), edema (pericardial and yolk-sac), cardiac and cranio-facial abnormalities, and exhibit enhanced anti-oxidative responses (Incardona et al., 2004; Wassenberg and Di Giulio, 2004).

The rapid development of genomics gives rise to numerous challenges and scientific opportunities to understand the potential risks of chemicals to humans' and ecosystems' health at a mechanistic level, enabling a more robust in-depth risk assessment. Considering the availability of DNA sequencing projects, genome-scale DNA sequence, and ESTs material for zebrafish; this information facilitates the development of resources to understand and identify the xenobiotics' mode of action at the molecular and cellular levels, thus characterizing the response and acclimatation of an organism to changes in the external environment (Coverdale et al., 2004).

The mechanisms underlying developmental toxicity of PAHs and dioxin type AhR agonists are under study. Identifying the pathways involved in PAH toxicity will provide a more robust, mechanistic-based tools for risk assessment of single compounds and complex environmental mixtures or environmental samples. As the AhR-mediated effects of PAHs could significantly contribute to their overall toxicity, mutagenicity and carcinogenicity, we aimed to characterize the profile of the genes involved in the AhR signalling cascade and to identify specific biomarkers of the action at sub-lethal concentrations of Benzo[a]pyrene, Benzo[k]fluoranthene, and β-Naphtoflavone during the early stages of zebrafish development. Whereas the induction of cardiovascular defects is very similar between PAH and pHAH-type AhR agonists, the roles of the AHR and *cyp1a* activity in the toxicity of PAH are less clear. In this study we attempted to further dissect the zebrafish mechanisms underlying PAH exposures using the genetic tools available to understand these toxic effects.

4.3 Material and Methods

4.3.1 Zebrafish embryo assays

Wildtype zebrafish were maintained at 28.5ºC (Kimmel et al., 1995) in a 12L:12D photoperiod in embryo water [90ug/ml of Instant Ocean (Aquarium Systems, Sarrebourg, France; http://zfin.org/), 0.58mM CaSO4.2H2O, dissolved in reverse osmosis purified water]. Zebrafish embryos were collected following adult mating and maintained at the same conditions as adults during the whole process. Animal stages were recorded as days or hours post-fertilization (dpf or hpf).

4.3.2 Reagents - embryo and eleutheroembryo treatment

Benzo[a]pyrene (B[a]Pyr; ≥ 96%), Benzo[k]fluoranthene (B[k]Fla; 98%) and β-Naphthoflavone (BNF, 90-95%) were purchased from Sigma-Aldrich (St. Louis, MO) (Figure 4.1). DMSO (99.5% GC) was obtained from Sigma-Aldrich. All dilutions are reported as nominal concentrations. Stock solutions were prepared in DMSO on the day of the experiment. Fertilized eggs were collected from zebrafish facility. Eggs were collected within the 1hpf (4-cell to 128-cell). Zebrafish embryos were exposed to 38.5ug/L, 33.2ug/L and 12.7ug/L of β-Naphtoflavone, Benzo[a]pyrene and Benzo[k]fluoranthene, respectively. Those concentrations were selected based on the results obtained for *cyp1a* induction in zebrafish exposures until saturating concentrations of those compounds in (Olivares et al., 2013), and correspond to the

EC_{50} values for the relative *cyp1a* mRNA abundance. Final DMSO concentration were 0.2% or lower in the exposure medium. The experiments start at the 4hpf and lasted 48h; after this period a large amount of embryos are still enclosed in the chorion. In addition, a longer exposure to B[a]Pyr (0.1mg/L, 0.4uM), designated L-B[a]Pyr from now on, was performed from 1 to 5 dpf. The experimental solutions were changed every day, 6 biological replicates were made per treatment. Negative controls were treated with the same proportion of DMSO. Untreated embryos were also collected at 1, 2, 3, 4, 5, 6 and 7dpf to determine the relative abundances of the *ahr2* and *cyp1a* transcripts.

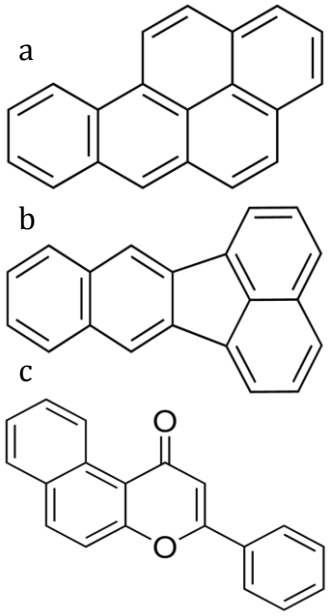

Figure 4.1 Structures of A: Benzo[a]pyrene; MW= 252.31g/mol; B: Benzo[k]fluoranthene; MW=252.31g/mol; C: β-Naphthoflavone; MW=272.30g/mol.

4.3.3 RNA extraction

Total RNA was isolated from 50 frozen embryos or eleutheroembryos using the Trizol reagent protocol (Invitrogen Life Technologies, Carlsbad, CA) and purified using standard methods following the manufacturer's protocol (RNeasy Kit; Qiagen). RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDrop ND-8000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality checked by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). RIN (RNA integrity) values ranged between 9.5 and 10 (maximal quality value in the Bioanalyzer). Microarray studies were performed using the commercial Agilent *D. rerio* (Zebrafish) Oligo Microarray v3 platform, using two-color strategy. The study included three biological replicates (independent embryos pools either untreated or treated with each studied compound), labelled and hybridized at the same time (results are deposited at GEO, reference: GSE41334).

4.3.4 Sample preparation for microarray analysis

The samples were prepared according to the Agilent Two-Color Microarray – Based Gene Expression Analysis protocol using Cyanine 3 (Cy3) and Cyanine 5 (Cy5) dye. As starting material, 200ng of total RNA for each sample was used. After the amplification, labelling and purification procedures, samples were evaluated for yield and effectiveness of Cy3 and Cy5 dye incorporation using the NanoDrop spectrophotometer.

Samples that presented at least a specific activity of 8 pmol Cy3 or Cy5 per μg cDNA and a quantity of 825ng labeled cRNA were used for the analysis. Fragmentation of the cRNA was performed according to the manufacturer instruction. A final volume of 100μl containing the fragmented cRNA was added to the 4x44K arrays and the hybridization proceeded for 17h at 65ºC. Microarray slides were washed according to the supplemental procedure Agilent protocol. The slides were kept on dark until scanning, using Agilent G2505C, and signals were extracted using the Agilent Feature Extraction Software v10.5.1.1.

4.3.5 Microarray data analysis

Raw data, was imported to the Robin, version 1.3.2 (Lohse et al., 2010) gene expression data analysis system version. Data from the three biological replicates of each condition were combined, resulting in an error-model weighted average of the three. The *p*-values for differential expression calculated by Robin were adjusted for multi-hypothesis testing using the Benjamini & Hochberg procedure, as implemented in the Bioconductor multitest package in R (http://www.bioconductor.org/packages/bioc/stable/src/contrib/html/multtest.html).
Genes for which the Benjamini & Hochberg-adjusted *p*-value was <0.001 within both comparisons were selected, resulting in the identification of a total of 2201 genes that showed robust expression changes during the time of exposure. Genes that were detected as differentially expressed were subjected to cluster analysis using the Pearson correlation algorithm implemented in MultiExperiment viewer MeV4 (Saeed et al., 2003) software. Gene ontology analyses were performed using Amigo! webpage (http://www.geneontology.org/); metabolic pathway information was obtained also from KEEG (http://www.genome.jp/kegg/kegg2.html) for zebrafish standard model organism.

4.3.6 Microarray validation and quantitation by qRT-PCR

Total RNA was extracted from the whole embryos, RNA was treated with DNAseI (Ambion, Austion, TX) to remove genomic DNA contamination. Quantities of 1µg were retro-transcribed to cDNA using Transcriptor First Strand cDNA Synthesis Kit (F. Hoffmann- La Roche, Basel Switzerland) and stored at -20ºC. Aliquots of 50ng 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). The selected gene primers used for qRT-PCR validation were designed from existing zebrafish nucleotide sequences.

Appropriate primers (Table 4.1) for 6 test genes (*cyp1a*, *gstp2*, *panx1*, *rxfp2*, *cyp4v2*, *rdh1*), were designed using Primer Express 2.0 software (Applied Biosystems) and the Primer-Blast server (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Amplification efficiencies were calculated as better than 90% for all tested genes as described (Pfaffl et al., 2002). House-keeping genes *eef1a1l1* and *ppiaa* were both selected as reference genes (Morais et al., 2007; Pelayo et al., 2012), as mRNA levels of neither gene changed upon treatment (Pfaffl et al., 2002). PCR products (amplicons) were sequenced in a 3730 DNA Analyzer (Applied Biosystems), and compared to the corresponding reference sequences at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Table 4.1 shows the actual sequences obtained from the amplification and their match to the corresponding sequences deposited in GeneBank.

Table 4.1 Sequences of primers used in this study for *q*RT-PCR.

Gene	Accession number	Primer sequence (5'-3') Forward	Reverse	Amplicon (bp)	Amplicon sequence
eef1a1l1	X77689	cgtctgccacttcaggatgtg	acttgcaggcgatgtgagcag	376	tacaaaattggaggtattggaactgtacctgt gggtcgtgtggagactggtgtcctcaagcct ggtatggttgtgaccttcgcccctgccaatgt aaccactgaggtcaagtctgttgagatgcac cacgagtctctgactgaggccactcctggtg acaacgttggcttcaacgttaagaacgtgtct gtcaaggacatccgtcgtggtaatgtggctg gagacagcaagaacgacccacccatgga ggctgccaacttcaacgctcaggtcatcatc ctgaaccaccctggtcagatctctcagggtt acgccccagtgctggattgccaca
ppiaa	AY391452	gggtggtaatggagctgaga	aatggacttgccaccagttc	179	gccgacgttgtccccagaactgcagagaat ttcaggcagttgtgcacgggtcagcctggct atggctacaagggatcctctttccatcgtgta attcctggtttcatgtgccagggaggtgactt cacaaaccacaatg
cyp1a	AB078927	ggttaaagttcaccgggatgc	ctgtggtgtgacccgaagaag	101	caggagaaatgctggatatgaccccagagt acgggttgaccatgaaacacaagcggtgt
gstp2	NM_001020513	cctgctgaatctgaaggtgct	gagctttgactttgggacgg	101	ttctccttcctgtctggactctttcccgtctctc aagagcttcgtggacaagatctctgc
panx1	NM_200916	tgcaagctagttgctgtggg	ggaaccaacatggcaaacatg	101	catcttccagctcctgagctacatcaaccttt cagtgtatttgctgctgttgccattgtg
rxfp2	NM_200443	agcgtcaccagctccacc	tggttgtccatgtcagccaa	101	atctacatggagacctcacgaaatcctgaat accccgcaaagaattctttaccacgactgtct
cyp4v2	NM_001077602	cagcggtttgctatcatgga	cccagtgggcgaagttctt	101	ggagaaggttattctggcttatattctgcgct actttaacatcgtagcatgtcaaaagaggg
rdh1	NM_198069	cgcactttctgcacgtcatc	tgtccactacacacgtgggc	101	ctcgaacacgttacgccgccggctgggac gcaaagttcctgtggatcccgctgtcgtatct

Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated by triplicates). To minimize errors on RNA quantification among different samples, Cp values for stress-related target genes (Cp_{tg}) were normalized to the average Cp values for *ppiaa*, used as reference gene for each sample, $corrCp_{tg} = Cp_{ppiaa}-Cp_{tg}$. Changes in mRNA abundance in samples from different treatments were calculated by the ΔΔ Cp method, (Pfaffl, 2001), using corrected Cp values from treated and non-treated samples ($\Delta\Delta Cp_{tg} = corrCp_{tg\_treated} - corrCp_{tg\_untreated}$). Fold-change ratios were derived from those values.

4.3.7 Statistical analysis

For transcriptomic analysis, genes were considered as differentially expressed using a confidence *p*-value <0.001. All statistical calculations for qRT-PCR data were performed using ΔΔCp values, as this parameter followed normal distributions (Levene's test). Differences among control and treated groups were analysed by Student's t-test (2 groups) or ANOVA plus Tukey's tests (more than 2 groups). No statistical differences were observed between the biological replicates of each treatment. Correlations between differentially expressed genes of each treatment (BNF, B[a]Pyr,

B[k]Fla and L-B[a]Pyr) were calculated using Spearman's correlation. Differences were considered statistically significant when *p*-value was <0.01.

4.3.8 Acridine Orange (AO) Staining

Apoptotic cells in zebrafish larvae were detected using AO (Tucker and Lardelli, 2007). Zebrafish embryos were exposed at 24hpf stage to 0.1mg/L of B[a]Pyr. At 48, 72, 96 and 120hpf apoptotic cells were stained with acridine orange (Sigma) by placing them in a 5ug/ml solution of the dye in embryo water for 1 hour, protected from light. The embryos were then washed repeatedly with embryos water, and again light protected to prevent bleaching. Images were acquired with a Nikon digital Sight DS-Ri1 camera and NIS Elements AR Software (version 3.0). Larvae were anesthetized with Phenoxyethanol 1:2000. All images were captured using identical exposure parameters. A parallel exposure to UV light (positive control) was conducted in order to validate the obtained measurements.

4.4 Results

4.4.1 Trancriptomic responses of zebrafish embryos to BNF and PAH exposure

The analysis of the four sets of microarrays identified 2201 unique sequences showing changes on their mRNA levels as a result of, at least, one of the treatments (Figure 4.2). The hierarchical clustering of microarray results clustered the replicates of each treatment, indicating the reproducibility of the observed changes. Comparing the different treatments, BNF-treated samples clearly separated from the rest, indicating that BNF and PAHs affected the transcriptome in different extends (Figure 4.2). Among PAH-treated samples, the lenght of the treatment (96 vs 24h, L-B[a]Pyr vs B[a]Pyr, respectively) appeared as more relevant than the nature of the PAH congener (B[a]Pyr vs B[k]Fla, Figure 4.2) The mutual relationship among the observed mRNA variations upon the different treatments is shown in the Venn diagram in Figure 4.3. A relatively small subset of genes (40) showed changes in all treatments, a number that increases up to 81 if only PAH treatments are considered. Most of these genes (39 out of 40, and 77 out of 81, respectively) showed increases, rather than decreases, in their mRNA levels upon the treatments, suggesting a general upregulation of at least a defined subset of promoters. Apart from these putative commonly regulated genes, BNF-affected showed very little overlapping with the rest of treatments, as 609 out of the 723 BNF-affected genes showed no changes upon PAH exposure (Figure 4.3). This overlap is much more

important among PAH treatments (332 genes changed in at least two of the treatments). Long term exposure to B[a]Pyr showed the highest number of specific changes, with 692 genes (out of 937) that did not showed changes by any treatments but L-B[a]Pyr (Figure 4.3). The results shown in Figure 4.2 and 4.3 demonstrate the different pattern of BNF-related changes in comparison to the PAH-induced ones, as well as the specificity of the long exposure to PAHs relative to shorter ones. They also suggest the presence of a regulatory cluster (a regulon) under the control of a BNF/PAH-inducible regulator, most likely the *ahr2*. Figure 4.4 plots fold changes of the 40 common genes by the three PAH treatments in front of the BNF exposure results. Most transcripts showed a 1.5-2.5 fold change (mean the log scale of the graph), except for two genes with a significantly higher induction (*cyp1a* and *sult6b1*) and a single one (*rlbp1b*, retinaldehyde binding protein 1b) showing a significant reduction on its mRNA levels (Figure 4.4). The observed variations in mRNA abundance for some selected genes were confirmed by qRT-PCR, showing a significant correlation (R^2=0.712) with the microarray data (Figure 4.5).

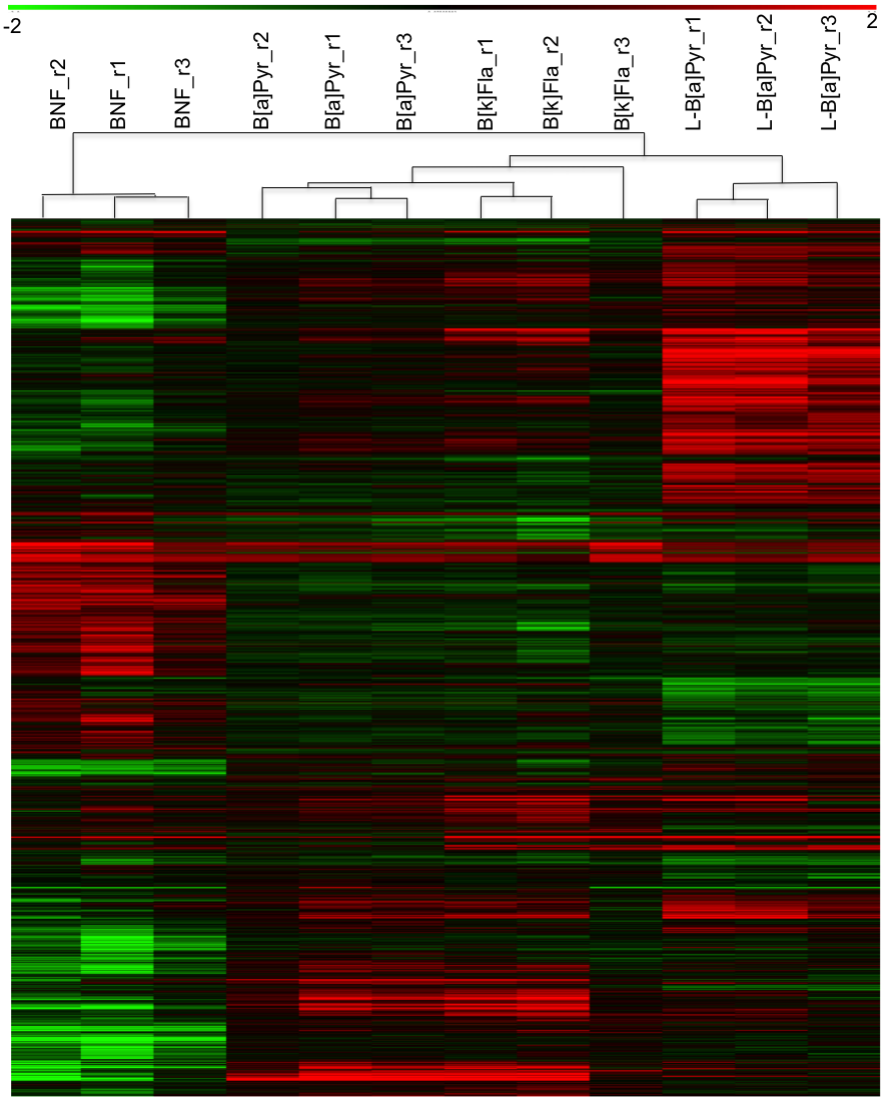

Figure 4.2 Heat map corresponding to the relative changes of the dioxin –like compounds *Danio rerio* responsive transcripts during the 48hpf for BNF, B[a]Pyr and B[k]Fla; L-B[a]Pyr states for the B[k]Pyr exposure during 96h. Only transcripts showing significative changes (p<0.001) are shown. Significative genes and samples (treatments) are hierarchically clusterd using Pearson correlation and average linkage.

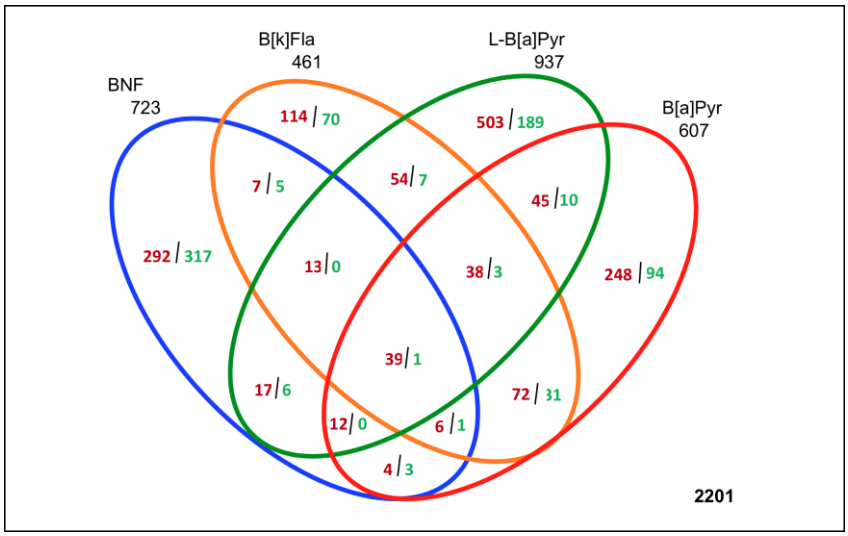

Figure 4.3 Venn diagram with the microarray results. Total number of up- and down- regulated genes of the 2201 transcripts differentially expressed.

Basal expression of *cyp1a* and *ahr2* transcripts, in the absence of any xenobiotic, was detectable, but very low, during the first 7 dpf (Figure 4.6). This low, but consistent presence of these transcripts is a pre-requisite to the use of zebrafish embryos to study *ahr2*-based transcriptional activation. In addition, their relatively low mRNA abundance at 1dpf suggests a small maternal contribution on the expression of *cyp1a* and *ahr2*, unlike other "canonical" nuclear receptors (Joore et al., 1994; Pelayo et al., 2012; Oliveira et al., 2013).

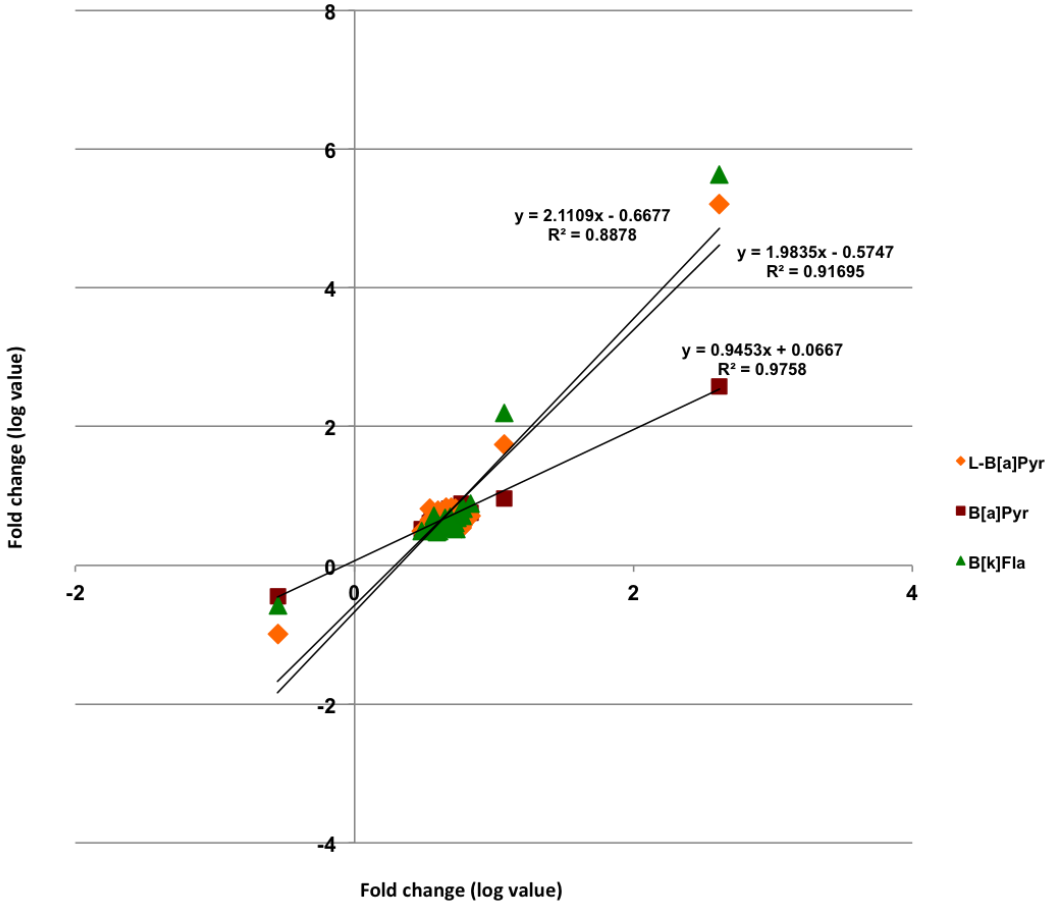

Figure 4.4 Lineal correlation between fold change (log 2 transformed) of zebrafish embryos exposed to BNF (x-axis) and the corresponding L-B[a]pyr, B[a]pyr and B[k]Fla treatment (y-axis) to zebrafish. Each dot corresponde to unique genes differentially expressed and shared by all the four different conditions, a total of 40 genes are common to the conditions. Regression lines, equations and r coefficients are indicated.

Dioxin-like compounds have in common the binding to the AhR receptor followed by the activation of AhR responsive genes. Moreover our results (Table 4.2) showed more than an elementary AhR pathway activation. The correlation between BNF and the others treatments is very moderated, in terms of fold change log2 comparisons of all the 2201 significative genes derived from the array analysis. The void correlation suggests that the PAHs may act through or activate other pathways that BNF does not.

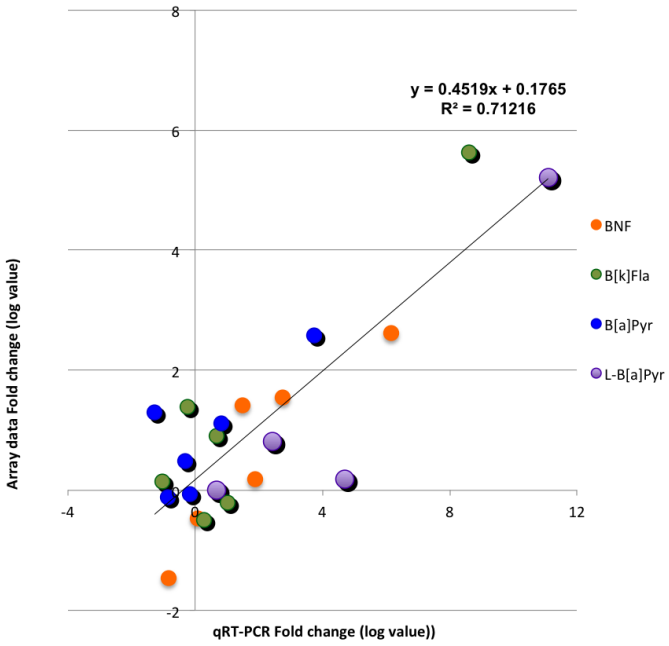

Figure 4.5 Correlation between the array data and the qRT-PCR data. Symbols correspond to individual conditions and each feature states for the expression of unique genes. Corresponding regression line between all the conditions and R^2 are also shown. mRNA relative abundance for selected genes: *cyp1a*, *gstp2*, *panx1*, *rxfp2*, *cyp4v2* and *rdh1*.

Table 4.2. Correlation between log FC values for the different conditions (2201 significativelly expressed genes).

	Spearman's rho			
	BNF	B[k]Fla	L-B[a]Pyr	B[a]Pyr
BNF	1			
B[k]Fla	0.057**	1		
L-B[a]Pyr	0.02	0.468***	1	
B[a]Pyr	-0.038	0.787***	0.423***	1

**. Correlation is significant at the 0.01 level (2-tailed).
***. Correlation is significant at the 0.0001 level (2-tailed).

Go enrichment terms analysis identified different structural and functional categories for over represented genes, within the L-B[a]Pyr condition. The functional profile of this set of zebrafish genes showed genes that are related to the mitochondrial cellular compartment and belong to the solute carrier family 25 (Table 4.3). In order to specify the function of the genes over-represented by this treatment, a different approach of the analysis was conducted. Those set of genes was loaded into the mouse genome informatics database, which endorse the data retrieve from zebrafish database. Not only genes related to the response to oxidative-stress, but also other genes related to the cell death, or regulation of the apoptotic process (Table 4.4) were detected, which is confirmed by the activation of *casp8* and *card14* genes.

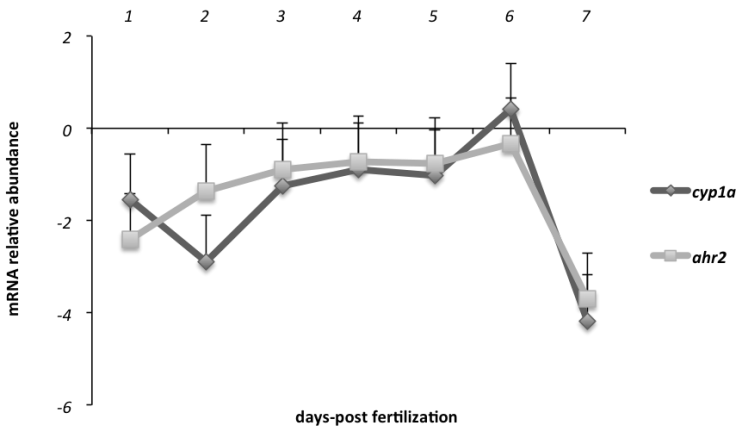

Figure 4.6 Differential contribution of *cyp1a* and *ahr2* gene expression, during the zebrafish early stages of development.

Table 4.3 Gene ontology analysis of transcripts significantly up-regulated by L-B[a]Pyr only in zebrafish eleutheroembryos.

Go term	Aspect	*p*-value	Sample frequency (n=272)	Background frequency (n=9066)	Genes
Cellular component					
GO:0044429 mitochondrial part	c	7.30E-04	17(6.2%)	134(1.5%)	*slc25a38a slc25a43 mrps31 ucp3 alas2 ucp2 slc25a47a agk slc25a25a slc25a33 alas1 sdha slc25a28 sdhb slc25a25b cox17 slc25a40*
GO:0005743 mitochondrial inner membrane	c	1.45E-03	12(4.4%)	70(0.8%)	*slc25a38a slc25a43 alas2 ucp2 slc25a47a slc25a25a slc25a33 sdha slc25a28 sdhb slc25a25b slc25a40*
GO:0005740 mitochondrial envelope	c	2.19E-03	15 (5.5%)	114(1.3%)	*slc25a38a slc25a43 ucp3 alas2 ucp2 slc25a47a agk slc25a25a slc25a33 sdha slc25a28 sdhb slc25a25b cox17 slc25a40*
GO:0005739 mitochondrion	c	2.36E-03	21 (7.7%)	213 (2.3%)	*slc25a38a slc25a43 mrps31 ucp3 alas2 ucp2 slc25a47a agk slc25a25a slc25a33 alas1 sdha hccs pi4k2a qrsl1 hccsa slc25a28 sdhb slc25a25b cox17 slc25a40*
GO:0019866 organelle inner membrane	c	2.69E-03	12(4.4%)	74(0.8%)	*slc25a38a slc25a43 alas2 ucp2 slc25a47a slc25a25a slc25a33 sdha slc25a28 sdhb slc25a25b slc25a40*
GO:0031966 mitochondrial membrane	c	4.72E-03	14 (5.1%)	106 (1.2%)	*slc25a38a slc25a43 ucp3 alas2 ucp2 slc25a47a agk slc25a25a slc25a33 sdha slc25a28 sdhb slc25a25b slc25a40*
GO:0031967 organelle envelope	c	4.71E-02	15 (5.5%)	146 (1.6%)	*slc25a38a slc25a43 ucp3 alas2 ucp2 slc25a47a agk slc25a25a slc25a33 sdha slc25a28 sdhb slc25a25b cox17 slc25a40*
GO:0031975 envelope	c	5.11E-02	15 (5.5%)	147 (1.6%)	*slc25a38a slc25a43 ucp3 alas2 ucp2 slc25a47a agk slc25a25a slc25a33 sdha slc25a28 sdhb slc25a25b cox17 slc25a40*
Molecular function					
GO:0033549 MAP kinase phosphatase activity	F	7.79E-02	4 (1.5%)	8 (0.1%)	*dusp5 dusp3b dusp6 dusp2*
GO:0004252 serine-type endopeptidase activity	F	8.86E-02	8(2.9%)	46 (0.5%)	*ctrl ela2 ela3l lonp2 htra1b ela2l try tmprss13a*

Table 4.4 Gene ontology analysis of transcripts significantly affected by L-B[a]Pyr using mouse genome informatics database.

Go term	Aspect	P-value	Sample frequency (n=166)	Brackground frequency (n=25495)	Genes
Biological process					
GO:0008152 metabolic process	1.19E-15	117 (70.5%)	9276/(36.4%)	*Lpin1 Med30 Sgk1 Tdh Rel Pcmtd1 Nfil3 Gale Dusp6 Pias1 Flad1 Ide Rps14 Tfap2c Nr4a1 Gnl2 Lipg Ucp3 Gclm Btg2 Cpb1 Ctrl Pah Ptpn22 Atf3 Alas2 Cela2a Cnksr1 Cox15 Il6ra Ovol1 Xpc Cox17 Klf3 Nudt22 Agk Runx3 Ercc2 Tspo Pi4k2a Ripk4 Mob3c Cpa2 Tsku Gss Cebpd Pycr1 Dusp5 Egln1 Casp8 Bmp4 Tceb3 Qrsl1 Dio1 Tapbp Etfa Esrp2 Rgs2 Surf1 Fkbp5 Dusp2 Enpp4 Rasd1 Plekhf1 Gmds Setdb2 Xiap Mmp9 Pdk2 Smek2 Aldh3b1 Agmo Rem1 B4galt1 Cybrd1 Flcn Alas1 Klf9 Tbc1d5 Rps18 Tnfrsf1a Psme2 Uevld Esrp1 Htatip2 Sdhb Sdha C1qtnf1 Txnrd1 St6galnac2 Atg4b Lonp2 Fkbp1b Myog Gna14 Ankk1 Trmt61a Inhbb Sqrdl Elane Spry4 Neil1 Smox Card14 Fgf7 Dhodh Nfs1 Tat Cebpb Irf7 Nox1 Gnpnat1 Trib3 Ppil1 Chac1 Cpa5 Bbc3*	
GO:0010941 regulation of cell death	8.49E-06	28 (16.9%)	1176 (4.6%)	*Sgk1 Rel Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Txnrd1 Inhbb Card14 Dhodh Cebpb Bbc3*	
GO:0010942 positive regulation of cell death	1.64E-05	17 (10.2%)	441 (1.7%)	*Dusp6 Nr4a1 Runx3 Ercc2 Tspo Bmp4 Ucp2 Plekhf1 Mmp9 B4galt1 Flcn Lta Htatip2 Txnrd1 Dhodh Cebpb Bbc3*	
GO:0008219 cell death	4.64E-05	31 (18.7%)	1528 (6.0%)	*Sgk1 Rel Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 Pdk2 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Txnrd1 Inhbb Card14 Dhodh Cebpb Trib3 Chac1 Bbc3*	
GO:0042981 regulation of apoptotic process	4.98E-05	26 (15.7%)	1113(4.4%)	*Sgk1 Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Inhbb Card14 Dhodh Cebpb Bbc3*	
GO:0016265 death	5.15E-05	31 (18.7%)	1535 (6.0%)	*Sgk1 Rel Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 Pdk2 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Txnrd1 Inhbb Card14 Dhodh Cebpb Trib3 Chac1 Bbc3*	
GO:0043067 regulation of programmed cell death	6.29E-05	26 (15.7%)	1126 (4.4%)	*Sgk1 Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Inhbb Card14 Dhodh Cebpb Bbc3*	
GO:0006979 response to oxidative stress	7.64E-05	12 (7.2%)	220 (0.9%)	*Ucp3 Gclm Ercc2 Pycr1 Egln1 Ucp2 Mmp9 Pdk2 Aldh3b1 Txnrd1 Neil1 Tat*	
GO:0044281 small molecule metabolic process	1.14E-04	34 (20.5%)	1864 (7.3%)	*Lpin1 Tdh Flad1 Ide Gnl2 Ucp3 Gclm Pah Gss Pycr1 Egln1 Qrsl1 Rgs2 Surf1 Rasd1 Gmds Pdk2 Smek2 Agmo Rem1 Flcn Tbc1d5 Tnfrsf1a Sdhb Sdha Txnrd1 Lonp2 Gna14 Fgf7 Dhodh Nfs1 Tat Gnpnat1 Trib3*	
GO:1901576 organic substance biosynthetic process	1.30E-04	57 (34.3%)	4320 (16.9%)	*Lpin1 Med30 Rel Nfil3 Pias1 Flad1 Rps14 Tfap2c Nr4a1 Gclm Btg2 Pah Atf3 Alas2 Cox15 Il6ra Ovol1 Klf3 Agk Runx3 Ercc2 Tspo Pi4k2a Gss Cebpd Pycr1 Dusp5 Egln1 Bmp4 Tceb3 Qrsl1 Rgs2 Surf1 Rasd1 Pdk2 Smek2 Agmo B4galt1 Flcn Alas1 Klf9 Rps18 Tnfrsf1a Htatip2 St6galnac2 Myog Inhbb Elane Card14 Fgf7 Dhodh Nfs1 Cebpb Irf7 Nox1 Gnpnat1 Trib3*	
GO:0006915 apoptotic process	1.37E-04	29 (17.5%)	1425 (5.6%)	*Sgk1 Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 Pdk2 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Inhbb Card14 Dhodh Cebpb Trib3 Chac1 Bbc3*	
GO:0019222 regulation of metabolic process	1.75E-04	60 (36.1%)	4712 (18.5%)	*Lpin1 Med30 Rel Nfil3 Dusp6 Pias1 Ide Rps14 Tfap2c Nr4a1 Lipg Gclm Btg2 Atf3 Il6ra Ovol1 Cox17 Klf3 Runx3 Ercc2 Tspo Tsku Cebpd Dusp5 Egln1 Casp8 Bmp4 Tceb3 Esrp2 Rgs2 Dusp2 Rasd1 Plekhf1 Xiap Pdk2 Smek2 Flcn Klf9 Tbc1d5 Tnfrsf1a Psme2 Esrp1 Htatip2 C1qtnf1 Atg4b Lonp2 Fkbp1b Myog Inhbb Elane Spry4 Neil1 Card14 Fgf7 Cebpb Irf7 Nox1 Trib3 Chac1 Bbc3*	

GO:0012501 programmed cell death	1.85E-04	29 (17.5%)	1445 (5.7%)	*Sgk1 Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 Pdk2 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Inhbb Card14 Dhodh Cebpb Trib3 Chac1 Bbc3*
GO:1901564 organonitrogen compound metabolic process	2.17E-04	29 (17.5%)	1456 (5.7%)	*Tdh Flad1 Ide Gnl2 Gclm Pah Alas2 Cox15 Agk Gss Pycr1 Egln1 Qrsl1 Tapbp Rgs2 Surf1 Rasd1 Rem1 Flcn Alas1 Tbc1d5 Txnrd1 Lonp2 Gna14 Smox Fgf7 Dhodh Nfs1 Tat*
GO:0048519 negative regulation of biological process	3.74E-04	46 (27.7%)	3196 (12.5%)	*Lpin1 Med30 Sgk1 Rel Nfil3 Dusp6 Pias1 Ide Rps14 Tfap2c Gclm Btg2 Ptpn22 Atf3 Il6ra Ovol1 Xpc Runx3 Ercc2 Tspo Tsku Cebpd Dusp5 Casp8 Bmp4 Rgs2 Rgs13 Dusp2 Rasd1 Ucp2 Xiap B4galt1 Flcn Lta Tnfrsf1a Fkbp1b Inhbb Elane Spry4 Card14 Cebpb Invs Trib3 Chac1 Bbc3 Cish*
GO:0006796 phosphate-containing compound metabolic process	3.77E-04	40 (24.1%)	2559 (10.0%)	*Lpin1 Sgk1 Dusp6 Flad1 Ide Gnl2 Ptpn22 Cnksr1 Il6ra Agk Runx3 Ercc2 Pi4k2a Ripk4 Mob3c Dusp5 Bmp4 Rgs2 Surf1 Dusp2 Rasd1 Gmds Pdk2 Smek2 Rem1 Flcn Tbc1d5 Tnfrsf1a Htatip2 Txnrd1 Fkbp1b Gna14 Ankk1 Spry4 Card14 Fgf7 Dhodh Nfs1 Gnpnat1 Trib3*
GO:0043068 positive regulation of programmed cell death	3.93E-04	15 (9.0%)	422 (1.7%)	*Dusp6 Nr4a1 Runx3 Ercc2 Tspo Bmp4 Plekhf1 Mmp9 B4galt1 Flcn Lta Htatip2 Dhodh Cebpb Bbc3*
GO:0055114 oxidation-reduction process	5.98E-04	22 (13.3%)	932 (3.7%)	*Tdh Pah Cox15 Pycr1 Egln1 Dio1 Etfa Surf1 Aldh3b1 Agmo Cybrd1 Flcn Uevld Htatip2 Sdhb Sdha Txnrd1 Lonp2 Sqrdl Smox Dhodh Nox1*
GO:0043065 positive regulation of apoptotic process	2.08E-03	14 (8.4%)	417 (1.6%)	*Dusp6 Nr4a1 Runx3 Ercc2 Tspo Bmp4 Plekhf1 Mmp9 B4galt1 Flcn Lta Dhodh Cebpb Bbc3*
Cellular component				
GO:0005739 mitochondrion	6.14E-08	37 (22.3%)	1635 (6.4%)	*Lpin1 Sgk1 Tdh Ide Rps14 Slc25a33 Ucp3 Mrps31 Alas2 Cox15 Cox17 Agk Slc25a28 Tspo Pi4k2a Rpl34 Pycr1 Casp8 Qrsl1 Etfa Esrp2 Surf1 Hccs Ucp2 Pdk2 Slc25a40 Alas1 Sdhb Sdha Slc25a43 Txnrd1 Sqrdl Dhodh Nfs1 Tat Bbc3 Slmo2*
GO:0044429 mitochondrial part	2.08E-07	22 (13.3%)	601 (2.4%)	*Lpin1 Slc25a33 Ucp3 Mrps31 Alas2 Cox15 Slc25a28 Tspo Etfa Surf1 Hccs Ucp2 Pdk2 Slc25a40 Alas1 Sdhb Sdha Slc25a43 Sqrdl Dhodh Nfs1 Bbc3*
GO:0043226 organelle	6.98E-05	101 (60.8%)	10079 (39.5%)	*Lpin1 Med30 Sgk1 Tdh Rel Nfil3 Pias1 Ide Rps14 Ubxn4 Tfap2c Nr4a1 Gnl2 Slc25a33 Ucp3 Mrps31 Ptpn22 Sdad1 Atf3 Alas2 Cox15 Ovol1 Xpc Cox17 Klf3 Agk Runx3 Slc25a28 Ercc2 Tspo Zfand2a Pi4k2a Rpl34 Cebpd Pycr1 Dusp5 Egln1 Casp8 Bmp4 Tceb3 Rrp12 Qrsl1 Dio1 Tapbp Etfa Esrp2 Rgs2 Surf1 Rgs13 Fkbp5 Dusp2 Hccs Rasd1 Ucp2 Plekhf1 Yipf1 Setdb2 Xiap Ric8b Pdk2 Smek2 Agmo B4galt1 Flcn Stard10 Slc25a40 Alas1 Klf9 Rps18 Tnfrsf1a Esrp1 Htatip2 Sdhb Sdha Slc25a43 Txnrd1 St6galnac2 Lonp2 Fkbp1b Myog Trmt61a Sqrdl Elane Neil1 Smox Fgf7 Dhodh Nfs1 Tat Cebpb Invs Irf7 Bcas3 Nox1 Gnpnat1 Trib3 Ppil1 Chac1 Bbc3 Slmo2 Fbxo32*
GO:0005740 mitochondrial envelope	7.29E-05	17 (10.2%)	488 (1.9%)	*Lpin1 Slc25a33 Ucp3 Alas2 Cox15 Slc25a28 Tspo Surf1 Hccs Ucp2 Slc25a40 Sdhb Sdha Slc25a43 Sqrdl Dhodh Bbc3*
GO:0044464 cell part	1.72E-04	127 (76.5%)	14396 (56.5%)	*Lpin1 Med30 Sgk1 Tdh Rel Pcmtd1 Nfil3 Dusp6 Pias1 Gipc2 Ide Rps14 Ubxn4 Tfap2c Mob1a Nr4a1 Gnl2 Slc25a33 Ucp3 Gclm Mrps31 Fgfr1op2 Ptpn22 Sdad1 Atf3 Alas2 Cnksr1 Cox15 Il6ra Ovol1 Xpc Cox17 Itgb4 Klf3 Mpzl2 Agk Runx3 Slc25a28 Ercc2 Tspo Zfand2a Pi4k2a Ripk4 Rpl34 Cebpd Pycr1 Dusp5 Egln1 Casp8 Bmp4 Tceb3 Rrp12 Qrsl1 Dio1 Tapbp Etfa Esrp2 Rgs2 Surf1 Cdc123 Rgs13 Fkbp5 Dusp2 Hccs Enpp4 Rasd1 Ucp2 Plekhf1 Yipf1 Gmds Setdb2 Xiap Ric8b Pdk2 Klhl24 Smek2 Aldh3b1 Agmo B4galt1 Cybrd1 Flcn Stard10 Slc25a40 Alas1 Cmbl Klf9 Tbc1d5 Rps18 Tnfrsf1a Esrp1 Htatip2 Sdhb Sdha Slc25a43 Txnrd1 St6galnac2 Atg4b Lonp2 Fkbp1b Myog Gna14 Trmt61a Inhbb Sqrdl Elane Spry4 Neil1 Smox Card14 Fgf7 Dhodh Nfs1 Tat Cebpb Invs Ier2 Irf7 Bcas3 Nox1 Gnpnat1 Trib3 Ppil1 Chac1 Bbc3 Slmo2 Cish Fbxo32*
GO:0005743 mitochondrial inner membrane	2.17E-04	14 (8.4%)	346 (1.4%)	*Slc25a33 Ucp3 Alas2 Cox15 Slc25a28 Surf1 Hccs Ucp2 Slc25a40 Sdhb Sdha Slc25a43 Sqrdl Dhodh*

Understanding the relationship between the zebrafish and human/mouse genomes will help to identify roles for human/mouse diseases (Brownlie et al., 1998). Due to the fundamental similarities among vertebrates, the identification of zebrafish genes can provide important insights about functions that are conserved in other vertebrates, including humans. Large segments of zebrafish chromosomes are syntenic with those of the human and mouse genomes, and many genes have been demonstrated to have a high degree of sequence homology (Barbazuk et al., 2000). One complication is the fact that fish, including zebrafish, have undergone gene duplication and subsequent diversion of gene expression patterns and functions (Van de Peer et al., 2002). This new approach was included in the analysis due to the fact that, mouse genome has a notable functional annotation.

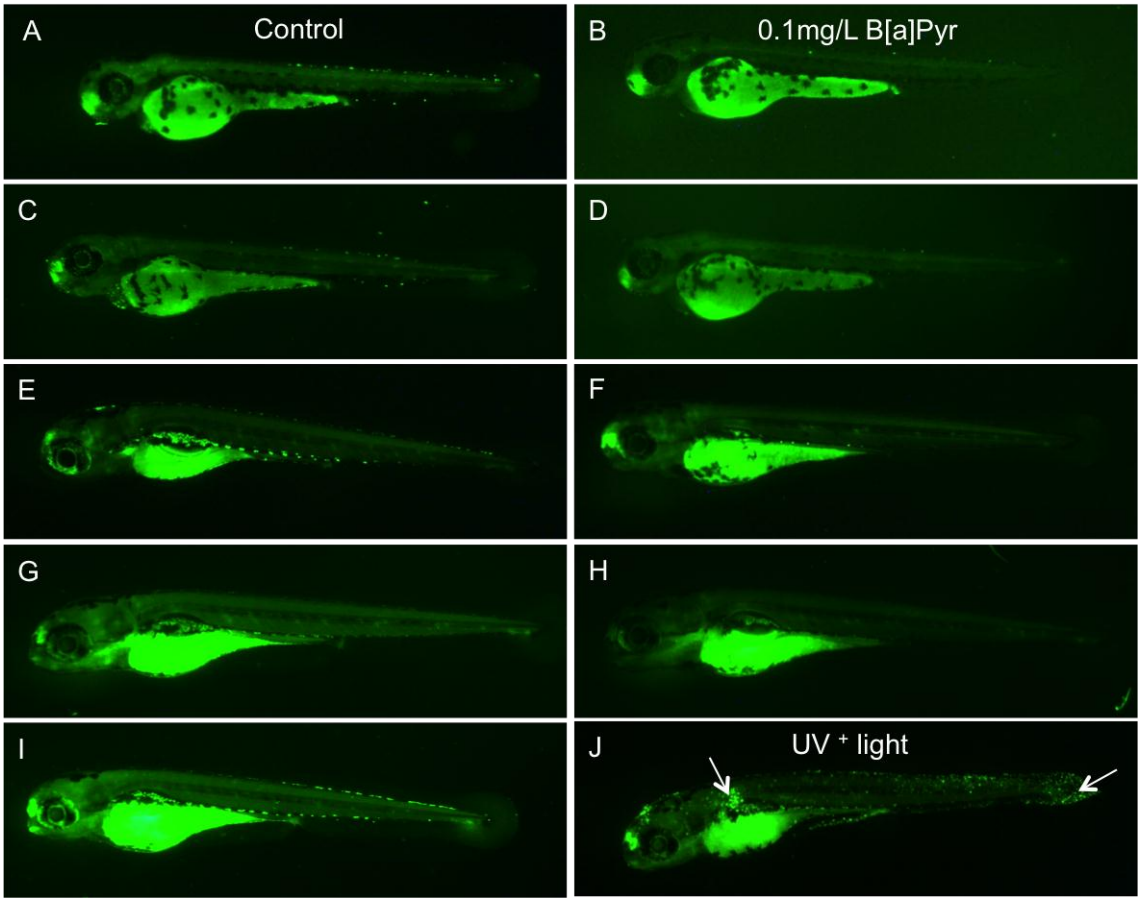

Figure 4.7 Cell death assay measured with the acridine orange staining in *Danio* rerio early stages of development after the B[a]Pyr 0.1mg/L treatment. A and B show embryos with 48hpf, C and D represents larvae with 72hpf, E, F, I and J are larvae with 96hpf, G and H states for 120hpf larvae. White arrow indicates the body parts in which the apoptotic process of the cells is visible.

The overall function analysis of the 3 PAHs conditions without the BNF-treated groups, showed an over-expression of the genes related to the oxidation-reduction process, for zebrafish the background genes present in our array (Table 4.5). The genes

exclusively related to the BNF treatment were grouped separatly from the PAHs – treated ones, which constitute a major fact. Due to the impossibility of having sigificative GO enrichment terms for those BNF set of up-regulated genes for this treatment, the analysis was conducted with the over and under expressed genes in the mouse database (Table 4.6). Those genes are related to the cellular Amino Acid (aa) metabolic process. The genes included in the analysis could be observed in the (Figure 4.8) where the metabolic routes are displayed.

The functional analysis results lead us to the assumption that the long term, B[a]Pyr exposure (until the 5dpf), induced cell death in zebrafish. To verify this observation, acridine orange staining onto L-B[a]Pyr treated eleutheroembryos was performed. In fact, results indicate that the treated samples do not show an obvious increase in the amount of death cells (Figure 4.7), besides that they present the regular AhR symptoms, such as the pericardial edema.

Table 4.5 Gene ontology analysis of transcripts significantly genes over expressed by the PAHs treatments zebrafish database.

Go term	Aspect	*P*-value	Sample frequency (n=617)	Brackground frequency (n=9066)	Genes
Biological process					
GO:0055114 oxidation-reduction process	P	9.74E-04	53 (8.6%)	382 (4.2%)	*cybrd1 akr1a1a mpx msrb1a cyp2y3 adhfe1 sccpdhb egln1 cyp2aa8 sdha pycr1 aldh9a1b uevld hgd ptgs1 cyp2x7 grhprb alox12 hmox1 cybb pah rdh8b sqrdl cyp2p7 cyp2aa4 cyp2aa7 smox txnrd1 dhodh mao agmo taldo1 plod2 msrb1b cyp2p6 dio1 cyp2aa2 gpd1c ptgs2b cyp24a1 acox3 cox15 hsd17b12a aldh8a1 sdhb cyp4v8 aldh3b1 cyp2p9 cyp7a1a nox1 dao.1 cyp1c1 cyp4v7*
Cellular component					
GO:0005576 extracellular region	C	6.72E-03	54 (8.8%)	417 (4.6%)	*otop1 cetp calub nppa stc1l cel.2 spaw fn1b bmp4 rspo3 pi4k2a tnfsf10 cyr61l1 b2m lft1 c1qtnf1 edn1 hbl4 pomcb thbs3b igf2b igfbp1b tnfb ctgfa ntn4 nbl1 arsk thbs3a bmp15 lipg inhbb ptgdsb tsku c1qtnf9 htra1b sst2 ccl1 plod2 apol1 ctsl1b masp2 furinb kazald2 lamb4 zp2 inhbaa f7 ins serpinc1 igfbp7 pdgfab lcn15 cxcl14 apoeb*
Molecular function					
GO:0016491 oxidoreductase activity	F	2.52E-04	60 (9.7%)	438/9067 (4.8%)	*cybrd1 akr1a1a mpx flj13639 msrb1a cyp2y3 adhfe1 sccpdhb egln1 cyp2aa8 dhdhl pdia2 bco2l sdha pycr1 aldh9a1b uevld hgd ptgs1 cyp2x7 grhprb alox12 hmox1 cybb pah rdh8b sqrdl cyp2p7 cyp2aa4 cyp2aa7 smox prdx5 txnrd1 dhodh surf1 mao dhrs3b agmo plod2 msrb1b cyp2p6 dio1 cyp2aa2 gpd1c ptgs2b cyp24a1 acox3 cox15 hsd17b12a aldh8a1 sdhb rdh1 cyp4v8 aldh3b1 cyp2p9 cyp7a1a nox1 dao.1 cyp1c1 cyp4v7*
GO:0004252 serine-type endopeptidase activity	F	2.98E-03	14 (2.3%)	46 (0.5%)	*ctrl ela2l rhbdf1 ela2 ela3l lonp2 htra1b masp2 habp2 furinb f7 try tmprss13a mst1*
GO:0017171 serine hydrolase activity	F	5.85E-03	15 (2.4%)	55 (0.6%)	*ctrl ela2l rhbdf1 ela2 scpep1 ela3l lonp2 htra1b masp2 habp2 furinb f7 try tmprss13a mst1*
GO:0008236 serine-type peptidase activity	F	5.85E-03	15 (2.4%)	55 (0.6%)	*ctrl ela2l rhbdf1 ela2 scpep1 ela3l lonp2 htra1b masp2 habp2 furinb f7 try tmprss13a mst1*

Table 4.6 Gene ontology analysis of transcripts significantly genes over or under expressed by the BNF treatment retrieved from the mouse genome database.

Go term	*P*-value	Sample frequency (n=166)	Brackground frequency (n=25495)	Genes
Biological process				
GO:0008152 metabolic process	1.19E-15	117 (70.5%)	9276/(36.4%)	*Lpin1 Med30 Sgk1 Tdh Rel Pcmtd1 Nfil3 Gale Dusp6 Pias1 Flad1 Ide Rps14 Tfap2c Nr4a1 Gnl2 Lipg Ucp3 Gclm Btg2 Cpb1 Ctrl Pah Ptpn22 Atf3 Alas2 Cela2a Cnksr1 Cox15 Il6ra Ovol1 Xpc Cox17 Klf3 Nudt22 Agk Runx3 Ercc2 Tspo Pi4k2a Ripk4 Mob3c Cpa2 Tsku Gss Cebpd Pycr1 Dusp5 Egln1 Casp8 Bmp4 Tceb3 Qrsl1 Dio1 Tapbp Etfa Esrp2 Rgs2 Surf1 Fkbp5 Dusp2 Enpp4 Rasd1 Plekhf1 Gmds Setdb2 Xiap Mmp9 Pdk2 Smek2 Aldh3b1 Agmo Rem1 B4galt1 Cybrd1 Flcn Alas1 Klf9 Tbc1d5 Rps18 Tnfrsf1a Psme2 Uevld Esrp1 Htatip2 Sdhb Sdha C1qtnf1 Txnrd1 St6galnac2 Atg4b Lonp2 Fkbp1b Myog Gna14 Ankk1 Trmt61a Inhbb Sqrdl Elane Spry4 Neil1 Smox Card14 Fgf7 Dhodh Nfs1 Tat Cebpb Irf7 Nox1 Gnpnat1 Trib3 Ppil1 Chac1 Cpa5 Bbc3*
GO:0010941 regulation of cell death	8.49E-06	28 (16.9%)	1176 (4.6%)	*Sgk1 Rel Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Txnrd1 Inhbb Card14 Dhodh Cebpb Bbc3*
GO:0010942 positive regulation of cell death	1.64E-05	17 (10.2%)	441 (1.7%)	*Dusp6 Nr4a1 Runx3 Ercc2 Tspo Bmp4 Ucp2 Plekhf1 Mmp9 B4galt1 Flcn Lta Htatip2 Txnrd1 Dhodh Cebpb Bbc3*
GO:0008219 cell death	4.64E-05	31 (18.7%)	1528 (6.0%)	*Sgk1 Rel Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 Pdk2 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Txnrd1 Inhbb Card14 Dhodh Cebpb Trib3 Chac1 Bbc3*
GO:0042981 regulation of apoptotic process	4.98E-05	26 (15.7%)	1113(4.4%)	*Sgk1 Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Inhbb Card14 Dhodh Cebpb Bbc3*
GO:0016265 death	5.15E-05	31 (18.7%)	1535 (6.0%)	*Sgk1 Rel Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 Pdk2 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Txnrd1 Inhbb Card14 Dhodh Cebpb Trib3 Chac1 Bbc3*
GO:0043067 regulation of programmed cell death	6.29E-05	26 (15.7%)	1126 (4.4%)	*Sgk1 Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Inhbb Card14 Dhodh Cebpb Bbc3*
GO:0006979 response to oxidative stress	7.64E-05	12 (7.2%)	220 (0.9%)	*Ucp3 Gclm Ercc2 Pycr1 Egln1 Ucp2 Mmp9 Pdk2 Aldh3b1 Txnrd1 Neil1 Tat*
GO:0044281 small molecule metabolic process	1.14E-04	34 (20.5%)	1864 (7.3%)	*Lpin1 Tdh Flad1 Ide Gnl2 Ucp3 Gclm Pah Gss Pycr1 Egln1 Qrsl1 Rgs2 Surf1 Rasd1 Gmds Pdk2 Smek2 Agmo Rem1 Flcn Tbc1d5 Tnfrsf1a Sdhb Sdha Txnrd1 Lonp2 Gna14 Fgf7 Dhodh Nfs1 Tat Gnpnat1 Trib3*
GO:0006915 apoptotic process	1.37E-04	29 (17.5%)	1425 (5.6%)	*Sgk1 Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 Pdk2 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Inhbb Card14 Dhodh Cebpb Trib3 Chac1 Bbc3*
GO:0019222 regulation of metabolic process	1.75E-04	60 (36.1%)	4712 (18.5%)	*Lpin1 Med30 Rel Nfil3 Dusp6 Pias1 Ide Rps14 Tfap2c Nr4a1 Lipg Gclm Btg2 Atf3 Il6ra Ovol1 Cox17 Klf3 Runx3 Ercc2 Tspo Tsku Cebpd Dusp5 Egln1 Casp8 Bmp4 Tceb3 Esrp2 Rgs2 Dusp2 Rasd1 Plekhf1 Xiap Pdk2 Smek2 Flcn Klf9 Tbc1d5 Tnfrsf1a Psme2 Esrp1 Htatip2 C1qtnf1 Atg4b Lonp2 Fkbp1b Myog Inhbb Elane Spry4 Neil1 Card14 Fgf7 Cebpb Irf7 Nox1 Trib3 Chac1 Bbc3*
GO:0012501 programmed cell death	1.85E-04	29 (17.5%)	1445 (5.7%)	*Sgk1 Dusp6 Rassf6 Nr4a1 Gclm Btg2 Runx3 Ercc2 Tspo Casp8 Bmp4 Dusp2 Ucp2 Plekhf1 Xiap Mmp9 Pdk2 B4galt1 Flcn Lta Tnfrsf1a Htatip2 Inhbb Card14 Dhodh Cebpb Trib3 Chac1 Bbc3*

Cellular component				
GO:0005739 mitochondrion	6.14E-08	37 (22.3%)	1635 (6.4%)	*Lpin1 Sgk1 Tdh Ide Rps14 Slc25a33 Ucp3 Mrps31 Alas2 Cox15 Cox17 Agk Slc25a28 Tspo Pi4k2a Rpl34 Pycr1 Casp8 Qrsl1 Etfa Esrp2 Surf1 Hccs Ucp2 Pdk2 Slc25a40 Alas1 Sdhb Sdha Slc25a43 Txnrd1 Sqrdl Dhodh Nfs1 Tat Bbc3 Slmo2*
GO:0044429 mitochondrial part	2.08E-07	22 (13.3%)	601 (2.4%)	*Lpin1 Slc25a33 Ucp3 Mrps31 Alas2 Cox15 Slc25a28 Tspo Etfa Surf1 Hccs Ucp2 Pdk2 Slc25a40 Alas1 Sdhb Sdha Slc25a43 Sqrdl Dhodh Nfs1 Bbc3*
GO:0005740 mitochondrial envelope	7.29E-05	17 (10.2%)	488 (1.9%)	*Lpin1 Slc25a33 Ucp3 Alas2 Cox15 Slc25a28 Tspo Surf1 Hccs Ucp2 Slc25a40 Sdhb Sdha Slc25a43 Sqrdl Dhodh Bbc3*
GO:0005743 mitochondrial inner membrane	2.17E-04	14 (8.4%)	346 (1.4%)	*Slc25a33 Ucp3 Alas2 Cox15 Slc25a28 Surf1 Hccs Ucp2 Slc25a40 Sdhb Sdha Slc25a43 Sqrdl Dhodh*
GO:0031966 mitochondrial membrane	2.28E-04	16 (9.6%)	465 (1.8%)	*Lpin1 Slc25a33 Ucp3 Alas2 Cox15 Slc25a28 Tspo Surf1 Hccs Ucp2 Slc25a40 Sdhb Sdha Slc25a43 Sqrdl Dhodh*
GO:0019866 organelle inner membrane	4.05E-04	14 (8.4%)	364 (1.4%)	*Slc25a33 Ucp3 Alas2 Cox15 Slc25a28 Surf1 Hccs Ucp2 Slc25a40 Sdhb Sdha Slc25a43 Sqrdl Dhodh*
GO:0031967 organelle envelope	3.15E-03	18 (10.8%)	711 (2.8%)	*Lpin1 Slc25a33 Ucp3 Alas2 Cox15 Slc25a28 Tspo Surf1 Hccs Ucp2 Slc25a40 Htatip2 Sdhb Sdha Slc25a43 Sqrdl Dhodh Bbc3*
GO:0031975 envelope	3.48E-03	18 (10.8%)	716 (2.8%)	*Lpin1 Slc25a33 Ucp3 Alas2 Cox15 Slc25a28 Tspo Surf1 Hccs Ucp2 Slc25a40 Htatip2 Sdhb Sdha Slc25a43 Sqrdl Dhodh Bbc3*
Molecular function				
GO:0003824 catalytic activity	2.93E-11	79 (47.6%)	5287 (20.7%)	*Lpin1 Sgk1 Tdh Pcmtd1 Gale Dusp6 Pias1 Flad1 Ddx49 Ide Gnl2 Lipg Gclm Cpb1 Ctrl Pah Ptpn22 Alas2 Cela2a Cnksr1 Cox15 Nudt22 Agk Ercc2 Pi4k2a Ripk4 Mob3c Cpa2 Gss Pycr1 Dusp5 Egln1 Casp8 Qrsl1 Dio1 Etfa Surf1 Fkbp5 Dusp2 Hccs Enpp4 Rasd1 Gmds Setdb2 Xiap Mkrn1 Mmp9 Pdk2 Aldh3b1 Agmo Rem1 B4galt1 Cybrd1 Alas1 Cmbl Uevld Htatip2 Sdhb Sdha Txnrd1 St6galnac2 Atg4b Lonp2 Fkbp1b Gna14 Ankk1 Trmt61a Sqrdl Elane Neil1 Smox Dhodh Nfs1 Tat Nox1 Gnpnat1 Trib3 Ppil1 Cpa5*
GO:0005488 binding	1.39E-07	114 (68.7%)	11084 (43.5%)	*Lpin1 Med30 Sgk1 Tdh Rel Nfil3 Gale Dusp6 Pias1 Flad1 Ddx49 Gipc2 Ide Rps14 Tfap2c Mob1a Rassf6 Nr4a1 Gnl2 Lipg Gclm Btg2 Cpb1 Mrps31 Pah Fgfr1op2 Ptpn22 Atf3 Alas2 Cnksr1 Il6ra Ovol1 Xpc Cox17 Klf3 Agk Runx3 Ercc2 Tspo Zfand2a Pi4k2a Ripk4 Mob3c Cpa2 Tsku Gss Cebpd Pycr1 Egln1 Casp8 Bmp4 Tceb3 Qrsl1 Dio1 Tapbp Etfa Esrp2 Rgs2 Rspo3 Rgs13 Fkbp5 Dusp2 Hccs Rasd1 Plekhf1 Gmds Setdb2 Xiap Mkrn1 Mmp9 Pdk2 Smek2 Agmo Rem1 B4galt1 Cybrd1 Flcn Lta Stard10 Alas1 Klf9 Rps18 Tnfrsf1a Psme2 Esrp1 Sdhb Sdha Txnrd1 Atg4b Lonp2 Fkbp1b Myog Gna14 Ankk1 Inhbb Elane Spry4 Neil1 Card14 Fgf7 Dhodh Nfs1 Tat Cebpb Invs Irf7 Nox1 Gnpnat1 Trib3 Chac1 Cpa5 Bbc3 Cish Fbxo32*
GO:0048037 cofactor binding	5.27E-05	13(7.8%)	259(1.0%)	*Sgk1 Tdh Gale Pah Alas2 Etfa Gmds Alas1 Sdha Txnrd1 Dhodh Nfs1 Tat*
GO:0016491 oxidoreductase activity	3.29E-04	20 (12.0%)	750 (2.9%)	*Tdh Pah Cox15 Pycr1 Egln1 Dio1 Etfa Surf1 Aldh3b1 Agmo Cybrd1 Uevld Htatip2 Sdhb Sdha Txnrd1 Sqrdl Smox Dhodh Nox1*
GO:0016635 oxidoreductase activity, acting on the CH-CH group of donors, quinone or related compound as acceptor	3.45E-03	3 (1.8%)	4 (0.0%)	*Sdhb Sdha Dhodh*

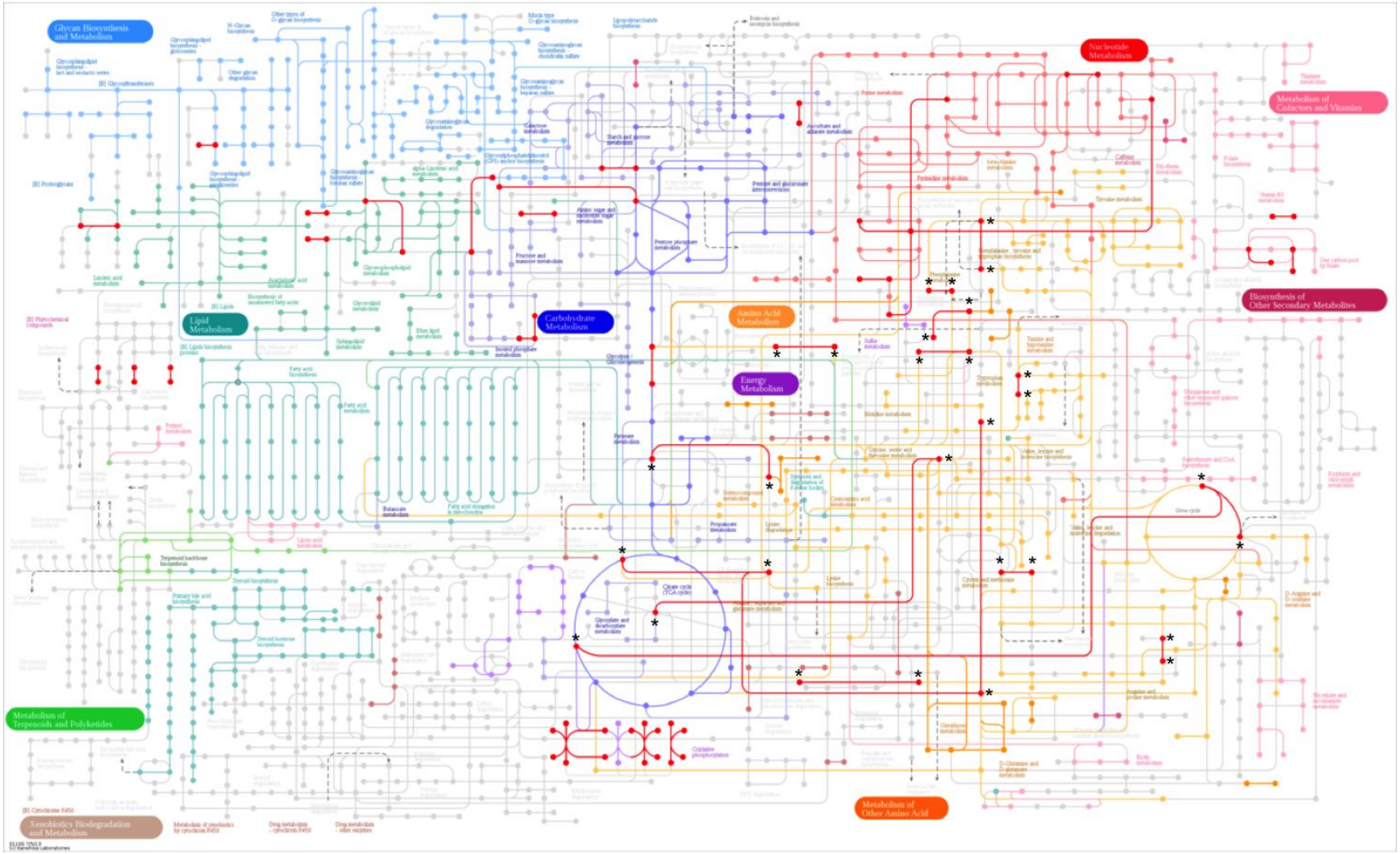

Figure 4.8 Networks of bio functions and involved genes differentially expressed upon exposure to BNF, generated using terms enrichment function (amiGO) for zebrafish. (*) states for genes related to the Amino Acid metabolism.

4.5 Discussion

4.5.1 Transcriptomic changes related to dioxin-like compounds' exposure

One of the primary aims of environmental quality studies is to understand the impacts of anthropogenic compounds such as organic pollutants on the ecosystem, in order to minimise or prevent adverse effects. In this work, we analysed the transcriptome changes on zebrafish embryos when exposed to three dioxin-like aromatic compounds, BNF, B[k]Fla and B[a]Pyr. In addition we compare the effects after a long exposure to B[a]Pyr (5 days) with the standard 48h-exposure. The first 48hpf constitute the most vulnerable stage of zebrafish development, in which most of the embryo's genome is activated.

Fish embryos and larvae are very sensitive to PAHs mixtures or dioxins, which can elicit a variety of common toxic effects, including pericardial and yolk sac edema, cranio-facial abnormalities and body axis defects (Peterson et al., 1993; Marty et al., 1997; Carls et al., 1999; Heintz et al., 1999; Incardona et al., 2005). Studies with individual PAHs species suggest that the mechanism of toxicity may vary with the PAH and is likely correlated with the number of rings in the molecule (Incardona et al., 2006). Studies with tricyclic PAHs suggest that many of the developmental effects elicited are AhR independent and may be secondary to effects on cardiac function (Incardona et al., 2005; Incardona et al., 2006). In contrast, studies with 4-ringed PAHs suggested that some of these compounds cause developmental toxicity through the AhR, the major target of dioxin-like toxicity in vertebrates (Incardona et al., 2006). However, some of the reported PAH-associated developmental effects can not be predicted solely from the hydrophobicity or the affinity to AhR of the compound, but they require toxicokinetic and tissue distribution data to explain the effects related to exposure to different PAHs (Incardona et al., 2006)

B[a]Pyr is widely distributed in various aquatic ecosystems often at high concentrations, particularly in the sediments (Li et al., 2006; Guo et al., 2007). B[k]Fla is also a representative PAH that appears in environmental samples at elevated concentrations. The natural compound BNF is a reference chemical known to be an effective inducer of *cyp1a* (Hahn, 1998) in vertebrates, including zebrafish. We tested compounds at environmentally typically concentrations, selected from previous results from our group (Olivares et al., 2013). For example, the concentration of B[a]Pyr in surface water of rivers and lakes from Hangzhou, China reaches the 10 μg/l mark (Zhu et al., 2004), 909 ng/L for Estuary of River Thames, UK (Law et al., 1997), or 390ng/L for Rain water in Netherlands (Van Noort and Wondergem, 1985).

PAHs induce their own metabolism by activating transcription of *cyp1a* genes through AhR binding (Nebert et al., 2004), but they also have the potential to cause AhR-dependent toxicity in the same manner as dioxins (Carney et al., 2004). Several interesting biological hypotheses may result from our analysis. All tested compounds induced changes on the zebrafish transcriptome, many of them likely related to activation of the AhR signalling pathway, including the early over-regulation of the genes related to the response to xenobiotics. In addition to the strong induction of the transcript for the classic dioxin biomarker *cyp1a*, the analysis revealed the activation of genes belonging to the AhR regulon, such as *ahr2*, *ahrrb*, *ugt1b5*, and the *cyp1* series *cyp1b1*, *cyp1c1*, and *cyp1c2*.

Besides, the activation of the AhR regulon, PAH-type AhR agonist cause developmental toxicity in fish, including teratogenesis (Carney et al., 2004), particularly after the first 2dpf (Carney et al., 2004; Billiard et al., 2006). Whether this teratogenic effect is related or not to the AhR activation is presently unclear (Billiard et al., 2006). Our analyses suggest that a significant part of the transcriptional changes differed between a presumed, non-toxic pure AhR activator, BNF, and two PAHs, B[a]Pyr and B[k]Fla, being the former known by its effective mutagenicity. B[k]Fla and B[a]Pyr 48h treatment resulted in an increase in transcripts for several related genes, which is consistent with some uncharacterized mitochondrial toxicity. This effect was particularly strong for the longer, 96h exposure to B[a]Pyr. In agreement with these finding, several studies indicate that mitochondria are major targets of PAH toxicity in mammalian cells. PAHs localize to the mitochondria and their presence correlates with a decrease in ATP production (Zhu et al., 1995), loss of mitochondrial membrane potential, changes in mitochondrial morphology (Li et al., 2003; Xia et al., 2004), and induction of the mitochondrial-dependent apoptotic pathway (Ko et al., 2004; Huc et al., 2006).

Mitochondria are responsible for the majority of cellular energy production in eukaryotic cells. They are also involved in cellular calcium homeostasis, cell signalling, and apoptosis (Wallace, 1999; Duchen, 2004). Disruption of normal mitochondrial function has been linked with a variety of diseases in humans (Chan, 2006). Each eukaryotic cell may include even thousands of mitochondria, each one containing several copies of the bacterial-like mitochondrial genome (mtDNA). Those circular chromosomes are believed to be especially prone to damage (Wallace, 1999; Izzotti, 2009), and their proximity to the electron transport chain makes them especially susceptible to oxidative damage (Musatov and Robinson, 2012). Some PAHs and, particularly, B[a]Pyr, are known to be activated by oxidative reactions into radical species able to produce DNA adducts (Cavalieri and Rogan, 1995). As mtDNA is considered structurally less protected from damage than the more compact DNA histone complex formed in the nucleus (Suliman et al., 2004), it is likely that mtDNA may be

the mitochondrial target of the observed PAH effects, although this hypothesis is still to be tested. Damage in the mtDNA can result in deleterious functional consequences (Copeland et al., 2002; Kroemer, 2006; Stuart and Brown, 2006). Our data may thus indicate that the integrity of mtDNA is affected by B[a]Pyr during the early stages of development, which have implications for normal mitochondrial function. In any case, we were not able to detect a massive cellular death even in conditions in which teratologic effects were apparent (Figure 4.9), indicating that PAH may disrupt the mitochondrial metabolism (either by affecting mtDNA integrity or by other mechanisms) without eliciting a large scale apoptosis.

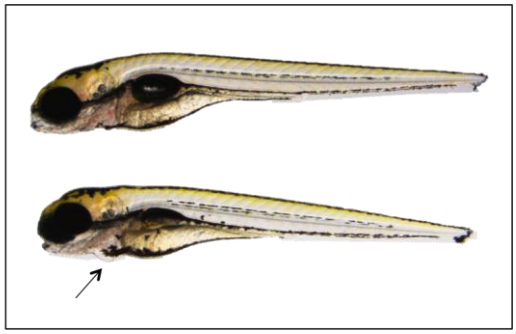

Figure 4.9 Typical deformation observed in 120hpf zebrafish larvae exposed to 0.1mg/L of B[a]Pyr. A) is a larvae control and B) represents a treated larvae, arrow indicate pericardial edema.

BNF treatment elicits a pattern of transcriptome changes very different from those observed with PAH, including several genes responsible to the metabolism of amino acids (aa), during the first 2 dpf. A similar effect has been already reported for immature female rainbow trout (*Oncorhynchus mykiss*) (Tintos et al., 2008), in which hepatic Glutamate dehydrogenase (GDH) and Aspartate Aminotransferase (Asp-AT) activities increased upon BNF treatment.

Our results may be ecologically relevant and they may have important implications. Fish mitochondria are already exposed to higher levels of oxidative stress, most likely due to peroxide production by the respiratory chain and episodes of environmental and physiological hypoxia (Abele and Puntarulo, 2004). Fish generally have lower mitochondrial antioxidant capacity than terrestrial vertebrates (Willett et al., 2001). Added oxidative stress may result in reduced bioenergetics fitness and subsequently, it may result in reduced survival of these fish. At the organism level, loss of mitochondrial integrity would results in a decrease energy production, and limits the energy allocation on growth and reproduction (Kooijman and Bedaux, 1996; Woodford et al., 1998).

Our results indicate that mitochondria are important targets of B[a]Pyr toxicity in zebrafish. Additional studies are needed to determine the consequences of these over-represented genes in terms of mediated mitochondrial apoptosis, aerobic metabolism, and organismal fitness. Because gene homology and retention of similar biochemical

function of proteins among phylogenitcally divergent organisms are well recognized, the results described here could aid in development of a rapid response model for predicting developmental toxicity of combustion-derived chemical compounds in a broad range of vertebrates. As mentioned above, PAHs are an important class of contaminants in the aquatic environment. Therefore, further investigation into how this group of pollutants may affect the mitochondria in aquatic animals is necessary. In addition, studies that attempt to determine the response and adaptation of fish chronically exposed to such external stress in areas heavily polluted with PAHs can add new insights into the mechanism behind such effects.

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5 - Transcriptomic response of zebrafish embryos to PAMAM dendrimers

Transcriptomic response of zebrafish embryos to PAMAM dendrimers

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5.1 Abstract

The progressive practical applications of engineered nanoparticles results in their ever-increasing release into the environment. Accurate assessment of their environmental and health risks requires the development of methods allowing their monitoring in different environmental compartments and the evaluation of their potential toxicity at different levels of organization. Toxic effects of third-generation (G3) and fourth-generation (G4) Poly(amidoamine) dendrimers (ethylenediamine cored, imine-terminated) were assessed on zebrafish embryos during the first two days post fertilization (dpf). Particle characterization by dynamic light scattering (DLS) showed no tendency to form aggregates in the assay conditions. G3 and G4 particles showed similar LC50 values (1.8mg/L and 2.7mg/L, respectively). Transcriptomic analysis at sublethal concentrations showed about 700 transcripts differentially expressed in at least one of the treatments. Transcriptome profiles were similar for both dendrimers, suggesting a similar mode of action. The overall transcriptome response showed a significant correlation with the already reported response to bacterial infection in zebrafish embryos, which is consistent with the activation of the innate immune response in by both G3 and G4. This unique biological response represents a yet unreported effect of these particles, and contributes to the characterization of the possible hazards of these nanomaterials for both human health and the environment.

Keywords: Dendrimer; zebrafish embryo, developmental toxicity, nanotoxicology, nanotranscriptomic.

5.2 Introduction

There is an increasing use and production of engineered nanoparticles (NPs) in numerous industrial applications. In biological sciences and medicine, novel approaches to drug delivery and formulation using nanomaterials are revolutionizing the future of some treatments due to the capacity to manipulate materials at small-scale (<100nm) reaching even the molecular and atomic sizes. This is creating a potential for new classes of products with distinctive mechanical, optical and electrical properties and functions (Schrand et al. 2010). It is foreseen that in the next few years we will assist to a steady succession of new nanopharmaceuticals entering the marketplace. Actually, the U.S. National Science Foundation predicts that nanotechnology will represent half of the pharmaceutical industry product line by 2015 (Lux Research 2006).

Dendritic polymer nanoparticles such as PAMAM dendrimers have a well-defined, mono dispersive and stable molecular architecture that is advantageous for target drug delivery. Dendrimers are composed of an initiator core with layers of radially repeating units (i. e. generations, G) attached to the core and outer surface of terminal functional units within a three-dimensional shape defined by the shape/size of the core. Succeeding generations (referred to as G0, G1, G2, etc.) have increased diameter and twice the number of terminal functional groups than their predecessor. Low generation PAMAM dendrimers tend to be present in relatively open forms whereas the higher generation dendrimers (G>4) exist in spherical three-dimensional structures. (Tomalia et al. 1990; Tomalia et al. 2007; Boas and Heegaard 2004; Svenson and Tomalia 2005). "Full generation" PAMAM dendrimers are terminated with an amine functional group, whereas "half-generation" PAMAM dendrimers terminate with carboxylic acid functionalities. PAMAM dendrimers have unique properties related with their capability of developing interior void space capable of hosting a high number of smaller guest molecules by surface induced congestion (Tomalia et al. 2007) and with their assumed lack of immunogenicity. These properties allow their use in imaging targeted diagnostic, drug and pesticide delivery applications, and in gene transfection protocols (Tomalia 1996). While dendrimers constitute an attractive drug carrier system, widely considered non-toxic, little information is available about their risks for human health (Singh and Nalwa 2007). Previous findings documented the potential ecotoxicity effects for PAMAM dendrimers to several aquatic organisms: *Vibrio fisheri*, *Daphnia magna, Thamnocephalus platyrus, Chlamidomonas reinhardtii* (Mortimer et al. 2008; Naha et al. 2009; Petit et al. 2010), including *Danio rerio* (Heiden et al. 2007).

Although nanopharmaceuticals are in principle not considered an environmental problem, but rather a human toxicity issue, the analysis of their toxic properties in

animal models is still necessary for clarifying the mechanisms underlying their possible toxic action. In this work, we use the zebrafish eleutheroembryo as a vertebrate model for assessing the toxicity of dendrimers, as this model species has been widely used for drugs and chemicals screenings and biomedical research in general. The easiness of breeding and handling, with an all-year-round reproductive cycle, the abundant knowledge on its physiology and genome, and the extended homology with the human genome configure an ideal in vivo, whole-animal model, which can be used as a high cost-effective complement to mouse models of human disease (Dooley and Zon 2000; Peterson et al. 2000; Spitsbergen and Kent 2003; Teraoka et al. 2003; Carvan Iii et al. 2005; Hill et al. 2005; Lieschke and Currie 2007; Kari et al. 2007), or for screening chemical libraries to identify potentially therapeutic new compounds (Peterson et al. 2000; Margolis and Plowman 2004; Burns et al. 2005).

Nevertheless, the release of nanopharmaceuticals into the environment and the possible increase of their environmental concentrations makes essential the development of methods for characterizing their biological and chemical properties, including physicochemical stability. In addition, it will be necessary to build up techniques for the pharmacological and toxicological screening, and for the pharmacokinetic and biodistribution profiling of these substances.

Microarrays have been increasingly used in ecotoxicological studies to determine the molecular mode of action of environmental pollutants and to identify new biomarkers as indicators of exposure and effect for risk assessment (Robbens et al. 2007). They have many applications in medical diagnosis, in the analysis of the progression and probable outcome of diseases, in the prediction of possible mechanisms of toxicity for new chemical compounds, and in the categorization of genes that share functional metabolic pathways so that they can be related to specific health adverse outcomes (Stoughton 2005).

Keeping in mind the rising concerns about the putative toxic effects of nanoparticles and their potential compatibility with the immune system, the aim of the present investigation was to increase our knowledge about the concrete genomic alterations underlying the toxic effects of two commercially available PAMAM dendrimers (G3, G4) using zebra fish embryos and microarray techniques for detecting alterations in multiple sets of genes. Acute ecotoxicological effects and transcriptomic analyses were performed after exposure of zebra fish embryos during the first two days after fertilization (Usenko et al. 2008; Yeo and Kim 2010; capitulo 3.docx). The obtained information will be of a very high value when determining possible hazards related with the release of dendrimers into the environment as well as their toxic effects when applied in health treatments..

5.3 Materials and Methods

5.3.1 Zebrafish maintenance

Zebrafish (*Danio rerio*) fertilized eggs were obtained by natural mating and raised at 28,5ºC (Kimmel et al. 1995) with a 12L:12D photoperiod in "embryo water": [90ug/ml of Instant Ocean (Aquarium Systems, Sarrebourg, France; http://zfin.org/), 0.58mM CaSO4.2H2O, dissolved in reverse osmosis purified water]. Animal stages were recorded as days or hours post-fertilization (dpf or hpf) as previously described (Kimmel et al. 1995).

5.3.2 Reagents and standards

Two PAMAM dendrimers (G3 and G4) with an ethylenediamine core were a courtesy of Dendritech, INC. Midlan MI, USA. The average molecular weight for G3 is 6909 kDa, and it contains approximately 32 functional surface groups (with –NH2 termini). The average molecular weight for G4 is 14215kDa and it contains approximately 64 functional surface groups (with –NH2 termini). Comparison of G3 and G4 dendrimers to other generations is listed in (table 5.1). 3,4-Dichloraniline Pestanal was purchased from Sigma-Aldrich (Seelze, Germany).

5.3.3 Particle characterization

Dynamic Light Scattering (DLS) was used as a method to determine the hydrodynamic size of the particles in solution using a Zeta sizer Nano (Malvern Instruments Ltd., UK). Measurements were performed in the solutions prepared in fish medium at different concentrations directly after preparation. The temperature of the cell housing was set to 25 (0.1ºC). Four independent measurements were taken with each measurement consisting of six runs, each of 20s duration.

5.3.4 Toxicity assay

Fertilized eggs were collected within 1hour post fertilization (4-cell to 128-cell stages) and distributed in 24-well cell culture plates (1 embryo/well). Initial exposure experiments were conducted to determine individual toxicity within a range of concentrations following the International Standard 15088 (ISO 2007) for zebrafish eggs. As positive control, a delimited concentration of a reference compound (3.7mg/L

of 3,4-dichloroaniline) was tested at the same time; in addition a negative control was set for each plaque. Mortality data was obtained after 4, 24 and 48h of exposure. Live embryos were observed to assess developmental progression (i.e., completion of gastrulation, formation of somites, proper heart beat and spontaneous movement), as well as alterations in morphology and signs of toxicity. LC_{50} values for both dendrimers were calculated assuming standard logistical curves. Dendrimer exposures for microarray analyses were performed with a concentration of approximately 1.5mg/L for each dendrimer. Six biological replicates consisting of 50 embryos were made for each nanomaterial treatment.

5.3.5 RNA extraction and microarray analysis

Total RNA was isolated from 50 embryos per treatment, using Trizol reagent protocol (Invitrogen Life Technologies, Carlsberg) and purified using standard methods following the manufacturer's protocol (RNeasy Kit; Qiagen). RNA concentration was measured by spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality checked in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RIN (RNA integrity) values ranged between 9.5 and 10.

Microarray studies were performed using the commercial Agilent *D. rerio* (Zebrafish) Oligo Microarray v3 platform, using two-color strategy. The study included three biological replicates (independent embryos pools either untreated or treated with each dendrimer generation), labelled and hybridized at the same time using Cyanine 3 (Cy3) and Cyanine 5 (Cy5) dye (Agilent Two-Color Microarray – Based Gene Expression Analysis protocol). As starting material, 200ng of total RNA for each sample was used. After the amplification, labeling and purification procedure, samples were evaluated for yield and effectiveness of Cy3 and Cy5 dye incorporation using the NanoDrop spectrophotometer. Samples that presented at least a specific activity of 8 pmol Cy3 or Cy5 per µg cDNA and a quantity of 825ng labeled cDNA were used for the analysis. Fragmentation of the cDNA was performed in the recommended blocking agent and a volume of 2x GE Hybridization Blocking Buffer (Agilent, Gene Expression Hybridization Kit). A final volume of 100µl containing the fragmented cDNA was added to the 4x44K arrays and the hybridization proceeded for 17h at 65ºC. Microarray slides were washed according to the supplemental procedure Agilent protocol. The slides were kept on dark until scanner, using Agilent G2505C, and signals were extracted using the Agilent Feature Extraction Software v10.5.1.1. The quality of the Microarray data was evaluated manually using the Quality Control report provided by Agilent Software.

5.3.6 Microarray data analysis

Raw data and feature extraction software pre-processed data from the Agilent Microarrays, were imported into the Robin (Lohse et al. 2010) gene expression data analysis system version. Data from the three biological replicates of each condition were combined, resulting in an error-model weighted average of the three. The p-values for differential expression calculated by Robin were adjusted for multi-hypothesis testing using the Benjamini & Hochberg procedure, as implemented in the Bioconductor multitest package in R (http://www.bioconductor.org/packages/bioc/stable/src/contrib/html/multtest.html).
Genes for which the Benjamini & Hochberg-adjusted *p*-value was <0.001 within both comparisons were selected, resulting in the identification of a total of 700 genes that showed robust expression changes during the time of exposure. Genes that were detected as differentially expressed were subjected to cluster analysis using the Pearson correlation algorithm implemented in MultiExperiment viewer MeV4 (Saeed et al. 2003) software. Gene ontology analyses were performed using Amigo webpage (http://www.geneontology.org/); metabolic pathway information was obtained also from KEEG (http://www.genome.jp/kegg/kegg2.html) for zebrafish standard model organism. Results have been deposited at the NCBI's GEO (Gene Express Omnibus) database, reference GSE41333.

5.3.7 Microarray validation and quantitation by qRT-PCR

Total RNA was extracted and measured from the whole body of the embryos. RNA was treated with DNAseI (Ambion, Austion, TX) to remove genomic DNA contamination. Quantities of 1μg were retro-transcribed to cDNA using First Strand cDNA Synthesis Kit (F. Hoffmann- La Roche, Basel Switzerland) and stored at -20ºC. Aliquots of 50ng 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). The selected gene primers used for qRT-PCR validation were designed from existing zebrafish nucleotide sequences.

Appropriate primers for 9 test genes (*ms4a17a.1, irg1l, tnfb, ch25h, agxtb, rx2, lhx1a, pax6b, mmp9*) were designed using Primer Express 2.0 software (Applied Biosystems) and the Primer-Blast server (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome, primer sequences in Table 5.1). Amplification efficiencies were calculated as better than 90% for all tested genes as described (Pfaffl et al. 2002). House-keeping gene *ppia2* was selected as reference gene (Morais et al. 2007; Pelayo et al. 2012), as mRNA levels of neither gene changed upon dendrimer

treatment. PCR products (amplicons) were sequenced in a 3730 DNA Analyzer (Applied Biosystems), and compared to the corresponding reference sequences at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated by triplicates). To minimize errors on RNA quantification among different samples, Cp values for stress-related target genes (Cp_{tg}) were normalized to the average Cp values for *ppia2*, used as reference gene for each sample, $corrCp_{tg} = Cp_{tg}-Cp_{ppia2}$. Changes in mRNA abundance in samples from different treatments were calculated by the ΔΔ Cp 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.

Table 5.1 Sequences of primers used in this study.

Gene	Accession Number	Primer Sequence (5'-3')		Amplicon length (bp)	Efficiency
		Forward	Reverse		
ef1α	X77689	CGTCTGCCACTTCAGGATGTG	ACTTGCAGGCGATGTGAGCAG	376	2.0
ppia2	AY391452	GGGTGGTAATGGAGCTGAGA	AATGGACTTGCCACCAGTTC	179	2.0
ms4a17a.1	NM_001017783	TGCAGAAATGCCCCAACAG	TTGCCTTCAGTGTACGCTGG	101	1.9
irg1l	NM_001077607	CACTGCTTTGCTGGATGGAG	GCTCTAAACGGACACGGGAG	101	1.9
tnfb	NM_001024447	ACAGATGCGGTGAGGGAAAA	GCTCCAAGGTAAATGGTGCTG	101	2.0
ch25h	NM_001008652	GGTGTACATCTTCCCGCTGAG	CAGCAAGATCCCAGACGACC	101	2.0
agxtb	NM_213162	TAACTGGAAGGAACTGCTGGC	TGAGCCCAATCCGCAATAC	101	1.9
rx2	NM_131226	CTCACCCATACGGTCCTTCAA	TGGACAGTGGCGAGGACAG	101	1.8
lhx1a	NM_131216	AGGCGGCTGATCTAACGCTA	AAGGCAAGGAGGTCTGAGGAG	101	2.0
pax6b	NM_131641	CGTGTCGTCGATAAACCGAGT	TCTGCCCGTTGAGCATTCTC	101	1.9
mmp9	BC160656.1	GCTGGCTCACGCTTATCCTC	GGGTTTGAATGGCTGGTCC	101	1.9

5.3.8 Statistical analysis

A logit regression was conducted to determine the lethal concentration 50 (LC_{50}) for each compound using SPSS 19 (SPSS Inc., Chicago, III) package. For transcriptomic analysis, genes were considered as differentially expressed using a confidence *p*-value <0.001. All statistical calculations for qRT-PCR data were performed using ΔΔCp values, as this parameter followed normal distributions (Levene's test). Differences among control and treated groups were analysed by Student's t-test (2 groups) or ANOVA plus Tukey's tests (more than 2 groups).

5.4 Results

5.4.1 Dendrimers characterization

Size distribution profiles indicated mean hydrodynamic diameters of 3.20 nm and 3.84 nm for G3 and G4 dendrimers, respectively (Supplementary Figure 5.1). These values are similar to the predicted ones for both particles (Supplementary Table5.1), indicating that no aggregates were formed even at concentrations 100 times higher than those used for zebrafish exposure. Although the low exposure concentration of G3 and G4 dendrimers precludes their direct measurement at the actual test concentrations, we concluded from these data that both molecules exist isolated in solution and that this is the form to which fish were exposed.

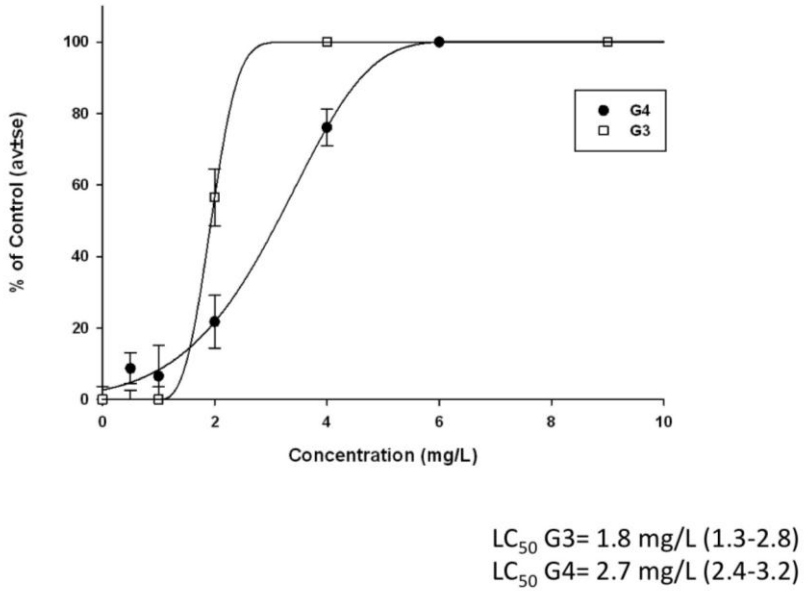

Figure 5.1 Effect of G3 and G4 on the survival of *Danio rerio* embryos during the 48h of exposure. Results are expressed as percentage of mortality.

5.4.2 Dendrimers effects on zebrafish development

Embryo toxicity tests met the standard validity criteria (egg mortality in the negative controls not exceeding 10% after the 48h incubation time and 100% mortality for the positive control 3,4-dicholoaniline). Mortality curves followed a standard sigmoidal function, with LC_{50} values of 1.83 mg/L for G3 and 2.73 mg/L for G4 (Figure 5.1, NOEC and LOEC values in supplementary table 5.2). To analyze sub-lethal effects

in embryos a concentration of 1.5 mg/L was used, below LC_{50} but high enough to elicit strong toxicity responses.

Transcriptomic analyses showed significant changes on mRNA abundance for 478 genes (376 up and 102 down) for G3-treated samples and for 372 genes (366 up and 6 down) for G4-treated ones. In total, 700 transcripts (596 up and 104 down) were affected by at least one of the treatments, 150 of them by both of them (Figures 5.2A and B), The significant proportion (21%) of genes affected by both treatments suggests a common mechanism of action. This is better seen in Figure 5.2C, which shows the correlation between fold-change results (significant or not, 43,000 points) for all features in the array for both treatments. The observed positive correlation confirmed the similar pattern of transcriptional changes induced by both treatments. The observed variation in mRNA abundance for some key genes was confirmed by qRT-PCR, whose results showed a very good correlation with the microarray data and corroborated the similar effect of both treatments (Figures 5.3A and B).

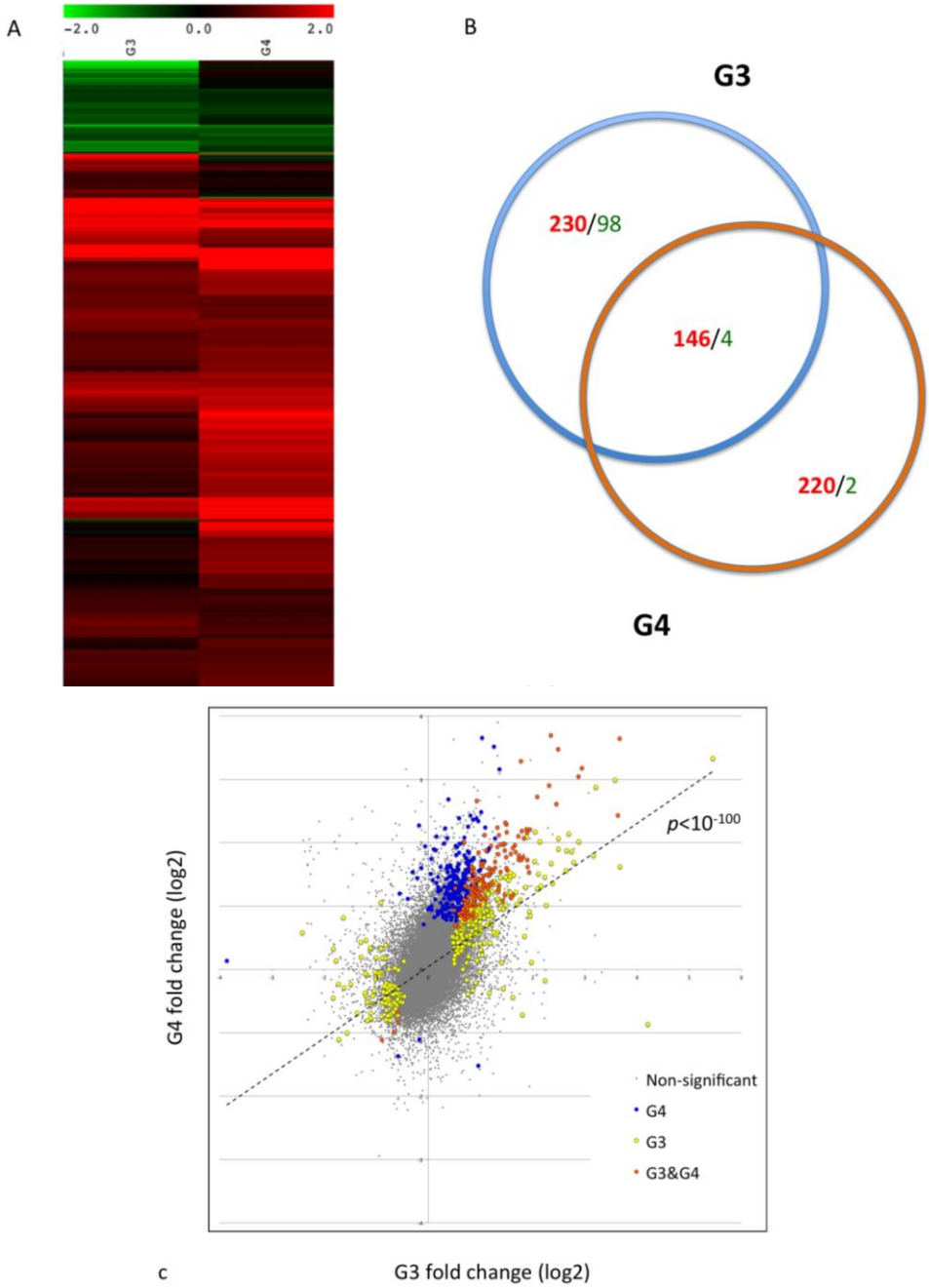

Figure 5.2 a) Heat-map and hierarchical clustering for exposures to G3 and G4. Red spots indicate genes that are upregulated in relation to the control groups, whereas green spots indicate genes that are downregulated with respect to control groups. b) Venn diagram with microarray results c) Comparison of transcriptome changes in the zebrafish embryo upon treatment with G3 (X-axis) and G4 (Y-axis). Results are presented as dual logarithms of fold change values. Significantly changed values are represented in blue (only G3), yellow (only G4) or orange (both). The resulting regression line and the corresponding p-value are also represented.

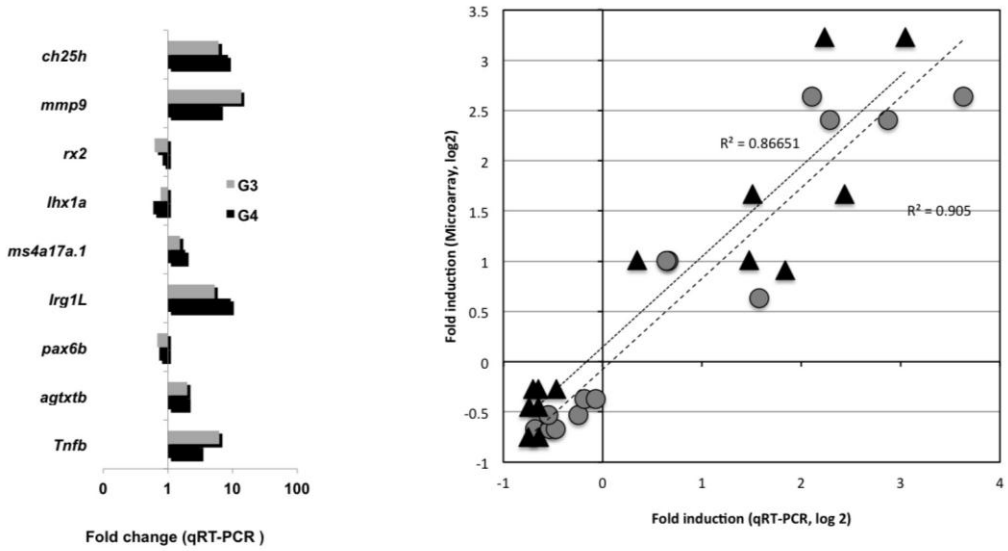

Figure 5.3 a) RT-qPCR data of genes shown as differentially expressed in the microarray analysis results, corresponding to G3 (grey) and G4 (black) treatments. Values correspond to fold changes relative to controls. B) Correlation between the array data and qRT-PCR data. Symbols correspond to individual biological replicates. Grey circles correspond to G3 treatments and black triangles to G4 ones. Corresponding regression lines (long- and short-dashed, respectively) and R^2 coefficients are also shown.

5.4.3 Functional analyses of G3 and G4 - induced transcriptomic changes

A total of 256 transcripts affected by at least one of the treatments corresponded to a functionally annotated zebrafish gene. Gene Ontology (GO) analyses revealed no significant enrichment in any functional category for underrepresented transcripts. GO enrichment analysis identified different functional and structural categories that were overrepresented in the total of affected transcripts (Table 5.2). Two main subsets were apparent, a functional one related to response to foreign body, either bacteria or other organisms, and a cellular component category related to cell-cell junction, specifically to occluding and tight junctions (Table 5.2). The fact that tight junctions are functionally related to inflammatory and immunological processes suggests that both subsets are mechanistically related and linked to cellular immunity response (Sawada 2013).

Table 5.2 Gene Ontology analysis of transcripts significantly affected by either G3 or G4 treatment in zebrafish embryos

GO Term	Aspect	P-value	Sample frequency (n=256)	Background frequency (n=18463)	Genes
Biological Process					
GO:0009617 response to bacterium	P	2.19E-05	10 (3.9%)	63 (0.3%)	rhogb mmp9 lect2l tnfrsf1a rnasel3 myd88 cpt1b cebpb cfb c1ql4l
GO:0051707 response to other organism	P	3.77E-05	11 (4.3%)	85 (0.5%)	rhogb atf3 mmp9 lect2l tnfrsf1a rnasel3 myd88 cpt1b cebpb cfb c1ql4l
GO:0051704 multi-organism process	P	7.77E-05	11 (4.3%)	91 (0.5%)	rhogb atf3 mmp9 lect2l tnfrsf1a rnasel3 myd88 cpt1b cebpb cfb c1ql4l
GO:0009607 response to biotic stimulus	P	7.77E-05	11 (4.3%)	91 (0.5%)	rhogb atf3 mmp9 lect2l tnfrsf1a rnasel3 myd88 cpt1b cebpb cfb c1ql4l
GO:0016042 lipid catabolic process	P	3.93E-02	6 (2.3%)	43 (0.2%)	pla2g12b irg1l oc90 irg1 pla2g7 pla1a
GO:0006629 lipid metabolic process	P	4.37E-02	16 (6.2%)	347 (1.9%)	pla2g12b ch25h degs2 irg1l hsd17b12a chpt1 agpat9l cyb5r2 oc90 mtp dhrs9 smpd2 acsf2 irg1 pla2g7 pla1a
Cellular Component					
GO:0070160 occluding junction	C	9.38E-04	8 (3.1%)	53 (0.3%)	cldn2 oclnb tjp3 cldni cldne oclna cldnf cldng
GO:0005923 tight junction	C	9.38E-04	8 (3.1%)	53 (0.3%)	cldn2 oclnb tjp3 cldni cldne oclna cldnf cldng
GO:0043296 apical junction complex	C	1.67E-03	8 (3.1%)	57 (0.3%)	cldn2 oclnb tjp3 cldni cldne oclna cldnf cldng
GO:0005911 cell-cell junction	C	1.97E-02	9 (3.5%)	103 (0.6%)	cldn2 cx28.9 oclnb tjp3 cldni cldne oclna cldnf cldng

The putative capacity of G3 and G4 dendrimers to elicit a foreign body response was tested by comparing the corresponding transcriptome changes with those observed in zebrafish embryos challenged by a *S. thyphimurium* infection during the first 48 hpf, the same period chosen for the dendrimer exposure assays (Stockhammer et al. 2009). The analysis showed a significant correlation between both sets of data for the two dendrimers, although the correlation was better for G3 ($p=2.5x10^{-62}$) than for G4 (Figure 5.4). Both correlations are more evident when the analysis is limited to genes annotated to the "response to other organism" functional category (GO:0051707, Figure 5.4, orange diamonds) or " tight junction" (GO:0005923, Figure 5.4, green dots). In these cases, *S. thyphimurium* challenge data also showed a better correlation with G3 than with G4 data.

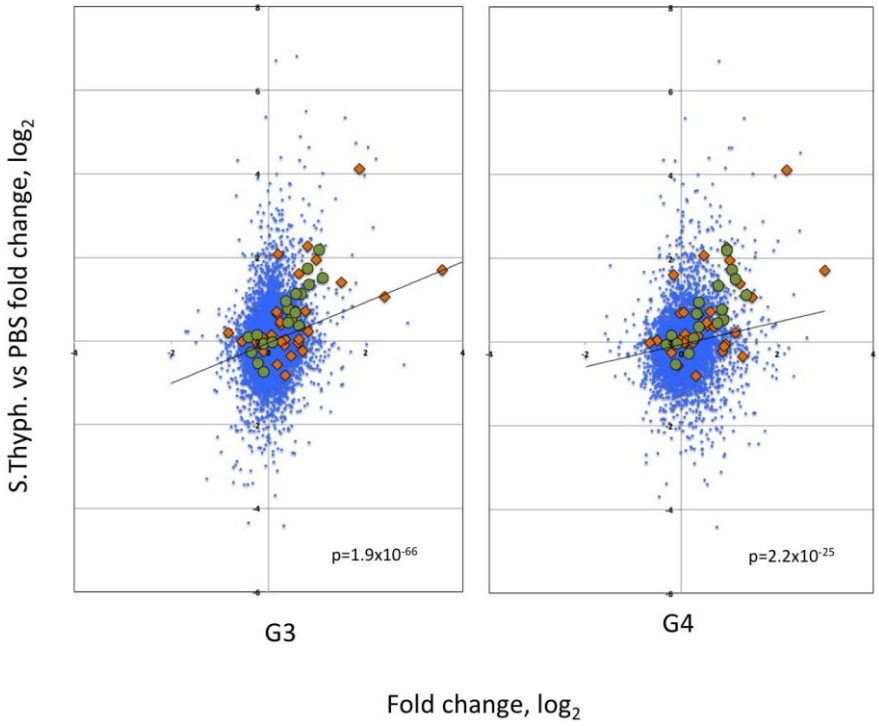

Figure 5.4 Linear correlation between fold change values (log2 transformed) observed in zebrafish embryos infected with *S. typhymurium* (X-axis) and the corresponding results from G3 (left) and G4 (right) dendrimers (Y-axis). Only genes unequivocally identified in all data sets are represented; transcripts represented by more than one feature in each of the dataset are presented as averaged values. Orange diamonds correspond to genes included in the "response to other organism" functional category (GO:0051707), green circles correspond to " tight junction" cell component category (GO:0005923). Regression lines and the corresponding p-values are indicated.

5.5 Discussion

Dendrimers are attracting great interest due to their unique properties as carriers of active molecules in aqueous media. As a consequence, a wide range of applications are foreseen in different sectors from medical treatments to pesticide administering, so that their presence in the environment is expected to be widespread in the future. Therefore, the evaluation of the toxicity of those macromolecules should be considered as a priority demand, given their possible risk to human health and the aquatic environment. Toxicity assays have been performed with several aquatic organisms, but very limited information is available on their effect on non-target species, and, to date, only limited studies have been carried out to assess the embryonic toxicity of dendrimers as engineered nanoparticles. The first work comparing the toxic effect of low generation (G3.5 and G4) PAMAM dendrimers on development of zebrafish embryos showed that G4 dendrimers, which have amine terminal functional groups, were toxic at the low µM concentration ranges, attenuating the growth and the development of the zebrafish embryos at sublethal concentrations; in contrast, G3.5

dendrimers, with carboxylic-acid terminal functional groups, did not exhibit toxicity at concentrations as high as 200uM (Heiden et al. 2007). Our results on G3 and G4 dendrimers, showing similar toxicity, would suggest that the differences in toxicity between G4 and G3.5 resulted from their different surface groups, rather than from their size.

The microarray results indicate that the analyzed PAMAM dendrimers acted as inductors of the natural immunity response. Microarray analyses were performed at dendrimer concentrations (aprox. EC_{50}) for which a toxic response was expected. We thus consider that the observed effects should be mechanistically related to the mortality observed at higher doses. In this context, it is remarkable that the oxidative stress response, a common marker of toxicity for many substances, including many nanomaterials (Yeo and Kang 2009; Choi et al. 2010), did not appear in our analysis, at least at any significant level. Instead, we found evidence of the induction of genes related to responses triggered by the contact with other organisms, particularly bacteria. Exposure to G4 and G3 induced expression of *tnfb*, *irg1l*, *mmp9*, and *mmp13*, all of them related to the innate immune response to bacteria, and known to be activated as a result of an infection (Stockhammer et al. 2009). In addition, genes implicated in tight-cell junction, including claudins *cldn2*, *cldni*, *cldne*, *cldnf*, and *cldng*, occludins *oclna* and *oclnb*, and the tight junction protein *tjp3* were also up-regulated. While the role of tight junction in fence and barrier functions are well known, participating as static components of innate immunity and constituting a physical barrier against allergens, pollutants and bacteria, it is believed that they are also involved in signal transduction and innate immunity (Sawada 2013).

Nanoparticles are known to stimulate and/or suppress immune responses, and that their compatibility with the immune system is largely determined by their surface chemistry (Dobrovolskaia and McNeil 2007). Zebrafish hematopoiesis is comparable anatomically, morphologically, and genetically to that of mammals (Amatruda and Zon 1999). Zebrafish embryos possess macrophages, which act as a bacterial defense mechanism (Herbomel et al. 1999; Crowhurst et al. 2002). The mammalian counterpart of zebrafish *mmp9*, MMP9, is mainly expressed in neutrophils and eosinophils, where it serves as a key marker for their differentiation (Dahlen et al. 1999). In the zebrafish embryo, *mmp9* transcripts are detected in unfertilized eggs, indicating maternal origin, and it is expressed throughout development, at least to 5dpf. During this stage, *mmp9* expression concentrates in the head region around the eye and to a lesser extent in the tail of embryo (Yoong et al. 2007). Our data suggests that *mmp9* is a good biomarker of the induction of the innate immune response in zebrafish, which includes cytokines, chemokines, and other proteins (Manicone and McGuire 2008).

In conclusion, our data show significant and specific toxic effects of PAMAM dendrimers on zebrafish embryos, likely related to the innate immune response. The

application of this type of nano engineering macromolecules to drug target delivery will depend on an accurate validation of throughput assays to account for the effects of charge, shape, size, and numerous other physicochemical characteristics. The development of methods for the prompt evaluation of the toxicity of dendrimers based on the use of fish embryo forms will help to bridge the gap between in vitro or cell culture models and in vivo mammalian models for rapid pre-clinical development. Zebrafish are also increasingly used for immunological research (Trede et al. 2004) and offer a unique opportunity to discover and study novel genes required for the control of normal vertebrate hematopoiesis and functioning of adult blood cells in health and disease. Our results also evidence that new regulations should be introduced in terms of safety and efficacy of these new products, since they affect important metabolic pathways concerned to the development as well as the immunological system.

5.6 Declaration of Interests

The authors declare no conflict of interests with the data presented in this work.

5.7 Acknowledgements

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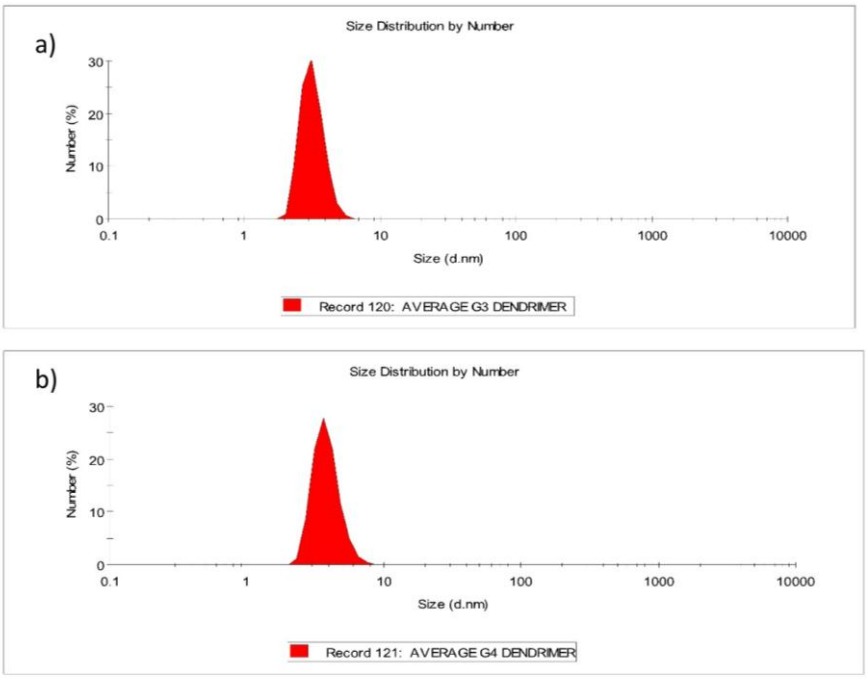
5.8 Supplementary material

Supplementary Table 5.1 Physical characteristics of PAMAM dendrimers.

Generation	Molecular weight (Da)	Diameter (nm)	Surface groups
0	517	1,5	4
1	1430	2,2	8
2	3256	2,9	16
3	6909	3,6	32
4	14215	4,5	64
5	28826	5,4	128
6	58048	6,7	256
7	116493	8,1	512
8	233383	9,7	1024
9	467162	11,4	2048
10	934720	13,5	4096

Supplementary Table 5.2 Effect concentrations (LC50) with corresponding 95% confidence intervals (CI), no-observed-effect concentrations (NOEC), and lowest-observed-effect concentration (LOEC) for the effects of dendrimers G3 and G4 on survival of Danio rerio eggs.

	LC50	NOEC	LOEC
	(mg/L of dendrimers in water)		
Survival (95% CI) for G3	1.835 (1.348-2.760)	1	2
Survival (95% CI) for G4	2.725 (2.363-3.188)	1	2



Supplementary Fig 5.1 Size distribuction of each dendrimer related to the number of particles determined by DLS method, 1a) represents the size for G3 and 1b) represents the size for G4.

6 - General discussion. General conclusions, considerations and research perspectives

6.1 General discussion

The environment is continuously loaded with chemical compounds released by urban communities and industry. The detection, identification and toxicological assessment of those substances are of major importance, as well as the understanding of their molecular mechanisms of toxicity. The prediction of toxicity of a given toxicant, either natural or anthropogenic, requires both a holistic view of toxic effects as well as the identification of specific biomarkers. Toxicogenomics is a major tool for this task, and it is being implemented in improved mechanistic toxicology screens, effective toxicity assessments, chemical safety assessments, and new drug discovery assays (Hayes and Bradfield, 2005). However, toxicogenomic studies require new approaches for the comprehensive organization and interpretation of large data sets. The use of DNA microarray technology has been central to the field of toxicogenomics, which enables the coverage of the transcriptional states of thousands of genes simultaneously.

In this thesis, we explored the characteristics of zebrafish at early stages of development to identify of the sublethal effects of newly and classical pollutants using toxicogenomics technologies. We focused in the effects of substances able to disrupt the cell and body homeostasis through binding to Nuclear Hormone Receptors and of new materials for whose mode of action is unknown. To achieve these goals we conducted several experiments aimed to:

1) Characterize the mRNA abundance of the members of two families of Nuclear Hormone Receptors (RAR and RXR), during the early embryo and larvae stages of zebrafish development, as well as the expression of their target genes (Chapter II).

2) Compare the transcriptome effects of two related compounds: the 9-cis Retinoic Acid and all-trans Retinoic Acid. The assessment between two isoforms of retinoic acid will be made in terms of gene expression in *Danio rerio* embryonic stages (Chapter III).

3) Assess the effects of dioxin-like compounds (Benzo[a]pyrene, Benzo[k]Fluoranthene and β-Naphtoflavone) on Zebrafish transcriptome. (Chapter IV).

4) Evaluate the toxicity and transcriptomic effects of the nanomaterial PAMAM (Polyamidomine) dendrimers, generations 3 and 4, on zebrafish embryos (Chapter V).

Alterations in gene expression are of particular interest as indicators of potencial adverse effects. In addition, the analysis of their altered behaviour allows the identification of the metabolic pathways involved in the toxic effects induced by a chemical compound. Differential gene expression may be associated to a protective

response (acclimatation or defense response), a toxic effect (ectopic expression), or a combination of both. Zebrafish embryos are very useful to identify pollutant-linked changes in gene expression and their possible meaning. Functional studies could be applied to a wide variety of genes, allowing to link gene transcription and translation to effects on the organism health status. Some major points were established within this research: (1) the identification of further suitable molecular markers as indicators of the mode of action, (2) the establishment of strong links between molecular and phenotypic effects in shortand long-term assays in embryos, (3) the definition of limitations of the model and (4) the development of tests that can be used for regulatory purposes.

A wide variety of man-made chemicals have been shown to mimic endogenous hormones. The presence of these compounds in the aquatic environment has been associated with a number of reproductive disorders, particularly in fish (Tyler et al., 1998). Most studies to date have investigated the effects of endocrine disrupting chemicals that act via estrogen, androgen and thyroid receptors, with focus on specific endpoints, for example, induction of vitellogenin or disruption of sexual differentiation (Hill Jr and Janz, 2003; Kazeto et al., 2004; Raldúa and Babin, 2009). The potential application of the zebrafish embryo model is primarily the screening for endocrine disrupting compounds, by measuring the effect of exposure on hormone-responsive genes. In a previous work, such a screening assay was applied to monitor the effect of suspected thyroid disruptions (Pelayo et al., 2012). This work identified genes in the hematopoietic process and in eye development as putative markers for thyroid disruption. The complex regulation, pleiotropic effects and multiple interactions that characterise the RXR/RAR-TR-PPAR regulatory systems have not yet been analysed in the embryo model, when compared with the knowledge of the "classical" endocrine disruptors. Therefore, a holistic assay is preferable over other, less global alternatives. Genomic tools for Zebrafish are very developed, there are commercial microarrays covering essentially all its genome, which has been fully sequenced and is being rapidly annotated. As was evidenced in the chapters II, III, IV and V, the ecotoxicogenomic technology provides the possibility of the establishment of a molecular profile and the characterization of each one of the tested substances. This information contributes decisively to the development of new molecular markers able to characterize the action of the toxicants.

A major conclusion of our work is the suitability of microarray technology to perform the link between molecular and cellular biomarkers with higher-level population and ecosystem responses; and the use of this knowledge to anticipate potential ecological risks for new chemicals and emerging technologies. Ideally, each observed changes on the levels of mRNA of the different genes can be interpreted as modifications of specific metabolic or regulatory modules within the cell.

Cell metabolism is adjusted to the presence of external impacts by modification of gene expression patterns. This process implies that genes sharing a common pathway or under the same control mechanism, change their regulation in a coordinated manner. The identification of such clusters of genes can give information of the cellular target of the pollutant or environmental stress. A schematic overview of the compounds and receptors studied in this work is described in Figure 6.1. The initial studies were conducted with all-trans and 9-cis Retinoic acid, endogenous compounds that act through the binding to the heterodimer RAR/RXR (chapters II and III). The expression of their respective target genes was assessed as well as their phenotypic effects on zebrafish eleutheroembryos at different times. Secondly, the differential transcriptomic effects of coplanar organic compounds (Benzo[k]fluoranthene, Benzo[a]pyrene and the natural compound β-Naphtoflavone) were also assessed in zebrafish. These substances have the common property of activating several cytochromes, including the *cyp1a* gene, under the control of the Aryl hydrocarbon Receptor, AhR, (chapter IV), so our goal was to describe differences on toxicity effects independent from this common mechanism. Last, toxic effects of two polyamidoamine dendrimers were analyzed as example of the assessment of the environmental risk associated to newly manufactured products (chapter V).

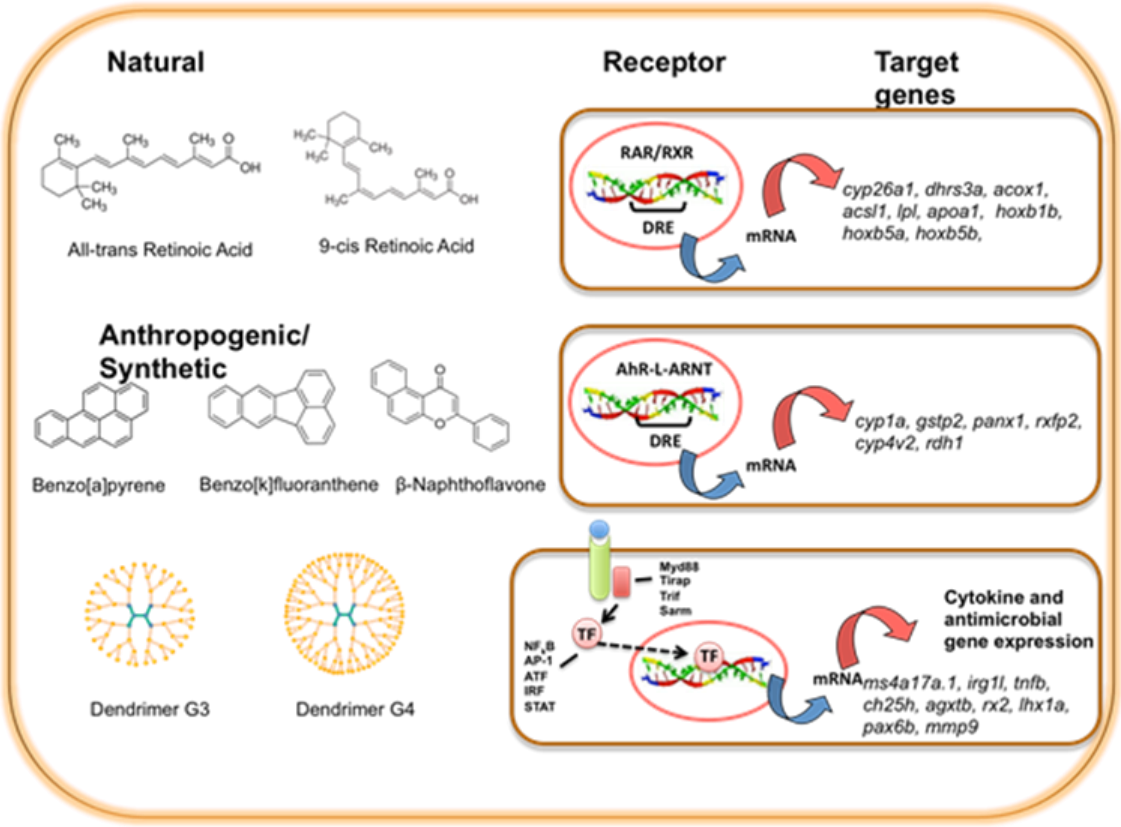


Figure 6.1 Schematic pattern recognition of receptors and effectors. Target genes associated to the activation of each receptor, which are considered as specific for each metabolic pathway.

The chapter (II) study is based on the expression of some target genes related to the RAR/RXR regulatory system after an exposure to a retinol derivative metabolite, the atRA. The first analysis conducted was specific to the determination of the maternal contribution of each RAR and RXR isoforms and to the evaluation of the basal variations of those NRs during the 5 days post fertilization. The RAR/RXR systems play an important role in the formation of the central nervous system (CNS) during the early development of zebrafish. The study shows that the proportions of RARs and RXRs transcripts change during the early period of development. Most of those changes occur in the 24hpf for both regulatory systems, where the transition between maternal to embryonic transcripts occurs. These changes do not affect the response of zebrafish embryos to exogenous RA, and the transcriptomic analyses showed that the development and RA-metabolism-related genes were particularly affected by atRA treatment. We concluded that zebrafish eleutheroembryos are very sensitive to the presence of exogenous atRA. Furthermore, in Chapter (III), due to the similar characteristics of atRA and 9cRA compounds, microarrays were performed in order to find some transcripts that characterize the individual action of each compound, as they presented comparable phenotypes in each period of exposure, being potent teratogens. Both compounds present similar phenotypical effects in longer exposures (72h) to high concentrations (µM) in comparison to the shorter ones (24h). The most evident morphological effects detected were the pericardial and yolk sac edemas, tail malformation, and a delay on the development. Transcriptomic analyses reveal a different mode of action for both compounds. Genes related to the translation process are induced by an atRA exposure and inhibited by a 9cRA exposure. Besides that, it was not possible to individualize the transcripts that characterize the singular action of each system RAR and RXR.

The results presented in chapter IV, showed the transcriptomic analysis conducted after an exposure of zebrafish embryos to dioxin-like compounds (Benzo[k]fluoranthene, Benzo[a]pyrene and the natural compound β-Naphtoflavone), due to their persistence and widespread presence in the aquatic environment. Their function is related to the AhR, the key transcription factor regulating specific phase I (compound modification, mainly oxidation) and phase II (compound conjugation) metabolic enzymes, among others (Daniel W. Nebert et al., 1993; Hankinson, 1995). The mechanism of toxicity may vary within the PAH and is correlated with the number of rings in individual PAHs (Incardona et al., 2006). PAHs and dioxins induce their own metabolism by activating transcription of *cyp1a* genes through AhR binding (D. W. Nebert et al., 2004). In our study, all the tested compounds induced changes on the zebrafish transcriptome at the 2dpf. The overall analysis revealed the activation of genes, common for all treatments belonging to the AhR regulon, such as *ahr2, ahrrb, ugt1b5,* and the *cyp1* series *cyp1b1, cyp1c1, and cyp1c2*. These analysis of the results suggested that a significant part of the

transcriptional changes differed between a presumed non-toxic pure AhR activator, BNF, and the two PAHs, B[a]Pyr (a known mutagen) and B[k]Fla. BNF treatment elicits a pattern of transcriptome changes very different form the ones observed with PAHs, including several genes responsible to the metabolism of amino acid, during the first 2 dpf. B[k]Fla and B[a]Pyr 48h treatment resulted in an increase in transcripts related with some uncharacterized mitochondrial toxicity. This effect was particularly strong for the longer, 96h exposure to B[a]Pyr, which affected the mitochondria cellular component and disrupt several important transcripts related to the apoptosis or cell death. Consistently with these findings, several studies indicate that mitochondria are major targets of PAH toxicity in mammalian cells. PAHs localize to the mitochondria and their presence correlates with a decrease in ATP production (Zhu et al., 1995), loss of mitochondrial membrane potential, changes in mitochondrial morphology (Li et al., 2003; Xia et al., 2004), and induction of the mitochondrial-dependent apoptotic pathway (Ko et al., 2004; Huc et al., 2006). Our results indicate that mitochondria are important targets of B[a]Pyr toxicity in zebrafish. As some of these toxic effects were not observed for BNF, we concluded that at least part of the toxic action of PAH might be independent from their interaction with the AhR.

The same transcriptomic approach was also applied to analyze the mode of action of newly developed nanoparticles, the generation 3 and generation 4 PAMAM dendrimers (chapter V). The survival rate and transcriptome profiles were analysed during the first two days post fertilization. Both compounds showed similar toxicity, and their toxicity is consistent with the activation of the innate immune response. The study of those particles was important due to their application for drug (or active substances) target delivery and their application for human diagnostic by imaging technologies. Unlike other nanomaterials, the studied dendrimers did not show an oxidative stress-related toxicity. Rather, we observed the up-regulation on several genes (*tnfb*, *irg1l*, *mmp9*, and *mmp13)* related to a response to other organisms. This transcriptomic profile was similar to the one observed in zebrafish embryos when challenged with an infection by *Salmonella* (Stockhammer et al., 2009). We concluded that our data evidenced the overregulation of several genes related to an immunological response.

From the examples depicted above, it is clear that transcriptional profiles may vary considerably according to the structural composition of the substances and dosis applied, as well as the time of exposure. At low concentrations, only those genes regulated by the specific target of the exogenous substances would be affected by its presence. Increasing concentrations would trigger expression of proper stress genes (oxidative stress-related enzymes, heat-shock proteins). Toxic levels would finally activate cell-or organism-survival functions, like the DNA-damage response, the heat-

shock complex or, in some cases apoptotic genes (Ljungman, 2010; Richter et al., 2010; Pina and Barata, 2011).

The most relevant ecotoxicological effects occur after long-term exposures to low toxic concentrations, although the studies often rely on short-term exposures and high concentrations, Figure 6.2. In this study we evaluate the differences between shorter and longer exposures periods, and perform exposures to concentrations that didn't have phenotypical effects, but which alter the expression of key genes related to the metabolic routs involved in the disruption of each NR studied. It is of great importance to differentiate if a given response measured at the molecular level is a specific target of a particular pollutant or a consequence of a general response. It is important to study the effect of chemicals in the same stage of development, to avoid misinterpretation of the results. Transcriptomic analysis deals with three fundamental factors: the nature of substance, its dose, and the time of exposure. Endocrine disruptors apparently are more selective in their mode of action, because their endocrine response depends upon the capacity of any given substance (natural or artificial, endogenous or exogenous) to interact with one or more components of the endocrine system. The simple interaction of the disruptor with the target promotes a cellular response: for example exogenous atRA (or other structurally related retinoid substances) elicits synthesis of proteins related to the retinoic acid metabolism. Finally prolonged exposure to atRA may inhibit the expression of some important genes related to the development, and more severe exposures lead to the organisms' cell death.

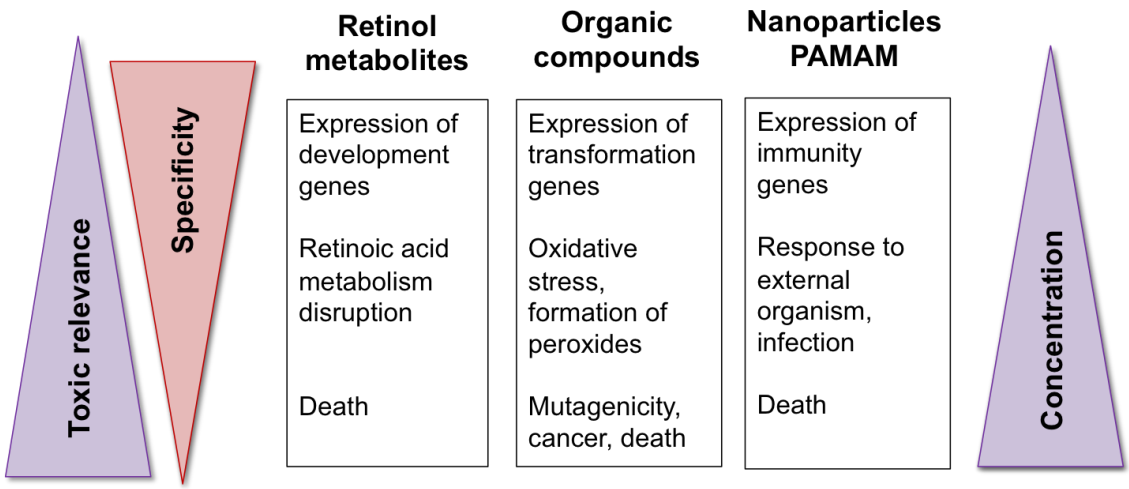


Figure 6.2 Graphic representation of the different responses along a putative concentration gradient of Retinol metabolites, organic compounds with dioxin-like activity, and Nanoparticles. The graph shows the inverse correlation of toxic relevance and specificity of response/effects.

Although it was possible to identify specific changes on the early stages of zebrafish transcriptome upon the selected compounds the approaches with the whole organisms face some additional problems. Transcriptome analyses of whole organisms

may not detect effects occurring on genes expressed in particular tissues, although they may detect general stress genes common to many tissues of the organism. Moreover the microarray technique does not provide information on the portion of the genes that become actually transcribed and ultimately translated into proteins.

The response to stressors follows three differentiated stages: the immediate early response, reflecting the primary response to the stressor through the interaction with its cellular targets; the stress response, at which the cell activates stress response mechanisms to cope with the damage produced by the stressor; and the accommodation/acclimation phase, at which a reduced number of genes and/or metabolic processes are affected to restore the normal function of the cell despite the presence of stressor (allostasis). The use of dynamic genomic tools to establish transcriptome changes across exposure periods and doses offers a promising framework to conduct such studies.

6.2 Research perspectives

This thesis rely on the study of the effects of some compounds that act through the binding to the RAR/RXR and AhR regulatory systems, the superfamily of the NRs is extended and other NRs exist and play important regulatory actions on the homeostasis of the organisms. Those NRs, such as the PPAR (Peroxisome Proliferator Activator Receptor), CAR (Constitutive androstane receptor), and PXR (Pregnane X receptor) are able to heterodimerize with the RXRs and are suitable to be the subject of study for the evaluation of their possible disruption.

6.3 Conclusions

- Techniques based on the quantification of mRNA levels of specific genes allow the detection of early warning signals of environmental stress or the presence of xenobiotics in aquatic organisms.
- The approaches presented in this thesis would contribute significantly to develop alternatives for experiments with adult animals and the establishment of economic, innovative and comprehensive testing approaches.
- Microarray data and qRT-PCR data show a good correlation. This study on microarrays allows concluding about their effectiveness and accuracy of the technique.
- The development of methods for the prompt evaluation of the toxicity of pollutants based on the use of fish embryo forms will help to bridge the gap between *in*

vitro or cell culture models and *in vivo* mammalian models for rapid pre-clinical development.

- Existing microarray studies in fish indicate a good experimental framework to identify specific targets of pollutants acting throughout the steroidogenic pathway and there is also some evidence that the former framework can also be used with success for those chemicals that alter the Retinoic and Retinoid system.

- Particularly, toxicogenomic studies are likely to reveal accurate results for new compounds, for instance, the identification of genes that may contribute for the determination of the mode of action, combined with morphological effects. These markers could be applied for the risk assessment in the registration of chemicals and the analysis of environmental samples.

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