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UNIVERSIDADE DO ALGARVE

Assessment of the mineralogenic toxicity of aquatic pollutants

Joana Rita Silva Caria

Thesis supervised by Vincent Laizé, PhD

Masters in Molecular Biology and Microbiology

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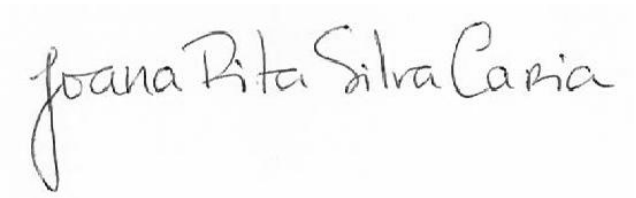
Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da lista de referências incluída.



Joana Rita Silva Caria

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Abstract

In ecotoxicology a major focus is in the aquatic environment, not only because it presents a great economic value to man but it is an ecosystem widely affected by the growing anthropogenic pollution. Most of the studies performed relate to adverse effects in development, reproductive or endocrine disruption but little is known about the possible effects in bone formation and skeletal development.

In this study, we set out to evaluate the effects of 8 aquatic pollutants on the skeletal development using an *in vivo* system, the zebrafish larvae aged 20 days post-fertilization, through chronic exposure. Several endpoints were considered such as the cumulative mortality, total length, occurrence of skeletal deformities and marker gene expression.

We were able to establish LD_{50} values for some pollutants, like 3-methylcholanthrene, lindane, diclofenac, cobalt and vanadate and found that the total length was not affected by any of the pollutants tested. Cobalt was the most harmful chemical to affect hatching time, severely affecting the ability of the zebrafish embryos to hatch and overall the number of deformities increased upon exposure to tested chemicals but no patterns of deformities were identified. We also propose that 3-methylcholanthrene has an osteogenic effect, affecting osteoblast and osteoclast function and that *op* levels can act as a mediator of 3-methylcholanthrene toxic stress to the osteoblast. In turn we found naphthalene to probably have a chondrogenic effect. Our results provided new insights into the potential osteotoxicity of environmental pollutants. Future studies should aim at confirming these preliminary data and at determining mechanisms of osteotoxicity.

Keywords: Ecotoxicology, osteotoxicity, aquatic pollutants, skeletal development, gene expression, zebrafish *Danio rerio* (Hamilton, 1822).

Resumo

Em ecotoxicologia, o meio aquático é uma área de grande importância não apenas porque representa um interesse económico para o Homem mas porque é um dos ecossistemas mais afectados pela crescente dispersão de poluição com origem antropogénica. A maioria dos estudos realizados neste campo dizem respeito a efeitos negativos no desenvolvimento, na reprodução ou na desregulação endrónica dos organismos mas pouco é conhecido sobre o impacto na formação do osso e o desenvolvimento esquelético.

Neste estudo propusemos a avaliação dos efeitos de 8 poluentes aquáticos na mineralização do esqueleto usando um sistema *in vivo,* larvas de peixe-zebra com 20 dias pós fertilização, com exposição crónica. Diversos parâmetros foram avaliados, sendo estes a mortalidade, o comprimento total, ocorrência de deformações esqueléticas e a expressão génica de um grupo de genes alvo de interesse.

Conseguimos chegar a valores para a LD_{50} para alguns dos poluentes, sendo estes o 3-metilcolantreno, lindano, diclofenac, cobalto e o vanádio e concluiu-se que o comprimento total medio não é afectado por nenhum dos poluentes testados. O cobalto foi o químico mais danoso á eclosão dos embriões de peixe-zebra, afectando severamente o processo de eclosão dos embriões. No geral, o número de malformações aumentou em consequência da exposição aos poluentes mas não foi possível identificar nenhum padrão na ocorrência de deformidades. Propomos que o 3- metilcolantreno tem um efeito osteogénico, afectando a função de osteoblastos e osteoclastos e que o gene *op* poderá actuar como mediador no stresse toxico induzido pelo 3- metilcolantreno nos osteoblastos. E por sua vez, o naftaleno possivelmente apresenta um efeito condrogénico. Os nossos resultados trazem uma nova perspectiva ao potencial osteotóxico de poluentes ambientais. Futura investigação deverá focar confirmação destes resultados preliminares obtidos e a determinação dos mecanismos de osteotoxicidade.

Palavras-chave: Ecotoxicologia, osteotoxicidade, poluentes aquáticos, mineralização óssea, expressão génica, peixe-zebra *(Danio rerio)*

Abbreviation List

AB	Alcian Blue
ALP	Alkaline phosphatase
AR-S	Alizarin Red S
BMP2b	Bone morphogenetic protein 2b
COL10A1	Collagen type X alpha 1
Dpf	Days post-fertilisation
EF1a	Elongation factor 1 alpha
EM	Embryo medium
Hpf	Hours post-fertilisation
MAFT	Transcriptional factor V-maf musculoaponeurotic fibrosarcoma oncogene homolog f (Avian)
MOA	Mode of action
NSAID	non-steroidal anti-inflammatory drug
OC	Osteocalcin
ON	Osteonectin
OP	Osteopontin
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-buffered saline
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SOD2	Superoxide dismutase 2
SOX9a	SRY-box containing gene 9a
TL	Total length

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I. Introduction

I.1. New frontiers in ecotoxicology

Ecotoxicology can be simply defined as the study of the impact of pollutants on organisms and their ecosystem (Relyea and Hoverman, 2006). It aims at characterizing the effects caused by harmful chemicals, at understanding their mode of action (biochemical and physiological), at determining how different types of organisms in one ecosystem will respond to chemical exposure and at establishing levels of toxicity for the different classes of chemicals (ECOTOX.). Ultimately, ecotoxicological research should aim at predicting the effects of these chemicals on a natural community under realistic conditions. Ecotoxicology is therefore of great importance when it comes to decision making concerning ecosystem protection and risk assessment (Chapman, 2002).

Commonly, the ecotoxicological research is applied to prospective and regulatory testing (e.g. Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) program in the European Union) to collect toxicity data on newly or already registered chemicals and to address a growing concern on emerging pollutants (e.g. pharmaceuticals) and complex wastes (e.g. effluents, ambient waters and sediments) mainly for compliance monitoring and remediation efforts (Daston et al., 2006; Pandard et al., 2006). Both regulatory and prospective testing is carried out using laboratory assays, focused in single species tests that normally measure biological endpoints such as survival, growth, development and reproduction and rely on whole-animal exposures (Fraysse et al., 2006). The most widespread used tool is the LD_{50} (dose lethal to 50% of the organisms) and the biological pathways commonly studied are those involved in chemical metabolism and detoxification (Fent, 2001). These methods provide useful species-specific data on the potential toxic effects of chemicals that may be extrapolated to the ecosystem. However, tests often do not consider the complexity of ecological processes and other environmental factors that can affect toxicity such as the chemicals bioavailability, the rate of chemical penetration, metabolism and excretion, the possible addictive effects of mixtures or the presence of nonchemical stressors (Daston et al., 2006). Chemical testing has mostly been carried out on teleost fish species (e.g. common carp *Cyprinus carpio*, fathead minnow *Pimephales promelas*, Japanese medaka *Oryzias latipes*, rainbow trout *Oncorhynchus mykiss* and zebrafish *Danio rerio*) not only because fish are one of the most abundant class of vertebrates in number of species, occupying the majority of aquatic habitats and have direct economic interest to man (Persoone

Guido, 1990) but also because aquatic environment is one of the most affected ecosystem by pollutants and anthropogenic chemicals and because aquatic organisms are often exposed throughout their whole life (Fent et al., 2006).

The challenges to ecotoxicology nowadays are the continuous exposure of animals to low levels and to mixtures of pollutants. There is an increasing need to understand how the long-term anthropogenic pollution is going to affect the environment and how can one predict effectively ways to deal with it. The impact of long-term exposure is gradual and sometimes hard to differentiate from the process of environmental change (Moore, 2002). At the same time there is a growing demand for reducing animal testing and improving animal welfare and for standardizing ecotoxicological models and criteria for building toxicological datasets (Raimondo et al., 2009). To make accurate predictions and tackle the challenges in regulation and environment protection, ecotoxicology needs to develop integrated models capable of associating changes in organism/ecosystem levels to a comprehensive mechanistic knowledge of chemicals impact on species, population and cellular organization (Carvan et al., 2008).

The fast development and evolution of the genomic area over the past decades has speed up the application of gene expression for understanding the mechanisms underlying the toxic response (Hamadeh et al., 2002). Which genes and pathway are targeted by chemicals and can pinpoint the unique signature of a stressor, is this variation adaptive and what are the ecosystem level consequences of the molecular transformation, are just some of the key questions that have been the focus of many studies (Snell et al., 2003).

I.2 The role of ecotoxicogenomics

The term *ecotoxicogenomics* was propose by Snape and collaborators (Snape et al., 2004) to describe the integration of genomic based science (transcriptomics, proteomics and metabolomics) into ecotoxicology, and could be described as the study of gene expression and molecular mechanisms involved in adaptive responses to environmental toxic exposures (see Fig.I.2.1). In aquatic ecotoxicology, the standardized methods rely on measuring whole-organism responses to chemicals, using endpoints that derive from the observed phenomena and can be associated to the status of populations. Endpoints such as mortality, growth, reproduction (e.g. fecundity, fertility and hatch), median lethal concentrations or no observed effect concentrations are a few examples.

Whilst this methods provide valuable information a collaborative approach using genomic data (e.g. gene profiling, DNA microarrays, report assays or biomarkers) is important to address the recognized need of (1) anticipating new chemicals ecological risk,(2) predicting toxicant response across diverse phylogenetic groups, (3) approximating how changes in one ecological level will influence other levels and (4) if toxicant responses differ with time of exposure (Snape et al., 2004; Watanabe and Iguchi, 2005).

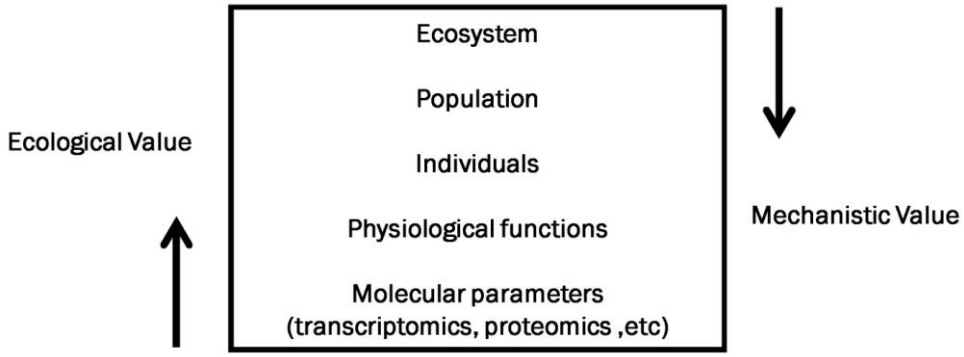


Fig.I.2.1. Proposed framework for ecotoxicogenomics (adapted from Snape et al., 2004).

Organism response to toxic compounds involves changes in the normal physiological setup and in gene expression patterns. This can be a direct result of the toxicant, e.g. through its binding to a transcriptional factor / nuclear receptor, altering the transcription of target genes, or it can be secondary, reflecting the response of the organism to cellular and molecular damage (De Wolf et al., 2004). Importantly, a mechanism of toxicity will generate a specific pattern of gene expression characterizing a mode/mechanism of action (MOA) for that chemical or class of chemical (Theodorakis, 2001). While some of these MOA are conserved across species, others can be species specific and a better description of toxicity pathways via genomic techniques can bring insights into this specificity and also validate the extrapolation of toxicity data from one species to another (Theodorakis, 2001). The information generated by the "omics" technologies together with the phenotypic endpoints of chemical effects will allow the classification of those chemicals and stressors to MOA classes. This way facilitating a more cost-effective and better testing approach and establishing priorities in the risk assessment protocols, for environment and human health (Miracle and Ankley, 2005). The importance of genomic techniques should however not be over emphasized. This field faces challenges namely in data collection, integration and interpretation, and conclusions drawn are only as

strong as their execution. A careful experimental design, e.g. with adequate sample size, chemical exposures, reduced survival or reproductive output and sample collections, is important for a proper interpretation of the genomic data obtained and to achieve a context-driven knowledge of the effects of chemicals on higher levels like populations and ecosystems and not only a mechanistic knowledge (Belfiore and Anderson, 2001). Additionally, the need for bioinformatics tools, the cost of some technology and the ecosystem complexity still makes the interpretation of genomic results a challenge (Poynton et al., 2008).

I.3 Zebrafish as a model in ecotoxicology

The aquatic environment is a sink for many anthropogenic chemicals and within aquatic food web, fish play a critical role in the nutrient and energy flow besides being an important natural and economic resource to man and acting as effective sentinels to aquatic pollution (Lammer et al., 2009). To assess the toxicity of the chemicals released in the environment it is necessary to collect appropriate data on the endpoints of toxicity, their dose-response and the toxicodynamics of the chemical. This data requires testing (acute and chronic exposures) with model vertebrates species, particularly fish (Hill et al., 2005a). One of the best-described and popular model vertebrate species is the zebrafish (*Danio rerio* Hamilton, 1822). What makes it so attractive is the level of available knowledge of its morphology and physiology, technology and approaches at hand (Kimmel et al., 1995; Rubinstein, 2006; Spence et al., 2008). Zebrafish is an important vertebrate model in development genetics, neurophysiology, biomedicine and ecotoxicology for decades and most recently it has emerged as a model for disease modeling and drug discovery pipelines (Zon and Peterson, 2005). It is a small, shoaling cyprinid, native to the rivers in India and South Asia, where it is found in shallow, slow-flowing waters. A number of unique characteristics have contributed to its popularity such as its rapid development, easy-low cost maintenance, large number of offspring, the transparency of embryos during early embryogenesis, access to experimental manipulation and genetic similarity to humans (Lawrence, 2007). In comparison to other toxicological vertebrate models, zebrafish is a small and robust fish enabling a large number to be kept in laboratory greatly reducing housing space and husbandry costs and because it has been widely used in laboratories the optimal breeding and maintenance conditions are well described (Spence et al., 2008; Westerfield, 1995) The zebrafish has

a short reproduction cycle, each female can spawn every 2-3 days several hundreds of eggs and under appropriate maintenance conditions can breed all year round making possible large-scale screens and testing, even in a small laboratory (Spence et al., 2008). An external development and a transparent chorion facilitating the manipulation and observation have largely contributed to a better knowledge of the development processes (Kimmel et al., 1995). They also allow the identification of phenotypic traits and other endpoints of toxicity during toxicity testing (Fraysse et al., 2006; Lammer et al., 2009; Seok et al., 2008). The development of fertilised eggs is rapid, its basic body plan is laid out after 24 hpf (hours post-fertilisation), the embryos hatch after 2-3 dpf (days post-fertilisation) and the organogenesis of major organs is completed at 5 dpf with the onset of external feeding (Spence et al., 2008).

In contrast to other model organisms, like rodents, the small size of larval and adult zebrafish minimizes maintenance costs but also reduces the volume of dosing solutions (experimental chemicals, drugs and pollutants) creating limited amount of waste for disposal and reducing the labware necessary to treat and maintain the fish alive throughout the duration of the assays (Hill et al., 2005b). In addition, the small size and transparency of zebrafish embryo enables the creation of high-throughput screens for toxicity testing (Peterson and Macrae, 2012; Rubinstein, 2006), preclinical drug discovery (Zon and Peterson, 2005) and small molecule screening (Murphey and Zon, 2006). The embryo transparency makes possible to detect functional and morphological changes in internal organs, in particular the skeleton, that can be highlighted through the use of transgenic lines or other reporter molecules (Zon and Peterson, 2005).

Zebrafish genomic tools have been perfected in the past few years, increasing its suitability as an experimental model: (1) Its genome has been almost entirely sequenced by the Sanger Institute and annotated through the trans-National Institutes of Health Zebrafish Genome Initiative; (2) full-length collections of zebrafish cDNAs are available on several DNA microarrays for expression profiling experiments; (3) tools for the generation of transgenic lines and for targeted mutations techniques have been developed; (4) probes and primers for the analysis of gene expression through *in situ* hybridization and quantitative real-time PCR have been developed; and (5) antisense morpholino oligonucleotides have been developed for functional genomics (Zon and Peterson, 2005).

In toxicology and ecotoxicology the use of zebrafish as a model, particularly embryonic stages, is very desirable to perform studies aimed at the understanding of toxic

mechanisms and possible adverse long-term effects and has a wide range of possible applications (see Fig.I.3.1.). One of the first applications of the zebrafish in ecotoxicology was the 96-h acute fish toxicity test (Nagel, 2002), to test chemicals for an appropriate environmental risk assessment according to the OECD (Organistion for Economic Co-operation and Development) guidelines (OECD, 2006, 1992). This test can also include screening for teratogenic effects (Weigt et al., 2011) along with other morphological, sub-lethal endpoints that can relate to effects like neurotoxicity, cardiotoxicity, reproductive toxicity, endocrine disruption, neurobehavioral toxicity, vascular toxicity or carcinogenicity (Scholz et al., 2008).

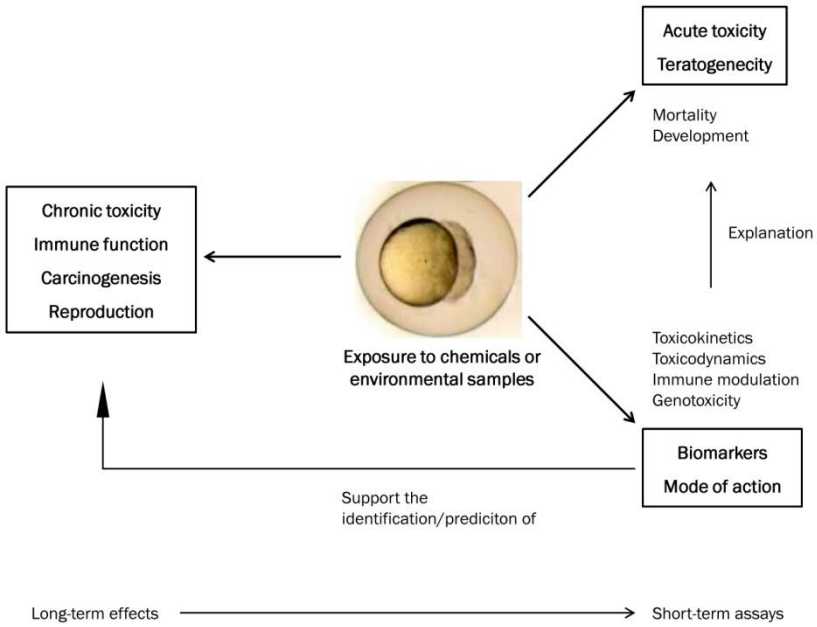


Fig.I.3.1. Schematic representation of the possible approaches and applications to the use of zebrafish embryo as a model in risk assessment of pollutants (Adapted from Scholz et al 2008).

Acute testing and the use of fish embryos is the most common practice, not only because of the evident benefits of using embryos for small-scale and high-throughput testing but also because the use of fish embryos is not clearly regulated in animal welfare legislations to a great extent (Table.I.3.2). Maybe for this reason there is a lack of knowledge when it comes to chronic toxicity data and its potential subtle effects (Fent et al., 2006). Chemical exposure in the long run can cause changes in the genetic patterns of a population by decreasing the genetic diversity and have a larger impact in the overall fitness of the individuals and higher-level effects that short-time assays cannot evaluate (Belfiore and Anderson, 2001). Though the fish embryo test can present several advantages, it falls short in giving us a look into the possible negative long-term effects of chemical exposure.

Although these approaches integrated with genomic tools have the potential to become very important and effective in predicting adverse long-term effects for risk assessment and chemicals registration, there are still several bottlenecks that need to be overcome in order to fully benefit from these applications of the zebrafish model. Work needs to be done towards the identification of genes, molecular markers indicators of the mechanism of action, establish the correlation between effects in short-time assays to possible long-term effects in the population and develop a comprehensive test that can be widely applied (Tyler et al., 2008).

Topic	*Application*	*References*
Toxicokinetics		
Bioconcentratrion	Analysis of bioconcentration on the embryo	Gorge and Nagel 1990; Wiegand et al. 1999; Schreiber et al. 2008
Toxicodynamics		
Transcriptomics	Effect of hypoxia	Ton et al. 2003
	Effect of 4-nonylphenol exposure	Hoyt et al. 2003
	Inhibition of shh-pathway by cyclopamine	Xu et al. 2006
	Effect of dioxin on gene expression in the zebrafish embryonic heart	Carney et al. 2006
	Comparison of transcription patterns in embryos exposed to 11 different compounds	Yang et al. 2007
Functional gene analysis	Role of AHR2 and CYP1A for the mediation of dioxin and benz[a]anthracene toxicity	Carney et al. 2004; Incandorna et al. 2006
	Protective function of *cyp1a, hmox1* and *nfe212* induction in embryos exposed to 3,4-dichloroaniline	Voelker et al. 2008
Proteomics	Differential response of zebrafish embryos to exposure with 17-beta-estradiol and 4-nonylphenol	Uses of zebraShrader et al. 2003
	Changes in yolk proteins as indicators of metabolic effects in embryos exposed to ethanol	Gundel et al. 2007
Prediction of adverse and long-term effects		
Immune modulation	Induction of pro-inflamatory responses and potencial use for analysis of immune modulation	Herbomel et al. 1999; Pressley et al. 2005; van der Sar et al. 2003; Watzke et al. 2007
Endocrine disruption	Induction of vitellogenin gene expression by estrogenic compounds	Muncke and Eggen 2006; Muncke et al 2007
Genotoxicity	Analysis of genotoxic effects by the assay in embryos exposed to river sediments	Kosmehl et al. 2006, 2007
	Analysis of genotoxic effects using the *rps1*, transgenic zebrafish	Amanuma et al. 2000
Chronic toxicity	Gene expression im embryos is affected in the same range as chronic effects in the early life stage test	Voelker et al. 2007

Examples refer to the zebrafish (Danio rerio) model, but can principally performed with other fish species as well

Table.I.3.2. Examples of applications of the fish embryo test in toxicology (adapted from Scholz et al., 2008).

For many substances, effects on zebrafish embryos have been assessed but only a few have looked at the possible osteotoxic effect. Studies have showed that tributyltin inhibits calcification of the supraoccipital bone and reduces the activity of alkaline phosphatase (ALP) and osteocalcin (OC) (Tsukamoto et al., 2004); 3-methylcholanthrene demonstrated to have an inhibitor effect on bone calcification and to reduce ALP activity, the rate of deposition of calcium in cells and a decrease in OC expression was also observed

(Naruse, 2002); vanadate has also been reported to impair the mineralization of the extra-cellular matrix in a gilthead seabream bone-derived cell line (Tiago et al., 2008a, 2008b) and to delay ossification and induce skeletal malformation in mice exposed in utero (Domingo, 1996); exposure to pesticides, in mice, have reduced bone growth (Pikaliuk, 1991); TCDD has been reported to disrupt skeletogenesis in exposed medaka fish (Dong et al., 2012) and cadmium exposed animals have shown bone loss (Bhattacharyya, 2009).

I.4 Skeletal development in teleost fish

Vertebrate endoskeleton is a specialized complex organ made of bone, a rigid, vascularized and mineralized connective tissue, and cartilage, a flexible connective tissue. It provides support and protection of vital organs as well as being a metabolically active tissue, acting as a reserve of inorganic ions, as source for hematopoiesis and adsorbing toxins (Jayakumar and Di Silvio, 2010; Lefebvre and Bhattaram, 2011; Marks and Odgren, 2002). Major skeletal cell types are the chondroblast in cartilage and the osteoblast and osteoclast in bone. Chondroblasts and osteoblasts have mesenchymal origin whereas osteoclasts are derived from the hematopoietic cell lineage (Karsenty and Wagner, 2002; Lefebvre and Bhattaram, 2011). The osteoblasts (bone-forming cells) are responsible for the synthesis of bone matrix and the regulation of mineralization, while the osteoclasts (bone-resorbing cells) degrade and resorbe the bone matrix (Fig.I.4.1). Teleost fish, like zebrafish develop bone tissue from mesenchymal cell condensations (membranous ossification) or from cartilage scaffolds (endochondral and perichondral ossification, where mineralization occurs within the cartilage model or from the perichondrium surrounding the cartilage model, respectively).

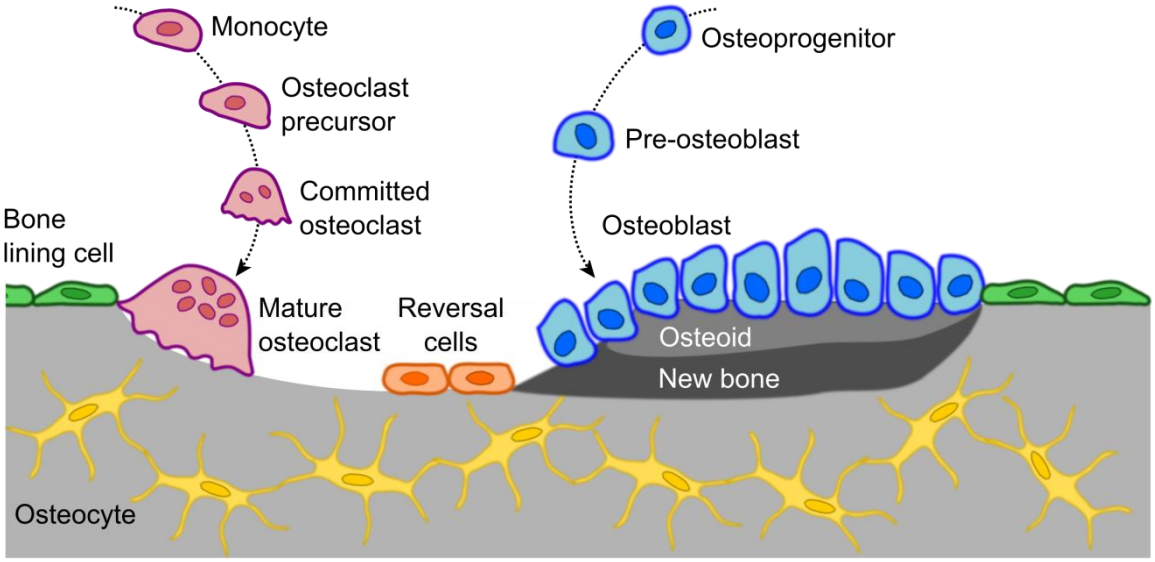


Fig.I.4.1. Schematic representation of bone remodeling. Osteocytes (yellow cells), which are embedded within mineralized bone, communicate via ramifications of dendritic processes and orchestrate the spatial and temporal recruitment of the cells that form (osteoblasts) and resorbe (osteoclasts) bone. Hematopoietic cells of the monocyte/macrophage lineage differentiate to mature osteoclasts (pink cells) and resorbe bone. During the reversal phase (orange cells), osteoblastic progenitors are recruited to the site of resorption, differentiate and synthesize osteoid, and mineralize the new bone matrix (blue cells). Adapted from Nicholls (2012).

Based on the presence of osteocytes, which are derived from mature osteoblast entrapped in the bone matrix and are involved in bone maintenance, teleosts can be divided into species that have cellular bone (or osteocytic bone) like the zebrafish, or acellular bone (or anosteocytic bone) like the medaka (Fig.I.4.2). Another specificity of teleost fish bone is the presence of two types of osteoclasts: (1) mononucleated cells, predominant in juveniles, observed early during skeletogenesis and still present in the adult fish in the craniofacial skeleton and vertebral column and (2) multinucleated cells, predominant in 40-day-old adults. Both types contribute to bone growth and bone resorption and remodeling, although the mononucleated osteoclasts resorbing activity is much lower (Marks and Odgren, 2002; Renn et al., 2006; Witten and Huysseune, 2010; Witten et al., 2001).

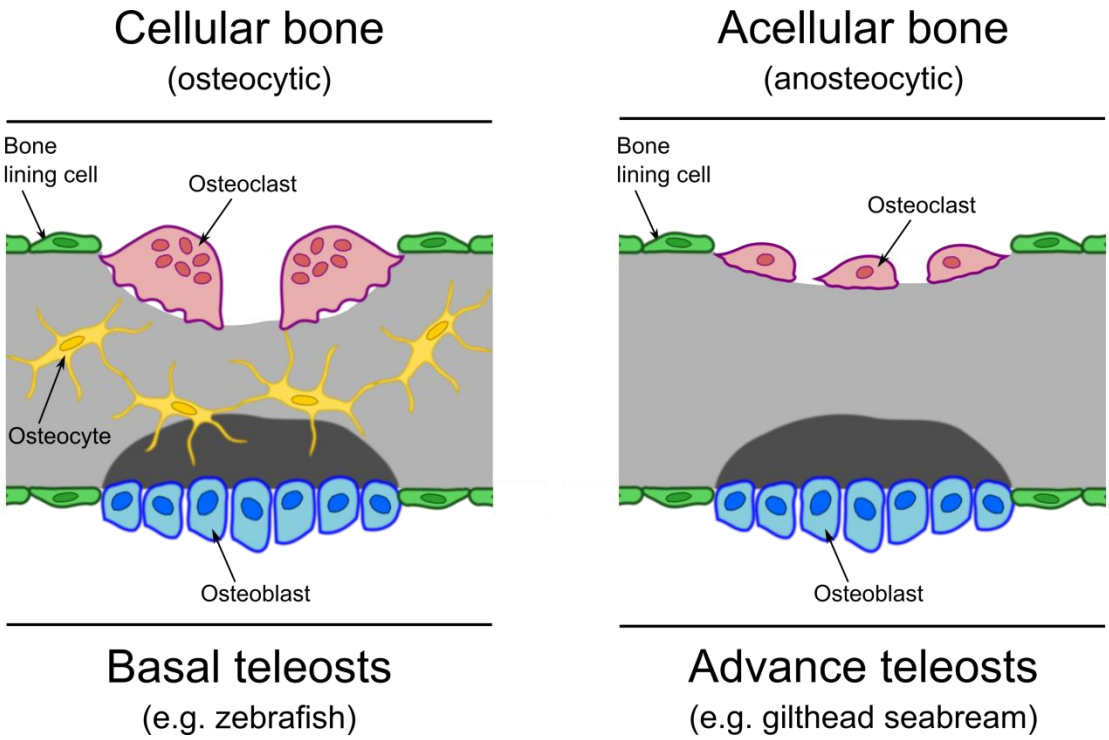


Fig.I.4.2. Main differences between cellular and acellular bone. Osteocytes (yellow cells), embedded in bone matrix, osteoclasts (pink cells) resorbing bone, osteoblasts (blue cells) laying down osteoid and bone lining cells (green cells). Adapted from (Witten *et al.*, 2010).

Skeletal organization as well as the mechanisms underlying skeletogenesis has been remarkably conserved throughout vertebrate evolution, from zebrafish to human. This similarity in ecotoxicology studies, using teleost species as models, is very important to help determine in the future what the level of risk for humans in case of exposure. For example, the zebrafish develops a simple pattern of early larval cartilages and bones as in other vertebrates; skeletal cell types (e.g. osteoblasts, chondrocytes and to some extents osteoclasts) are also similar; type of ossification (e.g. chondral and intramembranous) are also similar in zebrafish and in human (Javidan and Schilling, 2004). These similarities at the cellular level can also indicate that regulatory mechanisms behind bone formation and homeostasis are as well conserved between fish and higher vertebrates. Supporting this are numerous studies that show a significant degree of conserved functions of genes involved in bone metabolism and their pattern of expression during embryogenesis in higher vertebrates and zebrafish. For teleost fish this information comes mainly from expression studies, fish mutants and gene silencing techniques (Renn et al., 2006).

Looking specifically at skeletogenesis in zebrafish, larvae at 45-72 hpf exhibit cartilaginous structures such as the pharyngeal skeleton of the jaws and gills and the neurocranial skeleton. In the neurocranial cartilages, the first chondrifications occur parallel to the anterior end of the notochord (45-48 hpf) to form the parachordal cartilage followed by the trabecular cartilages. At the same time, the pharyngeal skeleton starts to chondrify forming the 7 pharyngeal arches, the jaw, hyoid and 5 branchial arches that will support the gills. The neurocranium bones are formed through perichondral ossification while the pharyngeal arches become calcified cartilage, which starts around 6 to 7 dpf. Intramembranous ossification is observed earlier, e.g. at 3 dpf the cleithrum and at 5 dpf the operculum (Javidan and Schilling, 2004). The axial skeleton, derived from the somitic mesoderm, is formed by the vertebrate column and associated fins. The vertebrate column is divided into 4 regions, the weberian vertebrae, the abdominal vertebrae (composed by centra, neural arches and spines, parapophyses and ribs), the caudal vertebrae (composed by centra, neural arches and neural spines and hemal arches and hemal spines) and the caudal fin vertebrae (Fig.I.4.3). The associated median fins include the dorsal, anal and caudal fins (Bird and Mabee, 2003).

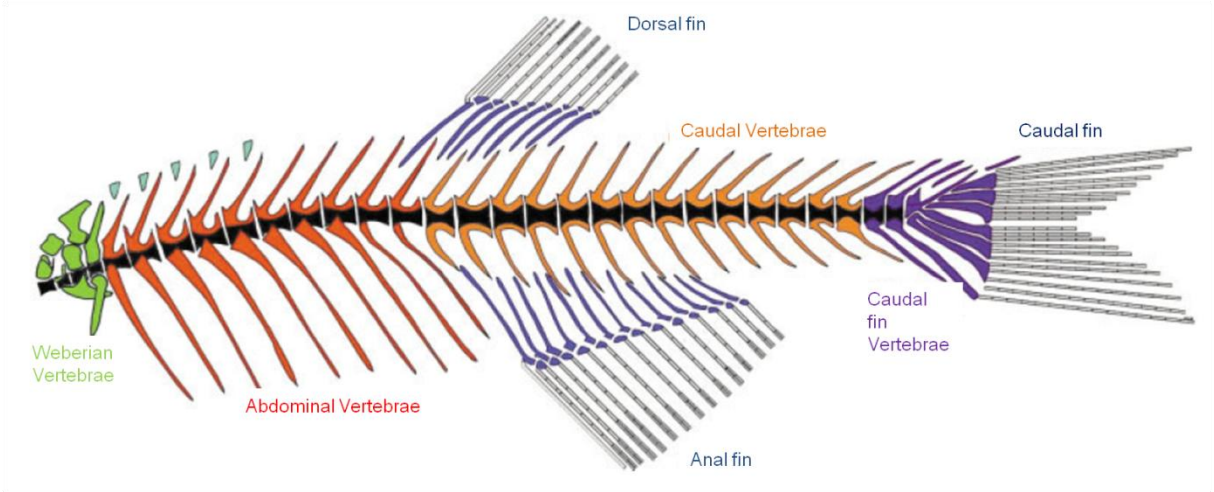


Fig.I.4.3. Schematic of the axial skeleton of the zebrafish. Centra are in black, the Weberian apparatus in green (vertebra 1-4), abdominal vertebrae are in red (vertebra 5-14), caudal vertebrae in orange (vertebra 15-28), the caudal fin in purple. (Adapted from (Bird and Mabee, 2003b)).

The ossification of the axial skeleton begins simultaneously in the weberian and caudal fin vertebrae (specifically the hypural 1 and parhypural), the anterior and posterior limits of the axial skeleton. The development proceeds then posterior and anteriorly from the weberian vertebrae meaning that the caudal vertebrae are the last to form; all the structures associated with the vertebrae follow the same pattern of development. Among

the fins, the caudal fin is the first to develop, followed by the anal and the dorsal fin is the last one to form (Inohaya et al., 2007).

II. Objectives and experimental setup

The main objective of this work was to evaluate the effect of pollutants on skeletal development and bone formation, and collect evidence on molecular pathways possibly involved. For this, developing zebrafish larvae were chronically exposed to 5 different concentrations of 8 pollutants throughout 20 days (Fig.II.1.).

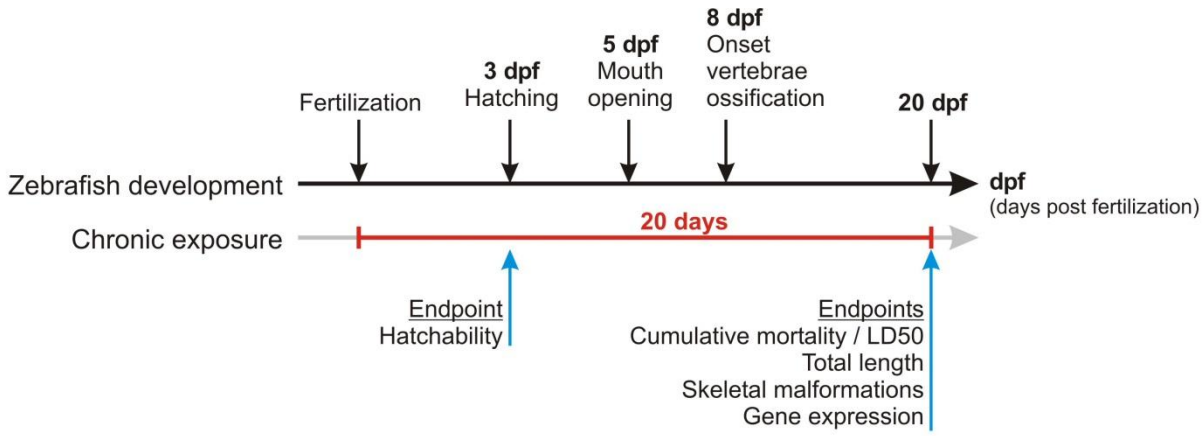


Fig.II.1. Schematic representative of the endpoints for this experimental work.

II.1. Chemicals used in the study

For this study 8 chemicals were chosen to be evaluated for their possible toxic effects in bone mineralization (Table II.1.1).

Table II.1.1. Summary of chemical classes used in this study

Chemical classes	*Type of toxicity study*	*References*
Heavy and transition metals		
Cobalt (II) chloride	Toxicity: dose response, effects on hatching and survival	Dave and Xiu, 1991
Cadmium chloride	Abnormal somite patterning and defects in axonogenesis	Hen Chow and Cheng, 2003
	Inhibition of neurogenesis in embryonic brain development	Chow et al.,2008
	Accumulation in tissues and genotoxic potential	Cambier et al., 2010
Vanadium (ammonium metavanadate)	Induction of cytotoxicity and oxidative stress in osteoblastic cells ***	Cortizo et al., 2000
	Stimulation of cell proliferation and impairment of extracellular matrix mineralization ****	Tiago et al., 2008
Pesticides and herbicides		
N-phosphonomethyl-glycine (Glyphosate)	Toxicity and effects of different environmental factors *	Tsiu and Chu, 2003
	Induction of oxidative stress and antioxidant response *	Lushchak et al., 2009
γ-1,2,3,4,5,6-hexachlorocyclohexane (Lindane)	Deformations, mortality, growth retardation and hatching rate	Gorge and Nagel, 1990
	Toxicity and effects on reproduction	Ensenbach and Nagel, 1997
PAHs		
Naphthalene	Morphological abnormalities occurring after cardiac dysfunction	Incardona et al., 2004
	Toxicity: induction of formation of ROS, oxidative tissue damage	Stohs et al., 2002
3-methylcholanthrene (3-MC)	Potential endocrine system disruption	Ota et al., 2000
	Inhibition of osteoclast formation **	Naruse et al., 2004
NSAID drugs		
Diclofenac (sodium salt)	Toxicity: effects on hatching and mortality, embryonic development	Haallare et al., 2004
	Effects on actin organization and muscle fiber alignment causing malformed somite phenotypes	Chen et al., 2011

** Examples refer to studies with other species that not zebrafish*
*** Study performed using mouse spleen cells and clonal osteogenic stromal ST2 cells*
**** Using cell lines osteoblast (MC3T3E1) and an osteosarcoma (UMR106)*
***** Using a bone derived fish cell line VSa13*

- *Polycyclic aromatic hydrocarbons (PAHs)*, persistent in aquatic environments, derived from waste materials with anthropogenic source like consumption of petroleum, transport activities, proximity to urbanized areas, oil spills or inadequate combustion of solid and fuel oils (Incardona et al., 2004). Research has suggested that PAHs can have an anti-estrogenic effect, acting as endocrine disruptors,

  3-Methylcholanthrene, a potent carcinogenic/mutagenic agent, very common in polluted urban air and also used in research as an experimental carcinogenic agent (Kallenborn and Hühnerfuss, 2001; Polat et al., 2013). It is metabolized in fish by the cytochrome P450 (Marionnet et al., 1997; Ota et al., 2000) and some research points to a inhibition of osteoblast proliferation and differentiation and inhibition of osteoclast formation (Naruse, 2002; Naruse et al., 2004).

  Naphthalene, a bicyclic aromatic compound with industrial and commercial applications, used in the synthesis of other materials, e.g. resins, surface active agents and synthetic tanning agents. Its diverse sources and usage makes it very

common to be expose to and a lot of acute and chronic effects been described in humans (Naphthalene, EPA). Naphthalene is metabolized under an oxidative process by the cytochrome P450 enzymes resulting in reactive metabolites that lead to oxidative stress causing DNA damage and tissue damages (Stohs et al., 2002) but no osteotoxicity effect has been described before.

- *Pesticides and herbicides*, chemicals usually found in aquatic environments and derived of human activities,

  Glyphosate (N-phosphonomethylglycine), a non-selective and post-emergent herbicide, best known as Roundup. Its toxicity, especially for animals, is considered low but due to its extensive use and persistence particularly in aquatic environments, the negative effects can have a bigger impact for aquatic species in particular fish. It inhibits plant growth through inhibition of the enzyme enolpyruvylshikimate phosphate synthase that produces essential amino acids (EPA; Alibhai and Stallings, 2001; Lushchak et al., 2009). Although no osteotoxicity effect is described, this herbicide concentration can have severe effects in population survival and other physiological factors.

  Lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane), an organochloride insecticide, used as an insecticide and fumigant, considered a possible human carcinogen and several sub-lethal effects have been reported such as biochemical changes in organisms and reproductive effects (EPA). Growth retardation and deformations have also been associated with lindane (Görge and Nagel, 1990).

- *Pharmaceuticals*, often derived of water wastes or non-treated residual waters.

  Diclofenac, a non-steroidal anti-inflammatory drug, considered a superficial water pollutant and relatively persistent in the environment being one of the most abundant pharmaceutical trace pollutants found in rivers. Diclofenac inhibits the cyclooxygenase activity, an enzyme that synthesizes prostanoids, mediators in the inflammatory and anaphylactic reactions, vasoconstriction and inflammatory response (De Felice et al., 2012).

- *Heavy metals*, due to their environmental persistence and the wide range of toxic effects, from carcinogenesis, neurotoxicity to development and reproductive toxicity (Blechinger et al., 2007) are considered very hazardous.

Cobalt, a natural element present in the environment although there can be cases of acute and chronic exposure due to its use in hard metal industry and pigment manufacture therefore air and aquatic pollution is a common effect (EPA Cobalt).

Cadmium, a non-essential heavy metal, highly toxic, commonly found in the environment because of its general use in industry and listed both in the U.S Environmental Protection Agency's list of priority pollutants and in the European Community's Black list of chemicals (Cheng et al., 2000). Cadmium's embryotoxicty and teratogenicity is well study, and it is known to cause itai-itai disease (Tsuchiya, 1969) and development defects and humans, as well as effects like reduction in embryo body size, growth retardation or morulee degeneration in chick or mice (Chen and Hales, 1994; De et al., 1993; Gilani and Alibhai, 1990). In adult fish there's also evidence of linkage between vertebral and maxillar deformities and cadmium exposure (Cheng et al., 2000).

Vanadate, a trace element with biological importance and also used in the metal industry (Tiago et al., 2008a). It plays a part in regulation of physiological processes like differentiation, cell growth, glucose and lipid metabolism and has insulin-like properties (Cortizo et al., 2000). Studies support vanadium role in the regulation of osteoblast-like cell proliferations and differentiation, the impairment of ECM mineralization and in having proliferative and anti-mineralogenic effects on fish chondrocytes (Laizé et al., 2009; Tiago et al., 2008a). The majority of the studies available are performed in fish cell lines so it will be interesting for us to test it in a whole fish test and evaluate its skeletogenic effects

II.2. Endpoints: mortality, total length, AR/AB staining and gene expression

Mortality was recorded throughout pollutant exposure until 20 dpf and the percentage of survival in relation to the control condition will be determined as well as the LD_{50} (**endpoint: LD_{50} value**). Growth (total length) will be determined at 20 dpf from micrographs (**endpoint: total length**). Following this procedure, skeletal deformities were determined from fish double stained with alizarin red (bone) and alcian blue (cartilage) (**endpoint: frequency of deformities**). A list of structures was defined to account for the number of deformities and later statistical analysis (Table II.3.1.). The potential effect of the chemicals on the expression of bone-related genes was assessed by quantitative real-time PCR (**endpoint: relative gene expression at 20 dpf**). The set of genes was as follows:

- *SRY-box containing gene 9a* (*sox9a*), a transcription factor essential for chondrocyte differentiation and chondrogenesis (Bi et al., 1999; Yan et al., 2002);
- *osteonectin* (*on/sparc*), a matricellular calcium-binding glycoprotein secreted by osteoblasts during bone formation, initiating mineralization and promoting mineral crystal formation (Redruello et al., 2005; Yan and Sage, 1999);
- o*steopontin* (*op/spp1*), an extracellular matrix protein involved in bone formation, remodeling and maintenance (Morinobu et al., 2003);
- *osteocalcin 1* and *2* (*oc1*, *oc2*), a marker of bone formation involved in calcium/hydroxyapatite binding and possibly energy metabolism (Gavaia et al., 2006; Lee and Karsenty, 2008; Singer and Eyre, 2008);
- *tissue non-specific alkaline phosphatase* (*tnap*) a marker of osteoblastic differentiation involved in pyrophosphate cleavage and phosphate production (Addison et al., 2007; Singer and Eyre, 2008);
- *collagen type X alpha1* (*col10a1*), an ECM structural protein expressed by hypertrophic chondrocytes during endochondral ossification (Simões et al., 2006) and also a maker for osteoblast differentiation (Eames et al., 2012);
- *bone morphogenetic protein 2b* (*bmp2b*) a molecule driving the differentiation of mesenchymal cells into osteoblasts toward bone formation; it has also been related to promotion of bone repair (Hassan et al., 2006; Wang et al., 2012).

A second set of marker genes was chosen based on the embryo toxicity test (DarT) that identifies possible marker genes for the mechanisms underlying the toxic effect:

- *Biotransformation: Cytochrome P450, family 1, subfamily A* (*cyp1a*) monooxygenase has an important role in the biotransformation of many xenobiotics (Sarasquete and Segner, 2000);

  aryl hydrocarbon receptor 2 (*ahr2*) a receptor for environmental contaminants and a mediator of chemical toxicity, altering gene expression and inhibiting cell cycle progression (Kung et al., 2009; Stevens et al., 2009);
- *Stress response: nuclear factor erythroid-derived 2* (*nfe2*) an important inducer of phase 2 detoxification enzymes (Motohashi and Yamamoto, 2004);

  transcription factor mafT (*maft*), activates the transcription of stress target genes (Katsuoka et al., 2005);

  heme oxygenase 1 (*hmox1*) that increases cell resistance to oxidative injury (Martin et al., 2004);

heat shock protein 70 (*hsp70*) a chaperone protein acts to stabilise denatured proteins, preventing the formation of harmful cytotoxic aggregates (Hofmann, 2005; Voelker et al., 2007; Weil et al., 2009);

- *Antioxidant response: glutathione peroxidase 1a* (*gpx1a*) has protective role against oxidative injury and death mediated by reactive oxygen species (Arthur, 2000);

  thioredoxin (*txn*) acts as a redutase in redox control, protects proteins from oxidative aggregation, regulates programmed cell death and stress response and is also involved in the modulation of the inflammatory response (Collet and Messens, 2010);

  superoxide dismutase 2 (*sod2*) prevents molecular damage by detoxifying superoxide (Hosoki et al., 2012; Yang et al., 2007).

Table II.3.1. List of skeletal structures analyzed per treatment

Structures
Anomalies of abdominal vertebrae
Fused vertebrae
Flatten vertebrae (platispondily)
Abnormal vertebrae
Anomalies of caudal vertebrae
Fused vertebrae
Flatten vertebrae
Abnormal vertebrae
Anomalies of caudal fin vertebrae
Fused vertebrae
Flatten vertebrae
Abnormal vertebrae
Anomalies of arches
Fused arches
Malformed arches
Altered n of arches
Scoliosis
Kyphosis
Notochord
Prognathism
Operculum
Branchial arches
Pectoral fin + rays
Dorsal fin + rays
Anal fin + rays
Epural
Parhypural +hypurals 1 and 2
Hypurals 3-5
Urostyle

III. Materials and methods

III.1. Materials

Stock solutions of naphthalene, glyphosate, lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane), cobalt (II) chloride, diclofenac sodium salt, cadmium chloride and vanadate (ammonium metavanadate), all from Sigma-Aldrich, were prepared in distilled water. Stock solution of 3-methylcholanthrene was prepared from the commercial solution (100 ng/µL in acetonitrile, Fluka) in embryo medium (EM, see composition (Westerfield, 1995)).

III.2. Fish maintenance and egg collection

A breeding stock of adult zebrafish (*Danio rerio*) aged between 6 and 24 months, kept at the EDGE-CCMAR facility, was used for egg production. Fish were held in a water re-circulating system (ZebTEC, Tecniplast) at a constant temperature of 28ºC, pH 7.7, conductivity of 600-700 µS, oxygen saturation of 70-80%, with 10% of water replaced daily and under a 10:14 h dark/light photoperiod. Fish were fed once a day with newly hatched *Artemia* nauplii and flake food (Benelux) twice a day. For egg production, male and female zebrafish were held together overnight in 1-L breeding tanks (Tecniplast), in a ratio of 2:1. Eggs were collected 30 to 45 min after the onset of light in the morning, which triggers spawning. Fertilized eggs were rinsed in water and selected under a dissecting microscope (unfertilized/coagulated eggs, eggs with asymmetries, formation of vesicles or damaged membranes were discarded).

III.3. Fish chronic toxicity test

The toxicity test was designed based on the OECD guidelines on Fish Embryo Toxicity (FET) test (OECD, 2006) and Fish Early-life Stage Toxicity (FEST) test (OECD, 1992) performed in semi-static test conditions throughout 20 days. After selection, 90 eggs per treatment were transferred into 10-cm plastic dishes (Sarstedt) containing approximately 50 mL of EM (control) or EM-containing chemical. At 1 day post-fertilization (dpf), 60 viable eggs per treatment were randomly chosen and placed into plastic cups containing 500 mL of EM or EM-containing chemical. The nominal test concentrations used per chemical are described in Table III.3.1.

Developing embryos were maintained until 20 dpf at a constant temperature of 28ºC and under a 10:14 h dark/light photoperiod using a Sanyo MIR 153 incubator. Larvae at 5

dpf (mouth opening) were fed once a day with newly hatched *Artemia* nauplii; larvae at 10 dpf were fed twice a day. EM was renewed every day to maintain water quality and chemical concentration. Dead eggs/embryos were recorded and removed. Coagulated eggs, non-hatched embryos, absence of heartbeat and/or absence of body movement were considered lethal effects.

Larvae at 20 dpf were euthanized with 2-phenoxy ethanol (1000 ppm in distilled water; Sigma-Aldrich).

III.4. Total length measurement

Total length of each larvae (TL, the length from the tip of the snout to the tip of the longer lobe of the caudal fin) was determined using Image J software (imagej.nih.gov/ij/) from high quality JPEG pictures acquired using a dissecting microscope (Leica MZ75) equipped with a color camera (Olympus Altra20). Pictures were taken using 0.63 x amplification and the fish were placed in a dorsal or lateral position.

III.5. Staining of bone and cartilage tissues

The acid-free double staining protocol followed in this work was based on the protocols described by Gavaia et al (Gavaia et al., 2000) and Walker and Kimmel (Walker and Kimmel, 2007).

Preparation of acid-free double stain solutions: Two different staining solutions were prepared and mixed just prior to staining. Part A contains Alcian Blue 8GX (AB; Sigma-Aldrich) for cartilage staining and part B contains Alizarin Red S (AR-S; Sigma-Aldrich) for bone staining. Part A (50 mL) is prepared by mixing 2.5 mL of AB stock solution (0.4% AB in 70% ethanol), 35 mL of 95% ethanol and 12.5 mL of 60 mM final $MgCl_2$. Part B is prepared by mixing 49 mL of AR-S stock solution (0.5% AR-S dissolved in distilled water) to 1 mL of 1% KOH to turn it deep purple.

Tissue fixation: Larvae were transferred into a 1.5-mL tube containing 1 mL of 4% paraformaldehyde (PFA, Carlo Erba Reagenti) in phosphate-buffered saline (PBS) and stored at 4ºC overnight (approximately 14 h) . Fixative was removed and larvae were washed 2 × 10 min with PBS and gradually dehydrated at room temperature with 70% and 50% ethanol, 10 min each.

Cartilage staining: After dehydration with 50% ethanol, larvae were stained with Alcian Blue solution for 2 to 3 h.

Neutralization/rehydration: Larvae were washed in 100% ethanol for 30 min and then hydrated through a decreasing ethanol series: 80%, 60% and 30% for 10 min each, at room temperature.

Bone staining: Larvae were stained with Alizarin Red solution for 30 min.

Clearing: Staining solution was removed and larvae were kept overnight in 1% KOH to remove the excess of pigmentation. This solution was then replaced by 0.5% KOH and 25% glycerol for further clearing. To decrease the exposure to KOH and speed up the process, a small amount of H_2O_2 was added to the tissues under an illuminated area until final clearing was reached.

Preservation: Stained and cleared larvae were washed in distilled water to remove any KOH residue and then gradually placed in increasing glycerol solutions to absolute glycerol for final storage.
Larvae were finally observed under a Leica dissecting microscope and malformations were recorded. Representative malformations were photographed using a digital camera.

III.6. RNA extraction and quantitative real-time PCR

RNA extraction: Total RNA was isolated from 20-dpf larvae following a protocol adapted from Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) and purified using the High Pure RNA Isolation kit (Roche) according to manufacturer protocol. RNA quantity and integrity was evaluated using an Experion RNA HighSens chip and electrophoresis system (Bio-Rad). RNA was stored at -80ºC.
Reverse transcriptase-polymerase chain reaction: 1 µg of total RNA was reverse transcribed to cDNA for 1 h at 37°C using 200 U/µL of M-MLV reverse transcriptase (Invitrogen), 40 U/µL of RNaseOUT (Invitrogen), [quantity] of oligo-d(T) universal primer [5'-ACGCGTCGACCTCGAGATCGATG$(T)_{13}$-3'] in a total volume of 20 µL.
Quantitative real-time PCR: To evaluate levels of gene expression, qPCR reactions were performed in triplicates using the StepOnePlus Real-Time PCR system (Applied

Biosystems). qPCR reactions contained 1x SsoFast EvaGreen Supermix (Bio-Rad), 0.2 µM of each gene-specific primer (described in Table III.6.1.) and as a template 1:10 dilution of cDNA. All PCR reactions were subjected to: 95°C for 1 min followed by 50 cycles (each cycle is 95°C for 5 s and 68°C for 15 s). Transcript levels were determined using the ΔΔCt method (Pfaffl, 2001) and normalized using elongation factor 1 alpha (EF1a) housekeeping gene.

III.7. Statistical analysis

One-way ANOVA (analysis of variance) combined to Tukey's multiple comparison test was performed to evaluate the significance of the differences between control and tested concentrations. The results for skeletal deformities were presented as percentages of fish affected and comparisons were made with chi-square test. Differences were considered significant for $p < 0.05$.

Table III.3.1. Nominal test concentrations

Substances	*Concentrations tested*
3-methylcholanthrene (3-MC)	*0, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M*
Naphthalene	0, 0.0001, 0.001, 0.01, 0.1, 1 mg/L
N-phosphonomethyl-glycine (Glyphosate)	0, 0.08, 0.4, 2, 10, 50 mg/L
γ-1,2,3,4,5,6-hexachlorocyclohexane (Lindane)	0, 0.8, 4, 20, 100, 500 µg/L
Cobalt (II) chloride ($6H_2O$)	0, 0.8, 4, 20, 100, 500 mg/L
Diclofenac (sodium salt)	0, 0.4, 2, 10, 50, 250 mg/L
Cadmium chloride	*0, 0.008, 0.04, 0.2, 1, 5* µg/L
Ammonium metavanadate	0, 0.04, 0.2, 1, 5, 25 mg/L

Table III.6.1. Primers used for qPCR

Gene name (acronym)	*Accession No.*	*Sequence (5'- 3')*
Elongation factor 1 alpha (EF1a)	NM_131263	Fw-AGCCCCTCCTGGCTTTCACCC Rv-TGGGACGAAGGCAACACTGGC
Osteonectin (ON)	AY239014	Fw-CTTCTTCCTGTTCTGCCTCGCTGG Rv-TCTCAGCAATAACATCCTCCACGACCT
Osteopontin (OP)	AY651247	Fw-GAACCTACACAGACCACGCCAACAG Rv-GGTAGCCCAAACTGTCTCCCCG
SRY-box containing gene 9a (SOX9a)	NM_131643.1	Fw-CGTCCATCTACGGTGTTCGCAT Rv-CGGACGGGCAGGGCGA
Tissue non-specific alkaline phosphatase (ALP)	NM_201007.1	Fw-TTCCTCTGCGGTGTCAAAGCCAA Rv-AAGCAGCACTCGGGGTGGCAT
Collagen, type X, alpha 1 (COL10A1)	NM_001083827	Fw-AGAAGGTGATGAAGGCCCCGCAGTAC Rv-CACCATCTTGTCCTGCAGGTCCAGGT
Bone morphogenetic protein 2b (BMP2b)	NM_131360	Fw GAGGAACTTAGGAGACGACGGGAACGC Rv TCTCGGGAATGAGTCCAACGGCAC
Osteocalcin 1 (OC1)	*	Fw-GAAGCGAACATGAAGAGTCTGACAGTCC Rv-GGAATCATCGCCGCCTATAAA
Osteocalcin 2 (OC2)	**	Fw-CCAACTCCGCATCAGACTCCGCATCA Rv-ATGTGCTGCTGAAGCGGAGTGTTGCT
Superoxide dismutase 2 (SOD2)	NM_199976.1	Fw-CTGGGGCTGGCTGGGCTTTG Rv-GCTTGGAAACGCTCGCTGA
Transcription factor mafT (MAFT)	AB167543	Fw-GAGGTAGAACGGCTCGGGG RV-GCTGTGGGGTCTTGACTATGGTG

**reconstructed from AY078413, gene and EH442597 **reconstructed from gene and ESTs*

IV. Results and Discussion

IV.1. 3-Methylcholanthrene

Zebrafish eggs were exposed chronically for 20 days to five different 3MC concentrations. The highest concentration, 10^{-6} M, early on reveled to be extremely toxic; within 24 h of exposure half of the eggs were unviable, coagulated and white, and after 96/120 h all hatched embryos were dead. As previously described by Seok et al. (Seok et al., 2008), an accumulation of fluid was observed in the pericardium and yolk sac of zebrafish embryos exposed to 10^{-6} M 3MC. No effect on time and rate of hatching was observed (i.e. 100 % by 72 hpf). LD_{50} for 3MC was 6.7×10^{-8} M in zebrafish larvae (Fig.IV.1.1). 3MC did not affect the average total length of zebrafish larvae in relation to our control, indicating no growth retardation upon 3MC exposure.

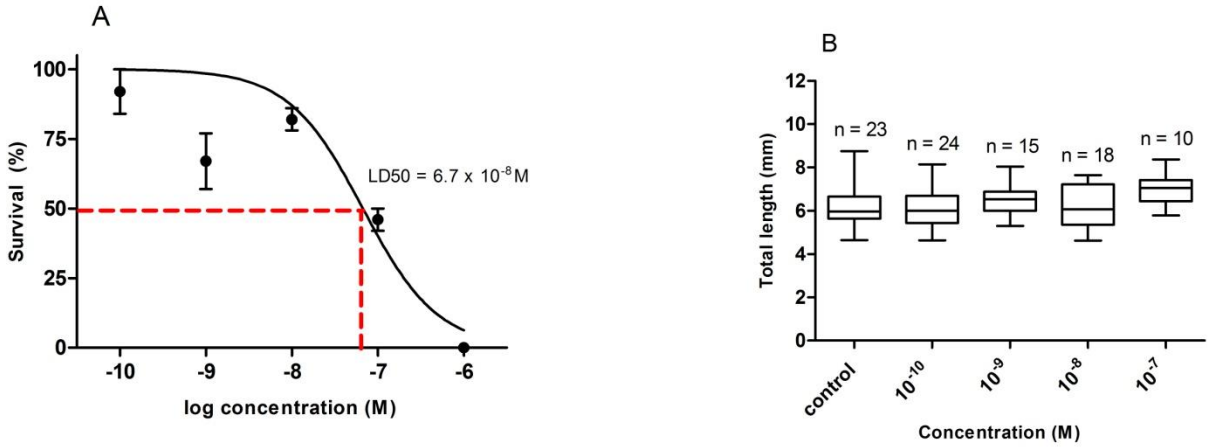


Fig.IV.1.1. Effect of 3MC on survival (**A**) and total length (**B**) of zebrafish larvae. LD_{50} value was calculated from the mathematical model fitting survival data in relation to 3MC concentration. Significance of differences in TL values was tested by one-way ANOVA followed by a Tukey's multiple comparison test $p<0.05$).

The occurrence of skeletal deformities upon exposure to 3MC was investigated in AB/AR-S double stained fish. Frequency and type of deformities has been listed in Table IV.1.1 and a representative set of photos are presented in Figure IV.1.2. A significant increase in the frequency of deformed fish (83% versus 36% in unexposed fish (Table IV.1.2.) was observed upon exposure to 10^{-7} M 3MC, with deformities in the caudal fin vertebrae, urostyle and hypurals 3-5. Fish exposed to 10^{-8}, 10^{-9} and 10^{-10} M 3MC exhibited deformities in the branchial arches and the cephalic area, but the frequency of deformed fish upon exposure to 3MC was not different from control fish. In the set of photos we can observe some examples of these deformities, like the ones found at the caudal fin area (**2**), involving the hypurals (**3**), and in the cephalic structures, like the operculum (**5**, **6**). About the number of deformities per fish, the number of deformed structures in deformed fish was similar in control and exposed fish. No particular pattern or prevalence of one type of deformity was identified. Despite not being life-threatening in larvae, the type of deformities observed upon 3MC exposure may influence fish locomotion and feeding in adult fish.

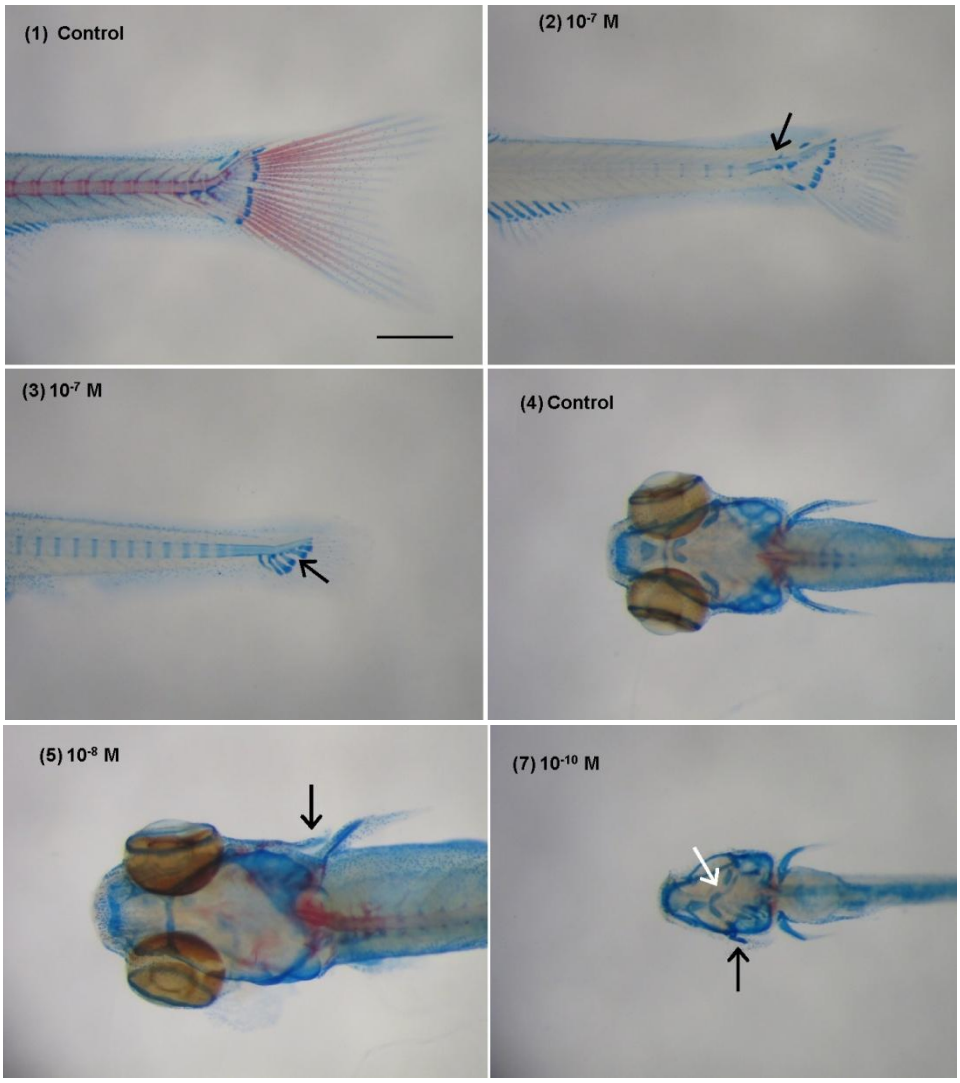


Fig.IV.1.2. Examples of malformations observed in 20-dpf zebrafish larvae exposed to 3MC. Larvae were double stained with alcian blue and alizarin red S and observed under a Leica stereo microscope. **1** Caudal vertebra and caudal complex of a control fish, **2** malformation of the last three caudal fin vertebra and the urostyle, **3** ectopic cartilage between hypural 2 and 3, **4** Cranial aspect of a control fish, **5** malformation of the right operculum, **6** "short" operculum on the left side (white arrow) and malformation of the branchial arches on the right side (black arrow). Scale represents 0.5 mm. *Note: some demineralization occurred in the time between staining and photo recording resulting in reduced alizarin red S coloration in some fish. In this fish less staining is therefore not the result of less bone and does not indicate growth retardation.*

Table IV.1.1. Skeletal structures affected upon exposure to 3MC

Structures	Control (n=11)		10^{-7} M (n=6)		10^{-8} M (n=9)		10^{-9} M (n=8)		10^{-10} M (n=9)	
	n	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*
Caudal fin vertebrae	1	9.1	1	16.7*						
Arches	1	9.1	1	16.7	4	44.4*	4	50*	2	22.2*
Cephalic					1	11.1*			2	22.2*
Urostyle			1	16.7*						
Notochord	1	9.1								
Parhypural + hypurals 1 and 2	1	9.1	1	16.7						
Hypurals 3-5			1	16.7*			1	12.5*		

*n the number of occurrences of malformation in the specific structure; % D percentage of deformed fish in the total fish observed; * significantly different from control (chi-square test, with 1 d.f., p<0.05)*

Table IV.1.2. Percentage of deformed fish and number of fish with 0, 1, 2 or more than 3 deformities

	T	*N*	*D*	*% D*	*% N*	*nr deformities*			
						0	*1*	*2*	*+ 3*
Control	11	7	4	36.4	63.6	7	4	0	0
10^{-7} M	6	1	5	83.3*	16.7	1	4	1	0
10^{-8} M	9	5	4	44.4	55.6	5	4	0	0
10^{-9} M	8	4	4	50.0	50.0	4	3	1	0
10^{-10} M	9	5	4	44.4	55.6	5	4	0	0

T** Total of fish observed; **N** number of fish without deformities; **D** number of fish with one or more deformities; **% D** percentage of deformed fish **% N** percentage of non-deformed fish; ** significantly different from control (chi-square test, with 1 d.f., p=0,05)*

In order to get insights into the molecular mechanisms triggering the skeletogenic effect of 3MC, the expression of several genes related to skeletogenesis and pollutant metabolism was analyzed by qPCR. Expression of *cyp1a, ahr2, nfe2, hmox1, hsp70, gpx1a and txn* genes could not be detected in any of the samples. Although we cannot exclude that primers may have failed to amplify target transcript (efficiency of gene-specific primers is currently being tested), it suggests that these transcripts are poorly/not expressed in 20-dpf larvae and not regulated upon 3MC exposure. Primers designed to amplify *mafT* and *sod2* exhibited an EFF% of approx. 127 and 123, respectively.

Only four genes had expression levels significantly altered by 3MC: *op*, *col10a1, oc1* and *oc2* (Figure IV.1.3). For all of them, gene expression was up-regulated upon exposure to 3MC (10^{-7} M for *op, col1a1* and *oc1*, and 10^{-8} M for *oc2*). All these genes are extracellular matrix proteins that have a role in osteoblast function and ECM mineralization, 3MC

exposure might interfere with bone formation and mineralization thus affecting later on bone remodeling. Some studies show us that an altered bone remodeling can set off several skeletal deformities (Witten and Huysseune, 2009; Ytteborg et al., 2010), therefore 3MC can influence the appearance of deformities by acting on bone remodeling.

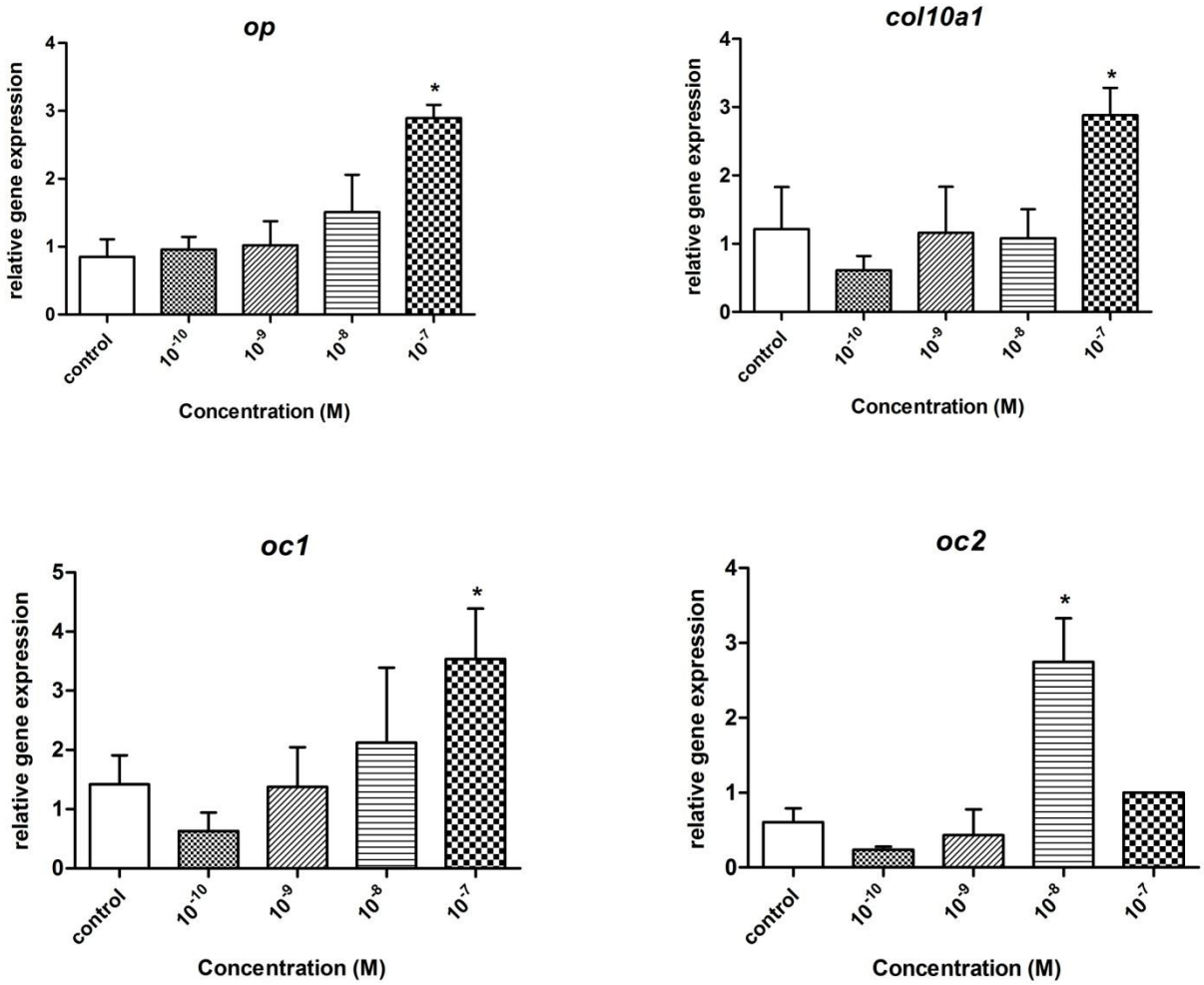


Fig.IV.1.3. Relative gene expression in 20-dpf fish exposed to different doses of 3MC. *op/spp1, osteopontin*; *col10a1, collagen type X alpha 1*; *oc1, osteocalcin 1*; *oc2, osteocalcin 2*. Values are presented as mean ± standard deviation calculated from 3 technical replicates for each pool of larvae; * indicate values significantly different from control (one-way ANOVA $p<0.05$ followed by Tukey's test).

Previous studies have shown that 3MC is a ligand of the aryl hydrocarbon receptor through which it inhibits osteoblast proliferation and differentiation, and bone formation *in vitro* (Naruse, 2002). While Naruse et al reported a down-regulation of ALP expression upon treatment of mouse osteoblast MC3T3 cells by 10^{-7} M 3MC, levels of expression remain the same in zebrafish larvae exposed to that concentration of 3MC. While levels of osteocalcin transcript were decreased in rat osteoblast ROB cells upon exposure to

10^{-7} M 3MC, expression of *osteocalcin 1* and *osteocalcin 2* was significantly up-regulated upon exposure to 10^{-7} M and 10^{-8} M 3MC, respectively. These differences might be related to the use of different experimental systems, i.e. different species – rat/mouse (mammals and terrestrial) vs. zebrafish (fish and aquatic animals) – different materials – whole larvae RNA vs. osteoblast RNA – and different tools – cell line (in vitro) vs. whole fish (in vivo) – where physiological response and detoxification processes are certainly different. In a study about the mechanisms involved in the stress-dependent regulation of bone formation under mechanical stress, Morinobu et al. indicated that expression levels of *osteopontin* were enhanced during bone formation under mechanical stress, and proposed that *osteopontin* may have a role in mediating stress signal to osteoblasts or cells in their lineage (Morinobu et al., 2003). In our fish system, expression of *op* gene was up regulated by 3MC and we propose that, as in mammals, *osteopontin* could serve as a mediator of 3MC toxic stress to the osteoblast.

According to Naruse et al., 3MC probably does not affect directly the expression of marker genes involved in osteoblast differentiation and function, but rather acts on osteoblast through an estrogen-dependent pathway (Naruse et al., 2002). Naruse *et al.* follow up research also reported a possible mechanism of action for 3MC through estrogen receptor-dependent pathways that resulted in the inhibition of osteoclasts-like cell formation through the inhibition of RANK (receptor activator of nuclear factor-κB ligand) expression in osteoclast supporting cells (Naruse et al., 2004). In light of these results, it would be of interest to assess the expression of osteoclast marker genes in zebrafish larvae exposed to 3MC and confirmed evidence indicating that skeletal deformities occurring in fish after 3MC exposure result from the alteration of mechanisms of bone remodeling.

IV.2. Naphthalene

None of the naphthalene concentrations tested triggered a lethal effect until 20 days of exposure and it was therefore not possible to determine the LD_{50} value for naphthalene in zebrafish larvae. Total length was also not affected upon exposure to naphthalene, indicating no growth retardation (Fig.IV.2.1). Hatching time and rate were also not affected, i.e. 100% hatching at 72 hpf for all conditions including the control.

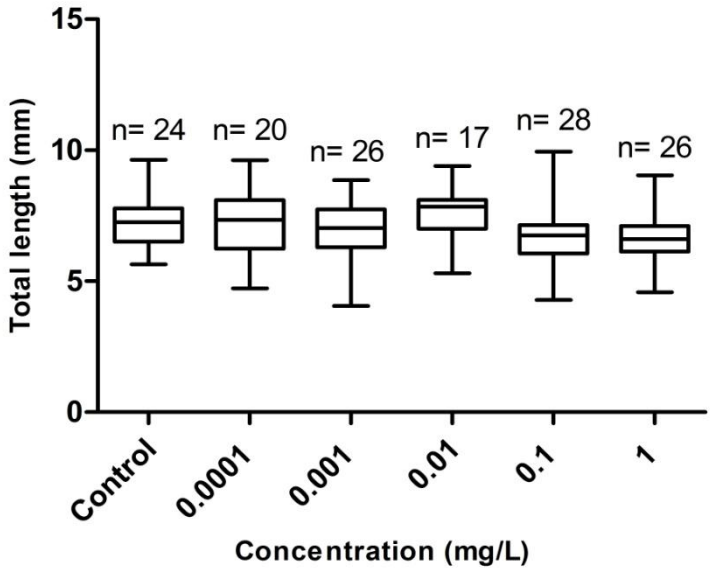


Fig.IV.2.1. Effect of naphthalene on the total length of zebrafish larvae at 20 dpf. Significance of differences in TL values was tested by one-way ANOVA followed by a Tukey's multiple comparison test $p<0.05$).

Skeletal deformities were identified in fish exposed to naphthalene and double stained with alcian blue and alizarin red S (see examples in Fig.IV.2.2).

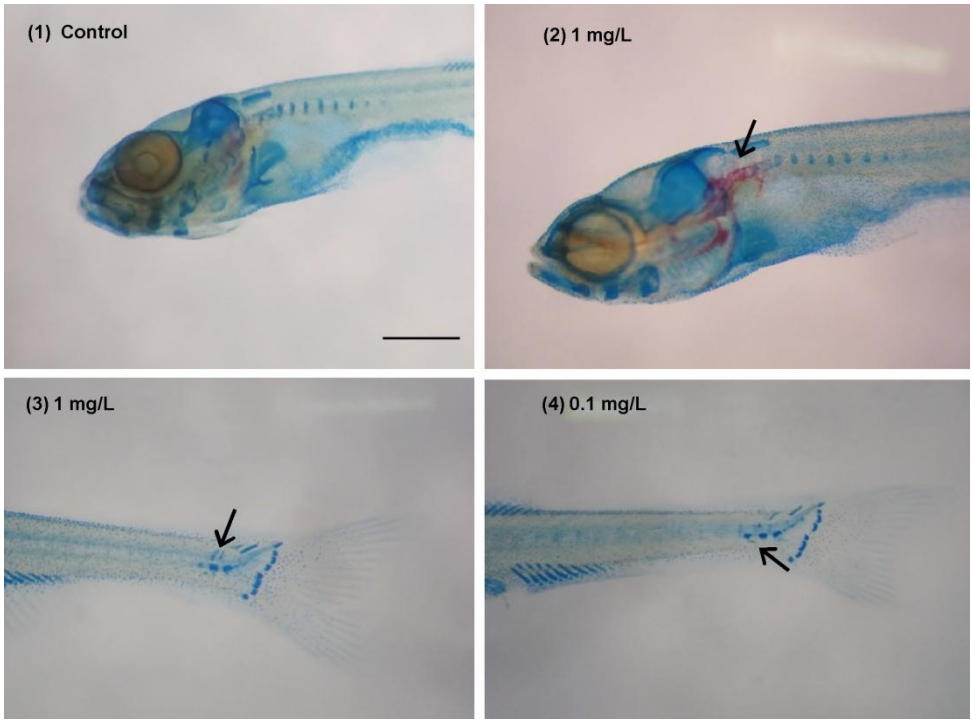


Fig.IV.2.2. Examples of malformations observed in 20-dpf zebrafish larvae exposed to naphthalene. Larvae were double stained with alcian blue and alizarin red S and observed under a Leica stereo microscope. **1** Cranial region of a control fish, **2** slight lordotic curvature in the first vertebrae, **3** two neural arches in the last vertebrae, **4** malformation of the hemal arch (s shape). Scale represents 0.5 mm.

In the set of photos we can observe some examples of these deformities, involving some spinal curvature (**2**) and malformations of the arches (**3, 4**). The frequency and structures affected by deformities have been listed in Table IV.2.1. Significant increase in the frequency of deformed structures were found at 1 mg/L, with deformities affecting the arches and urostyle; at 0.1 mg/L with deformities affecting the caudal fin vertebrae, branchial arches and the cephalic area; at 0.01 mg/L with deformities affecting the caudal fin vertebrae, urostyle and the parhypural + hypural 1 and 2 set; at 0.001 mg/L with deformities affecting the branchial arches, urostyle, notochord and parhypural + hypural 1 and 2 and hypurals 3-5; and finally at 0.0001 mg/L with deformities affecting the caudal vertebrae. In general, most deformities observed upon exposure to naphthalene appeared to affect the branchial arches and the caudal fin area.

Exposure to the highest concentration of naphthalene also significantly increased the number of deformed fish (66.7% versus 41.7% in unexposed fish; Table IV.2.2.), although this number was even higher for lower concentrations, i.e. 0.001 mg/L and 0.0001mg/L (91.7% and 70% deformed fish respectively). While only one deformity per fish was observed in control deformed fish, two and three deformities per fish were found in naphthalene-exposed fish, indicating that naphthalene not only promote an increase in the number of deformed fish but it also promote an increase in the number of deformity per fish.

Table IV.2.1.Skeletal structures affected upon exposure to naphthalene

Structures	*Control (n=12)*		*1mg/L (n=12)*		*0.1 mg/L (n=13)*		*0.01 mg/L (n=9)*		*0.001 mg/L (n=12)*		*0.0001 mg/L (n=10)*	
	n	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*
Caudal vertebrae											2	20*
Caudal fin vertebrae					1	7.7 *	1	11.1 *				
Arches	2	16.7	7	58.3 *	4	30.8 *	2	22.2	8	66.7 *	4	40
Cephalic					1	7.7 *					2	20
Caudal fin + rays	1	8.3	1	8.3	1	7.7						
Eporal	1	8.3					1	11.1				
Urostyle			1	8.3 *			1	11.1 *	1	8.3 *		
Notochord									1	8.3 *		
Parhypural + hypurals 1 and 2	1	8.3	2	16.7	2	15.4	2	22.2 *	4	33.3 *	1	10
Hypurals 3-5									2	16.7 *	2	20

n the number of occurrences of malformation in the specific structure **% D** *percentage of deformed fish in the total fish observed;*
** values statistically different from the control (chi-square test, with 1 d.f., p<0.05)*

Table IV.2.2. Percentage of deformed fish and number of fish with 0, 1, 2 or more than 3 deformities

	T	*N*	*D*	*% D*	*% N*	*nr deformities*			
						0	*1*	*2*	*+ 3*
Control	12	7	5	41.7	58.3	7	5	0	0
1 mg/L	12	4	8	66.7*	33.3	4	6	2	0
0.1 mg/L	13	6	7	53.8	46.2	6	6	1	0
0.01 mg/L	9	5	4	44.4	55.6	5	2	1	1
0.001 mg/L	12	1	11	91.7 *	8.3	1	7	4	0
0.0001 mg/L	10	3	7	70.0 *	30.0	3	4	2	1

T *Total of fish observed;* ***N*** *number of fish without deformities;* ***D*** *number of fish with one or more deformities;* ***% D*** *percentage of deformed fish;* ***% N*** *percentage of non-deformed fish;* ******* *significantly different from control (chi-square test, with 1 d.f., p=0.05)*

Although we observed some heterogeneity in the skeletogenic effect of naphthalene, i.e. lower concentrations being somehow more effective in promoting skeletal deformities, the general trend is that naphthalene exhibits a strong negative effect on skeletogenesis. Heterogeneity could result from the poor quality of the eggs used in this experiment, although eggs were screened before each pollutant exposure and bad spawns have been systematically excluded. It could also result from the mixing of spawns of different quality; eggs from different spawns were mixed in some few cases, when the size of one spawn was not enough to produce enough eggs.

In order to get insights into the mechanisms driving the skeletogenic effect of naphthalene, the expression of several genes related to osteogenesis and ECM mineralization was analyzed by qPCR. Expression of *cyp1a, ahr2, nfe2, hmox1, hsp70, gpx1a and txn* genes could not be detected in any of the samples (see previous chapter).

The expression of five genes was significantly altered upon exposure of zebrafish larvae to naphthalene (Figure IV.2.3). While expression of *sox9a* was down-regulated by approximately 3 folds at highest naphthalene concentrations (i.e. 1, 0.1 and 0.01 mg/L), expression of *col10a1* was down-regulated by approx. 2 folds at all concentrations. An opposite effect of naphthalene on osteocalcin expression was observed. While *oc1* was down-regulated (~4 times) at highest concentration, *oc2* was up-regulated (~3 times) at 0.1 mg/L. Finally, expression of *sod2* was up-regulated (~4 times) at lowest naphthalene concentration. The down-regulation patterns for *sox9a* and *col10a1* can indicate an effect on chondrocyte function and differentiation and maybe an effect on cartilage development upon naphthalene exposure. On the other hand, the up-regulation of *oc2* and *sod2* by lower concentrations can possibly reflect a stronger regulation with a lower concentration that does not induce a higher physiological toxicity response.

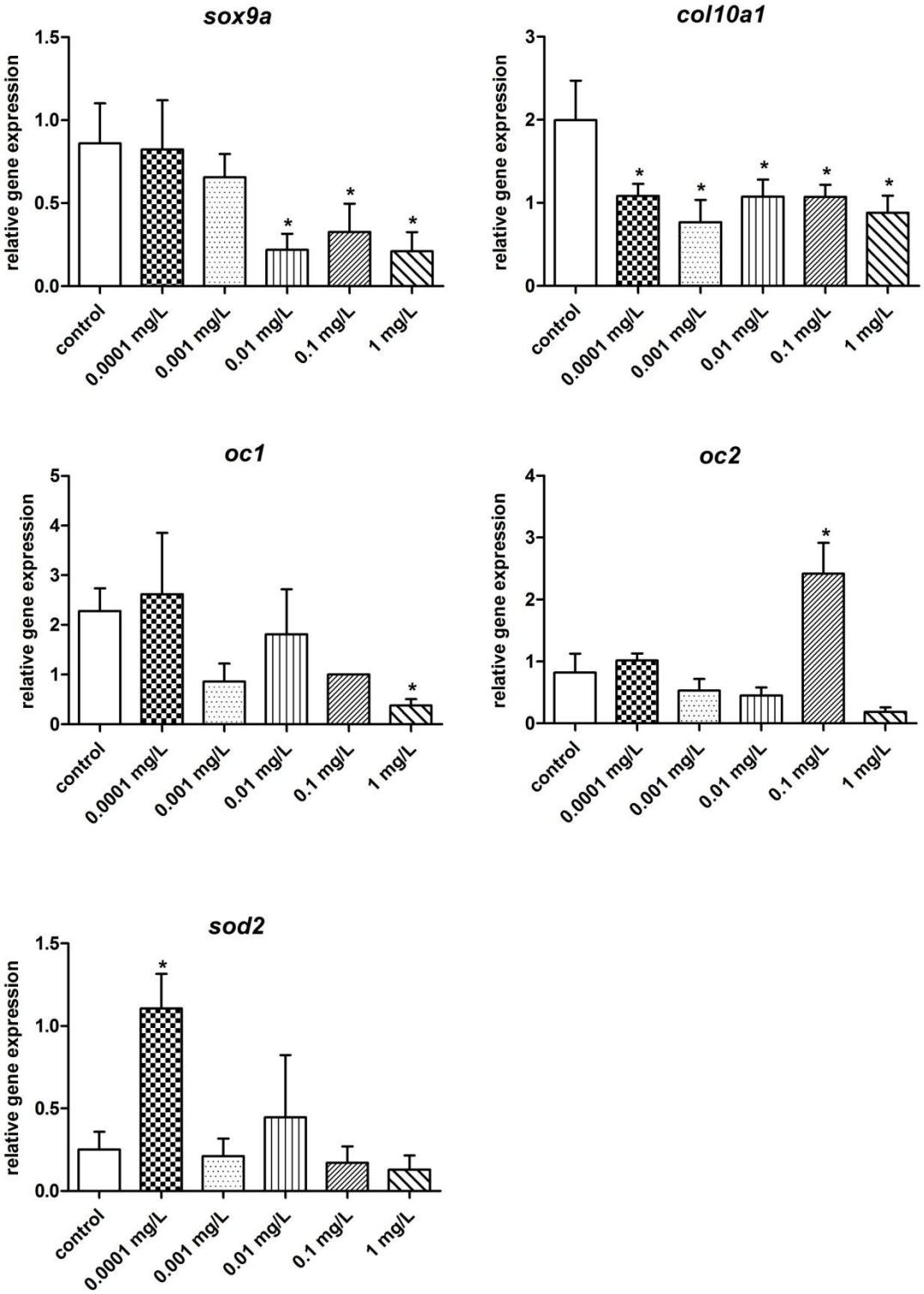


Fig.IV.2.3. Relative gene expression in 20-dpf fish exposed to different doses of naphthalene. *Sox9a, SRY-box containing gene 9a*; *col10a1, collagen type X alpha 1*; *oc1, osteocalcin 1*; *oc2, osteocalcin 2; sod2, superoxide dismutase 2*. Values are presented as mean ± standard deviation calculated from 3 technical replicates for each pool of larvae. * indicate values significantly different from control (one-way ANOVA $p<0.05$ followed by Tukey's test).

In the experience performed by Incardona et al, exposure to naphthalene is associated mainly to cardiac dysfunction, while toxicity from three-ring (or more) PAHs showed some effects in reducing head and jaw size and promoted smaller individual chondrocytes (Incardona et al., 2004). Our expression data showing a down-regulation by naphthalene of *sox9a* and *col10a1*, two genes associated with chondrocyte differentiation, would indicate that two-ring PAHs can also affect chondrocyte function and probably chondrogenesis; it would also explain the occurrence of skeletal deformities in naphthalene-exposed larvae. The opposite effect on the expression of *oc1* and *oc2* can be related to the fact that there are differently regulated, as exemplified by their different pattern of expression during development (Bensimon-Brito et al., 2012).The *oc1* maternal transcripts are detected from early developmental stages while *oc2* only starts to be expressed at 7 dpf. It is also possible that the up regulated result for *oc2* reflects some misreading during the qPCR and both isoforms follow a down regulation pattern on exposure to naphthalene. In a study exposing zebrafish embryos for 96 h to naphthalene, Wang et al show evidences supporting the MOA of naphthalene by inducing oxidative stress (Wang et al., 2009). One of the markers used is *sod*, its expression is first induced and after 2 days it is inhibited. Our study lasted for 20 dpf so we would expect for a close to control regulation or down-regulation; in fact all concentrations, except for 0.0001mg/L, showed a value close to the control while at 0.0001mg/L, *sod2* is significantly up-regulated. This could reflect a technical error or being this concentration so low, the inhibition pattern after 2 days of exposure reported by Wang et al. does not occur in our case leading to a continue response of the antioxidant system.

From only the expression of *sod2* we cannot demonstrate the influence of naphthalene in the antioxidant system and other implications, in the future it would be interesting to further test more genes involved in the antioxidant response. Also to support the expression of *sox9a* and *col10a1* obtained, it would be of interest to study the expression of more genes related to chondrocyte differentiation and acquire a more complete set of data.

Looking at the results of the two PAHs studied, 3MC and naphthalene, both seem to have different modes of action. While 3MC appears to affect osteoblast, and possibly osteoclast, function increasing the number of deformities through a defective bone remodeling process, naphthalene is likely to affect chondrocyte differentiation and

chondrogenesis. These evidences point to an osteogenic and chondrogenic effect by 3MC and naphthalene.

IV.3. Glyphosate

None of the glyphosate concentrations tested triggered a lethal effect until 20 days of exposure and it was therefore not possible to determine the LD_{50} value for glyphosate in zebrafish larvae. Total length was also not affected upon exposure to glyphosate indicating no growth retardation (Fig.IV.3.1). Hatching time and rate were also not affected, i.e. 100% hatching at 72 hpf for all conditions including the control.

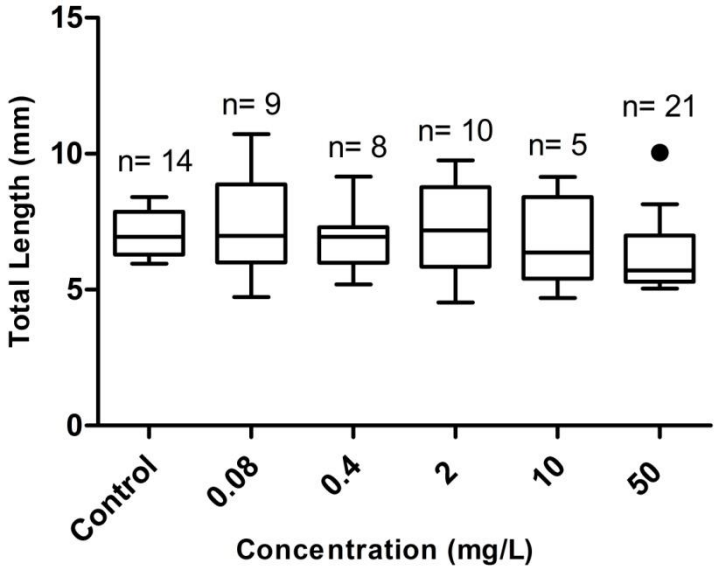


Fig.IV.3.1. Effect of glyphosate on the total length of zebrafish larvae at 20 dpf. Significance of differences in TL was tested by one-way ANOVA followed by a Tukey's multiple comparison test $p<0.05$).

From the observation of the stained fish, we were able to identify some skeletal deformities, which are exemplified in Fig.IV.3.2. In the set of photos, are represented deformities like the presence of scoliosis (**2**) and malformations in the notochord and hypural 1 (**3**, **4**). The frequency and skeletal structures affected by deformities are listed in Table IV.3.1. A significant increase of deformities in the caudal fin vertebrae, in the occurrence of kyphosis and scoliosis, in the urostyle and notochord were observed at 50 mg/L. Deformities in abdominal and caudal fin vertebrae, and branchial arches were observed in larvae exposed to 10 mg/L of glyphosate and an increase in the occurrence of kyphosis and scoliosis was noted. Caudal fin vertebrae, branchial arches and hypurals

3-5 were also significantly more affected in larvae treated with 2 mg/L, Deformities in abdominal vertebrae and branchial arches were observed in larvae exposed to 0.4 mg/L and an increase in the occurrence of scoliosis was noted. Finally, branchial arches and epural were significantly more affected in larvae treated with 0.08 mg/L of glyphosate.

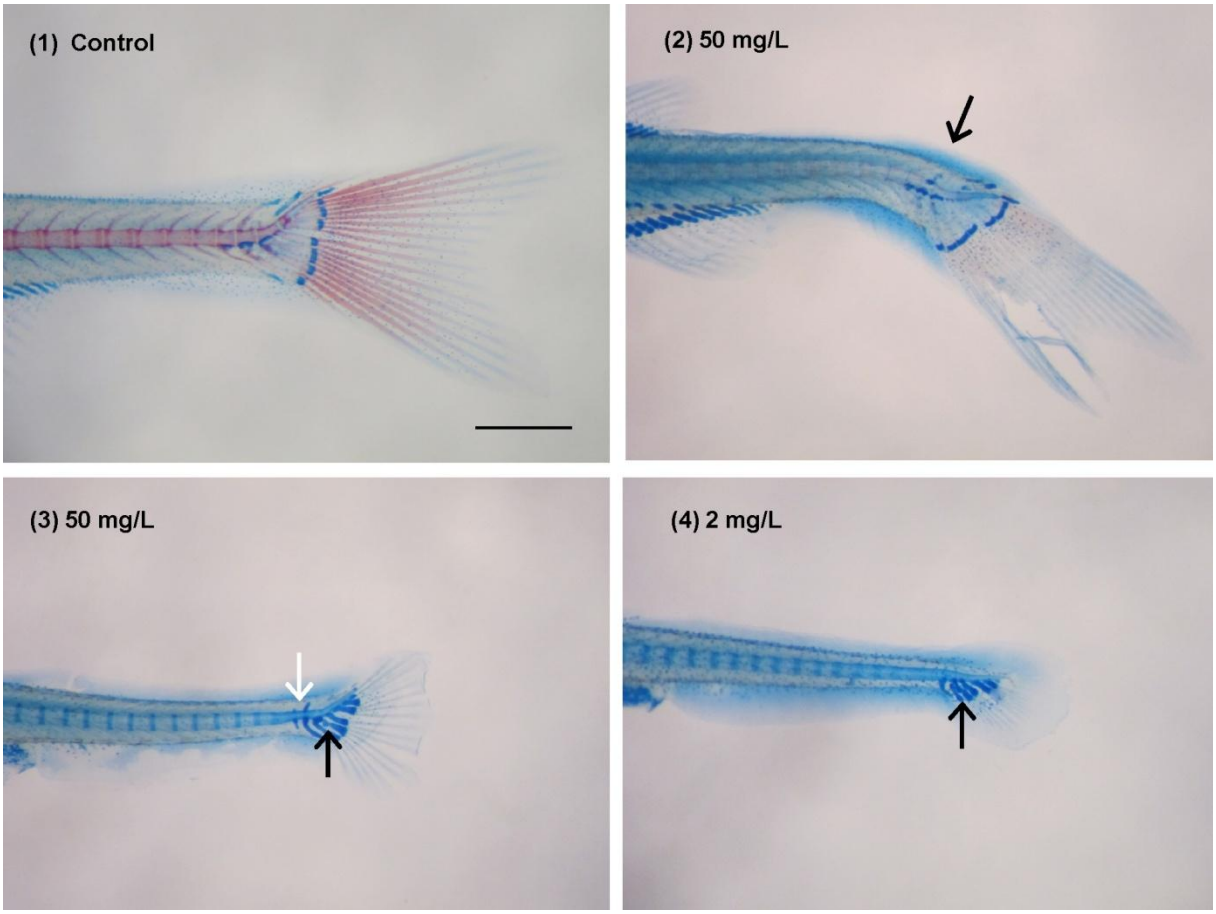


Fig.IV.3.2. Examples of malformations observed in 20-dpf zebrafish larvae exposed to glyphosate. Larvae were double stained with alcian blue and alizarin red S and observed under a Leica stereo microscope. **1** Caudal fin of a control fish, **2** caudal scoliosis, **3** malformations of the notochord (white arrow) and in the hypural 1, **4** malformation of the hypural 1. Scale represents 0.5 mm.

Table IV.3.1.Skeletal structures affected upon exposure to glyphosate

Structures	*Control (n=12)*		*50mg/L (n=11)*		*10mg/L (n=9)*		*2mg/L (n=10)*		*0.4mg/L (n=8)*		*0.08mg/L (n=8)*	
	n	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*
Abdominal vertebrae					1	11.1 *			2	25*		
Caudal fin vertebrae			1	9.1 *	1	11.1 *	1	10 *				
Arches	1	12.5			4	44.4 *	3	30*	3	37.5 *	2	25,*
Branquial arches											1	12.5 *
Kyphosis			3	27.3 *	1	11.1 *						
Scoliosis			4	36.4 *	1	11.1 *			1	12.5 *		
Eporal											1	12. 5*
Urostyle			1	9.1 *								
Notochord			1	9.1 *								
Parhypural + hypurals 1 and 2	1	12.5	1	9.1			1	10	1	12.5	1	12.5
Hypurals 3-5							1	10 *				

n the number of occurrences of malformation in the specific structure % D percentage of deformed fish in the total fish observed;
** values statistically different from the control (chi-square test, with 1 d.f., p<0.05)*

Exposure to 50 mg/L of glyphosate significantly increased the number of deformed fish (81.8% versus 37.5% in unexposed fish; Table IV.3.2.). Although to a lower extent, an increase in the number of deformed fish was also noted at 0.4 mg/L (75%) and 0.08 mg/L (62.5%). The increase in deformities can relate to the glyphosate exposure but we do not see that increase in a concentration-dependent way. [There is also an increase in the number of deformities per fish]. Although the presence of a specific pattern in the malformations is not noticeable, we would need more data to validate the statistical differences we found.

Table IV.3.2. Percentage of deformed fish and number of fish with 0, 1, 2 or more than 3 deformities

	T	*N*	*D*	*% D*	*% N*	*nr deformities*			
						0	*1*	*2*	*+ 3*
Control	8	5	3	37,5	63,5	5	3	0	0
50 mg/L	11	2	9	81,8*	18,2	2	7	3	0
10 mg/L	9	5	4	44,4	55,6	5	4	1	0
2 mg/L	10	6	4	40	60	6	4	1	0
0.4 mg/L	8	2	6	75*	25	2	4	1	1
0.08 mg/L	8	3	5	62,5*	37,5	3	5	0	0

T** Total of fish observed; **N** number of fish without deformities; **D** number of fish with one or more deformities; **% D** percentage of deformed fish; **% N** percentage of non-deformed fish; ** significantly different from control (chi-square test, with 1 d.f., p=0.05*

As for 3MC and naphthalene, the expression of several genes related to skeletogenesis and pollutant signaling were supposed to be analyzed by qPCR. Unfortunately, the quality of the RNA prepared from larvae exposed to glyphosate was not suitable to qPCR

analysis. Therefore we could not get insights into the molecular mechanisms driving skeletogenic effect of glyphosate. Although the types of skeletal deformities are not life-threatening in 20-dpf larvae, it can possibly influence adult fish fitness and locomotion.

IV.4. Lindane

Zebrafish eggs were exposed for 20 days to five different concentrations of lindane. Toxicity was observed for highest concentrations and LD_{50} was determined: 2.872×10^6 µg/L (Fig.IV.4.1). Hatching time and rate were not affected, i.e. 100% hatching at 72 hpf for all conditions. Total length of 20-dpf larvae was also not affected upon exposure to lindane (Fig.IV.4.1), indicating no growth retardation. Surprisingly Nagel et al. reported a decrease in larval growth upon exposure to 40 µg/L of lindane (Görge and Nagel, 1990). Differences in the protocols used (i.e. fish density, concentration range, chemical source) could explain the discrepancy between the two studies.

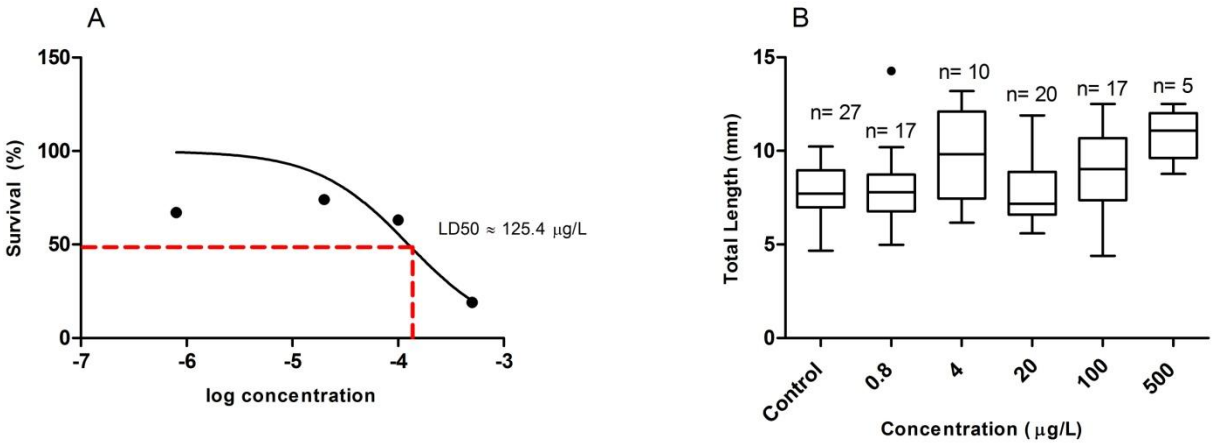


Fig.IV.4.1. Effect of lindane on survival rate (**A**) and total length (**B**) of zebrafish larvae. LD_{50} value was calculated from the mathematical model fitting survival data in relation to lindane concentration. Significance of differences in TL values was tested by one-way ANOVA followed by a Tukey's multiple comparison test $p<0.05$).

Skeletal deformities – e.g. malformations in the hypurals, the caudal fin vertebrae and the arches, and vertebral fusion – were observed in 20-dpf larvae exposed to lindane and stained with AB/AR-S; some of these malformations are illustrated in Fig.IV.4.2.

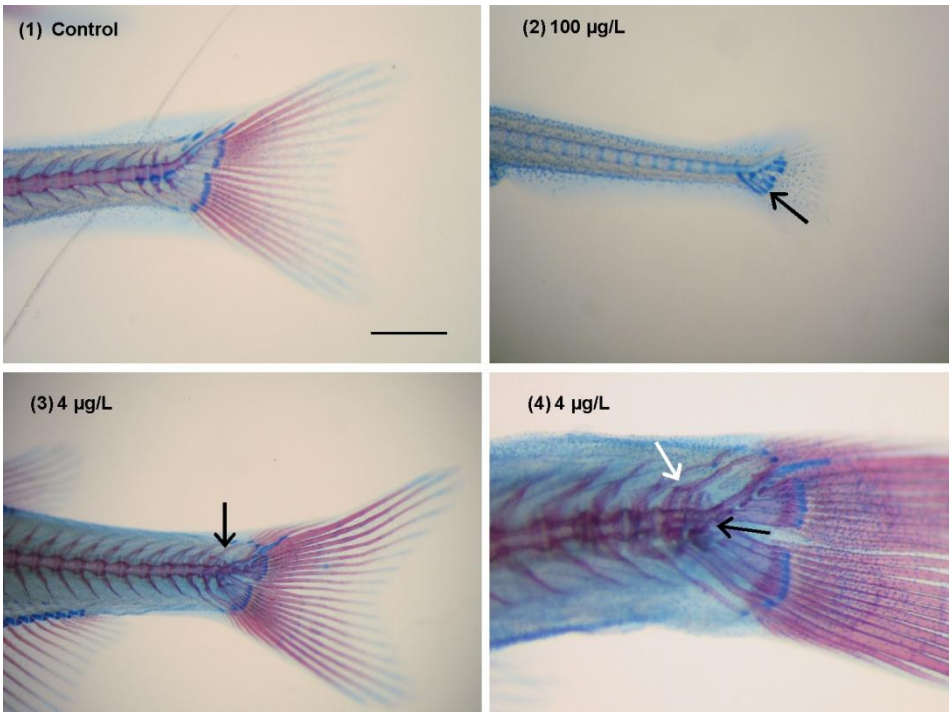


Fig.IV.4.2. Examples of malformations observed in 20-dpf zebrafish larvae exposed to lindane. **1** Caudal fin of a control fish, **2** malformation of the hypural 1 and parhypural, **3** scoliosis of the caudal vertebrae and malformation of the neural arch of the last vertebrae, **4** vertebral fusion, between the last vertebrae and the urostyle and the arches. Scale represents 0.5 mm.

The frequency and structures affected by deformities are listed in Table IV.4.1. A significant increase in the frequency of deformed structures was found at 500 µg/L in the arches; at 100 µg/L in structures such as the urostyle and parhypurals +hypurals 1 and 2; at 4 µg/L in the arches. Scoliosis was also observed; at 0.8 µg/L in the arches. Most of these deformities do not affect the survival of the larvae but may impair locomotion in adult fish. Exposure to lindane at 100 µg/L increased the number of deformed fish (66.7% versus 46.2% in unexposed fish; Table IV.4.2) but there was no noticeable increase in the number of deformities per fish upon exposure to lindane.

Table IV.4.1.Skeletal structures affected upon exposure to lindane

Structures	*Control (n=13)*		*500ug/L (n=5)*		*100ug/L (n=9)*		*20ug/L (n=10)*		*4ug/L (n=10)*		*0.8ug/L (n=9)*	
	n	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*
Caudal fin vertebrae	1	7.7					1	10	1	10		
Arches	5	38.5	3	60*	4	44.4	3	30	6	60*	2	22.2*
Scoliosis									1	10*		
Anal fin + rays	1	7.7					1	10			1	11.11
Urostyle					1	11.1*						
Parhypural + hypurals 1 and 2	1	7.7			2	22.2*	1	10	1	10		

n the number of occurrences of malformation in the specific structure ***% D*** *percentage of deformed fish in the total fish observed;*
** values statistically different from the control (chi-square test, with 1 d.f., p<0.05)*

Table IV.4.2. Percentage of deformed fish and number of fish with 0, 1, 2 or more than 3 deformities

	T	*N*	*D*	*% D*	*% N*	*nr deformities*			
						0	*1*	*2*	*+ 3*
Control	13	7	6	46.2	53.8	5	4	2	0
500 µg/L	5	2	3	60.0	40.0	2	3	0	0
100 µg/L	9	3	6	66.7*	33.3	3	4	2	0
20 µg/L	10	5	5	50.0	50.0	5	3	1	1
4 µg/L	10	4	6	60.0	40.0	4	2	4	0
0.8 µg/L	9	6	3	33.3	66.7	5	1	2	0

T *Total of fish observed;* ***N*** *number of fish without deformities;* ***D*** *number of fish with one or more deformities;* ***% D*** *percentage of deformed fish;* ***% N*** *percentage of non-deformed fish;* ******* *significantly different from control (chi-square test, with 1 d.f., p=0.05)*

The expression of several genes related to skeletogenesis and pollutant signaling was analyzed by qPCR. Expression of *cyp1a, ahr2, nfe2, hmox1, hsp70, gpx1a and txn* genes could not be detected in any of the samples. Few larvae were available for 2 of the concentrations, i.e. 500 and 4 µg/L, and priority was given to staining and identification of deformities. qPCR data is therefore not available for those concentrations. Significant differences in the expression of selected genes are presented in Figure IV.4.3.

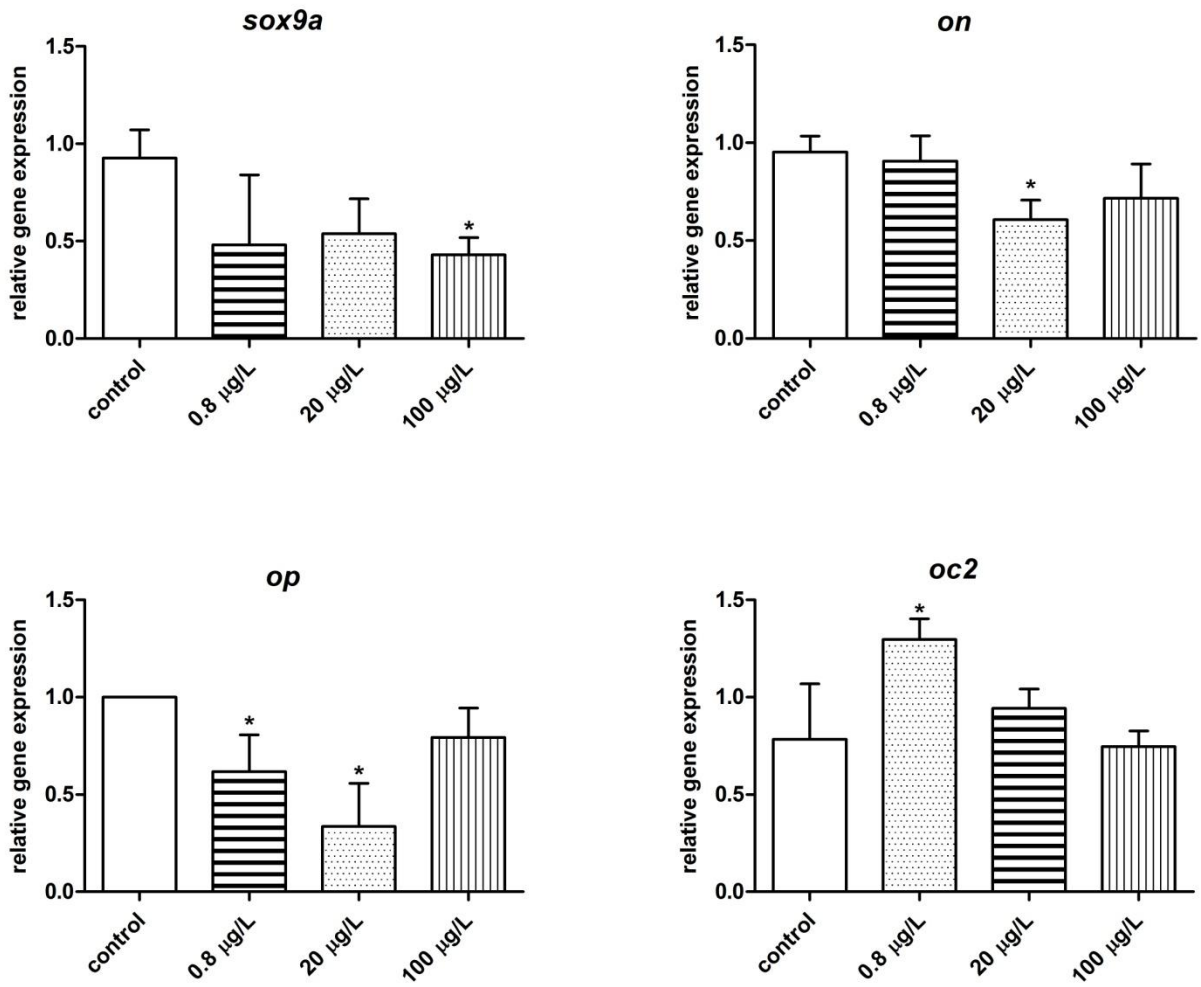


Fig.IV.4.3. Relative gene expression in 20-dpf fish larvae exposed to different doses of lindane. *Sox9a, SRY-box containing gene 9a*; *osteonectin (on/sparc); op/spp1, osteopontin*; *oc2, osteocalcin 2*. Values are presented as mean ± standard deviation calculated from 3 technical replicates for each pool of larvae; * indicate values significantly different from control (one-way ANOVA $p<0.05$ followed by Tukey's test).

While expression of *sox9a, on* and *op* appeared to be down-regulated by lindane at various concentrations, expression of *oc2* was up-regulated at 0.8 µg/L. Down regulation of *sox9a* expression at 100 µg/L may indicate that chondrocyte differentiation and possibly chondrogenesis may be affected. Down regulation of *on* expression at 20 µg/L may indicate that osteoblast matrix and possibly osteogenesis may be affected. Down regulation of *op* expression at 0.8 and 20 µg/L may also indicate defective extracellular matrix. On the contrary, up-regulation of *oc2* expression at 0.8 µg/L may indicate accelerated osteoblast differentiation. Altogether disruption of the expression of these marker genes is compatible with altered chondrogenesis and osteogenesis and coherent with the occurrence of skeletal deformities. A similar hypothesis was proposed by Dong et al. from medaka embryos exposed for 10 days to TCDD and presenting dysmorphic and

defectively hypural cartilage structures resulting from impaired differentiation and growth of cartilage and bone in axial structures. Their conclusion was that TCDD delayed and disrupted skeletogenic development, impairing both chondrogenesis and osteogenesis (Dong et al., 2012).

Once again, effective pollutant concentrations were not always the highest ones. Future studies should aim at determining whether this is true or resulting from technical artifacts (e.g. egg quality and number of technical/biological replicates). Data regarding the osteotoxicity of lindane are scarce and do not allow a deep discussion of our results. There's evidence that lindane is an endocrine disrupter affecting estrogen activity and reproduction process (Sang et al., 1999). Bone is part of the endocrine system (Karsenty and Oury, 2012; Karsenty, 2012) and estrogens are known effectors of bone; although this should be further studied, it is conceivable that lindane effect on skeletogenesis is related to the disruption of endocrine response in fish. In this regard, the expression of marker genes related to estrogen signaling should be determined upon exposure to lindane.

IV.5. Diclofenac

Zebrafish eggs were exposed for 20 days to five different concentrations of diclofenac. The highest concentrations, 250 and 100 mg/L early on revealed to be extremely toxic; all eggs exposed to 250 mg/L of diclofenac were unviable, coagulated and white after 72 h and eggs exposed to 100 mg/L did not hatch or died soon after hatching. Other concentrations (0.4, 2 and 10 mg/L) did not affect hatching time and rate (i.e. 100 % by 72 hpf), in disagreement with the study by Hallare et al (2004), where a delay in hatching time for embryos exposed to 1 and 2 mg/L has been reported. LD_{50} for diclofenac was 21.88 mg/L (Fig.IV.5.1). Diclofenac did not affect the average total length of zebrafish larvae in relation to our control (Fig.IV.5.1). The absence of growth retardation observed here is in contradiction with the shorter body length reported by Chen et al. in zebrafish embryos exposed to diclofenac (Chen et al., 2011). Discrepancy in diclofenac effect on growth could be related to the age of zebrafish embryos used in both studies, i.e. 72 hpf versus 20 dpf, and/or to the concentrations tested, i.e. 0 - 2000 ppm versus 0.4-10 mg/L.

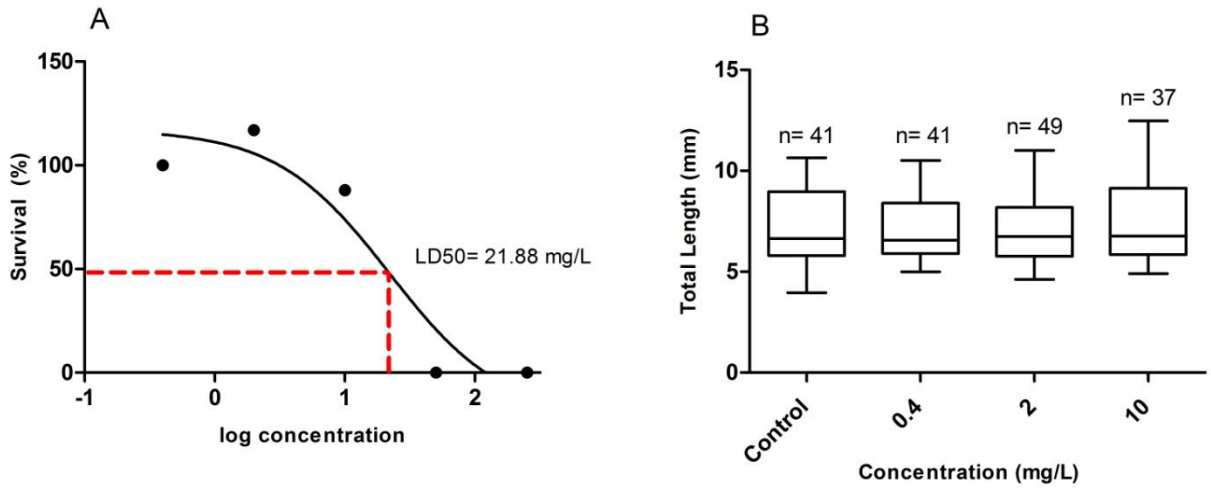


Fig.IV.4.1. Effect of diclofenac on survival rate (**A**) and total length (**B**) of zebrafish larvae. LD_{50} value was calculated from the mathematical model fitting survival data in relation to diclofenac concentration. Significance of differences in TL values was tested by one-way ANOVA followed by a Tukey's multiple comparison test $p<0.05$).

The occurrence of skeletal deformities upon exposure to diclofenac was investigated in AB/AR-S double stained fish. Frequency and type of deformities has been listed in Table IV.5.1 and a representative set of photos are presented in Figure IV.5.2.

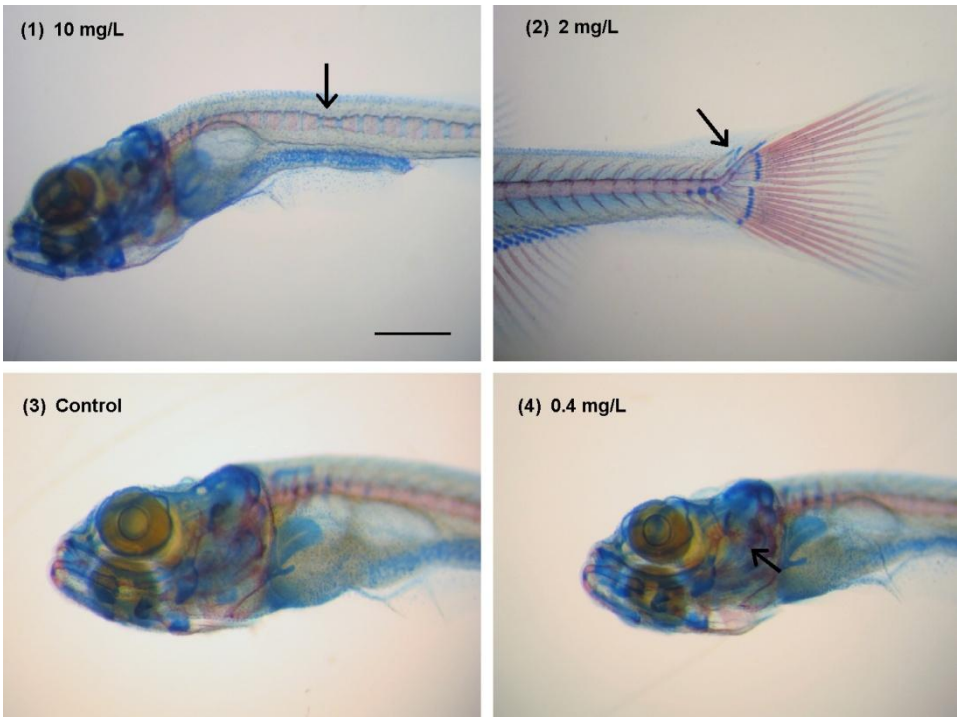


Fig.IV.5.2. Examples of malformations observed in 20 dpf zebrafish larvae exposed to diclofenac. Larvae were double stained with alcian blue and alizarin red S and observed under a Leica stereo microscope.**1** flatten vertebrae (platispondily), **2** malformation of the epural, **3** cranial region of a control fish, **4** malformation of the branchial arches, **5** malformation of the jaw (prognathism; arrow); control fish is placed above to evidence normal jaw. Scale represents 0.5 mm.

In this set of photos we can observe deformations like flatten vertebrae or platispondily (**1**), malformations in the epural and branchial arches (**2**, **4**) and malformations in the jaw (prognathism) (**5**). A significant increase in the frequency of several skeletal deformities was observed at 10 mg/L in structures like the abdominal vertebrae, arches, pectoral fin + rays and parhypural + hypurals 1 and 2; The occurrence of scoliosis was also observed; at 2 mg/L, deformities were observed in the arches, cephalic area, pectoral and dorsal fins + rays, epural and in the parhypural + hypurals 1 and 2; at 0.4 mg/L, deformities were observed in the cephalic area and in the parhypural + hypurals 1 and 2; The occurrence of scoliosis was also observed.

Table IV.5.1.Skeletal structures affected upon exposure to diclofenac

Structures	*Control (n=21)*		*10mg/L (n=17)*		*2mg/L (n=23)*		*0.4mg/L (n=21)*	
	n	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*
Abdominal vertebrae			1	5,9*				
Caudal vertebrae	2	9.5	1	5.9	2	8.7	1	4.8
Caudal fin vertebrae	3	14.3	1	5.9				
Arches	4	19	6	35.3*	10	43.5*	5	23.8
Cephalic					2	8.7*	2	9.5*
Kyphosis	1	4.8					2	9.5
Scoliosis			1	5.9*			2	9.5*
Pectoral fin + rays			1	5.9*	1	4.3*		
Dorsal fin + rays					1	4.3*		
Epural					1	4.3*		
Notochord	1	4.8	1	5.9			1	4.8
Parhypural + hypurals 1 and 2			1	5.9*	3	13*	4	19*

n the number of occurrences of malformation in the specific structure% D percentage of deformed fish in the total fish observed; values statistically different from the control (chi-square test, with 1 d.f., p<0.05)*

Table IV.5.2. Percentage of deformed fish and number of fish with 0, 1, 2 or more than 3 deformities

	T	*N*	*D*	*% D*	*% N*	*nr deformities*			
						0	*1*	*2*	*+3*
Control	21	12	9	42.9	57.1	12	5	3	1
10 mg/L	17	6	11	64.7*	35.3	6	9	2	0
2 mg/L	23	5	18	78.3*	21.7	5	15	3	0
0.4 mg/L	21	7	13	61.9*	33.3	8	11	2	0

T *Total of fish observed;* ***N*** *number of fish without deformities;* ***D*** *number of fish with one or more deformities;* ***% D*** *percentage of deformed fish;* ***% N*** *percentage of non-deformed fish;* ******* *significantly different from control (chi-square test, with 1 d.f., p=0.05)*

Exposure to 0.4, 2 and 10 mg/L of diclofenac significantly increased the number of deformed fish (64.7, 78.3 and 61.9% versus 42.9% in unexposed fish, see table IV.5.2.). About the number of deformities per fish, in the control most fish have zero deformities, while at all the concentrations most of the fish have at least one deformity. Although the types of skeletal deformities are not life-threatening in 20-dpf larvae, the type of deformities observed upon diclofenac exposure may influence, fish locomotion and feeding in adult fish Chen et al reported the adverse effect of diclofenac on actin polymerization and muscle fiber alignment in zebrafish, resulting in malformed somites (Chen et al., 2011), at concentrations similar to those used in this study; this effect coupled a skeletogenic effect will certainly impair the survival of a fish population in polluted environments.

The expression of several genes related to skeletogenesis and pollutant signaling was analyzed by qPCR. Expression of *cyp1a, ahr2, nfe2, hmox1, hsp70, gpx1a and txn* genes could not be detected in any of the samples (see chapter on 3MC). Primers designed to amplify *mafT* and *sod2* exhibited an EFF% of approx. 127 and 123, respectively.

Significant differences in the expression of *sox9a*, *on*, *oc1* and *oc2* genes were observed upon exposure to diclofenac (Figure IV.5.3). Expression of *sox9a* was down-regulated at 0.4 mg/L, but not at higher concentration; Expression of *on* was down-regulated at 2 and 10 mg/L, but not at lowest concentration; Expression of *oc1* expression was down-regulated at lowest and highest concentrations; finally, expression of *oc2* was down regulated at all concentrations.

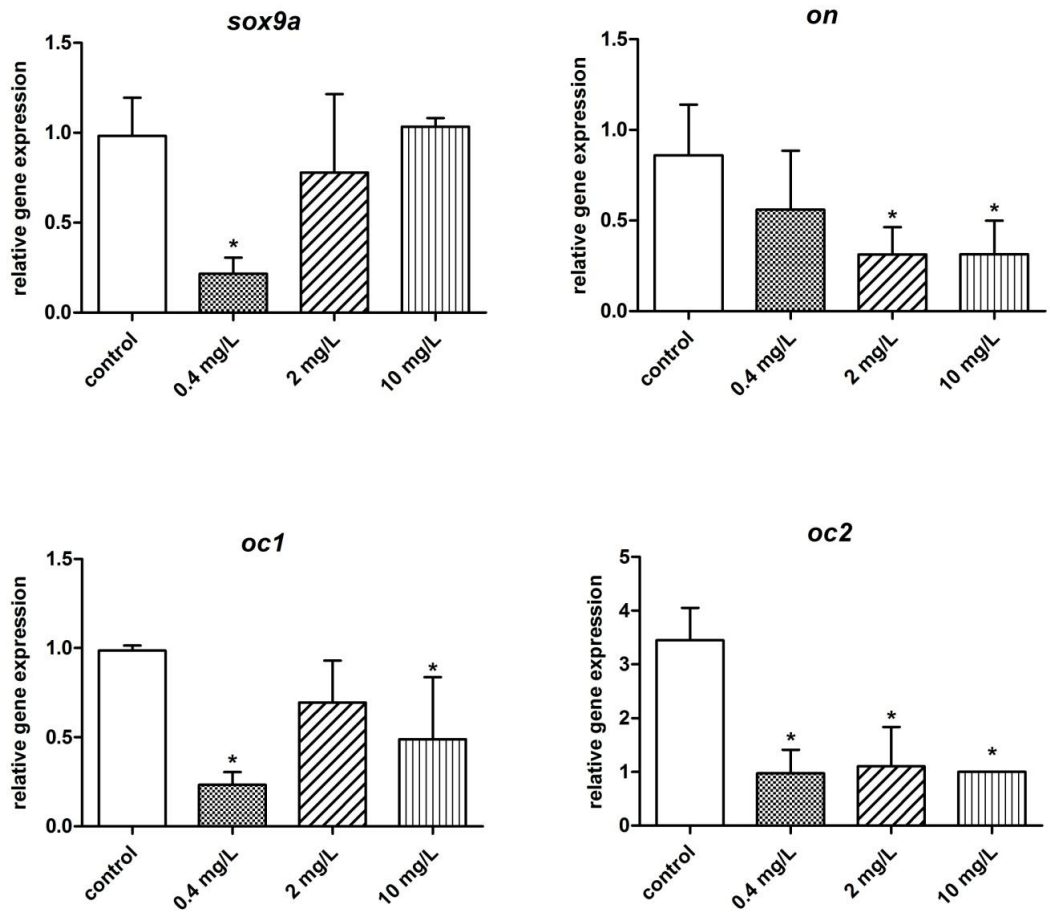


Fig.IV.5.3. Relative gene expression in 20-dpf zebrafish larvae exposed to different doses of diclofenac. *Sox9a, SRY-box containing gene 9a*; *osteonectin (on/sparc); oc1 osteocalcin 1*; *oc2, osteocalcin 2*. Values are presented as mean ± standard deviation calculated from 3 technical replicates for each pool of larvae; * indicate values significantly different from control (one-way ANOVA $p<0.05$ followed by Tukey's test).

While down-regulation of *sox9a* expression by diclofenac would indicate an alteration of chondrocyte differentiation, the fact that this effect is observed only at the lowest concentration is a bit puzzling. This can in fact be a true effect, and a lower diclofenac is more effective in inhibiting *sox9a* expression or can be a technical error. Future studies should aim at collecting additional data (more replicates and concentrations) to confirm or not this data. The down-regulation of *on, oc1* and *oc2* genes may indicate an alteration of osteoblast extracellular matrix and osteogenesis, and could explain the occurrence of skeletal deformities in larvae exposed to diclofenac. Mechanisms of diclofenac action have been associated with the inhibition of cyclooxygenase (*cox*) and DNA synthesis (Chen et al., 2011). Interestingly, inhibitors of cyclooxygenase have been associated to reduced bone mineralization in rat (Burdan et al., 2008; Dimmen et al., 2009) and

although this hypothesis should be further studied e.g. through the analysis of *cox* expression or enzymatic activity, we propose that skeletogenic effect of diclofenac may involve an inhibition of the cyclooxygenase. Finally, diclofenac concentrations used in this study are much higher than those predicted in the environment (0.54 µg/L; Hallare et al., 2004), but with evidence of concentrations much higher in Portuguese rivers than environmental levels (Gonzalez-Rey and Bebianno, 2012) and with significant results collected at 0.4 mg/L (our study), we cannot discard the possible negative effects of diclofenac pollution in aquatic ecosystems.

IV.6. Cobalt

Zebrafish eggs were exposed for 20 days to five different concentrations of cobalt chloride. The two highest concentrations, i.e. 0.1 and 0.5 g/L revealed to be very toxic; embryos were all dead after 7 days of exposure to 0.5 g/L of cobalt, and after 14 days of exposure to 0.1 g/L of cobalt. Hatching time and rate were severely affected: after 96 hpf, at 0.5 g/L there were still 39 non hatched embryos; at 0.1 g/L all embryos were non hatched; at 20 mg/L, there were 45 non hatched embryos; at 4 mg/L 32 embryos were not hatched and at 0.8 mg/L only 2 embryos were not hatched. During the daily change of medium and observation of the embryos we noticed that mechanical help was needed to the hatching since we started observing some embryos hatch upon slight touch by the pipettes or the medium change, reduced *in chorion* movements of the tail embryos was also observed in exposed fish. Jezierska et al reported that heavy metals often induce delay in the hatching process and increase death of newly hatched larvae (Jezierska et al., 2009); the hatching glands that produce chorionase, the enzyme needed for egg shell breakdown, may have their development and function impaired by heavy metals exposure which probably results in low chorionase activity/synthesis or in reducing the muscular movements that breakup the chorion. Any of these statements can be a possible reason for our observations during the hatching period. Larvae hatched through the mechanical method developed similarly to the control, although a higher mortality was observed between days 10 and 13 (data not shown). LD_{50} for cobalt was 5.705 mg/L (Fig.IV.6.1). The effects of heavy metals exposure also often translates into small body

size but from our results cobalt did not affect the average total length of zebrafish larvae in relation to our control, indicating no growth retardation upon cobalt exposure.

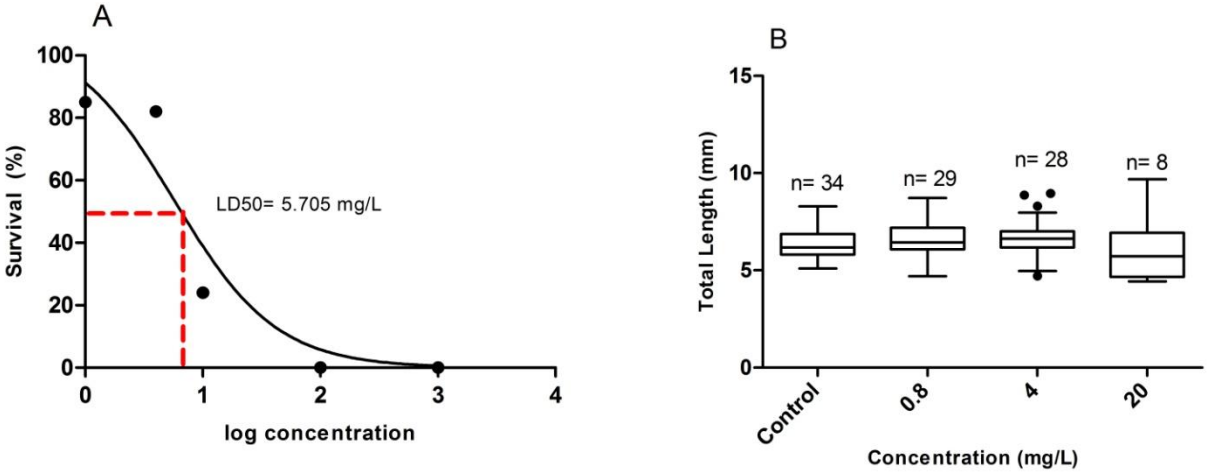
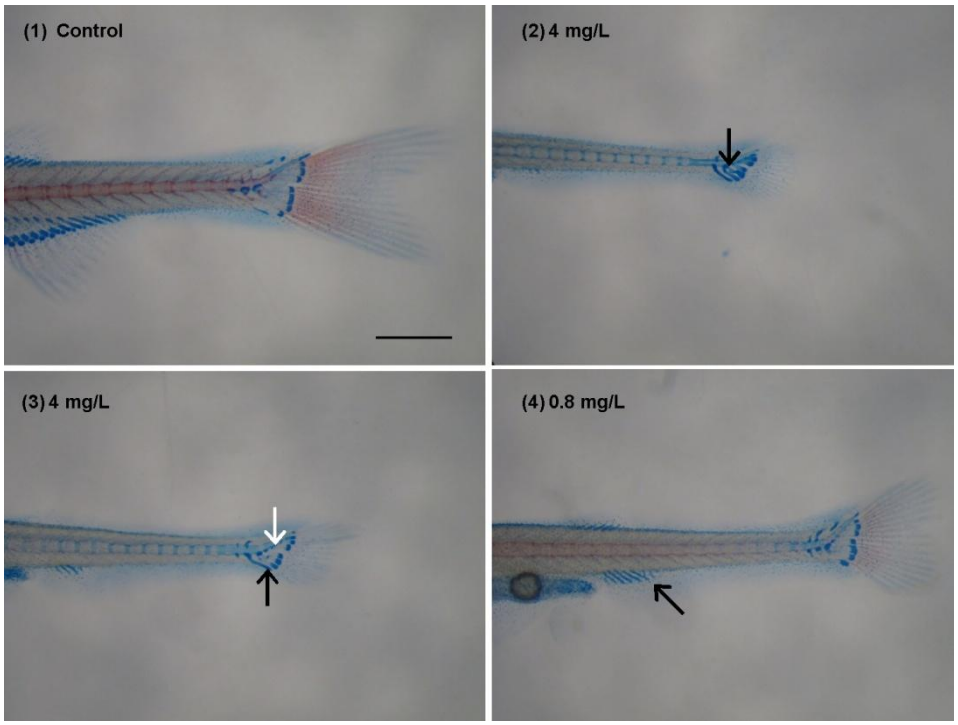


Fig.IV.6.1. Effect of cobalt on survival (**A**) and total length (**B**) of zebrafish larvae. LD_{50} value was calculated from the mathematical model fitting survival data in relation to cobalt concentration. Significance of differences in TL values was tested by one-way ANOVA followed by a Tukey's multiple comparison test $p<0.05$).

The occurrence of skeletal deformities upon exposure to cobalt was investigated in AB/AR-S double stained fish. Frequency and type of deformities has been listed in Table IV.6.1 and a representative set of photos are presented in Figure IV.6.2.



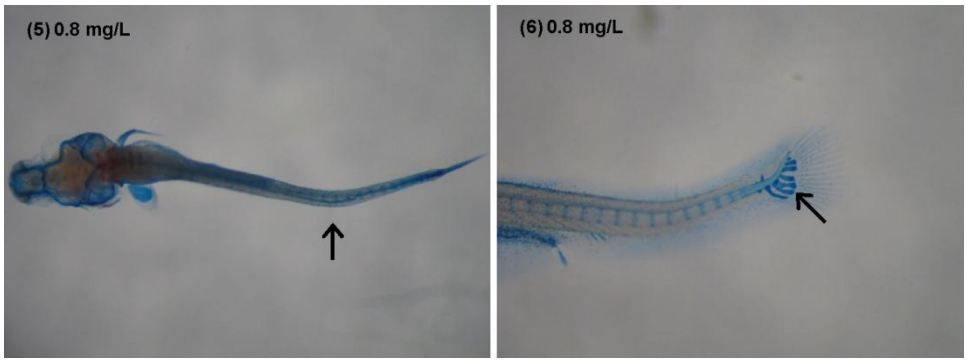


Fig.IV.6.2. Examples of malformations observed in 20-dpf zebrafish larvae exposed to cobalt. Larvae were double stained with alcian blue and alizarin red S and observed under a Leica stereo microscope. **1** caudal vertebrae and caudal fin of a control fish, **2** malformation of hypural 1, **3** malformation of hypural 3 (white arrow) and fusion between hypural 1 and parhypural (black arrow), **4** malformation of the pterygiophore (Y shape), **5** presence of scoliosis in the caudal vertebrae, **6** malformation of the parhypural and hypural 1. Scale represents 0.5 mm.

Photos above illustrate some malformations observed, e.g. malformations in the hypurals and parhypurals (**2**, **3**, **6**), occurrence of scoliosis (**5**) and malformations in the anal fin (**4**). We also noticed that fish exposed to cobalt chloride showed a slower skeletal development than control fish revealing possible development retardation upon cobalt exposure. Heavy metals are known to affect various metabolic processes in developing embryos and also activate energy-consuming detoxification pathways letting the exposure fish with less energy available for development and growth (Jezierska et al., 2009); this may account for the difference observed in fish exposed to cobalt. A significant difference in the frequency of skeletal deformities was observed at 20 mg/L in structures like the caudal vertebrae, the arches and in the presence of scoliosis; at 4 mg/L, affecting the arches, parhypural and hypurals and at 0.8 mg/L in structures like the caudal vertebrae, arches, the dorsal fin, the presence of scoliosis, parhypural and hypurals. Exposure to 0.8, 4 and 20 mg/L of cobalt significantly increased the number of deformed fish (62.5, 84.6 and 80.0%, respectively, versus 47.1% in unexposed fish; Table IV.6.2.). There was apparently no increase in the number of deformities per fish in deformed fish exposed to cobalt versus control fish.

Table IV.6.1.Skeletal structures affected upon exposure to cobalt

Structures	*Control (n=17)*		*20 mg/L (n=8)*		*4 mg/L (n=13)*		*0.8 mg/L (n=15)*	
	n	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*
Caudal vertebrae	5	29.4	1	12.5*	3	23.1	2	13.3*
Caudal fin vertebrae	1	5.9			1	7.7		
Arches	4	23.5	1	12.5*	10	76.9*	7	46.7*
Kyphosis			1	12.5*				
Scoliosis							1	6.7*
Dorsal fin + rays							1	6.7*
Anal fin + rays	1	5.9	1	12.5			1	6.7
Parhypural + hypurals 1 and 2	1	5.9	1	12.5	2	15.4*	3	20*
Hypurals 3-5					1	7.7*	1	6.7*

***n** the number of occurrences of malformation in the specific structure; **% D** percentage of deformed fish in the total fish observed; * significantly different from control (chi-square test, with 1 d.f., p<0.05)*

Table IV.6.2. Percentage of deformed fish and number of fish with 0, 1, 2 or more than 3 deformities

	T	*N*	*D*	*% D*	*% N*	*nr deformities*			
						0	*1*	*2*	*+ 3*
Control	17	9	8	47.1	52.9	9	5	2	1
20 mg/L	8	3	5	62.5*	37.5	3	5	0	0
4 mg/L	13	2	11	84.6*	15.4	2	7	3	1
0.8 mg/L	15	3	12	80.0*	20.0	3	7	4	1

T** Total of fish observed; **N** number of fish without deformities; **D** number of fish with one or more deformities; **% D** percentage of deformed fish; **% N** percentage of non-deformed fish; ** significantly different from control (chi-square test, with 1 d.f., p=0.05)*

The expression of several genes related to skeletogenesis and pollutant signaling was analyzed by qPCR. Expression of *cyp1a, ahr2, nfe2, hmox1, hsp70, gpx1a and txn* genes could not be detected in any of the samples (see chapter on 3MC). Primers designed to amplify *mafT* and *sod2* exhibited an EFF% of approx. 127 and 123, respectively. Few genes, i.e. *op* and *oc2*, had their expression significantly affected by cobalt (Figure IV.6.3); in both cases, expression appeared to be up-regulated by cobalt but only at 0.8 mg/L.

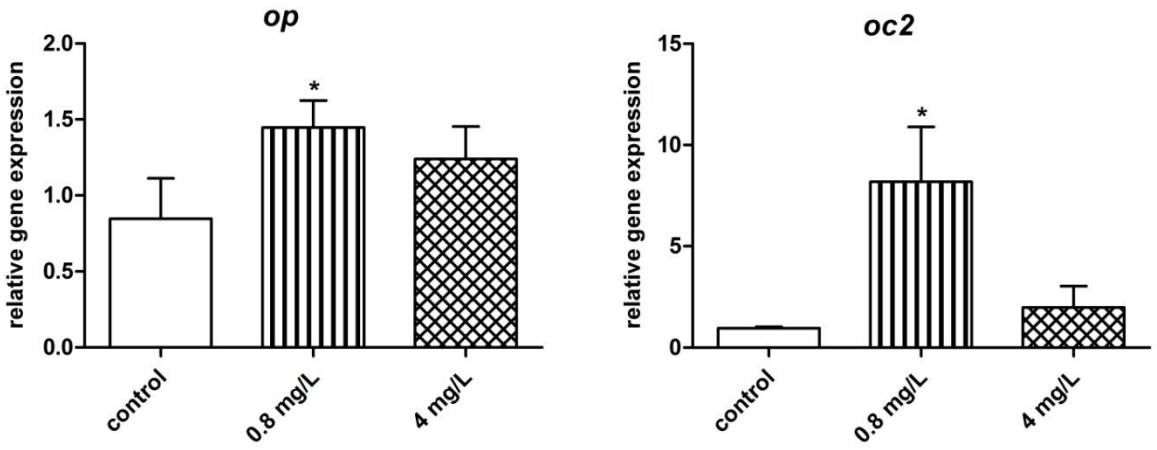


Fig.IV.6.3. Relative gene expression in 20-dpf zebrafish larvae exposed to different doses of cobalt. *op/spp1, osteopontin*; *oc2, osteocalcin 2*. Values are presented as mean ± standard deviation calculated from 3 technical replicates for each pool of larvae * indicate values significantly different from control (one-way ANOVA $p<0.05$ followed by Tukey's test).

These results are by themselves not sufficient to draw strong conclusions while an increase in *op* may due to a higher remodeling of the skeletal structures in fish exposed to cobalt, an increase in *oc2* expression could be related to a stimulation of bone formation. In the light of the study by Jezierska, we propose that the increase in oc2 expression could also be related to the stimulation of the energy metabolism in response to the higher demand in energy of organisms exposed to heavy metals such as cobalt (Jezierska et al., 2009).

The range of concentrations used in this study may be over the environmental levels but assuming that the effects at lower concentrations would be similar, we can expect that aquatic species would be affected by exposure to cobalt in a lower degree, affecting perhaps only the number of individuals impaired.

IV.7. Cadmium

It has not been possible to determine the LD_{50} value for cadmium in our system since none of the concentrations tested triggered a lethal effect until 20 days of exposure (results not shown). Total length was also not affected upon exposure to cadmium (Fig.IV.7.1). Higher doses should be tested in future studies to determine toxic limits and LD50.

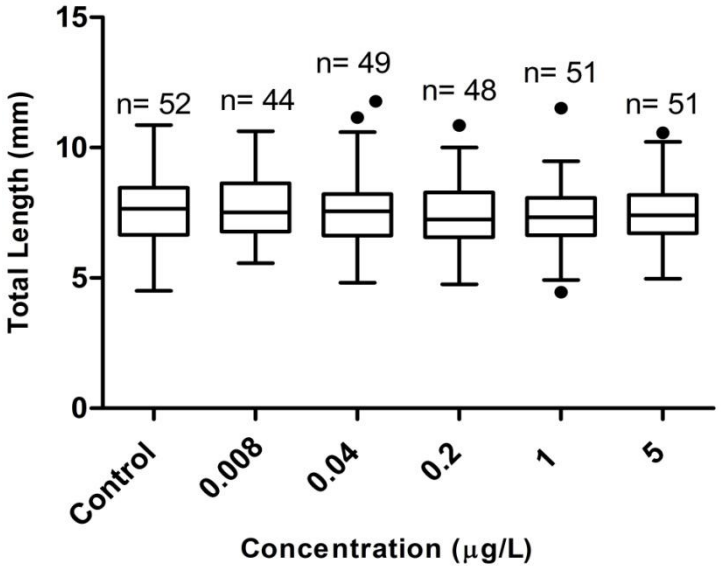
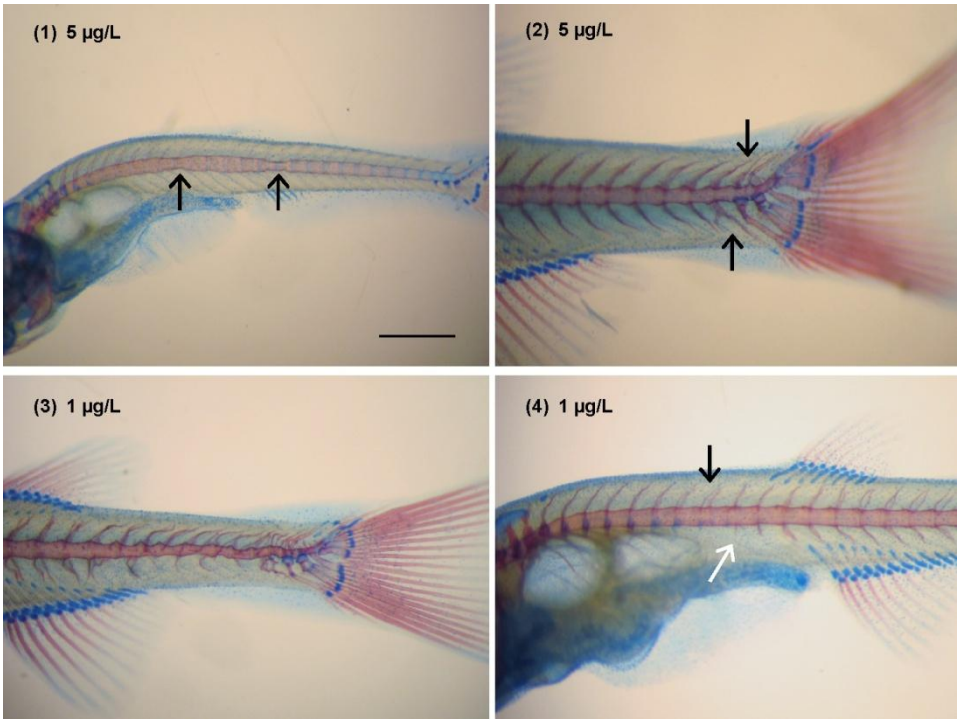


Fig.IV.7.1. Effect of cadmium on total length of zebrafish larvae. Total length variation between test conditions and control (TL results by one-way ANOVA analysis of variance followed by a Tukey's multiple comparison test $p<0.05$).

Although not as affected as for cobalt exposure, hatching time was slightly delayed: there were still 9, 8 and 11 embryos non-hatched at 72 hpf for 5, 1 and 0. 2 µg/L of cadmium, while all embryos were hatched by this time in control fish and fish exposed to lower concentrations. The occurrence of skeletal deformities upon exposure to cadmium was investigated in AB/AR-S double stained fish. Frequency and type of deformities has been listed in Table IV.7.1 and a representative set of photos are presented in Figure IV.7.2.



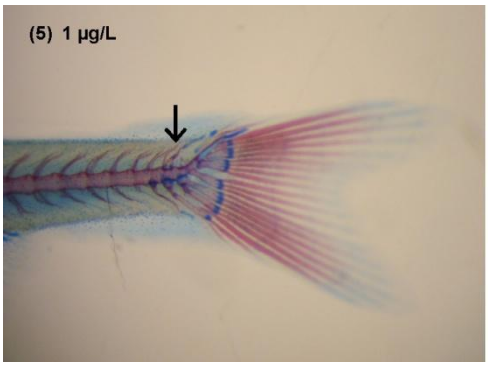


Fig.IV.7.2. Examples of malformations observed in 20-dpf zebrafish larvae exposed to cadmium. Larvae were double stained with alcian blue and alizarin red S and observed under a Leica stereo microscope. **1** abdominal and caudal vertebrae compression (platispondily), **2** malformation of the last neural and penultimate hemal arches, **3** severe malformation of the caudal vertebrae including neural and hemal arches and the urostyle, **4** fusion of abdominal vertebrae (black arrow) with malformation of hemal arches (white arrow), **5** fusion of neural hemal arches of the caudal fin vertebrae. Scale represents 0.5 mm.

In these photos, malformations were observed in vertebrae (**1**, **3**, **4**) and in the arches (**2**, **3**, **4**, **5**) in the two highest concentrations 5 and 1 µg/L.

Table IV.7.1.Skeletal structures affected upon exposure to cadmium

Structures	*Control (n=25)*		*5 ug/L (n=25)*		*1 ug/L (n=25)*		*0.2 ug/L (n=23)*		*0.04 ug/L (n=24)*		*0.008 ug/L (n=22)*	
	n	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*
Abdominal vertebrae			6	24*	2	8*	2	8.7*	1	4.2*	1	4.5*
Caudal vertebrae	2	8	4	16	5	20*	2	8.7	1	4.2	3	13.6
Caudal fin vertebrae	1	4	2	8								
Arches	14	56	21	84*	33	132*	23	100*	17	70.8*	15	68.2
Cephalic	1	4	8	32*	14	56*	6	26.1*	12	50*	4	18.2*
Scoliosis			1	4*								
Caudal fin + rays			2	8*					1	4.2*		
Pectoral fin + rays			1	4*								
Anal fin + rays			1	4*			1	4.3*				
Epural			1	4*							1	4.5*
Urostyle					1	4*					1	4.5*
Notochord	1	4					2	8.7				
Parhypural + hypurals 1 and 2			2	8*					1	4.2*		
Hypurals 3-5					1	4*						

n the number of occurrences of malformation in the specific structure % D percentage of deformed fish in the total fish observed;
** values statistically different from the control (chi-square test, with 1 d.f., p<0.05)*

A significant increase in the frequency of deformities was observed at 5 µg/L in structures like the abdominal vertebrae, the arches, in the cephalic area, in the caudal, pectoral and anal fins, in the occurrence of curvature (scoliosis), in the epural and in the parhypural and hypural 1 and 2; at 1 µg/L, in structures like the abdominal and caudal vertebrae, the arches, the cephalic area, the urostyle and the hypurals 3-5; at 0.2 µg/L, in

the abdominal vertebrae, the arches and cephalic area and malformations in the anal; at 0.04 µg/L, in the abdominal vertebrae, the arches and cephalic area, in the caudal fin and affecting the parhypural and hypural 1 and 2 and finally at 0.008 µg/L, in the abdominal vertebrae, in the cephalic area, epural and urostyle. Deformities observed in the cephalic area mostly affected the branchial arches throughout concentrations. Exposure to cadmium significantly increased the number of deformed fish in all of the concentrations used (84.0, 96.0, 78.3, 83.3 and 77.3% for highest to lowest concentration versus 56% in unexposed fish; Table IV.7.2.). The number of deformities per fish was also increased upon exposure to cadmium, with many individuals having 2 or more than 3 deformities, at higher concentrations of cadmium every fish is affected by more malformations than in unexposed fish. An incidence of malformations in the branchial arches was also noticed.

Table IV.7.2. Percentage of deformed fish and number of fish with 0, 1, 2 or more than 3 deformities

	T	*N*	*D*	*% D*	*% N*	*nr deformities*			
						0	*1*	*2*	*+ 3*
Control	25	11	14	56.0	44.0	11	12	2	0
5 µg/L	25	4	21	84.0*	16.0	4	9	8	4
1 µg/L	25	1	24	96.0*	4.0	1	7	12	5
0.2 µg/L	23	5	18	78.3*	21.7	5	6	8	4
0.04 µg/L	24	4	20	83.3*	16.7	4	12	7	1
0.008 µg/L	22	5	17	77.3*	22.7	5	12	4	1

T *Total of fish observed;* ***N*** *number of fish without deformities;* ***D*** *number of fish with one or more deformities;* ***% D*** *percentage of deformed fish;* ***% N*** *percentage of non-deformed fish;* ******* *significantly different from control (chi-square test, with 1 d.f., p=0.05)*

The expression of several genes related to skeletogenesis and pollutant signaling was analyzed by qPCR. Expression of *cyp1a, ahr2, nfe2, hmox1, hsp70, gpx1a and txn* genes could not be detected in any of the samples (see chapter on 3MC). Primers designed to amplify *mafT* and *sod2* exhibited an EFF% of approx. 127 and 123, respectively. From the qPCR results obtained, the statistical significant values are shown in Figure IV.7.3.

All the genes presented in Figure IV.7.3 had their expression significantly down regulated upon exposure to cadmium. While *sox9a, oc1* and *sod2* expression appeared to be slightly down-regulated and only at highest concentrations (1 or 0.2 µg/L), *op, col10a1* and *bmp2b* expression appeared to be strongly down-regulated for most, if not all, concentrations.

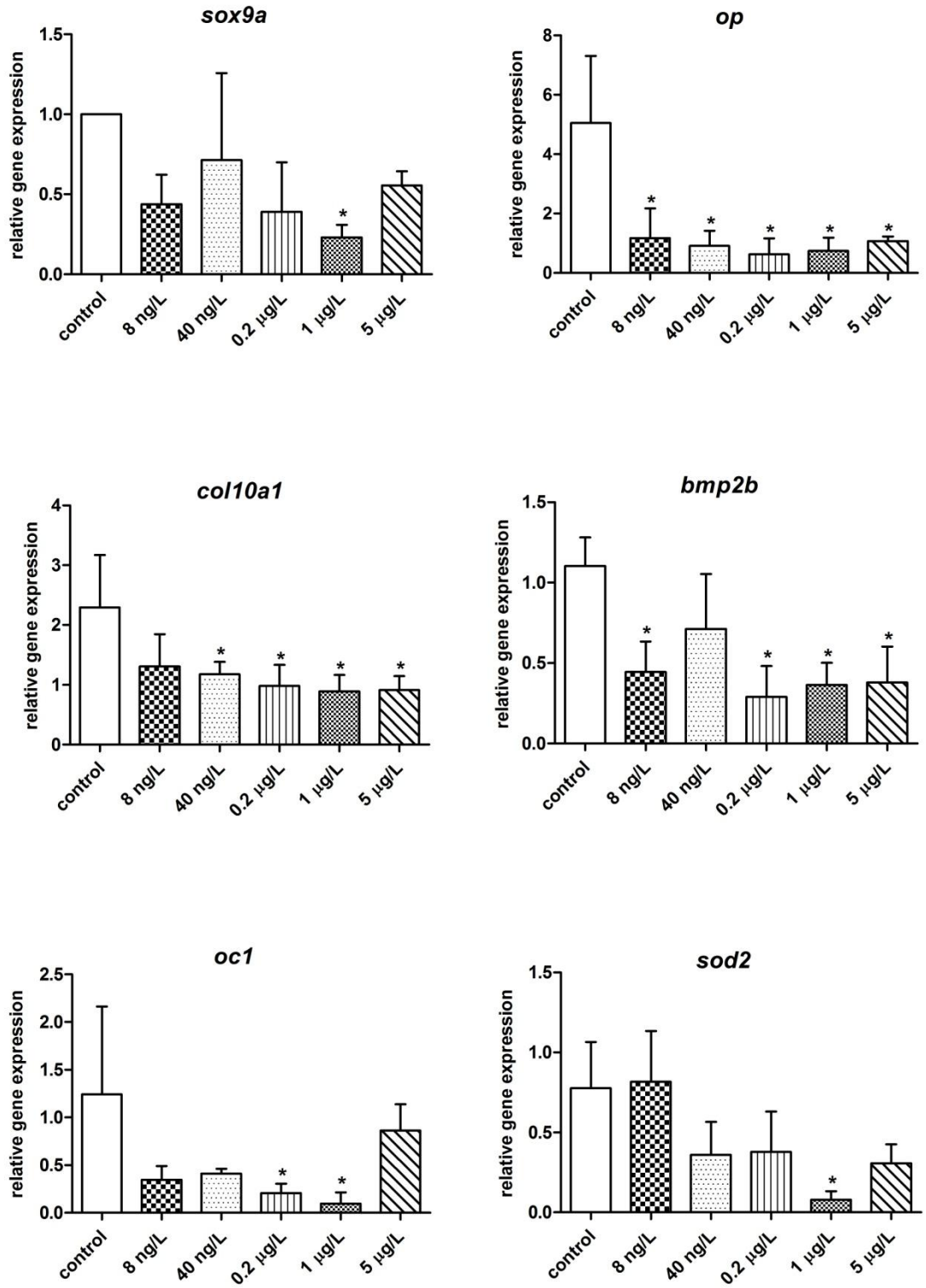


Fig.IV.7.3. Relative gene expression in 20-dpf zebrafish larvae exposed to different doses of cadmium. *Sox9a, SRY-box containing gene 9a; op/spp1 osteopontin*; *col10a1, collagen type X alpha 1; bmp2b, bone morphogenetic protein 2b; oc1, osteocalcin 1*; *sod2, superoxide dismutase 2*. Values are presented as mean ± standard deviation calculated from 3 technical replicates for each pool of larvae; * indicate values significantly different from control (one-way ANOVA $p<0.05$ followed by Tukey's test).

As seen before, the highest concentration is not always the effective concentration and a possible heterogeneity in the quality of the eggs could be the reason. If real, the down-

regulation of *sox9a* and *oc1* expression could indicate an alteration of chondrocyte differentiation and some impairment of osteogenesis respectively; and *sod2* down – regulation can reflect a higher oxidative stress imposed on the larvae. Down regulation of *op*, *col101a* and *bmp2b* expression is however convincing and would indicate a defective ECM formation and mineralization and altered osteogenesis. Jezierska et al reported that heavy metals, such as cadmium, can alter the synthesis and activity of enzymes, like the reduction of Ca^{2+} -ATPase activity, and have the ability to mimic calcium ions and occupy calcium-binding sites on calcium-dependent proteins, therefore affecting many different cellular functions and molecular mechanisms. BMP pathway has been associated with mechanisms of osteogenesis (Chen et al., 2004) and its alteration due to decrease expression of *bmp2b* would certainly account for the skeletal deformities observed upon cadmium exposure.

Studies have shown that long term exposure of rats to environmental cadmium levels (that match the environmental levels humans are exposed to), result in decrease bone mineral density and increased skeletal weakness (Bhattacharyya, 2009). Brzóska et al., exposing rats to 1 µg Cd/ml in drinking water, during 24 months reported lumbar vertebrae demineralized and a decreased in mechanical strength (Brzóska and Moniuszko-Jakoniuk, 2004a, 2004b). Showing evidences that even low cadmium levels can have a detrimental effect on rats and possibly humans. Also studies using organ cultures from chick embryos, exposed to cadmium at 2 to 20µM, reported several effects, like decreased in bone length and low collagen accumulation as well as degeneration of osteogenic cells and osteoblasts (Kaji et al., 1988; Miyahara et al., 1983, 1978).

Although the concentrations used here were very low to induced high mortality, there is evidence of cadmium effects on time of hatching and higher incidence of deformities upon exposure that coincide with effects already reported. Looking at the gene expression some patterns are interesting and can gives us new insight into to cadmium MOA but a more extensive set of results is needed.

IV.8. Vanadate

Zebrafish eggs were exposed for 20 days to five different concentrations of vanadate. Highest concentration (25 mg/L) killed all the fish by 9 days of exposure. Hatching time

and rate were not affected by other concentrations (i.e. 100 % by 72 hpf). LD_{50} for vanadate was 6.113 mg/L (Fig.IV.8.1). Vanadate did not affect the average total length of zebrafish larvae in relation to the control, indicating no growth retardation upon vanadate exposure.

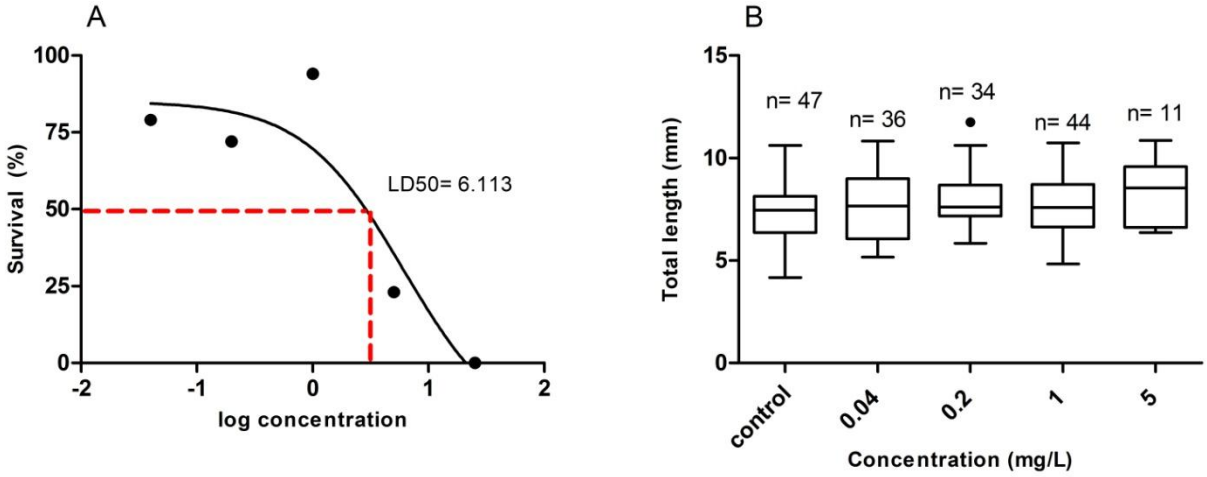


Fig.IV.8.1. Effect of vanadate on survival rate (**A**) and total length (**B**) of zebrafish larvae. LD_{50} value was calculated from the mathematical model fitting survival data in relation to vanadate concentration. Significance of differences in TL values was tested by one-way ANOVA followed by a Tukey's multiple comparison test $p<0.05$).

The occurrence of skeletal deformities upon exposure to vanadate was investigated in AB/AR-S double stained fish. Frequency and type of deformities are listed in Table IV.8.1 and a representative set of photos are presented in Figure IV.8.2.

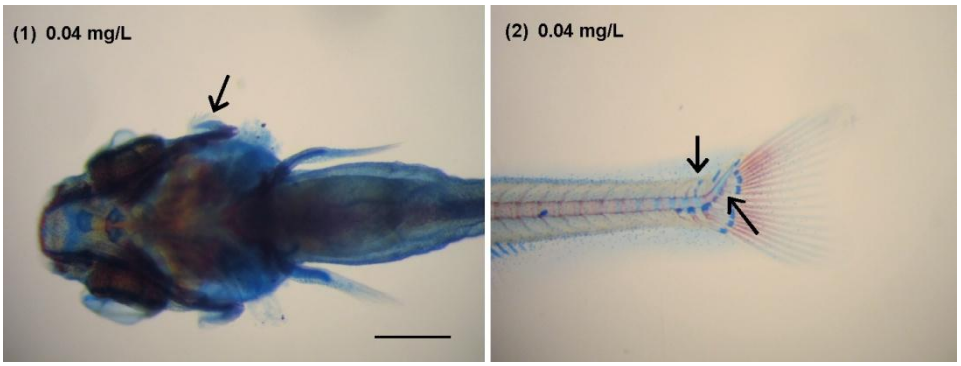


Fig.IV.8.2. Examples of malformations observed in 20-dpf larvae exposed to vanadate. Larvae were double stained with alcian blue and alizarin red S and observed under a Leica stereo microscope. **1** malformation of the operculum, **2** two neural arches in the last vertebrae and an ectopic structure between hypurals 2 and 3. Scale represents 0.5 mm.

Most representative malformations observed upon exposure to vanadate were found in the operculum (both sides) and in the hypurals with the occurrence of an ectopic

structure in between. A significant increase in the frequency of deformities, i.e. scoliosis and kyphosis, were found in fish exposed to the highest non-lethal concentration (5 mg/L). At 1 mg/L deformities were observed in the arches; axial curvatures, e.g. scoliosis and kyphosis, were also detected. At 0.2 mg/L, deformities were observed in the arches and at 0.04 mg/L, the occurrence of scoliosis was detected. Exposure to 5 mg/L of vanadate significantly increased the number of deformed fish (100% versus 60% in unexposed fish; Table IV.8.2.). Surprisingly, the number of deformed fish was lower in fish exposed to 0.2 mg/L of vanadate that in control; a technical problem is most probably at the origin of this result. The number of deformities per fish was apparently similar in deformed fish exposed to vanadate or left unexposed. Vanadate has been reported to increase bone formation, mineralization and mineral density in rats and other in vitro studies point to osteogenic properties (Laizé et al., 2009; Tiago et al., 2008b) Laizé et al. also refer the stimulatory effect of vanadate at low concentrations which can account for the low number of deformities at 0.2 mg/L comparing to higher concentration.

Table IV.8.1.Skeletal structures affected upon exposure to vanadate

Structures	*Control (n=20)*		*5 mg/L (n=20)*		*1 mg/L (n=20)*		*0.2 mg/L (n=14)*		*0.04 mg/L (n=16)*	
	n	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*	*n*	*% D*
Abdominal vertebrae	1	5								
Caudal vertebrae	1	5								
Caudal fin vertebrae	1	5			1	5	1	7.1		
Arches	16	80	9	81.8	20	100*	4	28.6*	11	68.8
Kyphosis			2	18.2*	1	5*				
Scoliosis			1	9.1*	1	5*			1	6.3*
Operculum									1	6.3
Dorsal fin + rays	1	5								
Parhypural + hypurals 1 and 2	2	10							1	6.3

n the number of occurrences of malformation in the specific structure % D percentage of deformed fish in the total fish observed; values statistically different from the control (chi-square test, with 1 d.f., p<0.05)*

Table IV.8.2. Percentage of deformed fish and number of fish with 0, 1, 2 or more than 3 deformities

	T	*N*	*D*	*% D*	*% N*	*nr deformities*			
						0	*1*	*2*	*+3*
Control	20	8	12	60.0	40.0	8	7	4	1
5 mg/L	11	0	11	100.0*	0.0	0	11	0	0
1 mg/L	20	6	14	70.0	30.0	6	12	1	1
0.2 mg/L	14	10	4	28.6*	71.4	10	4	0	0
0.04 mg/L	16	5	11	68.8	31.3	5	10	1	0

T *Total of fish observed;* ***N*** *number of fish without deformities;* ***D*** *number of fish with one or more deformities;* ***% D*** *percentage of deformed fish;* ***% N*** *percentage of non-deformed fish;* ******* *significantly different from control (chi-square test, with 1 d.f., p=0.05)*

Expression of several genes related to skeletogenesis and ECM mineralization was analyzed by qPCR. Expression of *cyp1a, ahr2, nfe2, hmox1, hsp70, gpx1a and txn* genes could not be detected in any of the samples (see chapter on 3MC). Primers designed to amplify *mafT* and *sod2* exhibited an EFF% of approx. 127 and 123, respectively. Few larvae were available for one of the concentrations, i.e. 5 mg/L, and priority was given to staining and identification of deformities. qPCR data is therefore not available for that concentration. Significant differences in the expression of selected genes are presented in Figure IV.8.3.

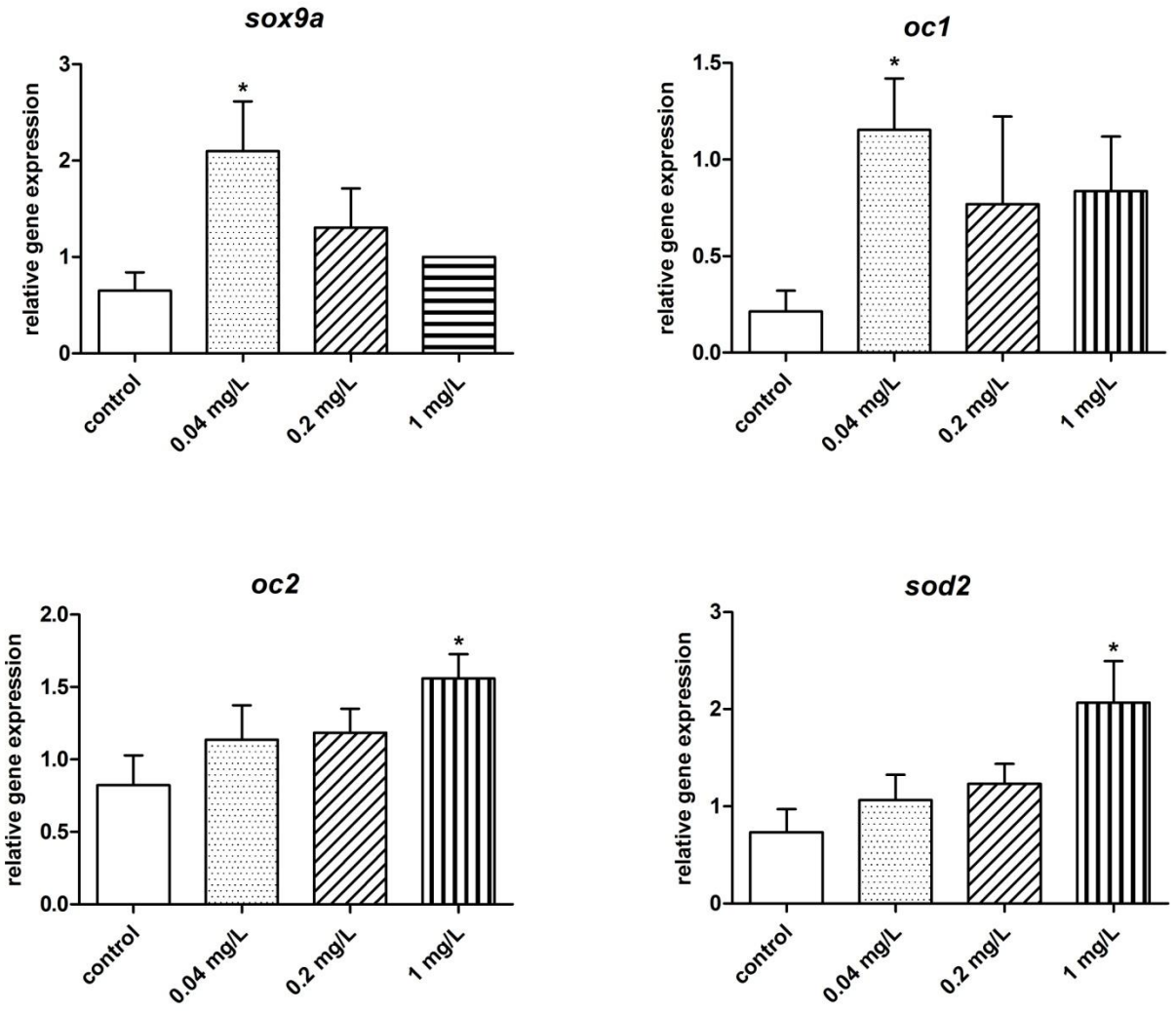


Fig.IV.8.3. Relative gene expression in 20-dpf zebrafish larvae exposed to different doses of vanadate. *Sox9a, SRY-box containing gene 9a; oc1, osteocalcin 1*; oc2, osteocalcin 2; *sod2, superoxide dismutase 2*. Values are presented as mean ± standard deviation calculated from 3 technical replicates for each pool of larvae; * indicate values significantly different from control (one-way ANOVA $p<0.05$ followed by Tukey's test).

Expression of *sox9a, oc1, oc2* and *sod2* genes appeared to be up-regulated upon exposure to vanadate, although only significantly at 0.04 mg/L (*sox9a* and *oc1*) and at 1 mg/L (*oc2* and *sod2*). The up-regulation of *sox9a* can indicate a proliferation of chondrocyte and differentiation and the *oc1* levels can reflect a stimulation of bone formation. The same can be assumed for the pattern for *oc2*, while an up-regulation of *sod2*, a marker for oxidative stress, may reflect the organism antioxidant system activity to suppress a higher vanadium concentration and prevent molecular damage. Laizé et al have described the regulatory role of some vanadium compounds in osteoblast-cell like proliferation and differentiation and the stimulatory effect at low concentrations and inhibitory at higher concentrations (Laizé et al., 2009). Looking at the patterns of expression obtained, we can see a tendency for up-regulation of genes by vanadate, related to chondrogenesis and osteogenesis. Comparing our *in vivo* results to results obtained using fish cell lines, we get opposite effects; while ours appear to indicate a stimulatory effect upon exposure, experiments with fish cell lines point to a decrease in ECM mineralization of chondrocytes and osteoblasts indicating an anti-mineralogenic effect, thus inhibiting osteogenesis.

V. Conclusions and future perspectives

This work mainly aimed at collecting basic and preliminary data on the possible osteotoxic effects of aquatic pollutants. For most of these pollutants, knowledge about mechanisms of toxicity is scarce, if not inexistent in the case of osteotoxicity; the validation and comparison of our results was indeed limited to the few existing data.

We were able to determine the LD_{50} for 3MC, lindane, diclofenac, cobalt and vanadate in our biological system, i.e. developing zebrafish larvae. For those pollutants with no LD_{50}, higher concentrations should be tested in future studies. None of the pollutant, at the concentrations tested, showed an effect on the average total length of zebrafish larvae, indicating that growth was not impaired upon pollutant exposure. On the contrary, hatching was shown to be affected upon exposure to some of these pollutants; among those, cobalt triggered the strongest effect, in a manner that hatching of embryos had to be assisted mechanical in order to break up the chorion. Cadmium also affected embryo hatching although to a lesser extent. It is worth to note that both pollutants affecting

embryo hatching belong to the same family of chemicals, heavy metals; although this should be further demonstrated, we propose that altered hatching time and rate may be a general characteristic of heavy metal toxicity. All the chemicals, at some of the concentration tested, increased the percentage of deformed fish and in some case the number of deformities per fish, e.g. for glyphosate, diclofenac or cadmium. Although deformities observed in the skeleton of pollutant-exposed zebrafish larvae were not lethal, they probably affected their fitness, locomotion and feeding ability and we cannot exclude that this could jeopardize the survival of the fish when adult. No prevalence of a particular deformity was observed for the pollutants tested in this work.

This study identified concentrations triggering skeletogenic effects. Interestingly, two PAHs tested within the scope of this work, 3MC and naphthalene, triggered skeletal deformities, although through different mechanisms: while 3MC probably affected osteoblast function and therefore osteogenesis, naphthalene appeared to alter chondrocyte differentiation and therefore chondrogenesis. Similar observations were done for heavy metals, i.e. cadmium probably affects chondrocyte differentiation (chondrogenesis) and ECM formation / mineralization, while vanadate appeared to stimulate chondrogenesis and osteogenesis. We therefore collected evidence that pollutants from a same family can trigger skeletal deformities through different mechanisms, which remain to be determined.

Future work should aim at confirming the present results and at determining the specific molecular mechanisms underlying the skeletogenic effect of these pollutants. Global gene expression analysis upon exposure of zebrafish larvae to pollutant could be assessed for example using a high throughput mRNA differential display strategy, a DNA microarray suitable to a toxicological approach or mass RNA sequencing for a more robust transcriptome analysis and a more precise measurement of the levels of transcripts. Signaling pathways affected upon exposure to pollutant could also be determined *in vitro* using transcription factor (TF) reporter constructions (e.g. QIAGEN Cignal assay), where repeats of TF binding elements are placed upstream of luciferase genes. Uncovering the mechanisms of pollutant action on skeletogenesis would allow predicting effects on natural populations and providing insights on how chemical exposure can affect humans and the ecosystems.

Finally, a protocol has been established within the scope of this work for exposing zebrafish larvae to water born chemicals during a period of 20 days in order to evaluate

their osteotoxicity. This protocol is not only novel, it has also been optimized in order to reduce the quantity of hazard wastes produced, validating the use of this in *vivo* system in osteotoxicity.

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