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Fish as a model to assess chemical toxicity in bone

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ABSTRACT

Environmental toxicology has been expanding as growing concerns on the impact of produced and released chemical compounds over the environment and human health are being demonstrated. Among the toxic effects observed in organisms exposed to pollutants, those affecting skeletal tissues (osteotoxicity) have been somehow overlooked in comparison to hepato-, immune-, neuro- and/or reproductive toxicities. Nevertheless, sub-lethal effects of toxicants on skeletal development and/or bone maintenance may result in impaired growth, reduced survival rate, increased disease susceptibility and diminished welfare. Osteotoxicity may occur by acute or chronic exposure to different environmental insults. Because of biologically and technically advantageous features – easy to breed and inexpensive to maintain, external and rapid rate of development, translucent larvae and the availability of molecular and genetic tools – the zebrafish (*Danio rerio*) has emerged in the last decade as a vertebrate model system of choice to evaluate osteotoxicity. Different experimental approaches in fish species and analytical tools have been applied, from *in vitro* to *in vivo* systems, from specific to high throughput methodologies. Current knowledge on osteotoxicity and underlying mechanisms gained using fish, with a special emphasis on zebrafish systems, is reviewed here. Osteotoxicants have been classified into four categories according to the pathway involved in the transduction of the osteotoxic effects: activation/inhibition of membrane and/or nuclear receptors, alteration of redox condition, mimicking of bone constituents and unknown pathways. Knowledge on these pathways is also reported here as it may provide critical insights into the development, production and release of future chemical compounds with none or low osteotoxicity, thus promoting the green/environmental friendly chemistry.

1. The use of fish in ecotoxicology

Several regulatory frameworks and agencies such as the European Environment Agency (EEA), the U.S. Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA), have developed and implemented regulatory measures, guidelines, toxicological databases, and prediction algorithms to avoid or limit the impact of anthropogenic chemicals released into the environment on the wildlife and human health. Relevant examples are the U.S. ToxCast program and the European Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) (Kim and Tanguay, 2013; Richard et al., 2016).

A number of eco-toxicity tests is usually conducted sequentially to assess the environmental risk for each compound. *In vivo* testing with multiple taxa allows an evaluation at ecosystemic level, *in vitro* and *ex vivo* testing provide specific knowledge on mechanisms, target tissues, etc., while *in silico* prediction reduces animal experimentation (Bradbury et al., 2004; Scholz et al., 2008). Current guidelines from the Organisation for Economic Co-operation and Development (OECD) regarding eco-toxicity testing require the use of different plant and animal species from a diverse set of environments (terrestrial, freshwater, estuarine and marine species) like the midges *Chironomus* sp. (OECD, 2011) or the crustacean *Daphnia magna* (OECD, 2012). The fish embryo is an ethically acceptable small scale analysis system with the complexity of a complete organism (Padilla et al., 2012). In particular, the zebrafish (*Danio rerio*; Hamilton, 1822) has emerged as an interesting vertebrate model in ecotoxicology (reviewed in Gustafson et al., 2012; Raldúa et al., 2012; He et al., 2014; Braunbeck et al., 2015). In brief, it is easy to breed and inexpensive to maintain, it has an external and rapid development, larvae are translucent, and females are highly fecund, while numerous biotechnological tools have been developed (*e.g.* transgenic and mutant lines, and *in vitro* cell systems; Howe et al., 2013; Gamse and Gorelick, 2016). Importantly, data from zebrafish toxicity tests are accepted by the FDA and required by the REACH.

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The Fish Embryo Test (FET) was introduced for chemical toxicity testing in 2005 (Braunbeck et al., 2005) and approved in 2013 by the OECD (OECD, 2013). The FET protocol is the same, independently of the fish used, and thus the OECD term FET will be used (and recommended) herein whatever the fish species is applied to, although zebrafish FET is also described as ZET or zFET in some cases. Nowadays, FET is the most commonly conducted test in vertebrates for environmental hazard and risk assessment, being better standardized than older guidelines (*e.g.* OECD, 1992, 2000; Braunbeck et al., 2015) and represents a promising alternative for replacement and/or refinement regarding the 3Rs principle (Beekhuijzen et al., 2015; Braunbeck et al., 2015). The identification of the mechanisms mediating chemical toxicity have benefited not only from the use of transgenic zebrafish lines, where specific cells, tissues and organs have been labeled with fluorescent markers (He et al., 2014), but also from *in vitro* and *ex vivo* approaches, which have been critical for evaluating cell/tissue/organ-specific toxicity. In the present review, we will focus on osteotoxicity, a toxic effect that has been somehow overlooked in comparison to hepato-, immune- and reproductive toxicity, but which has a critical impact on vertebrate viability, growth, health and welfare. We will define osteotoxicity and describe the suitability of (zebra)fish as a model to study underlying mechanisms. Compounds with reported osteotoxicity in fish and respective mechanisms of action (MoA) have been listed and some recommendations and future perspectives in the area of osteotoxicology will be provided based on current knowledge.

2. Osteotoxicity, skeletal development and (zebra)fish systems

Organ-specific toxicity has long been studied in zebrafish (Raldúa et al., 2012) and cardio-, hepato-, nephro-, neuro-, immuno-, thyroid, muscle and gastrointestinal toxicity have been recapitulated to some extent in the zebrafish (briefly reviewed in Peterson and MacRae, 2012). Osteotoxicity (or skeletal toxicity) can be generally defined as any negative effect inflicted to skeletal tissue during its development and/or maintenance, following acute or prolonged exposure to exogenous compounds (referred hereafter as osteotoxic compounds or osteotoxicants, independently of their ability to also induce toxic effects in other tissues, organs and/or systems) present in the environment or acquired through feeding or injury, and leading to morphological malformations and/or impairments on skeletal mineralization, remodeling and morphogenesis (Daston and Seed, 2007; Laizé et al., 2014; Stahlmann, 2003; Zur Nieden et al., 2004, 2010). Fish are especially vulnerable since most species are oviparous, i.e. eggs are laid in the water where they undergo most, if not all, their embryonic development. In addition, fish hatch at a much earlier developmental stage than other vertebrates, and undergo morphogenesis and skeletogenesis early during larval development. Moreover, fish are an important part of human diet and bio-accumulated pollutants may originate health problems for consumers, possibly inducing bone defects (*e.g.* poisoning with cadmium or organochlorides), if toxicity is not detected precociously. All these particularities have strengthened the position of fish as a suitable and valuable organism to assess risks associated with osteotoxicity, to study the underlying mechanisms, but also to serve as sentinel species (Warner and Jenkins, 2007). Nevertheless, an efficient use of fish as a model organism in osteotoxicity requires a deep and detailed knowledge on its skeletal development and maintenance.

2.1. Skeletal development and maintenance

Skeletal and bone maintenance are two complex processes involving a highly coordinated sequence of events at molecular, cellular and tissue level (see Hall, 2015 for more details). In addition to chondrocytes and osteoblasts (cartilage and bone forming cells, respectively), osteocytes and osteoclasts are also key players of bone maintenance and remodeling. Osteocytes are osteoblasts that became entrapped in the secreted osteoid matrix. They regulate the routine turnover of bony matrix responding to systemic stimuli through various mechanosensory mechanisms (Dallas et al., 2013). Osteoclasts are mono- or multinucleated bone resorbing cells, derived from the hematopoietic cell lineage, that are responsible for degrading the extracellular matrix components and resorption of bone minerals (Witten and Huysseune, 2009).

At the molecular level, a complex network of signaling pathways controling skeletal development and maintenance have been progressively identified and extensively reviewed in recent years including Szabo-Rogers et al. (2010), Mari-Beffa and Murciano (2010), Apschner et al. (2011), Eames et al. (2013), Kessels et al. (2014) and Mork and Crump (2015), among others. Briefly, all the genes regulating skeletal development and maintenance can be basically organized into five major functional groups: (i) osteoblast differentiation and maturation regulators, including *bmp2*, *atf4* and *runx2*; (ii) osteoblast/chondrocyte inhibitors, including *pthlh*, *twist*, *sox9a* and *sox9b*; (iii) bone formation factors, including *alpl*, *col10*, *spp1*, and *sp7*; (iv) osteoclast regulators, *tnfrsf11b* and *tnfsf11*; and (v) bone resorption factors, mainly *trap* and *ctsk* (Seemann et al., 2015).

2.2. Zebrafish

Unlike the classical models (mouse and chicken), the use of zebrafish as a model for bone research is relatively recent (Apschner et al., 2011). Nevertheless, although fish and tetrapod lineages diverged approximately 420 million years ago (Javidan and Schilling, 2004), skeleton formation and tissue mineralization (Mackay et al., 2013), skeletal tissues (cartilage and bone) and cell types (Witten and Hall, 2015; Witten et al., 2016) resemble in many aspects those found in mammals. The remarkable similarity of developmental events and molecular pathways leading to skeletogenesis, including the early formation of a cartilaginous anlage followed by bone formation through endochondral ossification, reflects the high evolutionary conservation in this process (Hall, 2015). Still, some differences between zebrafish and mammalian skeleton exist (reviewed in Laizé et al., 2014). In brief, (i) osteoclasts are mostly mononucleated in fish at early life stages, while multinucleated in mammals, (ii) hematopoiesis occurs in the head kidney in fish while the bone marrow is the hematopoietic organ *per se* in mammals (although an extra-medullary hematopoiesis can occur during fetal development, immune responses and pathological circumstances; Kim, 2010), (iii) ossification of the vertebrae occurs directly in fish, while mammals have cartilaginous precursor for these structures (Arratia and Schultze, 1992; Nordvik et al., 2005; Fleming et al., 2015) and (iv) fish present a higher diversity of cartilage types and intermediate skeletal tissues when compared to mammals (Benjamin, 1990; Witten et al., 2010, 2016). Nevertheless, the key regulators of bone formation have been conserved from fish to human, with orthologous genes sharing significant sequence similarities and largely overlapping expression patterns (Spoorendonk et al., 2010). Zebrafish skeleton also responds to similar external stimuli and has maintained most of the signaling and regulatory mechanisms found in mammals, making it an extremely useful model organism for performing large scale screens of molecules/chemicals that affect skeletal development and metabolism (Laizé et al., 2014; Apschner et al., 2011). The continuing improvement of the knowledge on zebrafish genome and the introduction of new technologies for targeted genome editing (Varshney et al., 2014) have facilitated the use of zebrafish in functional genomics studies. The abovementioned favorable biological features have also fostered the interest of the scientific community for the zebrafish as an advantageous organism in toxicology, and particularly on the negative impact that chemicals can have on skeletal development and bone formation (reviewed in Raldúa et al., 2012; Laizé et al.,2014 and Braunbeck et al., 2015). Accordingly, there has been a steady increase in articles mentioning the words “zebrafish” and “toxic” in their title during the last 15 years (Fig. 1) while a stagnation was

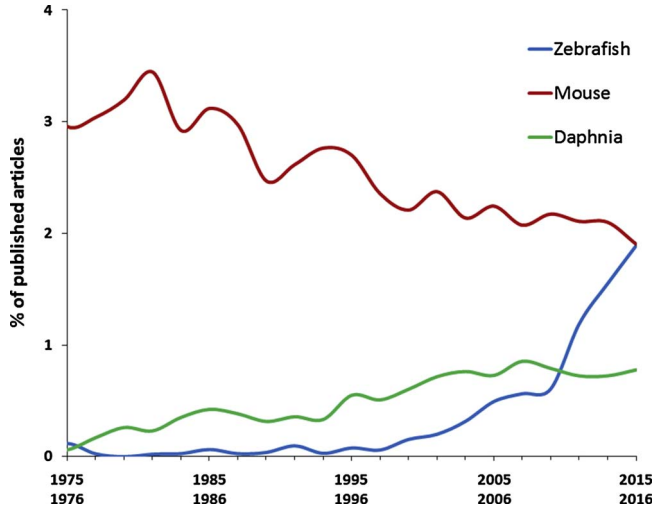

Fig. 1. Average percentage of published articles per two years within PubMed and Web of Science databases with the words "toxic*" and "mouse", "zebrafish" or "daphnia" in their title during the last 40 years.

observed in the use of other model species in toxicology like mouse or *Daphnia*. As a result, zebrafish is reaching an equal use to that of mouse model.

2.3. Fish systems for osteotoxicity

The widely diverse set of experimental systems initialy developed to study skeletogenesis and bone formation/mineralization in zebrafish (reviewed in Laizé et al., 2014) have been successfully applied to decipher the effect of several osteotoxicants (Fig. 2). Skeletal deformities generated through vanadate, cadmium and/or warfarin exposure were observed in several bone structures of the axial and appendicular skeleton (*e.g.* operculum, vertebrae, arches and caudal fin complex) and were commonly associated with fusion, compression and supranumerary elements (Fig. 2A–C). Mineralogenic effects (usually undermineralization or demineralization events) were also observed upon exposure to warfarin or cobalt (Fig. 2D and 2F). While developing larvae have been used to determine skeletogenic and mineralogenic effects (Fig. 2A–E), scale explants (*ex vivo*) (Fig. 2F), regenerating fin rays (Fig. 2G), and *in vitro* cell systems (Fig. 2H) were used to assess mineralogenic effects of compounds like prednisolone, cobalt or 3-methylcholanthrene (3-MC).

Although *in vitro* systems exhibit lower diversity of potential off-target sites than *in vivo* systems, lack the multicellular complexity and rarely recapitulate even a single target full range of functions (Peterson and MacRae, 2012), they represent a valuable first attempt for osteotoxicity screening in a high-throughput and ethically accepted manner (*Replacement*; Russell and Burch, 1959). Furthermore, they allow the identification of the cellular mechanisms underlying osteotoxicity (*e.g.* signaling pathways and target genes). *In vitro* mineralization is easily monitored through alizarin red S staining of the hydroxyapatite-like crystals deposited within the extracellular matrix (Pombinho et al., 2004). Cell-based assays allow the study of the MoA of ligand-activated transcription factors (nuclear receptors) including PXR, PPARs, ERs and AhR (Raucy and Lasker, 2013; Puy-Azurmendi et al., 2014), which recognize a wide range of structurally-unrelated ligands acting as osteotoxicants. Recently, a transactivation reporter assay has been developed for the common carp (*Cyprinus carpio*) PXR and compared with the human isoform under the exposure to various fungicides and pharmaceuticals (Lange et al., 2017), further confirming the differential hability of tested compounds to activate fish and human PXR isoforms previously reported (Ekins et al., 2008).

An *in vitro* approach might be used, for example, for identifying NF-κB signaling inhibitors that could impact bone resorption through osteoclast inhibition (Ashley et al., 2011) but also for analyzing BMP2-related osteoblast differentiation (Okada et al., 2009) and studying how compounds might affect the transcription of some of the 60 human genes regulated by PXR, some of them directly related with bone extracellular matrix (Ichikawa et al., 2006). Skeletal cell-type-specific *in vitro* tools are also reliable systems to characterize the specific cellular and molecular events by which a molecule induces an osteotoxic effect (Tiago et al., 2008, 2011; Zur Nieden et al., 2010; Fernández et al., 2015).

Ex vivo approaches provide an extra level of complexity, integrating the interaction of different cell types while still representing an ethical benefit (*Reduction*; Russell and Burch, 1959). The (zebra)fish scales are of particular interest since they appear to recapitulate the process of intramembranous ossification – i.e. osteoblast differentiation, acellular matrix deposition, mineralization and resorption by osteoclasts – and have been recently used as a non-invasive *in vitro* bioassay to demonstrate the estrogenic activity of different endocrine disruptors (Pinto et al., 2017). Scales are also technically advantageous since they can be easily harvested in large numbers with limited stress to the fish, can regenerate upon removal, and their matrix is translucent, thus facilitating imaging by microscopy (De Vrieze et al., 2014).

When a more integrative approach is required, animals (from embryonic to adult stages) are used, being the zebrafish one of the few vertebrates allowing *in vivo* approaches with the throughput necessary for the screening of large libraries of molecules (Peterson and MacRae 2012). The small size of the larvae and the fast development of its skeleton allow for high-throughput (in 96-well plate) and short duration (few days) assays. Exposure of larvae to chemicals is usually waterborne and mineralized structures are commonly detected through alizarin red S staining in 6–20 dpf zebrafish larvae (Walker and Kimmel, 2007; Bensimon-Brito et al., 2016). The zebrafish operculum is among the first bony structures to ossify (at 3 days post-fertilization), is flat shaped and localized at the surface of the fish head and has been successfully used to assess the anti-osteogenic effects of compounds like cobalt chloride (Tarasco et al., 2017). The ability of zebrafish to fully regenerate its caudal fin upon damage or amputation has also been used to gain insights into the mechanisms underlying *de novo* bone formation and mineralization, and to assess the activity of osteotoxic compounds (Cardeira et al., 2016).

Transgenesis and genome editing tools available for zebrafish have largely contributed to its recognition as a suitable model organism in ecotoxicology (see reviews by Cade et al., 2012; Huang et al., 2012; Hwang et al., 2013; Lee et al., 2014; Garcia et al., 2016). Mutant and transgenic lines have been developed and/or maintained by the Zebrafish Model Organism Database (ZFIN) at the Zebrafish International Resource Center (ZIRC; Howe et al., 2013). Transient knockdown of gene expression through the injection of Morpholino antisense oligos has also been a suitable approach for studying mechanisms of osteotoxicity, particular at early developmental stages (Blum et al., 2015). Various transgenic fish lines have been developed to study the toxic effects of compounds such as nanoparticles (Pan et al., 2013), heavy metals (Ng et al., 2015), bisphosphonates (Yu et al., 2016) or organic chemicals acting through nuclear receptors (NRs; Terrien et al., 2011; Gorelick and Halpern, 2011; Benato et al., 2014). Although their potential is considerable, they show a great variability on their sensitivity, and only very few are able to detect responses to environmentally relevant exposures (Lee et al., 2014). Bone specific transgenic reporter lines, which could be used in high throughput strategies aiming at identifying osteotoxicants in fish, have been recently established, *e.g.* the *Tg*(*cathepsin K*:mEGFP) for visualizing functional osteoclasts (To et al., 2012, 2015), the *Tg*(*collagen 10a1*:NlGFP) for osteoblast precursors (Renn et al., 2013), and the *Tg*(*osterix/sp7*:mCherry) or *Tg*(*osteocalcin*:EGFP) for early and late osteoblasts (Renn and Winkler, 2014), respectively.

The information gathered from the different experimental systems

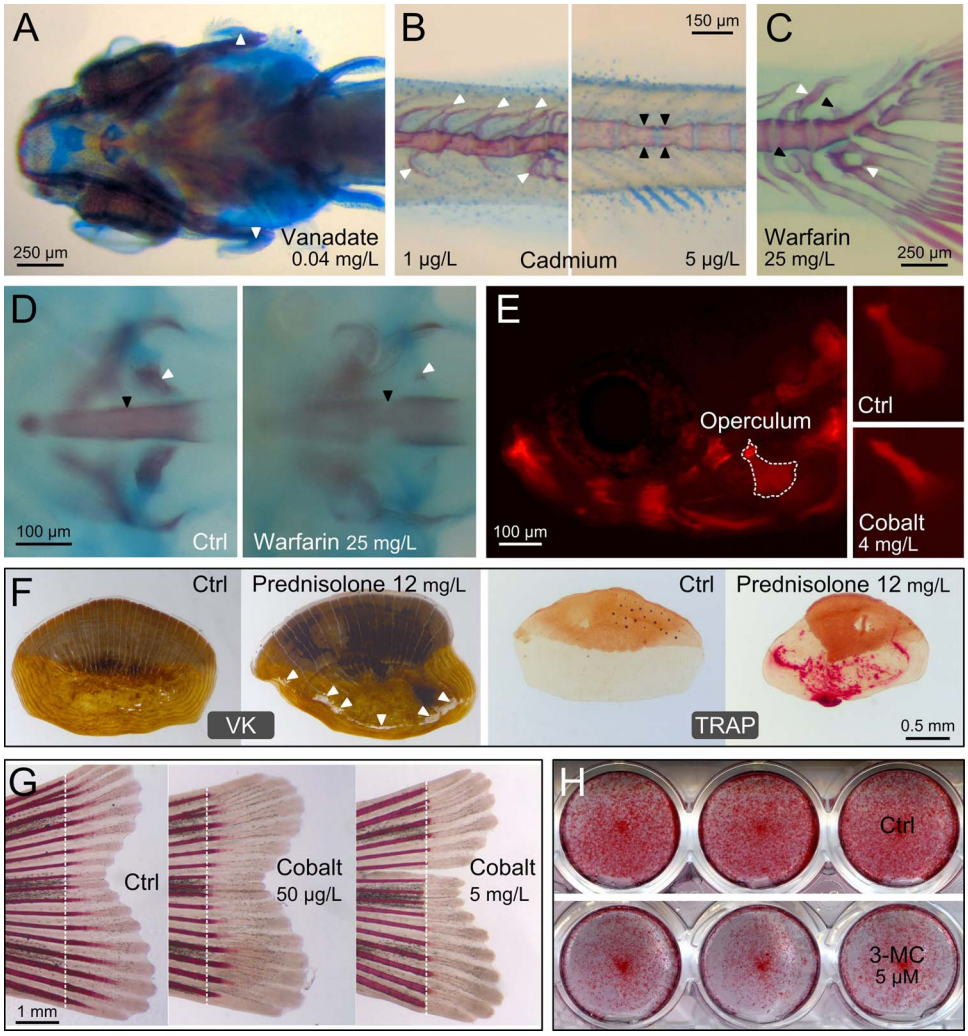

Fig. 2. Osteotoxicity in fish systems. **A**, deformed operculum (*white arrowheads*) of a 20-dpf old zebrafish exposed to 0.04 mg/L of ammonium metavanadate (dorsal view; alizarin red/alcian blue staining). **B**, deformed vertebrae (arch fusion and malformation, *white arrowheads*; platispondily, *black arrowheads*) of a 20-dpf old zebrafish exposed to 1 and 5 µg/L of cadmium chloride (lateral view; alizarin red/alcian blue staining); **C**, deformities in the caudal fin complex (fusion, *white arrowheads*; additional element, *black arrowheads*) of a 28-dpf old zebrafish exposed to 25 mg/L of sodium warfarin (lateral view; alizarin red/alcian blue staining); **D**, Undermineralized ceratobranchial 5 (*white arrowheads*) and extremity of the notochord (*black arrowheads*) of a 7-dpf old zebrafish larvae exposed to 25 mg/L of sodium warfarin during embryogenesis (from 1 hpf to 2.5 dpf; alizarin red/alcian blue staining); **E**, Underdeveloped operculum of a 6-dpf old zebrafish exposed for 3 days to 4 mg/L of cobalt chloride (lateral view; alizarin red staining); **F**, deformed, demineralized (*white arrowheads*) and over-resorbing (bright red coloration) scale of a 12-months old zebrafish exposed for 8 days to 12 mg/L of prednisolone (*VK*, von Kossa's staining; *TRAP*, tartrate-resistant acid phosphatase staining); **G**, anti-osteogenic effect of 5 mg/L and 50 µg/L of cobalt chloride evidenced in alizarin red-stained caudal fin of regenerating 3-months old zebrafish (lateral view; *dashed line* indicates the amputation plane); **H**, anti-mineralogenic effect of 5 µM of 3-methylcholanthrene (3-MC) evidenced in alizarin red stained cultures (triplicates) of gilthead seabream VSa13 cells. *Ctrl*, control. Prednisolone-exposed zebrafish scales pictures were kindly provided by Dr. Juriaan R. Metz. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used to assess osteotoxicity, MoA and signaling pathways, will provide essential and sufficient knowledge for the development of systems biology. The computational modeling of the complex network underlying osteotoxicity represents the ultimate tool for *in silico* chemical screening, to gather information on the potential threat of newly produced and/or released compounds, before performing any *in vitro*, *ex vivo* and/or *in vivo* screenings, and thus reducing the use of animals in research. This approach typically aims at identifying patterns of similarity (*e.g.* in structure, biological effect or MoA) between a compound of interest and other previously characterized (Peterson and MacRae, 2012). Such computational modeling has been experimentally applied to bone maintenance, but additional quantitative experimental validation is still required to build up on the insights already achieved (Trüssel et al., 2012).

3. Osteotoxicants and their corresponding mechanisms of action

MoA studies are an integral element of toxicology and their knowledge is used to understand and assess the risk of a molecule – in a particular chemical form and amount – to the environment and human health. This information is also critical to develop new drugs with none or limited undesired effects and/or no impact on off-target species. A representative case study of an osteotoxicant MoA is briefly presented here. Warfarin is an anticoagulant molecule commonly used at low concentrations to alleviate thromboembolic disorders (Chatrou et al., 2012) but also a popular rodenticide through the induction of bleeding when used in high concentrations. As a consequence of its extensive use, the release of warfarin in the environment might induce toxic effects in off-target species such as aquatic organisms (Fernández et al., 2014) and birds (Watanabe et al., 2010). In addition to hemorrhagic disorders, warfarin was able to induce other sub-lethal effects such as growth delay, ectopic calcification of soft tissues and increased skeletal deformities in zebrafish (Weigt et al., 2012; Fernández et al., 2014). Thus, a huge effort from the medical and scientific communities has focussed on the development of novel anticoagulants based on the MoA of old drugs like warfarin (Gómez-Outes et al., 2013).

Compounds that revealed some osteotoxicity in (zebra)fish systems have been listed in Table 1 together with additional information on their usage/origin, predicted half-life in aquatic environments and most frequently interacting genes, in order to provide insights into the mechanisms underlying their osteotoxicity. More than 40 different osteotoxicants (organized according to their chemical nature in 11 categories) have been identified using 6 different fish systems (larvae, juvenile and adult stages, cell culture, scales and caudal fin regeneration systems). Reported osteotoxic effects resulted from disrupted chondro- osteo- and osteoclastogenesis (impaired chondroblast, osteoblast and/or osteoclast differentiation and activity), altered extracellular matrix (ECM) mineralization/composition and/or physical properties, increased rate of skeletal deformities, and loss/gain of particular skeletal structures. The diverse nature of osteotoxicants here enumerated is representative of their uses, effects and their half-life in aquatic environments. The diversity of the MoA is also remarkable, with various pathways involved, even for compounds inducing the same bone phenotype. A first approach to unveil the MoA of

Table 1
List of osteotoxic compounds identified using fish systems and environmental risk assessment-related information (origin, half-life and interacting genes).

Molecules (abbreviation)	Fish systems	Skeletal and bone effects	Usage/origin	Half-life in model aquatic environments[a]	References	Most frequently interacting genes[b]
Polychlorinated dibenzodioxins (PCDDs)						
2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)	Developing medaka	Impaired chondro- and osteogenesis	Herbicide	21 to 161 d by volatilization	Dong et al., 2012	*ahr, cyp1a1, tiparp, cyp1b1, cyp1a2*
	Zebrafish larvae	Impaired development of cranial cartilage and dermal bone		3.5 to 5.9 h by photolysis	Burns et al., 2015	
	Zebrafish larvae	Impaired jaw growth			Teraoka et al., 2006	
Polycyclic aromatic hydrocarbons (PAHs)						
3-methylcholanthrene (3-MC)	Developing zebrafish	Increased rate of skeletal deformities	Toxicological research	280 h to 90 d by volatilization	Laizé (unpublished data)	*ahr, cyp1a1, cyp1a2, cyp1b1, nqo1*
	Regenerating zebrafish	Reduced *de novo* bone formation				
	Gilthead seabream bone cell lines	Reduced ECM mineralization				
Benzo[*a*]pyrene (BaP)	Developing medaka	Increased rate of skeletal deformities, reduced bone tissue and decreased osteoblast abundance	Coal tar, tobacco smoke	> 36 d by biodegradation	Zhao et al., 2013; Seemann et al., 2015	*ahr, cyp1a1, cyp1b1, cyp1a2, tp53*
	Adult zebrafish	Skeletal deformities			Corrales et al., 2014a	
Polybrominated diphenyl ethers (PBDEs)						
2,2′,4,4′-tetrabromodiphenyl ether (BDE47)	Developing medaka	Increased rate of skeletal deformities	Flame retardant	n. a.	Zhao et al., 2013	*ttr, casp3, cyp1a1, ern1, bdnf*
Steroids						
17β-estradiol (E2)	Cultured goldfish scales	Increased osteoclastic activity	Natural hormone	2 h by photolysis	Yoshikubo et al., 2005	*esr1, esr2, pgr, egf, tff1*
	Developing zebrafish	Disrupted cartilage formation			Cohen et al., 2014	
	Developing zebrafish	Impaired chondrogenesis			Fushimi et al., 2009	
17α-ethinylestradiol (EE)	Developing fathead minnow	Increased rate of vertebral deformities	Pharmaceutical drug	2 h by photolysis	Warner and Jenkins, 2007	*esr1, il1b, esr2, c3, igf1*
17α-methyltestosterone (MT)	Zebrafish	Increased incidence of skeletal abnormalities	Pharmaceutical drug	n. a.	Örn et al., 2000	*ar, esr1, spg1, cyp1a1, er*
	Mummichog larvae				Boudreau et al., 2005	
Dexamethasone	Developing zebrafish	Aberrant craniofacial cartilage development	Pharmaceutical drug	n. a.	Hillegass et al., 2008	*ins1, ins, nr3c1, tnf, il1b*
	Regenerating caudal fin of the common carp				Ochandio et al., 2015	
Hydrocortisone	Developing zebrafish	Aberrant craniofacial cartilage development	Pharmaceutical drug	n. a.	Hillegass et al., 2008	*nr3c1, hsd11b1, pomc, il1b, tat*
Prednisolone	Zebrafish scales	Increased bone resorption	Pharmaceutical drug	n. a.	De Vrieze et al., 2014	*il4, nr3c1, abcb1, ar, cxcl8*
Formestane	Developing zebrafish	Disrupted cartilage formation	Pharmaceutical drug	n. a.	Cohen et al., 2014	*cyp19a1, cyp11a1, cyp19a2, fshb, hsd17b1*
Organochlorines						
Dichlorodiphenyltrichloroethane (DDT)	Winter flounder larvae	Vertebral deformities at hatching	Insecticide	150 y	Smith and Cole, 1973	*esr1, ar, ptgs2, cyp19a1, pgr*
	Atlantic croaker larvae				Faulk et al., 1999	
Atrazine	Developing zebrafish	Increased skeletal deformities	Insecticide	> 200 d	Gorge and Nagel, 1990	*cyp19a1, fshb, cyp11a1, star, esr1*
Mirex	Fathead minnow	Vertebral column deformities	Insecticide	3.6 y by volatilization	Mehrle et al., 1981	*cyp2b1, cyp2b2, nr3c1, abcc6, abcg5*
			Fire-retardant	330 d by photolysis		
Kepone	Early stages of several fish species	Increased vertebral deformities	Insecticide	n. a.	Couch et al., 1979; Mehrle et al., 1981	*esr1, cyp19a1, hspa5, pgr, ltf*
Toxaphene	Several fish species	Reduced vertebral collagen	Insecticide	5 to 165 d by volatilization	Mayer et al., 1978.	*esr1, esrra, ar, pgr, cat*

(*continued on next page*)

Table 1 (*continued*)

Molecules (abbreviation)	Fish systems	Skeletal and bone effects	Usage/origin	Half-life in model aquatic environments[a]	References	Most frequently interacting genes[b]
				> 30 d by biodegradation		
Organophosphates						
Chlorpyrifos	Mummichog adults	Decreased yield strength of vertebrae	Insecticide	2 y by volatilization	Karen et al., 2001	*pon1, ache, bche, cyp3a4, cyp2b6*
	Grass carp adults	Increased deformities in scales		30–40 d by photo-transformation 16 to 72 d by hydrolysis 30 to 52 d by biodegradation	Jindal and Kaur, 2015	
Dylox	Goldfish	Decreased collagen formation	Insecticide	< 30 min to 510 d by hydrolysis 2.5 d by biodegradation	Kozlovskaya and Mayer, 1984	*ache, bche, app, casp12, casp3*
Organophosphates pesticides	African catfish juveniles	Loss of ceratobrachial bones	Pesticide	Few h to several d by hydrolysis 2 to 50 d by photolysis	Doherty et al., 2016	*ache, pon1, bche, cat, casp3*
Polychlorinated biphenyls (PCBs)						
Arochlor 1254	Brook trout fry	Decrease phosphorus and collagen, increase calcium	Electrical equipment	11 h to 9 d by volatilization	Mauck et al., 1978	*cyp1a1, cyp1a2, cat, cyp11a1, gsr*
PCB 118	Immature goldfish	Increased osteoclastic activity	Electrical equipment	> 60 d	Yachiguchi et al., 2014	*cyp1a1, cyp1a2, ahr, icam1, ocln*
Bisphenols						
Bisphenol A (BPA)	Cultured goldfish scales	Suppressed osteoclastic and osteoblastic activities	Plastics, epoxy resins	< 5 d by biodegradation	Suzuki and Hattori, 2003	*esr1, esr2, mapk1, mapk3, ar*
Benzopyrones						
Coumarin	Developing zebrafish	Increased rate of skeletal deformities	Perfumes	1.8 to 7.4 d	Weigt et al., 2012	*cyp2c9, vkorc1, esr1, cyp3a4, esr2*
Metals, transition metals, heavy metals and metalloids						
Arsenic (As)	Rohu fingerlings	Reduced biochemical and mineral contents	Biocides, alloys, semiconductors	n. a.	Palaniappan and Vijayasundaram, 2009	*as3mt, apoe, cat, mapk1, mapk3*
Boric acid	Developing zebrafish	Increased skeletal deformities	Antiseptic, insecticide, flame retardant	n. a.	Selderslaghs et al., 2012	*tnf, atf4, myod1, col1a2, runx2*
Cadmium (Cd)	Juvenile mosquito fish	Skeletal deformities	Component of electronic devices	n. a.	Sassi et al., 2010	*cat, casp3, mapk1, mapk3, hmox1*
	Developing zebrafish	head malformation, spinal curvatures			Cheng et al., 2000	
Chromium (Cr)	Common carp scales	Increased deformities in scales	Pigment for metal alloys, paints, cement, paper, rubber and other materials	n. a.	Çoban et al., 2013	*cat, cyp1a1, tp53, nqo1, gsr*
Copper (Cu)	Guppy embryos	Increased abnormalities	Electrical equipment, algaecide, therapeutic	n. a.	Lasienė et al., 2016	*atp71, app, atp7b, slc31a1, sod1*
	Zebrafish embryos	Twisted notochord			Almond and Trombetta, 2016	
Lead (Pb)	Rainbow trout	Induction of spinal deformities	Miscellaneous	1 to 2 d by volatilization	Hodson et al., 1978	*cyp1a1, mt1, mt2, alad, ptgs2*
	Indian major carp	Loss of bone minerals			Palaniappan et al., 2010	
Lithium chloride	Developing zebrafish	Increased skeletal deformities	Electrolytic reactions	n. a.	Selderslaghs et al., 2012	*gsk3b, ctnnb1, ccnd1, ache, axin*
Mercury (Hg)	Developing medaka	Increased developmental abnormalities	Miscellaneous	n. a.	Dong et al., 2016	*cyp1a1, nqo1, hmox1, abcc2, abcc2*
Selenium (Se)	Fathead minnow larvae	Increased rate of skeletal deformities, craniofacial and fin deformities	Electronics and trace element	n. a.	Schultz and Hermanutz 1990	*gpx1, cat, txnrd1, sepp1, hmox1*
	Developing zebrafish	Increased rate of total deformities			Thomas and Janz, 2016	

(*continued on next page*)

Table 1 (*continued*)

Molecules (abbreviation)	Fish systems	Skeletal and bone effects	Usage/origin	Half-life in model aquatic environments[a]	References	Most frequently interacting genes[b]
Strontium (Sr)	Developing zebrafish	Inhibition of bone mineralization	Fireworks, red colored smokes, rockets and special video screens	n. a.	Pasqualetti et al., 2013	*ryr1, casr, alpp, chrna7, gnaq*
Vanadium (V)	Gilthead seabream bone cell lines	Reduced ECM mineralization	Alloys and trace element	n. a.	Tiago et al., 2008, 2011	*cyp1a1, nqo1, cyp1a2, cyp1b1, mapk1*
Zinc (Zn)	Developing zebrafish	Increased tail malformations	Miscellaneous	n. a.	Choi et al., 2016	*mt1, parp1, mt2a, mapk3, hexb*
Miscellaneous/bioactive small molecules						
Acethydrazide	Zebrafish embryo	Cartilage and bone malformations in the head	Pharmaceutical drug	n. a.	Strecker et al., 2013	*col11a1, hoxb6, mapk10, mgp, pik3cg*
Disulfiram	Zebrafish embryo	Cartilage and bone malformations in the head	Pharmaceutical drug	14 h to 12 d by volatilization	van boxtel et al., 2010 ; Strecker et al., 2013	*cyp2e1, aldh2, mgmt, dbh, paprp1*
Dorsomorphin	Developing zebrafish	Reduced bone mineralization	BMP signaling inhibitor	n. a.	Yu et al., 2008	*tp53, ppargc1a, sirt1, adipoq, foxo1*
Ethyl tert butyl ether (ETBE)	Developing zebrafish	Craniofacial defects	Gasoline additive	3 h to 4 d by volatilization	Bonventre et al., 2012	*aldh2, cyp1a1, cyp2c6v1, cyp2e1, cyp3a23*
11.1 3-(2,3-dimethoxyphenyl)-1-(9-methyl-2-phenyl-9H-imidazo[1,2-*a*]benzimidazol-3-yl)-2-propen-1-one (IBIP)	Developing zebrafish	Increased bone mineralization	Pharmaceutical drug	n. a.	Bae et al., 2017	n. a.
Ketamine	Developing zebrafish	Increased rate of skeletal deformities	Pharmaceutical drug	n. a.	Felix et al., 2016	*il6, il1b, tnf, casp3, cyp2b6*
Sodium pentachlorophenate	Zebrafish embryo	Asymmetric cranial development	Biocide and disinfectant	h to d by photolysis > 7 d by biodegradation	López-Romero et al., 2012	*cyp1a1, esr1, fos, oval, thrb*
Tertiary amyl methyl ether (TAME)	Developing zebrafish	Craniofacial defects	Gasoline additive	4 h to 4 d by volatilization	Bonventre et al., 2012	*cyp2a6, mmp2, mmp9, wnt3a, wnt8a*
Warfarin	Developing zebrafish	Increased rate of skeletal deformities	Rodenticide and pharmaceutical drug	16 y by hydrolysis	Weigt et al., 2012; Fernández et al., 2015	*cyp2c9, vkorc1, cyp4f2, cyp3a4, alb*
Zearalenone (F-2 mycotoxin)	Developing zebrafish	Upward curvature of the body axis	Natural toxin	n. a.	Bakos et al., 2013	*esr1, cga, abcc1, tnf, il1b*

[a] Information taken from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/).

[b] On the basis of the number of interactions reported in the Comparative Toxicogenomics Database (www.ctdbase.org), and not considering whether the gene exists in fish species (*e.g. cyp1a1* and *cyp1a2*); *n.a.*, not available.

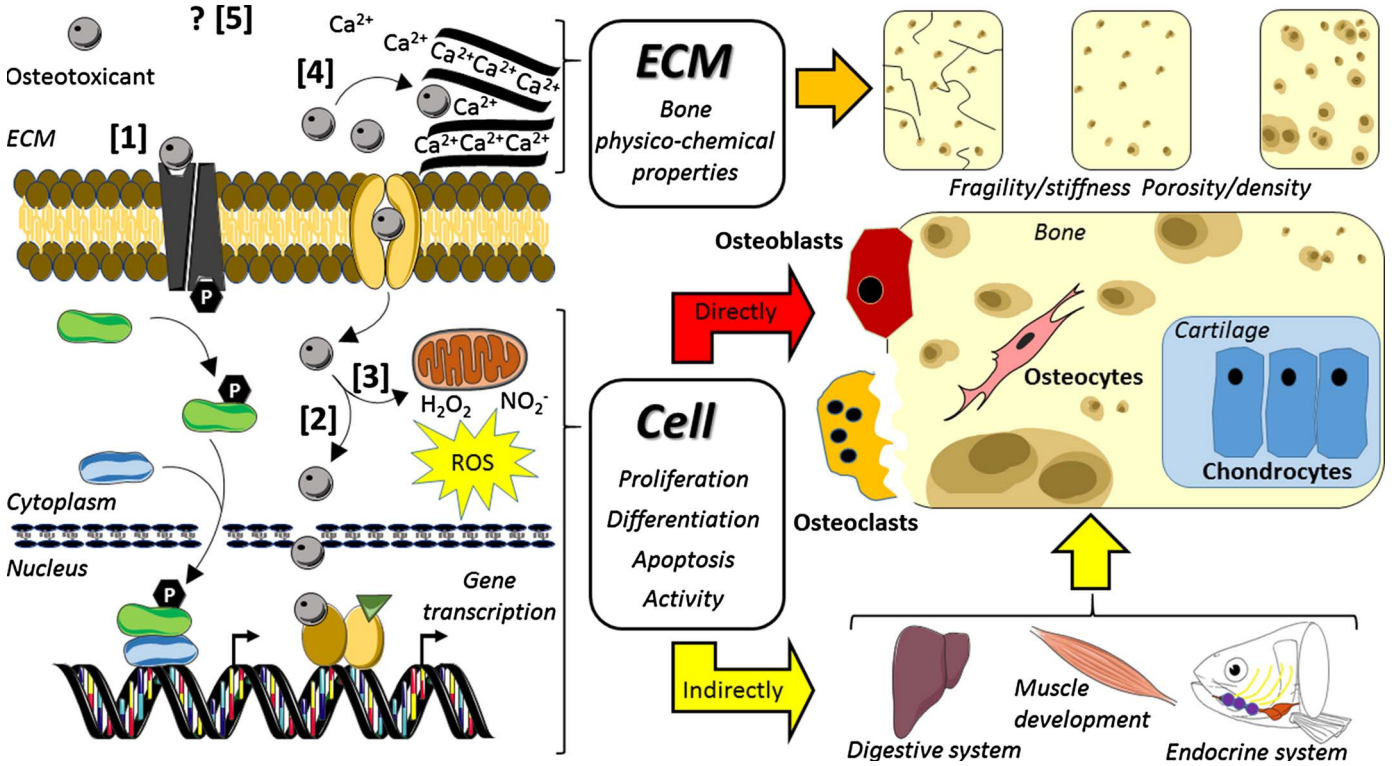

Fig. 3. Pathways underlying compound osteotoxicity. Compounds may [1] interact with membrane receptors and affect the phosphorylation of proteins regulating gene transcription when translocated to the nucleus, [2] bind and activate/inactivate nuclear receptors, [3] interfere with the redox status of the cell, [4] mimic components of the extracellular matrix (ECM) and/or [5] affect bone/skeletal formation or remodeling through pathways still unknown. Consequence of the alteration of these pathways by osteotoxicants are (a) the impairment of physico-chemical properties of the bone (*e.g.* fragility, stiffness, porosity, density) and (b) the alteration of cell proliferation, differentiation, apoptosis and/or activity of skeletal cells (chondrocytes, osteoblasts, osteocytes and/or osteoclast) in a direct manner or indirectly through a toxic effect on other tissues or organs.

osteotoxicants would be to study the compound-specific most-frequently-interacting genes recorded in a robust, publicly available database such as the Comparative Toxicogenomics Database (CTD; ctdbase.org; Davis et al., 2017). Although this approach might provide some useful insights on the specific pathways underpinning the osteotoxic effects, it may also suffer from uncomplete or fragmented information on specific molecular networks, or biased due to the nature of the few genes available and mostly described in other species (*e.g.* mammals). In the case of warfarin, the 5 most-frequently-interacting genes indicated in the CTD, *cytochromes 2c9*, *4f2* and *3a4*, *vitamin epoxide reductase complex subunit 1* (*vkorc1*) and *albumin* (*alb*), seem to be good candidates to integrate the MoA of warfarin (osteo)toxicity. These genes were identified within the scope of wide pharmacogenomics studies conducted to evaluate the convenient dose of warfarin for thromboprophylaxis in human patients (Lee et al., 2014). While *cyp3a4* codes for an enzyme involved in warfarin metabolism/detoxification, mutations in *cyp2c9* and *cyp4f2* have been associated with warfarin resistance (Gage and Lesko, 2008; Bress et al., 2012). Warfarin osteotoxicity has recently been linked to *pxr*, *vkorc1*, *vkorc1l1* and *ggcx* expression (ndez et al., 2014, 2015;) in zebrafish larvae and cultured cells, acting through warfarin-mediated blockage of vitamin K recycling by Vkorc1 and Vkorc1l1 and thus the inhibition of the transcriptional activity of Pxr by vitamin K (Tabb et al., 2003) and/or the γ-glutamyl carboxylation of vitamin K dependent proteins by the γ-glutamyl carboxylase (Ggcx), that requires vitamin K as a co-factor (Oldenburg et al., 2008). Similarly, *cyp1a* or *cyp1b1* have also been involved in the MoA of benzo[*a*]pyrene (BaP), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 17α-methyltestosterone (MT), among others (McManus et al., 1990; Westerink et al., 2008; Larsson et al., 2015). Cytochromes P450 are enzymes involved in the metabolism of different molecules. For example, Cyp1a is involved in phase I bioactivation of xenobiotics, catalyzing the first step in the metabolism of a number of polycyclic aromatic hydrocarbons (PAHs), such BaP (McManus et al., 1990). Their role in the osteotoxicity of these pollutants may be related to their metabolizing activity rather than driving the osteotoxic effects, which should be more related to the activation of the aryl hydrocarbon receptor (Ahr) and the transactivation and/or inactivation of downstream target genes such as *cyp1a*. Other studies have reported the activation of NRs (*e.g.* Pxr, Er and Rxr) through different osteotoxicants, and the transcriptional regulation of *cyps* by those NRs has also been extensively reported, for instance in the case of Pxr (*e.g.* Inui et al., 2014; Luckert et al., 2013; Sun et al., 2016).

A detailed analysis of the literature allowed us to classify osteotoxicants according to their MoA through i) the activation of membrane or nuclear receptor signaling cascade, ii) the alteration of redox conditions/processes, iii) the mimic (and thus the substitution) of natural components of skeletal tissues, and/or iv) the alteration of pathways that remain to be uncovered (Fig. 3). In some cases, osteotoxicants (*e.g.* toxaphene, chlorpyrifos, PCB 118 and selenium) may have multiple MoA. Toxaphene exposure increased the expression of genes targeted by two NRs, the constitutive androstane receptor (Car; *cyp2b10* and *cyp3a11*) and the Ahr (*cyp1a1* and *cyp1a2*) in the liver of mice (Wang et al., 2015). However, whether toxaphene osteotoxicity in fish is generated through its action on NRs or by altered Nadh-oxidase and succinoxidase activities remains to be determined (Mayer et al., 1978). Similarly, chlorpyrifos toxic effects seem to be mainly related to the inhibition of acetylcholinesterase (Karen et al., 2001). However, and particularly on the skeletal development, it may also be related to oxidative stress and mitochondrial dysfunction (Garcia-Reyero et al., 2016), activated Pxr (Kojima et al., 2011) and Ahr (Takeuchi et al., 2008) signaling pathways. The mechanisms underlying the osteotoxicity of PCB 118 are even more complex, affecting NF-κB (Yachiguchi et al., 2014), Ahr (Larsson et al., 2015), androgen receptor (Ar; Vinggaard et al., 2008), hypoxia-inducible factor 1α (Hif-1α; Clausen

Osteotoxicants	Nuclear receptors: Ahr	Ar	Car	Er	Err	Gr	Tr	Ppar	Pr	Pxr
3-methylcholanthrene	X									
17α-methyltestosterone		X		X						
Arochlor 1254	X						X			
Atrazine		?								
BDE47										X
Benzo[*a*]pyrene	X									
Bisphenol A		X		X	X		X	X	X	X
Chlorpyrifos	X									X
DDT				X						
Dexamethasone						X				
Dichlorvos		X								
Formestane				?						
Hydrocortisone						X				
PCB 118		X				X				X
Prednisolone						X				
Selenium						X				
TCDD	X									
Toxaphene	X		X							
Warfarin										X
Zearalenone				X						

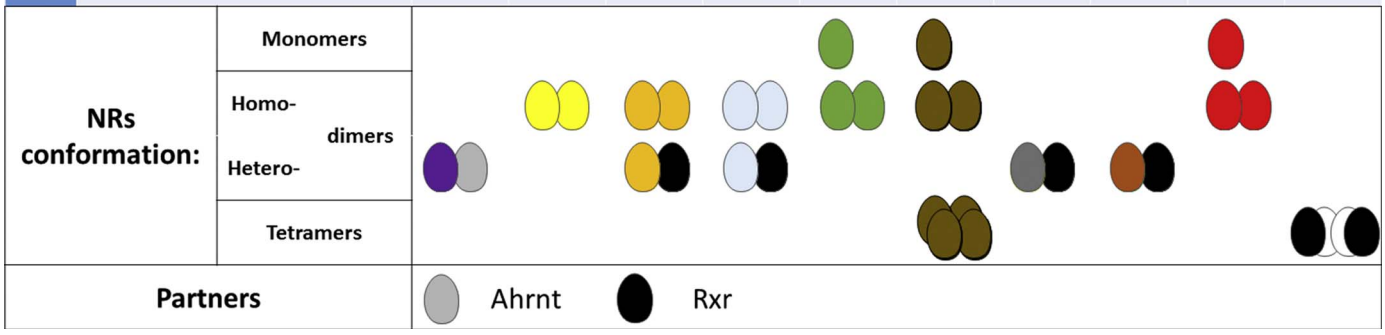

Fig. 4. Nuclear receptors bound by osteotoxicants (top panel) and conformational NR–NR interaction (bottom panel). Ahr, aryl hydrocarbon receptor; Ahrnt, Ahr nuclear translocator; Ar, androgen receptor; Car, constitutive androstane receptor; Er, estrogen recptor; Err, estrogen-related receptor; Gr, glucocorticoid receptor; Tr, thyroid hormone receptor; Ppar, peroxisome proliferator-activated receptor; Pr, progesterone receptor; Pxr, pregnane X receptor; Rxr, retinoid X receptor. ? denotes suggested, while X indicates experimentally demonstrated interaction.

et al., 2005), Pxr (Kretschmer and Baldwin, 2005) and/or glucocorticoid receptor (Gr; Antunes-Fernandes et al., 2011). In contrast, selenium exposure was reported not only to up-regulate *ahr2* expression in zebrafish (Thomas and Janz, 2016), and mRNA levels and activity of Grs in goldfish (Choi et al., 2015), but also to induce oxidative stress through oxidant-responsive transcription factors (nuclear factor erythroid 2-related factors a and b), and enzymes involved in cellular methylation (methionine adenosyltransferase a and ab). Arochlor 1254 was found to inhibit *in vitro* osteoblast proliferation and differentiation through Ahr (Herlin et al., 2015), while *in vivo* data suggested an alteration of Nrf2 and NF-κB pathways, oxidative stress markers (Wu et al., 2014) or the thyroid hormone metabolism and receptors (Giera et al., 2011).

3.1. Osteotoxicants impacting membrane or nuclear receptor signaling

Different membrane and nuclear receptors control proliferation, differentiation and/or activity of the different bone cell types (reviewed in Gartland et al., 2012; Imai et al., 2013; Rahman et al., 2015) and thus, any compound interfering with receptor signaling has the potential to alter skeletal/bone formation and maintenance. The particular NRs involved in osteotoxicant effects on bone formation and maintenance are compiled in Fig. 4. Different osteotoxicants can act through one or several different NRs (*e.g.* bisphenol A, PCB 118, and chlorpyrifos among others). Similarly, the signalling pathway drived by one NR can be affected by different osteotoxicants. In this sense, the most extensively affected are Pxr, Gr, Ahr and Ar. Furthermore, each NR can act as monomers, homo- or hetero-dimers, and/or as tetramers with themselves or another NRs as partners, such as the aryl hydrocarbon receptor nuclear translocator (Ahrnt) or the retinoid X receptor (Rxr).

3.1.1. Membrane receptors

Proliferation/differentiation and/or activity of bone cell types are tightly controlled by several types of membrane receptors (reviewed in

Gartland et al., 2012; Rahman et al., 2015). In particular, bone morphogenetic protein (BMP) type I and type II receptors – to which BMPs bind (Nohe et al., 2002; Little and Mullins 2009) – are essential to the control of skeletal development, among other biological processes (Rahman et al., 2015). If released in the environment, compounds with the ability to block BMPR-mediated signaling, such as dorsomorphin, a selective inhibitor of BMP type I receptor that blocks BMP-mediated SMAD1/5/8 phosphorylation (Yu et al., 2008), could drastically affect osteoblast differentiation and therefore bone formation. Oppositely, the release in the environment of compounds such as IBIP (3-(2,3-dimethoxyphenyl)-1-[9-methyl-2-phenyl-9H-imidazo[1,2-α]benzimidazol-3-yl]-2-propen-1-one), a potent activator of osteoblast differentiation through the increase of SMAD phosphorylation that is reverted by a BMP inhibitor (Bae et al., 2017), may also induce osteotoxic effects through BMP signaling.

3.1.2. Aryl hydrocarbon receptor

Ahrs are involved in the transcriptional responses to aromatic hydrocarbons, driving the expression of xenobiotic-metabolizing enzymes. They are expressed in chondrocytes, osteoblasts and osteoclasts (Sharan et al., 2011; Cedervall et al., 2015; Yu et al., 2015). TCDD is an exogenous Ahr ligand proposed to induce jaw malformations through a down-regulation of *shha* and *shhb* in an Ahr2 dependent manner (Teraoka et al., 2006) or an auto-induction mechanism of *ahr2* gene through TCDD-activated Ahr1 and Ahr2 (Bak et al., 2017). Nevertheless, a recent *in vitro* data suggested that impaired chondrogenesis and osteogenesis *in vivo* through TCDD exposure (Dong et al., 2012), may not involve Ahr in a local context (Cedervall et al., 2015). Other underlying mechanisms have been proposed such as the up-regulation of *cyp1a* expression by Ahr2/Ahr repressor heterodimer (Prasch et al., 2003), the modulation of retinoid signaling (Toyoshiba et al., 2004) or the alteration of Sox9 signaling pathway, a known target of TCDD through Ahr2 signaling pathway (Xiong et al., 2008; Goodale et al., 2012).

Benzo[*a*]pyrene (BaP) is another well-known ubiquitous polycyclic aromatic hydrocarbon (Zhang et al., 2006) that affects chondrocytes, osteoblasts and osteoclasts through Ahr (Voronov et al., 2008; Jayasundara et al., 2015) resulting in the alteration of steroid hormone metabolism (Jayasundara et al., 2015) and a decrease of circulating levels of 17β-estradiol (E2; Zhao et al., 2013), a steroid hormone involved in bone development and maintenance through estrogen receptors. A transgenerational osteotoxic effect was also observed in zebrafish (Corrales et al., 2014a) and medaka (Seemann et al., 2015) upon exposure to BaP. The use of the *Tg*(*sp7:mCherry*) transgenic line (Renn and Winkler, 2009) confirmed a decrease in the osteoblast population of BaP-exposed fish. The alteration of *sox9a/b* expression may represent the mechanism underlying BaP osteotoxicity, but both BaP accumulation in the egg yolk-sac (Hannah et al., 1982) and changes in promoter DNA methylation (Corrales et al., 2014b) might be also the responsible for transgenerational toxicity.

Finally, mineralogenic, osteogenic and skeletogenic effects were also observed in several fish systems (*in vitro* cell systems, developing zebrafish larvae and regenerating caudal fin) upon exposure to 3-methylcholanthrene (3-MC), a polycyclic aromatic hydrocarbon, which osteotoxicity was associated with Ahr activation (Laizé et al. unpublished data).

3.1.3. Estrogen receptors

Estrogens play a key role in bone formation (Gao et al., 2013) and chondrogenesis (Fujita et al., 2004; Fushimi et al., 2009), among other processes, being mainly mediated by estrogen receptors (Erα and Erβ; reviewed in Manolagas et al., 2013). By affecting estrogen metabolism and/or Er signaling, exogenous estrogens and xenoestrogens (compounds that mimic estrogens) found in the aquatic environment (*e.g.* 17β-estradiol (E2), estrone (E1) and the synthetic steroid 17α-ethynylestradiol (EE2); Rajapakse et al., 2002) can impair skeletal development and bone formation in fish such as inhibiting chondrogenesis, potentially through Er-independent mechanisms (Fushimi et al., 2009). Some of the skeletogenic effects of estrogens may also result from an increase in alkaline phosphatase (Alp) activity (Yoshikubo et al., 2005).

Exposure to the synthetic estrogen EE2 (a primary component of oral contraceptives), commonly found in the effluents of wastewater treatment plants (Warner and Jenkins 2007), down-regulated the expression of BMP-mediated genes (Yamamoto et al., 2002), activated G-protein-coupled Protein Kinase C (PKC) in chondrocytes (Sylvia et al., 2000) and altered the expression of *mmp13* (Tankó et al., 2008), the last leading to matrix degradation. These specific signaling pathways may be involved in the osteotoxicity of estrogenic compounds (Abzhanov and Tabin, 2004, Jayasinghe and Volz, 2012; Cohen et al., 2014).

Although with low affinity, persistent organochlorines such as chlordecone (CD) and dichlorodiphenyl-trichloroethane (DDT) have the ability to bind Ers (Donohoe and Curtis, 1996), being the Er-dependent osteogenic potential of DDT recently demonstrated (Strong et al., 2015). The mycotoxin zearalenone has also the capacity to bind Ers (Bakos et al., 2013), as well as G protein–coupled receptor 30 (GPR30; reviewed in Prossnitz and Barton, 2014). Exposure to zearalenone alters expression of estrogen-responsive genes (Bakos et al., 2013), as well as genes involved in DNA repair, cell cycle control, glycolysis, blood coagulation, iron-storage processes and cytoskeleton development/maintenance (Woźny et al., 2012).

Bisphenol A (BPA), is a well-known endocrine disruptor that affects skeletal development and bone maintenance through altered Er signaling (Yamaguchi et al., 2015; Tohyama et al., 2015). In addition, it is also a modulator of the activity of signaling pathways related to progesterone receptor, peroxisome proliferator-activated receptor, estrogen-related receptors, Ar, thyroid hormone receptors and Pxr (Funabashi et al., 2004; Naciff et al., 2005; Oshida et al., 2015). Despite the removal of BPA from consumer products, its substitutes such as bisphenol S (BPS) and bisphenol F (BPF) are also suspected to disrupt the endocrine system (reviewed in Rochester and Bolden, 2015).

Among the endocrine disruptors showing osteotoxicity through Ers and/or estrogen metabolism, formestane – an aromatase inhibitor that blocks the conversion of androgenic hormones into estrogens – impaired zebrafish craniofacial development in a way that resembles the phenotype of fish exposed to an excess of estrogens, indicating that estrogen homeostasis is a key point for skeletal development (Cohen et al., 2014) and a target for osteotoxic compounds.

3.1.4. Androgen receptor

Androgen receptor (Ar) signaling is essential to bone maintenance, in particular to trabecular bone mass homeostasis (reviewed in Manolagas et al., 2013). 17α-methyltestosterone (MT) – a testosterone derivative used to treat male androgen deficiency – triggered abnormal skeletogenesis in early-life stages of mummichog (Boudreau et al., 2005). Mechanisms of action are unclear and can be multiple since MT can be aromatized to 17α-methylestradiol (ME2), a potent Er activator (Hornung et al., 2004), and can therefore interfere with Ar and/or Er signaling (Boudreau et al., 2005).

Atrazine, a widely used herbicide in the US but banned in Europe, has been reported to increase axial deformities in zebrafish (Görge and Nagel, 1990). Whether atrazine interferes with Ar signaling is still unknown, but altered androgen metabolism was observed in fish exposed to atrazine (Moore and Waring, 1998; Spano et al., 2004). On the contrary, Dichlorvos – an insecticide that shortens ceratobranchial bones in African catfish (*Clarias gariepinus*; Doherty et al., 2016) – was found to affect Ar activity in a cell-based screening assay (Tavassoli et al., 2007), while also inhibiting acetylcholinesterase and inducing oxidative stress *in vivo* (Liu et al., 2015; Doherty et al., 2016).

3.1.5. Glucocorticoid receptors

Glucocorticoid receptors (Grs) mediate the transcriptional

Table 2
List of redox pathways affected by osteotoxicants.

Osteotoxicant molecules	Redox pathways										
	Vitamin C	Lysyl oxidase	Tyrosinase	Retinal to RA conversion	Lipid peroxidation	Hydrogen peroxide	Hydroxyl radical	Superoxide anion radical	GST	HSP	Others
Mirex	X										
Kepone	X										
Copper	X	X	X								
Copper pyrithiones					X						
Disulfiram		X		?							
Acetic acid hydrazide		X									
Chromium					X	X	X				
Mercury									X	X	
Vanadium						X	X	X			
Zinc											X
Boric acid											X

GST, glutathione S-transferase; HSP, heat shock proteins; RA, retinoic acid. ? denotes suggested, while X indicates experimentally demonstrated.

regulation of genes engaged in the proliferation, differentiation, and apoptosis of bone cells (osteoblasts, osteocytes, osteoclasts; Moutsatsou et al., 2012). They are off-target receptors of pharmaceutical drugs developed to treat non-bone related diseases and found to induce osteotoxic effects. In one hand, dexamethasone and hydrocortisone impaired the anterior growth and migration of the pharyngeal cartilages in zebrafish, possibly through the transcriptional activation and increased activity of matrix metalloproteinases (MMPs), and subsequently through increased degradation of collagen (Hillegass et al., 2008). The co-treatment of zebrafish with these drugs and the Gr inhibitor RU486 decreased the severity of craniofacial deformities and collagen degradation, confirming a Gr-dependent osteotoxic effect of these drugs. On the other hand, prednisolone was able to induce an osteoporotic phenotype in zebrafish regenerating scales, showing altered mineral content, enhanced matrix breakdown, and altered expression profile towards the enhancement of osteoclast activity and matrix resorption (De Vrieze et al., 2014).

3.1.6. Pregnane X receptor

Pregnane X receptor (Pxr) regulates the transcription of genes involved in xenobiotic detoxification, cholesterol and bile acid metabolism, and bone development (Tabb et al., 2003; Makishima 2005; Chen et al., 2012; Azuma et al., 2010, 2015). In bone, Pxr controls the expression of genes encoding ECM proteins and promotes collagen accumulation (Ichikawa et al., 2006). Since Pxr has the ability to bind to a great diversity of ligands, including steroids and xenobiotics (Ekins et al., 2008), the presence of those ligands might modulate the transcription of Pxr-targeted genes in a wider set of tissues and cell types than previously thought, as recently reported by *in situ* hybridization procedures in a fish species model in ecotoxicology, the *Solea senegalensis* (Marques et al., 2017). In medaka, 2,2′,4,4′-tetrabromodiphenyl ether (BDE47) can activate the Pxr signaling pathway and subsequently up-regulate the expression of *cytochrome P450 2B* (*cyp2b*) and *cyp3a*, enhancing the elimination of endogenous hormones such as triiodothyronine (T3) and thyroxine (T4), which are critical to skeletal development (Gogakos et al., 2010), and/or regulating the metabolism of a great number of endogenous and exogenous ligands for other nuclear receptors (Zhao et al., 2013). Since BDE47, as well as other polybrominateddiphenyl ethers, have been classified as persistent organic pollutants, new brominated flame retardants (BFRs) have been developed. Although neither osteotoxicity nor Pxr activation have been reported, recent studies have demonstrated that new BFRs triggered adverse effects related to immunotoxicity and endocrine disruption in rainbow trout (*Oncorhynchus mykiss*; Giraudo et al., 2017), suggesting that specific research effort on these issues are required.

Warfarin is another compound that affects Pxr signaling by binding directly to Pxr (Rulcova et al., 2010), or blocking the recycling of vitamin K (VK; Oldenburg et al., 2008), which is a natural ligand for Pxr (Tabb et al., 2003). An increased rate of skeletal deformities has been reported in zebrafish larvae exposed to sodium warfarin, while the expression of *pxr* and several genes targeted by Pxr was altered (Fernández et al., 2014, 2015). However, skeletal phenotypes promoted by warfarin could also be the consequence of the reduced γ-carboxylation of several ECM proteins important for bone development, in response to the limited recycling of VK, a critical co-factor of the γ-glutamyl carboxylase (Fernández et al., 2014).

3.2. Osteotoxicants altering a redox condition/process

Oxidation and reduction (redox) conditions are central to skeletal development and bone maintenance. In fact, mechanosensing, a key process that tightly controls bone development/maintenance, depends on the redox balance (reviewed in Knapik et al., 2014). In Table 2, the different pathways involved in the redox condition that can be altered by osteotoxicants are summarized. Mirex and Kepone are osteotoxic compounds that alter collagen production probably by interfering with vitamin C metabolism (Mehrle et al., 1981), an antioxidant molecule involved in fish skeletal development (Darias et al., 2011). Similarly, copper, a cofactor of cellular enzymes such as tyrosinase, lysyl oxidase, and ascorbate oxidase (Szauter et al., 2005; Setty et al., 2008), is also involved in the maintenance of a proper redox condition and any alteration of its homeostasis will probably induce abnormal bone development/function (de Bie et al., 2007). Curiously, the osteotoxic effects of disulfiram and acetic acid hydrazide seem to be also related to copper homeostasis. Although the abnormal axial skeletogenesis observed upon exposure of zebrafish to disulfiram has been initially associated with an inhibition of retinal conversion into retinoic acid (Marsh-Armstrong et al., 1995), a recent study proposed that both disulfiram and acetic acid hydrazide induce skeletal abnormalities through a modification of copper homeostasis and the subsequent inhibition of lysyl oxidase activity, needed for cross-linking collagen and elastin (Strecker et al., 2013). More recently, zebrafish embryos exposed to copper pyrithiones exhibited severely twisted notochord and increased lipid peroxidation end products, further suggesting that oxidative stress may underlie copper osteotoxicity (Almond and Trombetta, 2016). Chromium was found to alter scale morphology in the scaly carp (*Cyprinus carpio*; Çoban et al., 2013) while affecting the expression of genes related to oxidative stress in rat liver (Madejczyk et al., 2015). Although mechanisms underlying chromium effects in fish remains to be elucidated, the alteration of the redox conditions could

play a role on its osteotoxicity.

Exposure to mercury triggers an oxidative stress in the marine medaka (*Oryzias melastigma*; Wang et al., 2013), increasing *hsp70* expression (a general biomarker of oxidative stress condition) in a dose-dependent manner (Li et al., 2014). Nevertheless, an effect of mercury on endocrine system and transcription of steroidogenesis-related genes such as *cyp19a*, *hsd3b*, *cyp17* and *hsd17b* has also been reported in zebrafish (Wang et al., 2016).

Vanadium is a transition metal known to increase oxidative stress and trigger genotoxic effects. An increased production of reactive oxygen species (ROS) was observed in mammalian cells exposed to vanadate (Ding et al., 1999), while an anti-mineralogenic effect was demonstrated in fish upon vanadium exposure (Tiago et al., 2008). Skeletal cell lines exposed to metavanadate showed increased cell proliferation through the MAPK pathway, while an inhibition on differentiation/mineralization through a putative PI–3K/Ras/Erk signaling was found (Tiago et al., 2008). The transcriptome of vanadate-exposed cells revealed the involvement of signaling pathways related to insulin activity, in agreement with the insulin-mimetic properties of vanadium compounds, and confirmed the central role of MAPK and Bmp pathways in ECM mineralization (Tiago et al., 2011). Zinc is another transition metal with reported osteotoxicity and ability to alter redox conditions. A transcriptomic approach revealed within the cytokine–cytokine receptor interaction pathway an overexpression of *ogfrl2*, *edar* and *tnfsf13b,* being proposed to be at the origin of the morphological defects observed in zebrafish exposed to zinc (Choi et al., 2016).

Finally, boric acid, which is used in antiseptics, pesticides and flame retardants, is involved in bone metabolism associated with magnesium, calcium, and vitamin D and was shown to induce abnormal skeletogenesis in zebrafish (Selderslaghs et al., 2012). Besides antioxidant properties, boric acid inhibits peptidases, proteases, proteasomes, arginase and transpeptidases, modulating thyroid hormone metabolism and *hox* gene expression (Wéry et al., 2003; Tepedelen et al., 2016).

3.3. Osteotoxicants mimicking natural compounds

Compounds with the capacity to substitute calcium (Ca) or phosphorus (P) in the bone lattice can alter the biochemical composition, and thus the physical properties of the skeleton (stiffness and strength, among others). Cadmium (Cd), a divalent cation mostly used in batteries, was found to be responsible for the Itai–Itai disease, a severe osteoporosis-like defect observed in humans exposed through contaminated water and food (Ha et al., 2016). By depleting calcium and phosphorus levels in bony tissues, Cd induces demineralization (decrease in the ash weight/dry weight ratio and the percentage of non-organic components content), increases collagen solubility, and interferes with the crystallization of the main bone mineral (hydroxyapatite) and osteoblast activity (Sassi et al., 2010). Although studies in fish suggested the absence of oxidative stress upon exposure to Cd (Sassi et al., 2013), reactive oxygen species (ROS) were produced in mammalian osteoblastic cells when exposed to Cd, inducing apoptosis (activating caspase-3) and decreasing *alp* expression (Ha et al., 2016). The mechanisms of action involved are not fully understood yet and may involve damage to kidney leading to a secondary effect on bone, a stimulation of bone resorption by osteoclasts and/or an inhibition of bone formation by osteoblasts (Ha et al., 2016).

Exposure to lead (Pb) also decreased bone crystallinity, and Ca, Mg, and P contents. This seems to be due to the high efficiency of bones to store lead by the ability of Ca hydroxyapatite to exchange cations (Suzuki et al., 1981) and by the low solubility of lead-to-lead phosphates in Pb-hydroxyapatite (Palaniappan et al., 2010). In fact, the quantification of Pb content in opercular bone has been proposed as a suitable monitoring procedure for Pb environmental exposure (Hodson et al., 1978).

Bone accumulation and osteotoxicity has also been observed for lithium (Poust, 1973). Although initially lithium was suggested to cause substantial apoptosis in the neuroepithelium of the cranial neural folds (Giles and Bannigan, 2006), more recent results suggested phosphatidylinositol and the Wnt/GSK-3 pathways as two possible targets for lithium (Oruch et al., 2014).

Finally, strontium has been indicated as a modulator of bone turnover *in vivo* (Pasqualetti et al., 2013; Vezzoli et al., 1998). Strontium osteotoxicity might be due to an action on osteoblast activity through the modulation of Ca absorption at the intestinal level, or a competition with Ca for a common receptor, *e.g.* the extracellular calcium-sensing receptor (Pasqualetti et al., 2013). Nevertheless, the MoA of strontium still needs to be better characterized.

Besides changing bone mechanical properties, these chemical elements could trigger an abnormal skeletogenic phenotype by accumulating in muscle, altering enzymatic activities and electrolyte levels and inducing a paralysis, that ultimately would lead to skeletal deformities through the mechanostat theory (Jezierska et al., 2009).

3.4. Osteotoxicants with an unknown MoA

Although some widespread compounds have known osteotoxicity, their MoA remains poorly or not understood. Arsenic is considered one of the top pollutants of environmental concern by EPA (US EPA, 2001) and induces a significant reduction of the biochemical and mineral content in skeletal tissue, decreasing α-helical and random coil structures and increasing β-sheet structures (Palaniappan and Vijayasundaram, 2009). Recently, an altered expression of *insulin-like growth factor 1* (*igf1*) and its *receptor 1* (*igf1r1*) in muscle has been associated to arsenic exposure during embryogenesis (Szymkowicz et al., 2017) that might be, at least in part, responsible of its osteotoxicity. Similarly, gasoline additives such as ethyl tert butyl ether (ETBE) and tertiary amyl methyl ether (TAME) exhibit developmental toxicity in aquatic organisms and upon exposure, craniofacial abnormalities in fish were observed. MoA is largely unknown although a dysregulation at transcriptional level of Wnt ligands (*wnt3a* and *wnt8a*) and matrix metalloproteinases (*mmp2* and *mmp9*) has been reported in zebrafish (Bonventre et al., 2012).

Abnormal skeletogenesis during early development has also been reported upon exposure of zebrafish to ketamine. In this case, osteotoxicity may be related to i) an up-regulation of *sonic hedgehog a* (*shha*), which has been involved in cell fate determination and embryonic patterning during early vertebrate development, and ii) a down-regulation of *noggin-3*, a Bmp antagonist (Felix et al., 2016). Nevertheless, since ketamine may also inhibit voltage-sensitive channels (Felix et al., 2016), more data is needed to decipher its MoA during development.

More examples of osteotoxicants with poorly understood MoA can be found: i) sodium pentachlorophenate, which osteotoxicity was tentatively associated with altered retinoic acid signaling (López-Romero et al., 2012), ii) dylox, that may affect collagen formation through the inhibition of esterases (Kozlovskaya and Mayer, 1984) and acetylcholinesterases (Guimarães et al., 2007), and iii) phostoxin, that triggers the loss of ceratobrachial bones in response to altered acetylcholinesterase activity (Doherty et al., 2016).

The multiplicity of molecular players can also be an obstacle to the understanding of compound MoA. Coumarin, for example, has been reported to alter transcriptional regulation through different NRs (Uehara et al., 2008), affect cholinesterase activity (de Souza et al., 2016) and induce oxidative stress (Al-Majedy et al., 2016). Getting additional insights on the mechanisms underlying compound osteotoxicity should be an imperative for a proper environmental risk assessment. In this sense, cell-based assays may provide rapid, efficient, economic, ethically acceptable and high throughput screening method to identify the specific pathways activated by each compound (reviewed in Raucy and Lasker, 2013). Commercial solutions are readily available. For example, the Cignal Reporter Assays (QIAGEN; www.sabiosciences.com) enables the screening of up to 45 different signaling

pathways simultaneously, and the Transcription Factor (TF) Activation Profiling Plate Arrays (Signosis; www.signosisinc.com) can monitor the activity of up to 96 transcription factors. Although large scale screening methodologies are interesting from a pharmaceutical and ecotoxicological point of view (Ratajewski et al., 2015), particular limitations exist in relation to the use of species-specific TF probes (Signosis assay) or binding sites (QIAGEN assay), which may not reflect accurately the potential activation/inhibition of related pathways in other species. This is the case for Pxr, as previously mentioned due to a highly divergent evolution, showing significant differences in ligand specificity across species (Ekins et al., 2008). The implementation of OMICS technologies applied to toxicology, *e.g.* toxico-genomics, -epigenomics, -transcriptomics, -proteomics, and/or -metabolomics, may provide useful insights into the most relevant mechanisms of toxicity at single cell, tissue or organism levels (McHale et al., 2014; Parker et al., 2015; Bock et al., 2016), in particular those underlying osteotoxicity, as previously illustrated for vanadate and warfarin.

4. The complexity of an acurate osteotoxicity risk assessment and the unveiling of its underlying mechanism at the field

Environmental risk assessment by governmental institutions requires information about the compound, its concentration in the nature and the potential lethal and sublethal toxic effects. Laboratory unifactorial scale tests are regularly run applying different approaches, model species and tecnologies to get such information. However, the simultaneous occurrence of different osteotoxic molecules is often observed in the environment, particularly in aquatic environments where most of anthropogenic and potentially toxic compounds eventually end up. Thus, environmental protection is not only challenged with the identification and quantification of all the different compounds present in a sample, but also with the accurate risk assessment of mixtures (ECETOC TR 80, 111). In mixtures, osteotoxic effects may be independent or synergistic and may or may not affect similar structures, while prospective risk assessments may require a comparative approach, *e.g.* the comparison of phenotypes at a polluted site versus a reference (unpolluted) site. There are numerous reports of skeletal alterations in fish exposed to industrial effluents (*e.g.* Bengtsson and Larsson, 1986; Härdig et al., 1988; Mayer et al., 1988 and Lindesjöö and Thulin, 1992). A high incidence of skeletal deformities has been reported in fish inhabiting rivers receiving the effluents of washing and bleaching operations in pulp mills. These effluents contain a complex mixture of chemicals including dioxins and furans, extractive components from woods such as terpenes, sterols, phytosterols, lignin, and lignin phenolic residuals, chlorinated compounds from bleaching such as chlorophenols, and aromatic compounds from washing (Hewitt et al., 2006). Systematic samplings made on natural environments exposed to industrial effluents revealed that skeletal deformities are very frequently observed, with a clear correlation between the incidence of skeletal deformities and the distance of the sampling from the industrial plant (Bengtsson et al., 1988), while a large range of skeletal structures were affected (*e.g.* vertebral column and cranial bones), in various aspects (*e.g.* size and mineralization density) and to different extents.

Only few studies transposed industrial effluents exposure to lab conditions in order to study the underlying mechanisms. Mayer et al. (1988) showed that fish exposed to industrial effluents in laboratory conditions developed vertebral deformities, presenting a depletion of vitamin C stores and a disruption of collagen metabolism as a possible cause for the observed alterations in biochemical and mechanical properties of vertebrae. Similarly, Mehrle et al. (1982) investigated the mechanical response of bones from striped bass (*Morone saxatilis*) and how it could be related with body burdens of selected inorganic (arsenic, lead, cadmium, and selenium) and organic (Arochlors 1248, 1254, and 1260) contaminants at different freshwater basins from USA. More recently, Brian et al. (2005) demonstrated that several estrogenic toxicants (including BPA) act together in a predictable additive manner to produce effects *in vivo* when they are present in multichemical mixtures at concentrations that are not individually effective. These results highlight the need for testing osteotoxicity of multichemical mixtures when pollutants are present at lower concentrations than those unifactorially identified as osteotoxicants. Moreover, pollutants may have effects on skeletal development and/or homeostasis indirectly, by affecting other organs and systems with which the bone is interacting (see Fig. 3). Abnormal muscle activity/growth have been associated with skeletal deformities through the mechanostat theory (Kranenbarg et al., 2005; Jezierska et al., 2009). While thyroid hormones are known to play an important role in skeletal development and bone maintenance (reviewed in Bassett and Williams, 2016), liver secreted IGF-I has been reported to affect skeletal cells proliferation and differentiation through the growth hormone (GH)/IGF axis (Locatelli and Bianchi, 2014). In this sense, toxic effects on digestive system, thyroid hormones or muscle development, among others, might trigger secondary osteotoxicity.

In addition to the indirect osteotoxic effect and the synergistic interaction that osteotoxicants might have in natural environments, abnormal skeletal development and/or bone maintenance can result from other factors rather than exposure to specific pollutants (*e.g.* environmental factors like temperature, ocean acidification and/or water flow, genetic background, nutrition, among others; Boglione et al., 2013; Pimentel et al., 2014; Crespel et al., 2017) and thus, the multi-factorial nature of abnormal skeletogenesis should be kept in mind when on-field monitoring is performed. Such circumstances imply that for a proper diagnosis of the osteotoxic effects encountered in nature, the development of integrative studies at laboratory scale must be conducted in order to confirm that the hypothetical presence of a specific compound has an osteotoxic effect.

5. Conclusions and future developments

Osteotoxicity in vertebrates is an issue that needs to be further addressed in future risk assessment policies related to human health and ecological systems, as it is already the case for neurotoxicity, cardiotoxicity and hepatotoxicity (Rubinstein 2006; Peterson and MacRae 2012; Planchart et al., 2016). Since the teratogenicity of a compound is considered as the most dangerous characteristic (Zur Nieden et al., 2004), and although the prenatal development toxicity study (OECD, 2001) already provides developmental hazard and dose-response information on potential osteotoxicants by evaluating manifestations of abnormal development (*e.g.* skeletal malformation and/or variations; Daston and Seed, 2007); evaluation of endpoints such as skeletal deformities, skeletal variations, increased/decreased bone mineral density (BMD), ossification delay and/or advance, skeletal cells proliferation, differentiation and/or activity, mRNA levels comparison of osteogenic genes and/or osteogenic transcription factors activation/repression may enhance embryo- and osteo-toxicity compound screening (Daston and Seed, 2007; Zur Nieden et al., 2004; Zur Nieden et al., 2010; Braunbeck et al., 2015; and present review). Thus, a more accurate and predictive risk assessment of chemical compounds is expected. In this sense, teleost fish, and in particular zebrafish, represents a suitable, non-expensive, high throughput model to evaluate the likelihood of a compound to be osteotoxic. Some knowledge towards a better understanding of the mechanisms underlying osteotoxicity has already been generated in zebrafish using several *in vivo* and *in vitro* systems. Nevertheless, further developments are needed to achieve a broader acceptance, wider implementation, as well as more reliable results when using this model in order for lab experiments to be more representative of what might be occurring in nature, independently of which lab is performing the study, thus leading to more reproducible results. In this sense, the existence of different protocols in zebrafish husbandry, variable diets and housing conditions, inadequate or lack of disease surveillance and the use of different strains, have been recently proposed as the major drawbacks of standardization and validation of

zebrafish in toxicity assays (Planchart et al., 2016). From an ecological point of view, zebrafish housing/lab conditions (*e.g.* density, nutritional and physiological condition) should be as close as possible to those in natural environments, while understanding how fish physiological response against an osteotoxicant might depend on the environmental conditions should be an essential and key goal to be pursued in future studies. Moreover, in addition to fostering interactions between *in vitro* toxicologists and biomedical scientists using fish models, it is important to integrate toxicology data from *in silico*, *in vitro*, *ex vivo* and *in vivo* approaches for successful risk and safety assessments of chemical compounds. A better understanding of the similarities existing between the structure and the function of zebrafish and mammalian skeletons is also critical for the implementation of fish as a suitable model to assess osteotoxicity. Likewise, since metabolic capacity and toxicokinetics in fish are different from those in other animals, such differences across vertebrates deserve further research efforts to be able to extrapolate a nominal concentration spiked in exposure medium to an internal dose in the embryo and a dose relevant for risk analysis (Noyes et al., 2016). Future effort should focus on the identification of reliable biomarkers of osteotoxicity to expand observations from short-term assays (embryo exposure) to long-term effects (adult phenotype and transgenerational effects). The development and implementation of different experimental approaches and global screening methodologies will allow for the early characterization of compounds on the industrial/pharmaceutical production pipeline for a “green chemistry”, its fate after being released in the environment, an accurate risk assessment, as well as the implementation of regulatory measures by national and international entities. New data and extended knowledge on osteotoxic phenotypes and molecular mechanisms affected will also contribute to advance towards the ultimate goal: the enrichment and implementation of computational systems for predictive purposes.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

All authors worked on all aspects of the manuscript.

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