Effects of Marine Persistent Organic Pollutants on Early Life Development and Metamorphosis of Echinoids

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Everyone is a genius.
But if you judge a fish on its ability to climb a tree,
it will live its whole life believing that it is stupid.

A. Einstein

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CHAPTER 1.

General introduction, objectives and thesis outline

1.1. Marine environment

The marine environment represents a vast ecosystem with an immense biodiversity and productivity (Jenssen 2003). It is estimated that more than 2 billion people world-wide depend on seas and coastal habitats as a source of food and income (Bowen and Depledge 2006). Despite the obvious importance of marine ecosystems, they face continuous challenges from multiple stressors directly and indirectly imposed often by human activities. The most prominent threats include: overexploitation, habitat destruction, pollution, acidification and global climate change (Hendriks et al. 2010; Ofiara and Seneca 2006; Suchanek 1994).

As a consequence of the enormous volume of oceans and seas, water soluble pollutants will be highly diluted. Consequently, readily degradable pollutants will hardly pose a threat. Only in estuaries with high input of pollutants from rivers and local anthropogenic sources could the concentrations of such compounds reach levels high enough to pose a risk. However, the class of pollutants that hardly degrade and are hydrophobic, are not diluted in the seawater and can accumulate in sediments and biota. Therefore these compounds, classified as persistent organic pollutants (POPs), are of particular concern (Jones and de Voogt 1999; Moore et al. 2002; Reish et al. 2000; Rice 2004).

1.1.1. Persistent organic pollutants (POPs)

POPs are carbon containing chemical substances that persist in the environment, bioaccumulate through the food web, and pose the risk of causing adverse effects to human health and the environment (Ritter et al., 1995). POPs are very stable, and have long environmental half-lives. This leads to their persistence in soils, sediments, water and air. This class of pollutants is also lipophilic resulting in the bioconcentration in lipids and fat, leading to the bioaccumulation of POPs in the marine and human food chain. Most POPs are not acutely toxic, but can become toxic after chronic exposure and during sensitive life stages (Daughton 2004; Jones and de Voogt 1999).

- Main sources

Most POPs have been extensively produced and used because of their good technical qualities as pesticides, flame retardants or industrial chemicals. Examples of currently used POPs include brominated flame retardants (BFRs) such as polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA), as well as heptadecafluorooctane sulfonic acid (PFOS), triclosan (TCS), bisphenol A (BPA) and alkyltin compounds. Some POPs are no longer produced but are still found in the environment, such compounds include polychlorinated biphenyls (PCBs) and chlorinated pesticides such as DDT and DEE. POPs can also unintentionally be produced, for example in thermal processes (*e.g.* dioxins and

furans) and as impurities in deliberately manufactured compounds (*e.g.* PCBs) (Jones and de Voogt 1999; UNEP, 1999).

The major inputs of POPs to the marine environment, particularly coastal and estuarine areas, originate from primary sources such as contaminated riverine inflow as well as municipal and industrial waste water and local atmospheric deposition. Secondary sources also significantly contribute to current environmental concentrations of POPs. Examples of such sources of POPs include dumping sites for contaminated harbor sediment that become a major chronic source of contamination to biota (Larsson et al. 2000; Lohmann et al. 2007; Skei et al. 2000). In addition, POPs are prone to long range atmospheric transport, and the immense ocean surfaces result in a great influx of POPs to the marine systems (Jurado and Dachs 2008; Strukov 2001).

- Environmental persistence

The physico-chemical properties of POPs (*e.g.* high log Kow, low vapor pressure and long half-lives due to their stability) results in the accumulation of these compounds in estuarine and marine sediments that act as a repository of POPs. Also due to their ubiquitous atmospheric transport, POPs can be found in all the oceans ranging from the Arctic to the Antarctic, as well as from intertidal to abyssal areas (GESAMP, 1990; Shahidul Islam and Tanaka 2004). Despite the strict control imposed on emissions in the last decades, PCB concentrations in biota are still relatively high, suggesting that there may be a long lasting problem (OSPAR 2006). PCB levels in fish collected from the Belgian North Sea and the Western Scheldt (The Netherlands) ranged between 20-3200 ng PCBs/g wet weight (ww) and for sea stars from 26 to 83 ng PCBs/g ww (sum of 25 PCBs) (Voorspoels et al. 2004). In the case of BFRs, PBDE concentrations in the North Sea samples ranged from 0.02 to 1.5 ng/g wet weight (sum of 8 PBDEs) in benthic invertebrates, while in the Scheldt estuary concentrations were up to 30 times higher, reaching values as high as 30 ng/g wet weight (sum of 8 PBDEs) (Voorspoels et al. 2003). HBCD and TBBPA concentrations in sea stars collected from the Western Scheldt varied between <30-84 and <1-2 µg/kg lipid weight, respectively (Morris et al. 2004). Perfluorinated alkyl compounds (PFACs) such has PFOS also are widespread contaminants in the environment. Although they are very persistent, they do not accumulate in lipid. Due to their amphiphilic nature they accumulate in places where fatty acids accumulate such as blood and cell membranes. Levels in soft tissues of shrimp, crab, and sea star collected in the Western Scheldt estuary ranged from 19 to 520 ng/g, from 24 to 877 ng/g, and from 9 to 176 ng/g (ww), respectively (De Vijver et al. 2003). TCS is a biocide that is widely used in personal care products. The concentrations in marine sediment and cod fish liver collected in Norway ranged from 0.02-14 ng/g dw and 1-96 ng/g lipid weight (lw), respectively (Fjeld et al., 2004).

- Why of environmental concern?

In the past decades, the scientific community placed increasing attention on the adverse health effects associated with exposure to the ubiquitous POPs. As a result of the increasing awareness, in 2001 a treaty was signed in Stockholm for the protection of human health and the environment from POPs. In this Stockholm Convention on POPs priority was given to a selection of POPs called the 'dirty dozen' (UNEP, 2001). Despite these efforts POPs continue to pose an environmental risk due to their high persistence and toxicity, especially for organisms that are associated with sediments (benthic species) and organisms at the top of the food chain that are relatively high exposed via bioconcentration and biomagnification, respectively (Fair et al. 2009; Magnusson et al. 2006; Moore et al. 2002; Vethaak et al. 2005). Several studies link exposure to POPs with adverse effects on reproduction and early life development of fish, amphibians, birds and mammals (Murk et al. 1996; Ostrach et al. 2008; Peterson et al. 1993; Shahidul Islam and Tanaka 2004).

1.2. Early life development

There is growing evidence that early life stages (ELS) of vertebrates are susceptible to adverse effects of POPs at much lower concentrations than those causing effects in juvenile and adult individuals (Black et al. 1988; Foekema et al. 2008; Gutleb et al. 1999; Hutchinson et al. 1998; Peterson et al. 1993; Van Leeuwen et al. 1990; Warren et al. 1995). This developmental window in the life cycle of vertebrates is particularly sensitive to the effects of POPs since vital biochemical and molecular mechanisms take place during tissue differentiation and organization (Ensenbach 1998; Laale and Lerner 1981; Schulte and Nagel 1994) (Fraysse et al. 2006). Toxic effects may occur e.g. via disruption of vitamin A and thyroid hormone levels (Brouwer et al. 1998; Brouwer et al. 1989), and both are very important for development.

- Thyroid hormones

Thyroid hormones (THs) are important to regulate differentiation, growth, and metabolism during the early life development of many animal groups (Dussault and Ruel 1987) (OPPENHEIMER et al. 1987). These hormones are also responsible for the regulation of metamorphosis of amphibian and flatfish species (Inui and Miwa 1985; Kanamori and Brown 1996; Klaren et al. 2008), and interestingly, they also induce echinoid metamorphosis from free swimming larvae into a benthic juvenile (Chino et al. 1994; Heyland et al. 2004). Consequently, the disruption of TH function and/or signaling by thyroid hormone disrupting compounds (THDC) is of obvious concern (Brar et al. 2010; Gutleb et al. 2000; Schriks et al. 2006; Zoeller 2005). Especially hydroxy metabolites of POPs such as PCBs and PBDEs have been shown to mimic thyroid hormones and compete with TH for binding to

plasma transport proteins and to the thyroid hormone receptor (TR) (Boas et al. 2006; Brouwer et al. 1999; Brouwer et al. 1998; Brucker-Davis et al. 2003; Colborn 2002; Goldey et al. 1995; Marchesini et al. 2008; Miller et al. 2009; Murk et al. 1994a; Murk et al. 1994b). These compounds are also able to interfere with the sensitive metamorphic process of amphibians and flatfish, resulting in dramatic changes in the animal's body and cellular organization (Gutleb et al. 2000; Gutleb et al. 1999; Gutleb et al. 2007b; Soffientino et al. 2010).

- *Cellular efflux pumps*

One of the first line defense mechanisms of organisms against toxic compounds are the cellular efflux pumps which can transport contaminants and endogenous metabolites out of the cells (Germann 1993; Miller et al. 2002; Schinkel and Jonker 2003; Tatsuta et al. 1992). These pumps are mostly located in tissues that are involved in excretion of xenobiotics (*e.g.* gills, liver, hepatopancreas, kidney and intestines) (Smital et al. 2004). This defense mechanism is commonly known as Multi Xenobiotic Resistance (MXR) (Bard 2000; Kurelec 1992). Its principle is based on the Multi Drug Resistance (MDR) mechanism first described in cancer cell lines resistant to anti-cancer drugs such as vinblastine (VIN) (Ambudkar et al. 1999). In the case of MXR, the pumps thought to be responsible for the efflux transport in aquatic organisms belong to the permeability glycoprotein (P-gp), and multidrug resistance-associated protein (MRP) ATP-binding cassette (ABC) transporters (Bard 2000; Epel et al. 2006). MXR has been identified in gills and larvae of several marine organisms, such as fish, mussels, echinoids, sponges and marine worms (Kurelec 1992; Kurelec et al. 1996). It has, however, been shown that some toxic compounds can also inhibit efflux pumps in aquatic organisms, and this disruption of the MXR mechanism can lead to the intracellular accumulation of toxic substrates (Bosnjak et al. 2009; Smital et al. 2004; Smital et al. 2000).

1.3. Echinoids as an animal model

Echinoderms (*e.g.* echinoids) are benthic marine invertebrates that play a crucial role in marine and costal ecosystems (Brusca 1990). In their natural habitat they are exposed to POPs associated with the sediment and pore water, as well as via their food such as dead organic matter. This puts them at risk for toxic effects of POPs (Boese et al. 1996; Coteur et al. 2003; Morris et al. 2004). Invertebrates are commonly used in toxicity studies to evaluate toxic effects of POPs and other chemicals (Lagadic and Caquet 1998). Echinoids are also regularly used as animal model in marine ecotoxicological studies such as fertilization- and short term larval toxicity bioassays (Warnau 1996). Echinoids are easy to maintain under laboratory conditions (Kelly 2002; Schipper et al. 2008), have a large number of progeny, their transparency allows an easy observation of embryos and their development is

relatively fast. Furthermore, echinoids are deuterostomes making them more closely related to vertebrates than other invertebrate groups from a phylogenetic perspective. Interestingly, echinoids have an endocrine system similar to vertebrates (Lavado et al. 2006; Porte et al. 2006) and TH have been shown to induce echinoid metamorphosis (Chino et al. 1994; Heyland et al. 2004). According to the European Commission (Directive 86/609/EEC), the use of vertebrates for experimental and other scientific purposes should be avoided, and therefore, echinoid bioassays also have the advantage of being an ethical alternative to vertebrate studies. For the research presented in this thesis, the echinoid *Psammechinus miliaris* was selected as test species since it is easily found in Dutch coastal areas and can be easily reared under laboratory conditions (Kelly et al. 2001).

1.3.1. *P. miliaris* early life development

P. miliaris fertilized eggs hatch within 20 hours post-fertilization (hpf) releasing a planktonic ciliated blastula that reaches the gastrula stage 1 day post-fertilization (dpf). Between 2-4 dpf, the larvae develop into a 4-armed pluteus stage and once the digestive tract is fully developed they start free-feeding. Around 10 dpf, larvae reach the 6-armed pluteus stage and by 13 dpf the larvae reach the last larval stage, the 8-armed pluteus. Once the larval body is fully developed, a structure known as the echinoid or sea urchin rudiment starts developing. This rudiment is a structure resulting from the fusion of the aminiotic sac (vestibule) with the hydrocoel located on the left side of the larval body next to the stomach (Cameron and Hinegardner 1974; Cameron and Hinegardner 1978; Chino et al. 1994). Once the sea urchin rudiment is fully formed, the larva becomes competent and settles in the substrate where the larval body is fully reabsorbed and metamorphosis is completed (Fig. 1). In echinoids, the development of the rudiment as well as the metamorphic process from planktonic larva into a juvenile is induced by THs (Chino et al. 1994). In analogy to vertebrates, it is expected that early life development of echinoids (*e.g. P. miliaris*) is the period most sensitive to adverse effects of POPs (Buono et al. 2012; Novelli et al. 2002; Roepke et al. 2005).

In the research performed for this thesis, the early life development of *P. miliaris* was divided in two main developmental periods ranging from embryo until the early 8-armed pluteus stage and from the middle 8-armed pluteus stage to the completion of metamorphosis into a juvenile (Fig. 1). Three echinoid bioassays were developed to study effects of POPs during the most relevant stages of echinoid early life development:

1) Echinoid prolonged ELS bioassay - The assessment of toxic effects in the ELS is recognized as an essential element in environmental hazard assessment for chemicals (Foekema et al. 2008; Fraysse et al. 2006; Van Leeuwen et al. 1990) and several guidelines are available for (eco) toxicity testing (*e. g.* OECD 1992; 1998; EPA 1996; ASTM, 2004). The exposure and observation period for

the standard echinoid ELS test normally lasts between 48 and 96 hpf. For POPs, however, the duration of the observation period of standard fish and amphibian embryo bioassays has been shown to be too short for POPs like PCBs that only become toxic after a longer time. As a consequence, the standard ELS assays can lead to underestimation of the toxic effects (Foekema et al. 2008; Gutleb et al. 1999; Gutleb et al. 2007a). Therefore, it is to be expected that the currently available echinoid ELS bioassays also underestimate developmental effects of POPs because of the narrow window of observation of the echinoid early life development.

2) Echinoid metamorphosis bioassay - As THs play a crucial role regulating metamorphosis in echinoid as well as amphibian and flatfish species (Inui and Miwa 1985; Kanamori and Brown 1996; Klaren et al. 2008), and this metamorphic process is particularly sensitive to adverse effects caused by POPs (Balch et al. 2006; Cary Coyle and Karasov 2010; Kitamura et al. 2005; Veldhoen et al. 2006). It is expected that also the TH induced echinoid metamorphosis is sensitive to interference by certain POPs. Therefore, an echinoid metamorphosis assay could be a potential tool to assess disruption of TH function using an invertebrate species;

3) Echinoid cellular efflux pump bioassay - Efflux pumps have been shown to be inhibited by contaminants (*e.g.* certain POPs) leading to intracellular accumulation of toxic substrates otherwise effluxed from the cellular compartment (Bosnjak et al. 2009). Echinoid larvae are also interesting models for the detection of the inhibition of efflux pumps by POPs. *S. purpuratus* larva start expressing efflux pumps already half hour post fertilization (Hamdoun et al. 2004). These efflux pumps are homologous to at least one P-gp and two MRP pumps (Bosnjak et al. 2009; Hamdoun et al. 2004) and have been shown to be suitable to investigate efflux transporter activity (Epel et al. 2006). As echinoid larvae also are transparent they could be suitable models for the detection of the inhibition of efflux pumps by POPs using fluorescence techniques.

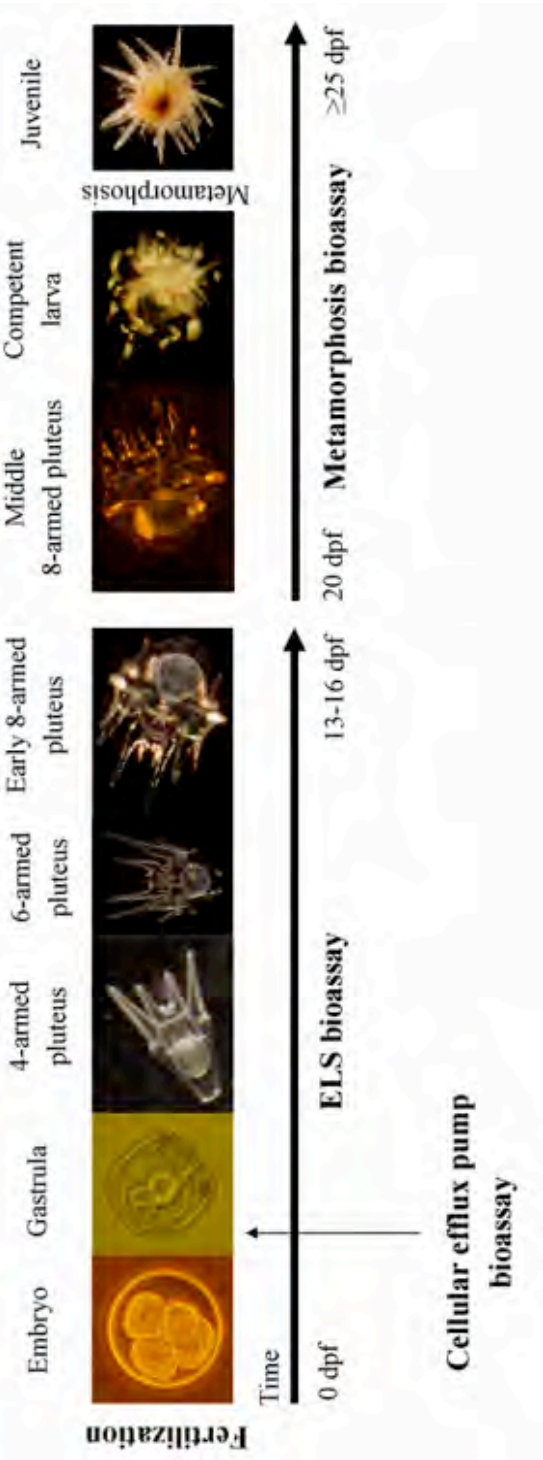

Figure 1. *P. miliaris* early life development stages from fertilization to completion of metamorphosis into juvenile. Arrows indicate the developmental stages exposed during the respective bioassays

1.4. Thesis outline

The present PhD project aimed at the development and application of bioassays covering the major development stages and most relevant endpoints of the early life stages of echinoids to assess toxic effects of POPs on early development. For that purpose, three main objectives were outlined:

1. Development of an ELS bioassay with a prolonged observation period, a metamorphosis assay and a cellular efflux pump inhibition assay as functional ecotoxicological in vivo bioassays to evaluate adverse effects of marine POPs using the sea urchin Psammechinus miliaris;

2. Assess the effects of environmentally relevant marine POPs as individual compounds and mixtures on echinoid early life development;

3. Evaluate the suitability and applicability of echinoids as an (eco)toxicological invertebrate animal model to assess the effects of POPs on early life development.

In Chapter 1, background information on the topic of the present thesis is given, the aims are defined and a short outline of the thesis is presented.

Chapter 2 describes the development of the echinoid prolonged ELS (p-ELS) to evaluate the effects of POPs during echinoid early life development with an observation period that lasts between 13 and 16 dpf. The development of the p- ELS bioassay with *P. miliaris* allowed to assess both acute and delayed effects of POPs (*i.e.* TCS, TBBPA, HBCD) on the rate of development and the occurrence of malformations.

In chapter 3 the echinoid metamorphosis bioassay was developed and validated by demonstrating that *P. miliaris* metamorphosis was induced by the TH thyroxin (T4) and delayed by the TH synthesis inhibitor thiourea (TU). Both acceleration and delay of metamorphosis as well as the development of malformations were included as endpoints in the metamorphosis bioassay, and the test was further validated by testing compounds known to interfere with TH function (e.g. PBDEs, TBBPA, TCS).

The development of the larva cellular efflux pump inhibition bioassay is described in chapter 4. First, the activity of efflux pumps in *P. miliaris* larvae at the gastrula stage was evaluated following the exposure to the model inhibitor verapamil (VER) using the efflux pump substrate calcein-AM that becomes fluorescent only when metabolized by intracellular enzymes. The accumulation of calcein-AM in *P. miliaris* larvae at the gastrula stage upon co-exposure to POPs was quantified for

compounds recently demonstrated to inhibit the cellular efflux pumps in vitro (*i.e.* PFOS, pentachlorophenol (PCP), BPA, TCS, nanoparticles P-85®, o,p'-DDT, HBCD). In addition, the effect of inhibition of the efflux pump on the toxicity induced by an efflux pump substrate was tested in an acute toxicity assay with sea urchin larvae.

In chapter 5, the effects of environmentally relevant mixtures on echinoid early life development was investigated. Marine organisms are not exposed to single POPs but to mixtures. Accordingly, mixture effects on the early life development of *P. miliaris* were evaluated. In chapter 4 the toxicological consequence of efflux pump inhibition was investigated by exposing *P. miliaris* larvae to an efflux pump inhibitor in combination with an efflux pump substrate. In chapter 5 the combined toxic effects of a field-based marine contaminant mixture were assessed applying the newly developed echinoid p-ELS and metamorphosis bioassays and compared to the effects of single compounds. The field-based mixture showed significant toxicity in the *P. miliaris* ELS and metamorphosis bioassays, and special attention was given to the alkylcompounds TPT and DBT.

In chapter 6 a general discussion, concluding remarks as well as some future perspectives are presented on the relevance of the newly developed bioassays to study the ecotoxicological effects of marine POPs.

Chapter 7 summarizes the content of the present thesis.

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CHAPTER 2.

Early life developmental effects of marine persistent organic pollutants on the sea urchin *Psammechinus miliaris*

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Abstract

A new 16-day echinoid early life stage (ELS) bioassay was developed to allow for prolonged observation of possible adverse effects during embryogenesis and larval development of the sea urchin *Psammechinus miliaris*. Subsequently, the newly developed bioassay was applied to study the effects of key marine persistent organic pollutants (POPs). Mortality, morphological abnormalities and larval development stages were quantified at specific time points during the 16-day experimental period. In contrast to amphibians and fish, *P. miliaris* early life development was not sensitive to dioxin-like toxicity in the prolonged early life stage test. Triclosan (TCS) levels higher than 500 nM were acutely toxic during embryo development. Morphological abnormalities were induced at concentrations higher than 50 nM hexabromocyclododecane (HBCD) and 1000 nM tetrabromobisphenol A (TBBPA). Larval development was delayed above 25 nM HBCD and 500 nM TBBPA. Heptadecafluorooctane sulfonic acid (PFOS) exposure slightly accelerated larval development at 9 days post fertilization (dpf). However, the accelerated development was no longer observed at the end of the test period (16 dpf). The newly developed 16-day echinoid ELS bioassay proved to be sensitive to toxic effects of POPs that can be monitored for individual echinoid larvae. The most sensitive and dose related endpoint was the number of developmental penalty points. By manipulation of the housing conditions, the reproductive season could be extended from 3 to 9 months per year and the ELS experiments could be performed in artificial sea water as well.

1. Introduction

Anthropogenic pollutants are ubiquitously present in marine and estuarine environments (Bowen and Depledge, 2006), which may present a risk to both biodiversity and productivity of marine ecosystems, as well as human marine resources (Jenssen, 2003). Persistent organic pollutants (POPs) tend to accumulate in the sediment and food web, thus reaching concentrations that potentially cause toxic effects (Moore et al., 2002; Magnusson et al., 2006; Fair et al. 2009). Such POPs include: polychlorinated biphenyls (PCBs), dioxin-like compounds, triclosan (TCS), perfluorinated alkyl compounds (PFACs), and brominated flame retardants (BFRs) such as hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA). For example, PCB levels in sediment collected from the Western Scheldt river ranged from 105-400 ng/g dry weight (dw) ∑PCB (Covaci et al., 2005). In sea stars (Echinodermata) collected from the Belgian North Sea and Western Scheldt (The Netherlands), PCB levels ranged between 680-2500 ng/g lipid weight (lw) $\sum PCB_{23}$ (Voorspoels et al., 2004). Concentrations of the BFRs HBCD and TBBPA in sediment ranged from <0.6-99 and <0.1-3.2 µg/kg dw, respectively, while in sea stars levels varied between <30-84 and <1-2 ng/g lw, respectively (Morris et al., 2004). TCS levels in marine sediment and cod fish liver collected in Norway ranged from 0,02–14 ng/g dw and 1-96 ng/g lw, respectively (Fjeld et al., 2004). Levels of perfluorooctanesulfonic acid (PFOS) in sediment collected from aquatic systems in The Netherlands varied between 0.5 and 8.7 ng/g dw (Kwadijk et al., 2010). In sea stars from the Western Scheldt estuary, PFOS levels varied between 9-176 ng/g ww (De Vijver et al., 2003).

Early life stages (ELS) of vertebrates are regarded as more sensitive to toxic effects of POPs when compared to juvenile and adult life stages (Van Leeuwen et al., 1990; Warren et al., 1995; Hutchinson et al., 1998). Furthermore and depending on the duration of the test observation period, effects on ELS's may even be seriously underestimated when studied with standard fish ELS (OECD, 1992; EPA, 1996;) and the amphibian Frog Embryo Teratogenesis Assay – Xenopus (FETAX) (ASTM, 1991). This has been shown with the dioxin-like PCB 126 and PCB 77 for tadpoles and fish larvae, where embryo exposure during 4 days post fertilization (dpf) caused delayed effects 12-15 days after the end of exposure and in some cases, after the onset of metamorphosis (Gutleb et al., 1999; Gutleb et al., 2007; Foekema et al., 2008). Delayed effects resulting from exposure to PCB 126 included the development of edema, misformed eyes and tail, and absence of gut coiling in tadpoles (Gutleb et al., 1999); in pre-metamorphic fish larvae, the development of edema in the abdominal region was also observed (Foekema et al., 2008).

Echinoderms are marine invertebrates that play a key role in marine ecosystems (Brusca and Brusca, 1990). They live in close contact with sediment, where POPs tend to accumulate, thus making

them particularly at risk for toxic effects of such pollutants (Boese et al., 1996; Coteur et al., 2003; Morris et al. 2004). Similarly to vertebrates, it has been shown that echinoderm ELS are the most sensitive life stages to the toxic effects of POPs (Pagano et al., 1985; den Besten et al., 1989). However, for practical reasons, the commonly used echinoid ELS bioassays only include a narrow window of the entire early life development, with an observation period that normally lasts between 48 and 96 hours post fertilization (hpf).

This study aims at extending the exposure and observation period of echinoid ELS bioassays in order to effectively evaluate the potential adverse effects posed by POPs to echinoid early life development. For this purpose, the echinoid *Psammechinus miliaris* was selected since it can easily be reared in laboratory conditions securing the availability of mature individuals.

The newly developed echinoid ELS bioassay has an observation period of 16 dpf from the cleavage stage until the 8-armed pluteus developmental stage. The development of the prolonged ELS bioassay required: 1) a novel rearing and exposure method for the entire 16-day test period; 2) selection of practical and relevant end points that allow quantification of toxic effects; 3) validation of the assay by testing a range of environmentally relevant POPs.

The POPs selected to be tested were PCB 126, tetrachlorodibenzodioxin (TCDD), TCS, HBCD, TBBPA and PFOS due to their ubiquitous presence in the marine environment, and their tendency to accumulate in the sediment and food webs. In addition these compounds have been shown to be able to adversely affect early life development of both fish and amphibians (Gutleb et al., 1999; Kuiper et al., 2007; Foekema et al., 2008; Oliveira et al., 2009; Deng et al., 2009; Shi et al., 2010). Lastly these compounds possess different chemical structures and different mechanisms of toxicity which is relevant to evaluate the responsiveness and sensitivity of the newly developed ELS bioassay. To determine the effectiveness of the exposure method, internal concentrations of the test compounds were measured.

2. Materials and methods

2.1. Adult animals

Sea urchins (*Psammechinus miliaris*) were collected from the Eastern Scheldt (The Netherlands) and maintained in fiber glass tanks (L*W*W in cm = 200*80*30) under controlled conditions (i.e. temperature, photoperiod and food availability) with a flow rate of 150L per day (corresponding to a renewal of half of the tank volume) for at least 2 months prior to use in the ELS

bioassays. Sea urchins maximum stocking density was 40 individuals per m^2 and they were fed *ad libitum* with freshly dissected mussels (*Mytilus edulis*) and TetraMin® (Tetra).

2.2. Adult aquaculture

In the present study, the natural reproductive season of *P. miliaris* was successfully extended from 3-4 months (Kelly, 2000) to 8-9 months per year under laboratory conditions. Mature adult animals collected from the field during May and June were kept in the laboratory under the temperature and photoperiod that mimic field conditions during the start of the reproductive season in spring (*i.e.* 13±1ºC water temperature, photoperiod 15:9 (L:D)). In late September, when animals were no longer producing mature gametes, the water temperature and photoperiod were slowly decreased by 1ºC and 1 hour per week, respectively, until winter conditions of 7ºC and a photoperiod of 8:16 (L:D) were reached. Animals were kept at winter conditions for at least 3 weeks to promote gametogenesis. Following this period, both temperature (1ºC per week) and photoperiod (1h per week) were slowly increased to values mimicking the prevailing field conditions in spring (*i.e.* 13±1ºC water temperature; photoperiod 15:9 (L:D)), when *P. miliaris* becomes mature. After this procedure animals, were mature from mid-January until October, thus securing availability of both eggs and sperm to perform the ELS bioassay.

2.3. Gamete collection and fertilization

The eggs and sperm used in the present study were collected from the freshly dissected gonads of a single pair of adult *P. miliaris* individuals. The average wet weight and test diameter of the adult individuals was 36±10g and 4.3±0.4cm, respectively. For transport eggs, were kept at 17±1ºC in filtered sea water (FSW; 0.2 μM) or artificial sea water (ASW) prepared using Instant Ocean® synthetic salts (Spectrum Brands, Inc.) and previously aged under continuous aeration for at least 1 week (salinity 31±1 Practical Salinity Units (PSU), $O_2 \geq 90\%$, pH 8±0.15). Sperm was collected "dry" and kept in ice. Fertilization took place within 4 hours of gamete collection according to a procedure based on the method by Environment Canada (1992) for echinoid fertilization. Fertilization success was at least 90% as indicated by Environment Canada (1992) and EPA (2002) for bioassay validation.

2.4. Test method

Fertilized eggs were randomly divided into glass beakers containing 500 ml of FSW or aged ASW (aerated for at least 1 week), and spiked with the appropriate test concentrations; a solvent control was also included (dimethyl sulfoxide (DMSO) at 0.1% v/v). Larval density was ± 0.5 larvae per ml. Test beakers were placed in an environmentally controlled room at 19±1°C, with a photoperiod of 16:8 (L:D) throughout the entire test period.

Embryos and larvae were exposed to POPs by the addition of test compound in 0.1% v/v DMSO from 0 to 16 days post fertilization (dpf). Twice a week 50% of the exposure volume was removed by inserting a PVC tube (Diameter (Ø) - 60 mm) fitted with a 90 µm pore size nylon mesh at the base in the test beaker. Inside this tube, a hose (Ø 5 mm) was placed to gently siphon the water and prevent mechanical damage to the larvae. Subsequently, the removed water was replaced with new sea water at the respective test concentration.

From 2 dpf and onwards, the larvae were fed a diet of microalgae (*Dunaliella sp.*) at densities of 1500, 2500, and 4000 cells/ml for the 4, 6 and 8-armed pluteus stage, respectively (Kelly et al., 2000) (Fig 1).

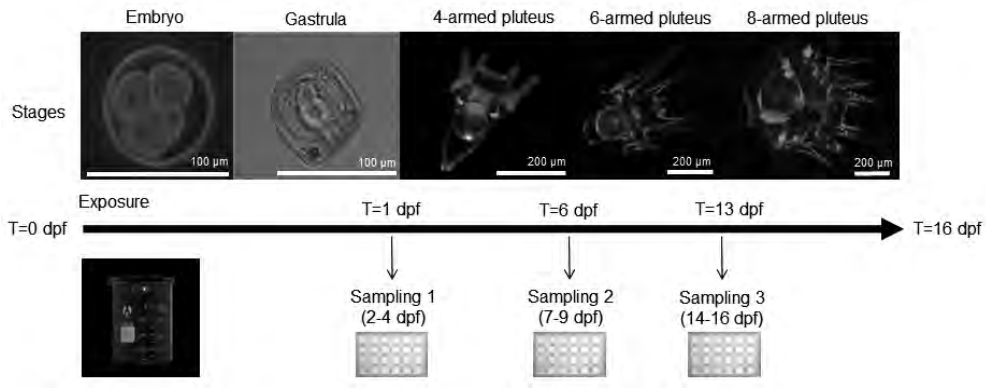

Figure 1. *P. miliaris* ELS bioassay experimental design. Following fertilization embryos were exposed in glass beakers containing 500 ml of test volume at a density of 0.5 larvae/ml. At 3 specific time points (1, 6 and 13 dpf) a total of 20 larvae per replicate (n=2) were sampled into a 24 wells-plate at a density of 1 larvae/ml. In the 3 subsequent days to sampling 1 (2-4 dpf), 2 (7-9 dpf) and 3 (14-16 dpf) larvae were scored for developmental stage, morphological abnormalities and mortality. All experiments were independently performed twice (A and B) and the internal concentrations in larvae were determined in experiment B.

In order to quantify potential toxic effects for each test concentration, 20 larvae were sampled per replicate and placed in 10 wells of a 24 well-plate containing 2 larvae in 2 ml of sea water. This sampling was performed at 1, 6, and 13 dpf (Fig. 1). In the 3 subsequent days following sampling 1 (2-4 dpf), 2 (7-9 dpf) and 3 (14-16 dpf) the larvae were scored daily for developmental stage, morphological abnormalities and mortality. Results for larval morphological abnormalities and mortality were reported as the average percentage of 3 repeated observations during the 3 subsequent days to sampling 1, 2 and 3. At 72 hpf, the percentage of morphological abnormalities and mortality was also calculated to illustrate that toxic effects might be underestimated when using the standard 72h echinoid early life development test in comparison with the 16-day echinoid ELS bioassay. *P. miliaris* larvae were considered morphologically abnormal when at least one of the following characteristics was observed: presence of short or abnormal arms and edema. It should be noted that the term edema used to describe the swelling/enlargement observed in sea urchin larvae does not necessarily correspond to the definition of edema used for vertebrates (*e.g.* blue-sac disease in fish larvae) since sea urchins are osmoconformers. Images of damaged larvae shown in figure 2 do not result from exposure to a specific test compound. All other larvae were classified as normal (Fig. 2).

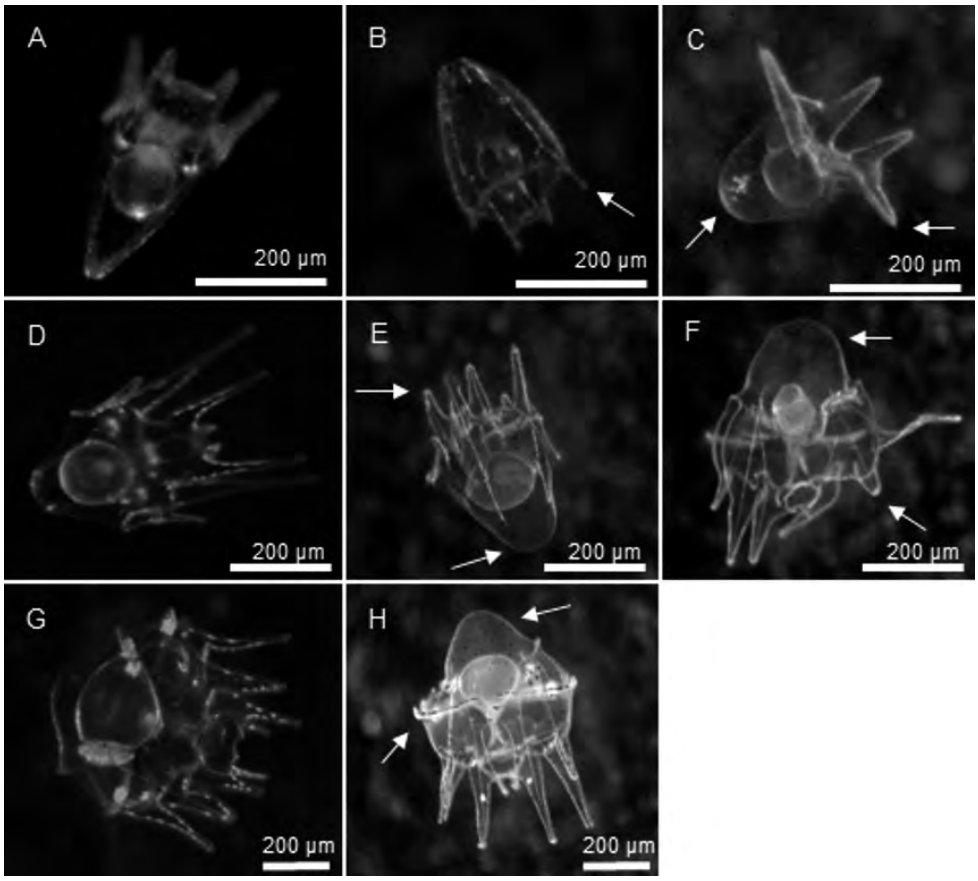

Figure 2. *P. milliaris* larvae at the 4, 6 and 8-armed pluteus stage with normal (A; D; G, respectively) and abnormal morphology (B; C; E; F; H). Arrows indicate the presence of short or abnormal arms (B; C; E; F), and edema (B; C; E; F; H). It should be noted that the type of larval damage presented in the figure is not an effect resulting from exposure to a specific test compound.

A second approach used to evaluate toxic effects on larval development were the "penalty points" following the same principle as introduced for quantification of tadpole development (Gutleb et al., 2007). At 16 dpf, larvae that develop normally should be at the 8-armed pluteus stage, each larva that was at an earlier developmental stage was accredited a penalty point depending on the number of developmental stages lacking at 16 dpf. Based on this method, larvae were accredited 0 penalty points when they were at the expected 8-armed pluteus stage, 1 point at the 6-armed pluteus, 2 points at the 4-armed pluteus, and 3 points for all earlier developmental stages.

Test concentrations were selected based on literature and pilot tests performed prior to the reported ELS experiments (Table 1). Each test compound was tested in two independent experiments (A and B) in duplicate between August 2008 and March 2010 with the following exceptions: TCDD was only tested once to confirm the absence of effects observed for the dioxin-like PCB 126 in the *P. miliaris* ELS bioassay and PFOS was only tested once due to time constraints and a lack of indication of any effect. For TBBPA only 2 concentrations were used in experiment A since it was a pilot test. Experiment B is a repetition of experiment A with the exact same endpoints and concentrations (except for TBBPA).

2.5. Test Compounds

Stock solutions of PCB 126 (3,3',4,4'5-pentachlorobiphenyl; CAS: 57465-28-8; purity 99.1%; Promochem), TCDD (2,3,7,8-tetrachlorodibenzodioxin; CAS: 9014-42-0; purity ≥98%; Accustandards) TCS (triclosan; CAS: 3380-34-5; purity ≥97%; Sigma-Aldrich), TBBPA (tetrabromobisphenol A; CAS: 79-94-7; purity 97%; Aldrich Chemical), HBCD (hexabromocyclododecane technical mixture; kind gift from Professor Åke Bergman; Stockholm University, Sweden) and PFOS (heptadecafluorooctane sulfonic acid potassium salt; CAS: 2795-39-3; purity ≥98%; Fluka) were prepared in DMSO (CAS: 67-68-5; purity 99,9%, Sigma-Aldrich) and stored in the dark at room temperature.

2.6. Chemical analysis of internal POP concentrations

In order to assess the actual internal exposure to the test compounds, the internal concentrations of PCB 126, TCS, TBBPA and PFOS were determined in larvae at the end of experiment B. Assessment of the internal HBCD concentrations was attempted but samples were lost due to instrument failure during analysis. It was chosen to measure the internal concentrations (which actually is quite unique for such extremely small animals) because we are of opinion that internal concentrations are more relevant for comparison to the field situation as in reality exposure to POPs takes place mostly via the food and not via the water.

To collect the larvae without exposure water, the test volume was reduced at 16 dpf from 500 ml to 100 ml and brought up again to 500 ml with non-spiked sea water. The following day the number of larvae per ml in each replicate was counted before they were collected by centrifugation (500*g* for 30 seconds), transferred into a HPLC vial with the minimum of sea water (≤ 150 µl) and frozen at -20°C. Due to the small size of the larvae and the incomplete removal of the sea water when

collecting them, it was not possible to determine the weight of the larvae used to measure internal concentrations. To have an indication of the larvae weight we conducted another experiment and collected larvae 16 dpf, which is the same age and developmental stage as the larvae collected to measure internal concentrations. The estimated larvae wet weight was 0.13 mg/larva.

The internal concentrations of the dioxin-like PCB 126 were determined by bioanalysis using an *in vitro* reporter gene assay for dioxin-like toxic potency in H4IIE rat hepatoma cells (Murk et al., 1998; Foekema, 2008). The *in vitro* TCDD-equivalent (TEQ) levels were directly quantified in this DR-H4IIE.Luc assay (also referred to as DR CALUX), which has been chemically validated before and produces a response that is linear with the concentration of compounds with dioxin-like toxicity, such as PCB 126. The potency of PCB126 is 0.1 compared to TCDD, therefore the concentration of PCB126 is 10 times higher than the TEQ determined (Murk et al., 1998; Besselink et al., 1998; Stronkhorst et al., 2002; Hoogenboom et al., 2006).

For TCS analysis, after 80 ng of PCB 112 in 1 ml iso-octane was added to the extract as an internal standard, the samples were extracted using dichloromethane and concentrated to 1 ml. Analysis was performed using an Agilent 6890 GC coupled to a 5973 MS Detector using a 50 m CPSil8 GC-column. TCS was quantified on m/z 288 using m/z 290 as a qualifier ion. The limit of detection (LOD) was 1 ng TCS.

TBBPA was analyzed by adding 100 ng 13C labeled TBBPA to the samples in 1 ml methanol. Samples were extracted using methanol and subsequently concentrated to 1 ml. Analysis was performed by LC-MS (Thermo Finnigan Surveyor LC coupled to a LCQ Advantage Ion-trap MS) using a symmetry C18 column for separation, and the LOD was 5 ng TBBPA. PFOS was analyzed by adding 1 ml 13C labeled PFOS as the internal standard, after which they were extracted using acetonitrile, followed by LC-MS analysis. The LOD was 0.3 ng PFOS. See Kwadijk et al., 2010 for further details.

2.6.1. Quality assurance/Quality control

Blanks were performed with each series of samples. Triclosan recoveries were between 70 and 100%. Calibration curves consisted of 7 points between 5 ng/ml and 500 ng/ml with R^2>0.99. Some traces of triclosan were detected in the blanks but they were below the limit of quantification (5 ng/ml). TBBPA recoveries were between 70 and 100%. Calibration curves consisted of 8 points between 5 ng/ml and 1000 ng/ml with R^2>0.99. No TBBPA was detected in the blanks. PFOS

recoveries were between 70 and 100%. Calibration curves consisted of 8 points between 0.3 ng/ml and 500 ng/ml with R^2>0.99. No PFOS was detected in the blanks.

Table 1. Average percentage of morphological abnormalities (±SD) from 3 repeated observations of 20 larvae per replicate (n=2) corresponding to 14-16 dpf (sampling 3); and percentage of morphological abnormalities (±SD) from 1 observation of 20 larvae per replicate (n=2) at 72 hours post fertilization. Average internal concentrations per larvae (±SD) (n=2), R^2 indicates the correlation between nominal and internal concentration.

Test compound							
PCB 126	**Nominal [nM]**		**0**	**0.0003**	**0.003**	**0.03**	**0.3**
R^2 = 0.92	TEQ [ng/larva]		< LOD	< LOD	0.002	0.022	0.06
Morphological abnormalities (%)	Exp. A	72 hpf	17±3	12±4	10±0	21±3	14±1
		14-16 dpf	6±4	12±4	9±6	11±8	11±4
	Exp. B	72 hpf	8±4	13±4	20±4	15±7	13±10
		14-16 dpf	4±5	13±5	7±4	3±3	9±5
TCS	**Nominal [nM]**		**0**	**125**	**250**	**500**	**1000**
R^2 = 0.98	Internal [ng/larva]		< LOD	0.90±0.07	1.44±0.35	3.72±2.14	(ND)
Morphological abnormalities (%)	Exp. A	72 hpf	10±0	12±10	8±4	100±0***	100±0***
		14-16 dpf	3±3	5±0	1±2	100±0***	100±0***
	Exp. B	72 hpf	15±6	20±8	17±20	83±4**	100±0**
		14-16 dpf	15±5	13±4	14±4	82±2***	100±0***
HBCD	**Nominal [nM]**		**0**	**9**	**25**	**50**	**100**
Morphological abnormalities (%)	Exp. A	72 hpf	5±0	10±14	13±11	15±0	28±5
		14-16 dpf	8±8	13±8	11±4	3±3	68±8***
	Exp.B	72 hpf	25±2	30±5	31±1	32±5	43±9*
		14-16 dpf	20±3	26±7	31±5	33±3	77±5***
TBBPA	**Nominal [nM]**		**0**	**150**	**500**	**1000**	**1500**
R^2 = 0.94	Internal [ng/larva]		< LOD	0.35±0.06	0.89±0.26	0.86±0.82	1.42±0.59
Morphological abnormalities (%)	Exp. A	72 hpf	8±12	2±3	(ND)	(ND)	16±10
		14-16 dpf	0±0	2±2	(ND)	(ND)	59±25**
	Exp. B	72 hpf	0±0	11±2	8±11	11±2	13±2
		14-16 dpf	12±4	13±7	15±5	15±8	28±10
PFOS	**Nominal [nM]**		**0**	**93**	**186**	**372**	**743**
R^2 = 0.91	Internal [ng/larva]		< LOD	0.20±0.02	0.47±0.01	0.51±0.08	2.51±1.89
Morphological abnormalities (%)	Exp. A	72 hpf	7±3	14±0	6±5	2±3	5±0
		14-16 dpf	5±0	4±4	7±4	3±5	3±6

Statistical analysis done by two-way ANOVA with Bonferroni's Multiple Comparison Test *p <0.05; **p <0.01; ***p <0.001; (ND) Not determined.

2.7. Statistics

Statistical analysis was performed using GraphPad Prism software (version 5). To determine EC_{50} values, a sigmoid dose–response curve was fitted through the experimental data. The significance of differences between the treatments was determined using a one-way or two-way ANOVA followed by Dunnett's Multiple Comparison Test and Bonferroni post test, respectively. The correlation between nominal and internal concentrations of test compounds was analyzed by simple linear regression.

3. Results

3.1. Development of a prolonged ELS bioassay for sea urchins

3.1.1. Experimental set up

For the successful development of the 16-day echinoid ELS bioassay, a dedicated experimental set up was developed. This set up allowed larvae to be exposed to treatments in a relatively small test volume of 500 ml during a period of 16 days and extended the observation of larval development until the 8-armed pluteus stage. The relatively long observation period implies that exposure water needs to be refreshed at least twice a week in order to both maintain water concentrations of test compounds, and water quality at suitable levels for larval survival. An important aspect of the developed method was the individual quantification of toxic effects on key larval developmental stages. This was achieved by sampling 20 larvae from each replicate test beaker (n=2) at 1, 6, and 13 dpf into a 24 well-plate (Fig. 1). During the 3 subsequent days after each sampling, larvae could be scored for developmental stage, morphological abnormalities and mortality during the 3 subsequent days after each sampling.

3.1.2. Early development of unexposed larvae

Unexposed eggs hatched in less than 24 hours following fertilization, and 1 dpf larvae were at the gastrula stage (Fig. 1). Sampling 1 (2-4 dpf) was performed at 1 dpf and in the following day (2 dpf) larvae were at the 4-armed pluteus stage thus reaching the free-feeding stage. By the end of the observation period of sampling 1 (4 dpf), the larvae remained at the 4-armed pluteus stage. When sampling 2 was performed (6 dpf), all larvae were at the 4-armed pluteus stage, but by the end of the observation period (9 dpf), 50% had reached the 6-armed pluteus stage. Finally, at the time of sampling 3 (13 dpf) the majority of larvae had were at the 8-armed pluteus stage. By the end of the

observation period (16 dpf), at least 90% of the control larvae had reached the 8-armed pluteus stage and at least 80% of the larvae developed normally. For sampling 1, 2 and 3 at least 80 % of control larvae developed normally, with the exception of sampling 1 for HBCD exposed larvae in experiment B where 70% of controls were normal (Fig. 7), and for TCDD exposure where approximately 75% were normal (Fig. 4).

For practical reasons and to prevent the risk of variations in natural sea water quality the larval rearing method was adapted to use aged ASW instead of natural FSW. No differences in the rate of larval development were observed between natural FSW and ASW, but the survival in the control groups was more reproducible when using artificial sea water. Likewise, no clear differences were observed in both morphological abnormalities and rate of development between exposures to PCB 126 in experiment A, performed with natural FSW, and experiment B with ASW (Table 1 and 3).

3.2. Effects of test compounds

3.2.1 Internal concentrations

This study firstly aims at the development of a relevant ELS bioassay used to assess the effects of POPs originating from different sources of exposure (*e.g.* also from food). Therefore, the internal concentrations were measured in larvae since they were considered to be more relevant than water concentrations for comparison with environmental levels. However, EC_{50} values reported in the present study are based on nominal concentrations since measured internal concentrations could not be expressed on wet-weight or lipid-weight basis. Evaluation of estimated EC_{50}'s for morphological abnormalities should be done with caution due to the step dose response curves obtained, particularly for HBCD and TBBPA, but also for TCS.

All internal concentrations, determined by either bioanalysis (*i.e.* PCB 126) or chemical analysis (*i.e.* TCS, TBBPA, PFOS) revealed a strong linear relation with the nominal doses, with R^2 values ranging from 0.91 to 0.98 (Table 1). The internal PCB 126 TEQ levels in *P. miliaris* larvae determined with bioanalysis ranged from below the LOD for the control larvae to 0.06 ng TEQ/larva (equivalent to 0.6 ng PCB126/larva) in the 0.3 nM PCB 126 dose group. TCS and TBBPA internal levels ranged from below the LOD for the control larvae to 3.72 and 1.42 ng/larva, respectively. The internal concentration of larvae exposed to 1000 nM TCS could not be determined since the embryos died before hatching and no larvae could be collected.

3.2.2. Dioxin-like PCB 126

Exposure to the dioxin-like PCB 126 did not cause any dose related effects on larvae survival, morphological abnormalities (Fig. 3), or developmental penalty points (Table 3) for both experiments. Larvae hatched normally in all dose groups, with larvae reaching the gastrula stage 1 dpf. For sampling 1 (2-4 dpf) no dose related effects were observed. At the end of the observation period of sampling 2 (9 dpf) approximately 50% of the larvae were at the 6-armed pluteus stage, which was similar to control larvae. Finally, at the end of the observation period of sampling 3 (16 dpf), at least 90% of the larvae were at 8-armed pluteus stage in all dose groups.

Because of the unexpected absence of toxic effects resulting from exposure up to and including 0.3 nM of the dioxin-like PCB 126 (Fig. 3; Table 3), a short term exposure to the actual dioxin 2,3,7,8-tetrachlorodibenzodioxin (TCDD) was performed from 0 to 4 dpf, equivalent to sampling 1 in the 16 day ELS assay, to further investigate the previously observed lack of dioxin-like sensitivity. Results for sampling 1 (2-4 dpf) indicated a slight statistically significant increase (20%) in morphological abnormalities at the highest exposure concentration of 3 nM TEQ only (Fig. 4). The observed effects included the absence or abnormal shape of the arms (Fig. 2), while no increase in mortality was observed.

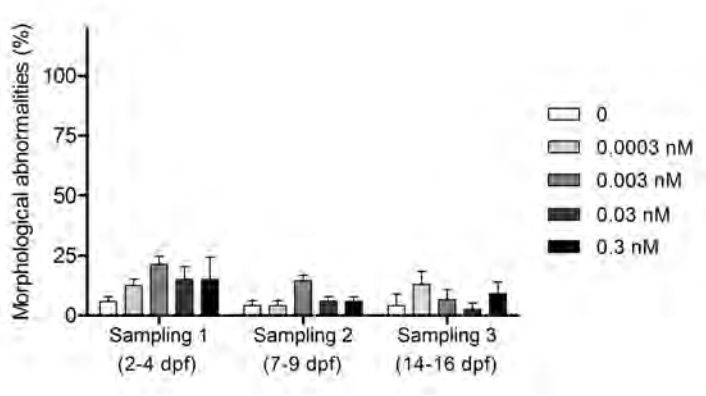

Figure 3. Morphological abnormalities (%) of *P. miliaris* larvae exposed to PCB 126 from 0-16 dpf (experiment A). Each bar represents the average percentage from 3 repeated observations of 20 larvae for each sampling plus the standard deviation (n=2).

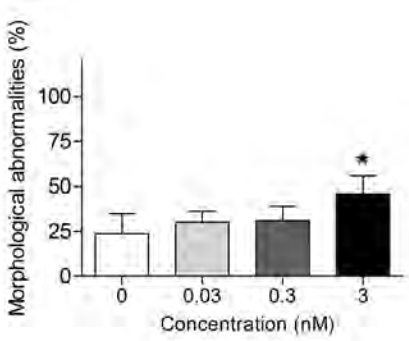

Figure 4. Morphological abnormalities (%) of *P. miliaris* larvae exposed to TCDD from 0-4 dpf. Each bar represents the average (%) from 3 repeated observations of 20 larvae plus the standard deviation (n=2). *p <0.05 (One-way ANOVA with Bonferroni's Multiple Comparison Test).

3.2.3. Triclosan

Exposure of fertilized eggs to 500 nM (experiment A) or 1000 nM (experiment B) Triclosan (TCS) resulted in complete hatching failure. Of the larvae surviving 500 nM TCS in experiment B, more than 80% developed morphological abnormalities (p<0.001) (Fig 5). Observed abnormalities were related to the development of the arms (i.e. short or deformed) and a slight edema around the larval body, which appears to result from abnormal skeletogenesis of skeletal rods (Fig. 2). The lowest estimated EC_{50} values obtained from sampling 1 (2-4 dpf) ranged between 308 (95% C.I. 107-886 nM) and 313 nM TCS (95% C.I. 128-568 nM) for experiment A and B, respectively (Table 2). These larvae were also delayed in their development (Fig. 6), thus resulting in a higher number of developmental penalty points at 16 dpf (Table 3). More than 90% of the larvae exposed to 250 nM TCS or lower developed normally and reached the 8-armed pluteus stage at 16 dpf.

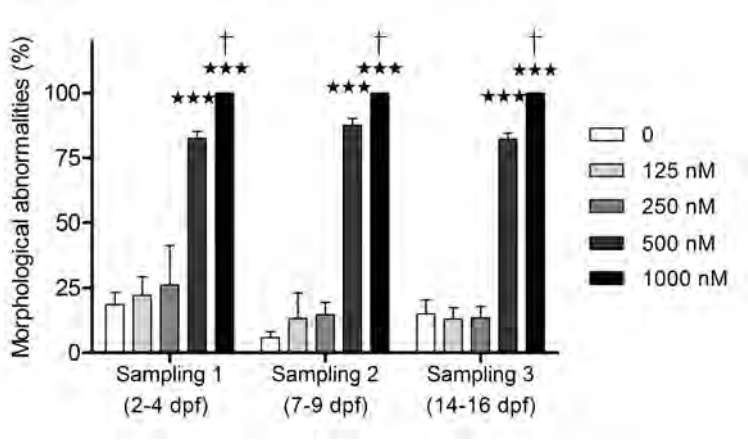

Figure 5. Morphological abnormalities (%) of *P. miliaris* larvae exposed to TCS from 0 -16 dpf (experiment B). Each bar represents the average (%) from 3 repeated observations of 20 larvae for each sampling plus the standard deviation (n=2). ****p*<0.001 (Two-way ANOVA with Bonferroni's Multiple Comparison Test). (†) No hatching occurred.

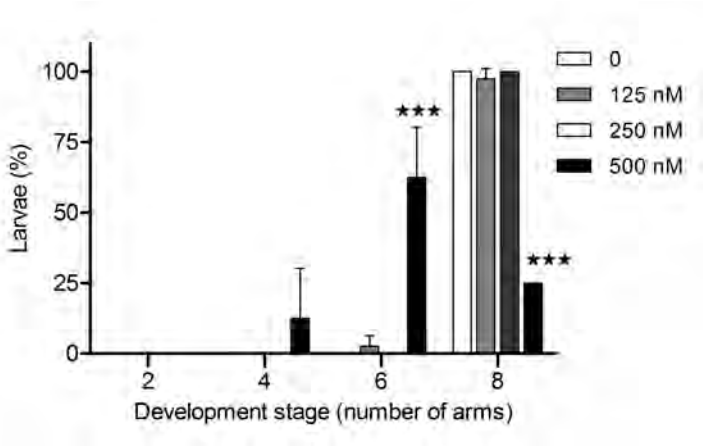

Figure 6. Developmental stages of *P. miliaris* larvae at 16 dpf exposed to TCS (experiment B). Each bar graph represents the average percentage of larvae at a given development stage plus the standard deviation (n=2). ****p*<0.001 (One-way ANOVA with Dunnett's Multiple Comparison Test). Values for 1000 nM TCS are not included since no hatching occurred.

3.2.4 HBCD

Exposure of fertilized eggs up to the highest HBCD test concentration of 100 nM did not cause any adverse effects on hatching success in both experiment A and B (data not shown). However, larvae exposed to 100 nM HBCD developed a significantly higher percentage of morphological abnormalities in sampling 1 (2-4 dpf) and 3 (2-4 dpf) in both experiment A ($p< 0.01$ and $p<0.001$ respectively) and B ($p<0.05$ and $p<0.001$ respectively), while this effect was only statistically significant for sampling 2 (7-9 dpf) in experiment B ($p<0.001$). Morphological abnormalities in larvae exposed to HBCD included the presence of short or deformed larval arms and a slight edema around the larval body that appears to result from abnormal skeletogenesis of skeletal rods as observed for TCS (Fig. 2). The estimated EC_{50} values were the lowest for sampling 3 (14-16 dpf) varying between 88 (95% C.I. 72-109 nM) and 54 nM HBCD (95% C.I. 31-95 nM) for experiment A and B, respectively (Table 2). Larvae in the 50 nM HBCD group had a slightly higher number of developmental penalty points, which strongly increased in the 100 nM HBCD group (Table 3) where most of the larvae were arrested in the 4-armed pluteus stage (Fig. 8).

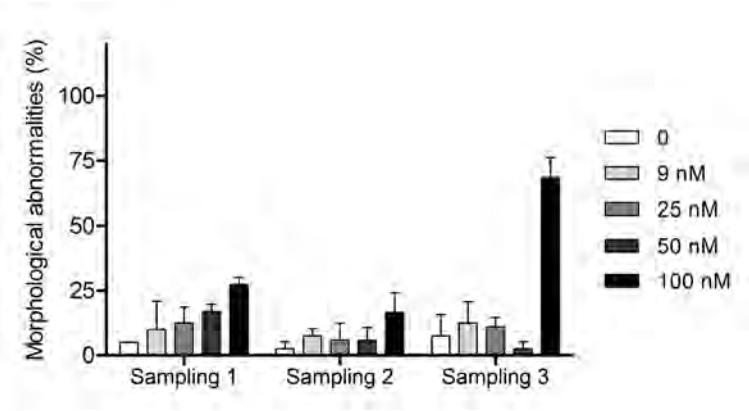

Figure 7. Morphological abnormalities (%) of *P. miliaris* larvae exposed to HBCD from 0 -16 dpf (experiment B). Each bar represents the average (%) from 3 repeated observations of 20 larvae for each sampling plus the standard deviation (n=2). *$p<0.05$; ***$p<0.001$ (Two-way ANOVA with Bonferroni's Multiple Comparison Test).

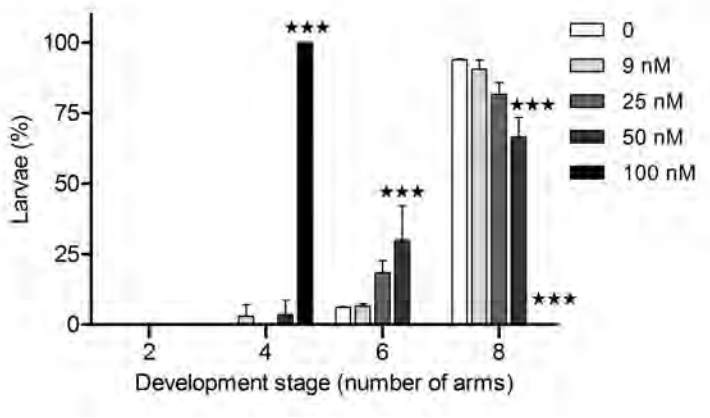

Figure 8. Developmental stages of *P. miliaris* larvae at 16 dpf exposed to HBCD (experiment B). Each bar graph represents the average percentage of larvae at a given development stage plus the standard deviation (n=2). ****p* <0.001 (One-way ANOVA with Dunnett's Multiple Comparison Test).

3.2.5 TBBPA

Even the highest TBBPA exposure (1500 nM) did not cause adverse effects on hatching success; larval development was normal during the observation period of sampling 1 (2-4 dpf) and 2 (7-9 dpf). However, sampling 3 (14-16 dpf) revealed a statistically significant increase in the percentage of morphologically abnormal larvae exposed to 1500 nM TBBPA in experiment A (p<0.01) (Fig. 9), while in experiment B the observed increase was not statistically significant. Abnormal larvae displayed shorter or deformed arms and a mild edema around the larval body, which appears to result from abnormal skeletogenesis of skeletal rods as previously mentioned for TCS and HBCD (Fig. 2). The estimated EC_{50} value for sampling 3 (14-16 dpf) was around 1500 nM TBBPA in experiment B. However, this value could not be precisely quantified due to the steep dose response curve since the concentration below 1500 nM TBBPA did not induce a clear effect. Also, the development stage of larvae at 16 dpf was significantly delayed (Fig. 10), which resulted in a significant increase in the number of developmental penalty points for the 1500 nM exposure group in both experiments (Table 3).

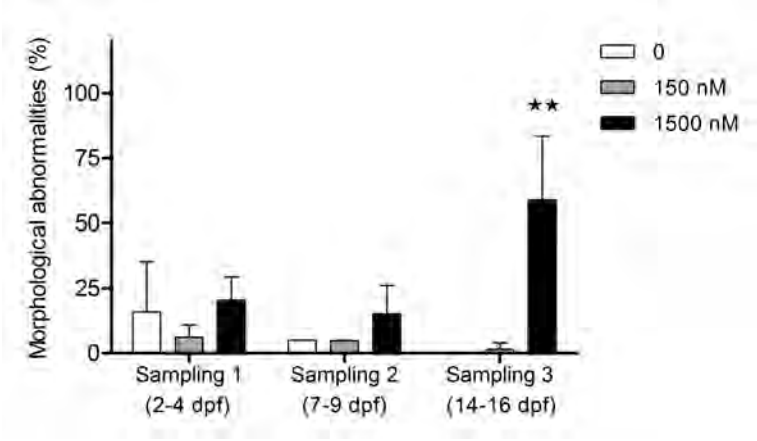

Figure 9. Morphological abnormalities (%) of *P. miliaris* larvae exposed to TBBPA from 0 -16 dpf (experiment A). Each bar represents the average (%) from 3 repeated observations of 20 larvae for each sampling plus the standard deviation (n=2). ***p*<0.01 (Two-way ANOVA with Bonferroni's Multiple Comparison Test).

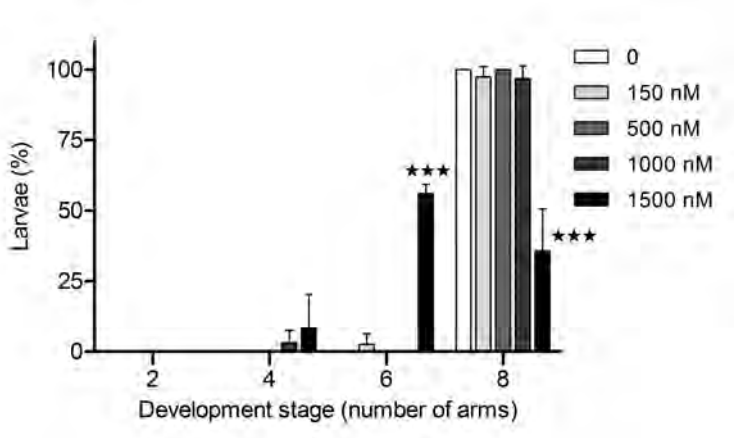

Figure 10. Developmental stages of *P. miliaris* larvae at 16 dpf exposed to TBBPA (experiment B). Each bar represents the average percentage of larvae at a given development stage plus the standard deviation (n=2). ****p* <0.001 (One-way ANOVA with Dunnett's Multiple Comparison Test).

3.2.6. PFOS

Exposure of fertilized eggs to PFOS concentrations up to 743 nM had no effects on hatching success, nor did it induce any morphological abnormalities in larvae (Fig. 11). At 9 dpf, a small dose related acceleration in larval development was observed (Fig. 12), which resulted in negative values

for the number of developmental penalty points. However, by the end of experiment (16 dpf), such advancement of larval development was no longer observed even when expressed as developmental penalty points (Table 3).

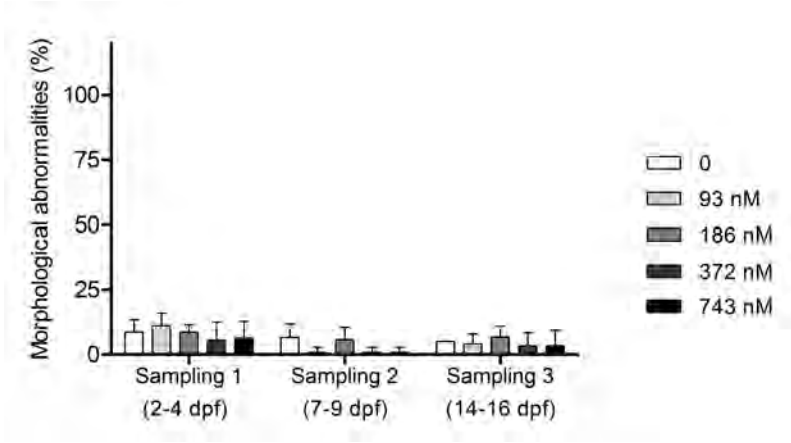

Figure 11. Morphological abnormalities (%) of *P. miliaris* larvae exposed to PFOS from 0 -16 dpf. Each bar represents the average (%) from 3 repeated observations of 20 larvae for each sampling plus the standard deviation (n=2).

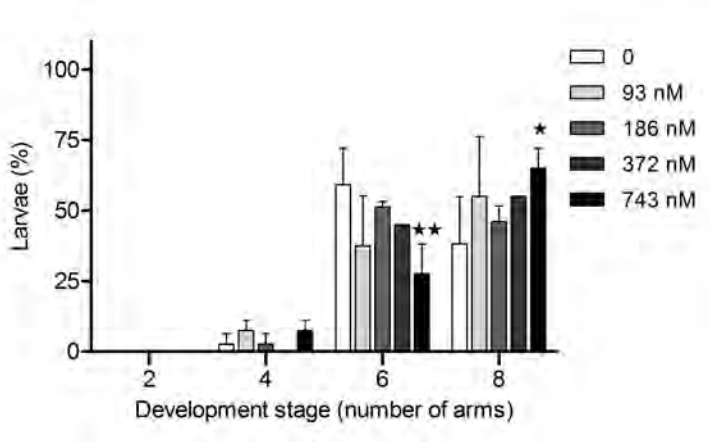

Figure 12. Developmental stages of *P. miliaris* larvae at 9 dpf exposed to PFOS. Each bar represents the average percentage of larvae at a given development stage plus the standard deviation (n=2). **p* <0.05; **p <0.01 (One-way ANOVA with Dunnett's Multiple Comparison Test).

Table 2 Morphological abnormalities EC_{50} values estimated for Triclosan (TCS) and HBCD.

Test compound	TCS						HBCD					
Experiment	A			B			A			B		
Sampling	1	2	3	1	2	3	1	2	3	1	2	3
EC 50 (nM)	308	321	339	270	313	336	NC	NC	88	70	63	54
95% C.I. (nM)	107-886	104-984	107-1077	128-568	133-735	142-778	NC	NC	72-109	32-153	35-114	31-95

NC – Not possible to calculate.

4. Discussion

This study describes the successful development of a novel 16-day echinoid ELS bioassay for the detection and evaluation of developmental effects resulting from exposure to environmentally relevant marine POPs (*i.e.* PCB 126, TCDD, TCS, HBCD, TBBPA, PFOS). In this prolonged ELS bioassay, *P. miliaris* embryos and larvae were continuously exposed to test compounds for 16 days and toxic effects were quantified at different time points using the following endpoints: larval development stages, morphological abnormalities and mortality.

4.1. Adult maturation

Adult *P. miliaris* are easy to aquaculture under laboratory conditions. However, for the successful application of the ELS bioassay, it is important to have the appropriate culture conditions that enable gonadal maturation of *P. miliaris*, in order to obtain good quality eggs and sperm during extended periods of the year. Methods for artificial maturation have been developed for certain sea urchin species such as *Paracentrotus lividus* (Spirlet et al., 2000) but not yet for *P. milliaris*. In this study, we developed a method to successfully induce gonadal maturation in *P. miliaris* under laboratory conditions by manipulating water temperature and photoperiod, thus extending the fertile period from 3-4 months (Kelly, 2000) to 8-9 months per year.

4.2. Development of ELS bioassay

Our study demonstrates that *P. miliaris* is a suitable test species to perform a prolonged ELS bioassay since larvae can be successfully reared under laboratory conditions in a relatively small volume and specific endpoints can be quantified. The most sensitive toxic endpoint is the larval development stage quantified as penalty points at 16 dpf (Table 3). This means that the effects of non-acutely toxic compounds can be underestimated in the commonly used echinoid early life development bioassays with an observation period of 48 to 72 hours only (Warnau M, 1996; Bellas et al., 2008; Durán and Beiras, 2010). To further investigate the potential underestimation of non-acute toxicity resulting from short term early life development toxicity tests, we compared the average percentage of larval morphological abnormalities at 72 hpf with the values obtained between day 14 and 16 post fertilization corresponding to sampling 3. As observed in table 1, morphological abnormalities caused by exposure to 100 nM HBCD at 72 hpf were either not statistically significant (experiment A), or showed a lower *p* value (experiment B), compared to the results obtained for sampling 3 (14-16 dpf). A similar pattern was observed for TBBPA, where in experiment A 1500 nM

TBBPA did not cause any statistically significant effect at 72 hpf, while for sampling 3 (14-16 dpf) it was significant. However, for experiment B this difference was not statistically at any sampling point. Consequently, another experiment would have to be performed to confirm the significance of this finding. In the case of TCS, no differences were observed between 72 hpf and sampling 3 since this compound was acutely toxic. The delayed toxicity observed for HBCD and TBBPA is in accordance with previously reported observations for prolonged ELS bioassays with amphibians and fish. Also, with these vertebrate larvae, the effects of non-acutely toxic POP's could be seriously underestimated depending on the duration of the test observation period (Gutleb et al., 1999; Gutleb et al., 2007; Foekema et al., 2008).

As previously stated in the results section, the steep dose response curves obtained for TCS, HBCD and TBBPA require a careful interpretation of estimated EC_{50} values. In order to obtain more accurate EC_{50}s, extra data points should be included to increase the accuracy of obtained values. The results of the *P. miliaris* prolonged ELS also indicate that only one scoring per sampling would be sufficient instead of the 3 days observation period following each sampling, as was done in our experiments. No statistically significant differences were observed between the first and third day after sampling which makes the scoring less time consuming.

4.3. Effects of test compounds

4.3.1 Internal concentrations

Since semi-static exposure can result in fluctuating water concentrations, the TCDD-equivalents (TEQs) and chemical levels in exposed *P. miliaris* larvae were measured for internal dose assessment of PCB 126, TCS, TBBPA and PFOS. Because the absence of PCB 126 effects was unexpected, an attempt was made to determine the internal TEQ-levels and compare them to the internal TEQ effect levels in fish larvae reported by Foekema et al. (2008) to show delayed mortality at very low levels. Although internal concentrations could be determined by bioanalysis, which requires less material than chemical analysis, they could not be expressed on larva wet weight or lipid weight basis. Therefore, larval internal concentrations had to be expressed based on the number of larvae, which is difficult to compare with the levels in the fish larvae. However, the internal levels clearly reflected the nominal concentrations (Table 1), demonstrating that the method used led to an effective exposure of the larvae to the test compound.

4.3.2 Dioxin induced developmental effects

In the *P. miliaris* ELS assay, no dose related effect could be observed for PCB 126 at test concentrations up to 0.3 nM, which is equivalent to 0.03 TEQs (Fig. 3), while for TCDD, only at 3 nM TEQs was there a slight but statistically significant effect (Fig. 4). These results are unexpected because in the prolonged ELS with amphibians and fish, exposure to PCB 126 induced effects at concentrations as low as 0.0008 and 0.0003 nM TEQs, respectively, after only 4 days of exposure (Gutleb et al., 1999; Foekema et al., 2008). Dioxin and dioxin-like toxicity in vertebrates is mediated by the aryl hydrocarbon receptor (AhR) (Safe, 1990; Safe, 2001) in the presence of the aryl hydrocarbon receptor nuclear translocator (ARNT) (Whitlock, 1993). In larvae of the related echinoid *Strongylocentrotus purpuratus* the expression of an AhR vertebrate orthologous gene has been shown within 36 hours post-fertilization, and an ARNT vertebrate orthologous gene within 48 hours post-fertilization (Howard-Ashby et al., 2006). However, it is has not yet been shown that this putative AhR has the same activity in echinoids as in vertebrates.

Therefore, the reason behind this apparent lack of sensitivity to dioxin and dioxin-like toxicity is not obvious. Nonetheless, one can speculate that it can be due to a low affinity of the specific echinoid AhR isoform towards dioxin and dioxin-like compounds, similarly to what is suggested by Lavine and co-authors (2005) for the lower sensitivity of two amphibian AhR isoforms (*i. e.* AhR1α; AhR1β) to halogenated aromatic hydrocarbons in relation to other vertebrates.

4.3.3 Triclosan developmental effects

No eggs hatched at test concentrations >500nM TCS in experiment A ($\geqq$ 500 nM in exp. B) (Fig. 5), indicating that P. miliaris embryos are more sensitive to this compound than medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) in which egg hatchability failed at 2160 and 2400 nM, respectively (Ishibashi et al., 2004; Oliveira et al., 2009). The larvae that did hatch at 500 nM TCS in experiment A were able to further develop, but an increase in morphological abnormalities and delayed development were observed. This is in the same order of magnitude as rainbow trout (*Oncorhynchus mykiss*) larvae, which showed a statistically significant decrease in survival following continuous flow-through exposure to 246 nM TCS for 35 days post hatch (Orvos et al., 2002).

The lowest estimated EC_{50} values were observed for sampling 1 (2-4 dpf) in both experiments (Table 2), indicating that embryo and very early larval development are possibly the most sensitive stages for acute effects induced by TCS exposure. This is supported by further experiments conducted with *P. miliaris* larvae, where an acute effect on survival was only observed at 5000 nM TCS when

exposure begins at 20 dpf and larvae are at the late 8-armed pluteus stage (Anselmo et al., in prep.). To improve the accuracy of estimated EC_{50}s, more data points are needed between the 500 and 1000 nM TCS doses to confirm the maximum response obtained at 500 nM in experiment A since between the EC_0 and EC_{100} there is only a 2-fold difference.

4.3.4 HBCD developmental effects

The developing larvae were particularly sensitive to HBCD exposure, with a NOAEC for larval development expressed as penalty points at 16 dpf of 25 nM HBCD, and a NOAEC for morphological abnormalities of 50 nM HBCD. Studies reporting effects of HBCD in aquatic organisms are rather limited, especially for early life developmental effects. In a study conducted with zebrafish embryos exposed to HBCD during 96h post fertilization, a statistically significant reduction in hatching success was observed at 1558 nM HBCD, an increase in developmental abnormalities occurred at concentrations ≥ 156 nM HBCD and a reduction in both survival and heart rates was evident at 78 nM HBCD (Deng et al., 2009). These effects occurred in the same order of magnitude as in sampling 1 (2-4 dpf) of our study with a LOAEC of 100 nM HBCD (Fig. 7). The mechanism suggested to be behind the observed effects in zebrafish appears to be related to the induction of oxidative stress, which then leads to apoptosis (Deng et al., 2009).

Table 3. Average total penalty points/group of 20 animals at 16 dpf (n=2). Larvae were accredited 0 penalty points when they were at the expected 8-armed pluteus developmental stage, 1 point at the 6-armed pluteus stage, 2 points at the 4-armed pluteus stage, and 3 points for the earlier development stages.

PCB 126							
Nominal concentration (nM)			**0**	**0.0003**	**0.003**	**0.03**	**0.3**
Total penalty points		Exp. A	1±1.41	0.5±0.71	1.5±2.12	1.5±0.71	0±0
		Exp. B	1±1.41	0.5±0.71	1±1.41	0±0	0±0
TCS							
Nominal concentration (nM)			**0**	**125**	**250**	**500**	**1000**
Total penalty points		Exp. A	0.5±0.71	0±0	0±0	†	†
		Exp. B	2±1.41	1±1.41	0.5±0.71	28±1.4***	†
HBCD							
Nominal concentration (nM)			**0**	**9**	**25**	**50**	**100**
Total penalty points		Exp. A	0.5±0.71	4±1.41	1±0	5±4.24	49±1.41***
		Exp. B	3.5±2.12	6±0	7±0*	13±0***	44.5±0.71***
TBBPA							
Nominal concentration (nM)			**0**	**150**	**500**	**1000**	**1500**
Total penalty points		Exp. A	0±0	1±1.41	ND	ND	31±4.24***
		Exp. B	3±1.41	1.5±0.71	3±1.41	9±1.41	19.5±6.36**
PFOS							
Nominal concentration (nM)			**0**	**93**	**186**	**372**	**743**
Total penalty points	Exp. A	9 dpf	-6±2.83	-9.5±4.95	-7.5±3.54	-11±0	-11.5±0.7
		16 dpf	1.5±2.12	1.5±2.12	3.5±2.12	1±1.41	0±0

Statistical analysis done by one-way ANOVA with Dunnett's Multiple Comparison Test. *p <0.05; **p<0.01; ***p <0.0001; (†) No hatching occurred; (ND) Not determined.

4.3.5 TBBPA developmental effects

Exposure to 1500 nM TBBPA caused an increase in larval developmental abnormalities, especially during sampling 3 (14-16 dpf). Also, the number of developmental penalty points significantly increased by the end of the test period (16 dpf) (Table 3). This LOAEC is in the same range as in a study conducted with the amphibian *Xenopus tropicalis*, where embryos exposed up to 36h to 1839 nM TBBPA showed a clear (93%) and time dependent increase in morphological abnormalities mostly related to the eyes and the appearance of pericardial edema and a slightly increase in mortality (6%) (Shi et al., 2010).

In vertebrates, the appearance of edema as a result of exposure to toxic compounds, known as blue sac disease, has also been observed for fish early life stages exposed to TCDD and bisphenol A (Spitsbergen et al., 1991; Honkanen et al., 2004) as well as TBBPA. Zebrafish embryos exposed to 1500 nM TBBPA revealed an increase in morphological abnormalities. Similarly to what was observed for amphibians, morphological abnormalities in zebrafish embryos included edema of the pericardial region as well as in the cranial yolk sac region. The etiology of this abnormality is not completely clear but is usually associated with a vascular or osmoregulatory dysfunction (Kuiper et al., 2007). To our knowledge, the occurrence and mechanisms originating edema in invertebrates such as sea urchins has not been well studied. Furthermore, since sea urchins are osmoconformers, it is less plausible that what we name edema in sea urchin larvae has the same etiology as in vertebrates (*e.g.* blue-sac disease in fish larvae).

4.3.6 PFOS developmental effects

No obvious effects were observed upon exposure up to 743 nM PFOS. The only observed effect was an acceleration of development at ≥ 372 nM PFOS, with the majority of larvae already at the 8-armed pluteus stage at 9 dpf instead of being at the 6-armed stage as in the control group (Fig. 12). A similar advance in development was observed in *Rana pipens* tadpoles exposed to 56 and 186 nM PFOS, however, this effect was both small and qualitative (Ankley et al., 2004). The acceleration in development caused by PFOS could be related to the considerable structural similarity of PFOS with fatty acids, which are known to be essential for the normal growth and development of juvenile bivalves (Caers et al., 1998). Therefore, PFOS could stimulate development at low (non-toxic) concentrations by mimicking the action of fatty acids. Such an effect could have consequences in later development stages when the fatty acids are needed for specific functions. This, however, will still need to be studied further.

5. Applicability of the prolonged echinoid ELS bioassay

Based on the findings with the chosen test compounds, we conclude that the newly developed 16-day echinoid ELS bioassay is a tool with an obvious potential in marine toxicology. In follow-up studies the approach could be further fine-tuned to suit specific needs such as less: scorings (only the 1st day after each sampling), more replicates, more doses to accurately assess EC_{50}s, and measuring water concentrations.

The use of echinoids in toxicological studies could contribute to the European Union animal welfare act (86/609/CEE) aimed at reducing the number of vertebrate animals used in toxicity studies. Echinoids, such as *P. miliaris*, show a considerable degree of homology with vertebrates from a physiological point of view (Lavado et al., 2006; Porte et al., 2006) and have been used in marine toxicity testing (Schweitzer et al., 1997; Schipper et al., 2008). Therefore, echinoids are a useful and ecologically relevant animal group that should be taken into account to assess the effects of POPs.

With the very early exposure of fertilized eggs in our study, we tried to mimic maternal transfer of toxic compounds to the eggs. This however, still may give an underestimation of the potential effects, since the embryos have already undergone the first cleavages before exposure occurs, and the adult animals were not affected by toxic compounds thus produced healthy eggs. Therefore, it would be interesting to investigate effects resulting from maternal exposure to POPs on egg quality and early life development due to maternal transfer of POPs to the egg. We could then compare effects resulting from maternal transfer with waterborne exposure based on larvae internal POPs concentrations. .

Further development of *P. miliaris* as an animal model for toxicological studies will also include a new metamorphosis assay focusing on thyroid hormone disrupting effects (Anselmo et al., in preparation).

6. Conclusion

We demonstrated that the 16-day *P. miliaris* ELS bioassay is sensitive to toxic effects on larval morphology and development. Of all the compounds tested, the most toxic was HBCD, while unexpectedly both the dioxin-like PCB 126 and TCDD surprisingly did not induce clear toxicity up to 0.3 nM TEQ. The most sensitive endpoint was the number of developmental penalty points at 16 dpf (e.g. HBCD), while survival as an endpoint proved to be rather insensitive for non-acute toxicity. Quantification of hatching success should also be included as an endpoint since TCS had a clear effect on hatchability, which was not done in the present study.

The newly developed 16-day echinoid ELS bioassay could be applied for environmental risk assessment focusing on the marine environment and to determine the ecological status of coastal areas within the framework of the EU Water Directive (2000/60/EC).

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CHAPTER 3.

Novel echinoid metamorphosis bioassay detects thyroid hormone disrupting effects of persistent organic pollutants.

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Abstract

Persistent organic pollutants (POPs) can disrupt the thyroid hormone (TH) dependent metamorphosis in vertebrates. Similarly, echinoids have a TH induced metamorphosis, making them potential model organisms to study TH disruption. This study describes the development of an echinoid metamorphosis bioassay using the sea urchin *Psammechinus miliaris*. Larvae were exposed to test compounds from the 8-armed pluteus stage until metamorphosis completion. Thyroxine (T4) accelerated metamorphosis (EC_{50} 0.12 and 0.09 nM experiment A and B, respectively), whereas the inhibitors thiourea (TU) (IC_{50} 0.1 and 0.04 mM experiment A and B, respectively) or potassium thiocyanate (KSCN) delayed metamorphosis (IC_{50} <0.1 mM). Polybrominated diphenyl ethers (PBDEs) strongly accelerated metamorphosis (EC_{50} 219 nM), while tetrabromobisphenol A (TBBPA) and triclosan (TCS) delayed it (IC_{50} 97 and 418 nM, respectively). Echinoids are promising marine model organisms for ecotoxicological studies and further insight into TH function may contribute to reduce the use of vertebrates to study TH disruption.

1. Introduction

During early life development thyroid hormones (THs) have an important role in the regulation of key signaling pathways in tissue growth and differentiation, as well as in metabolism in a wide variety of animal groups ranging from invertebrates (*e.g.* echinoids) to mammals (Heyland and Moroz 2005; Jugan et al. 2010; Silva 2001; Tan and Zoeller 2007; Yen 2001). TH function is a rather complex system where regulatory mechanisms are comprised of a cascade of events which include TH synthesis, transport and metabolism. In vertebrates the thyroid gland synthesizes the THs thyroxine (T4), and to a lesser extent, triiodothyronine (T3) (Köhrle et al. 2002; Kuiper et al. 2005; Schuur et al. 1997). THs are released by the thyroid gland into the blood stream and, given their low water solubility, are transported to target tissues bound to plasma proteins, the most important of which are transthyretin (TTR) and thyroxine binding globulin (TBG). Once T4 reaches target tissues it enters the cell via membrane transporters and is converted into T3 by iodothyronine deiodinases type I and II (D1 and D2) (Kuiper et al. 2005; Schuur et al. 1997). The action of T3 is mediated by nuclear receptors known as thyroid hormone receptors (TRs) (Samuels and Tsai 1974). In vertebrates, T3 is regarded as the active TH since it is able to bind to the TRs with an affinity nearly 10 times higher than that of the pro-hormone T4 (Franklyn 1988; KISTLER et al. 1975; Miwa and Inui 1987; Samuels and Tsai 1974).

Disruption of TH function is a major concern since it can lead to abnormal development, altered growth patterns, adverse effects on brain development and neuropsychological deficits in mammals (Dussault and Ruel 1987; Gauger et al. 2003; Murk et al. 1998) as well as abnormal development and physiological disturbances in amphibians and fish (Brar et al. 2010; Gutleb et al. 2000; Schriks et al. 2006). In vertebrates undergoing a TH dependent metamorphosis, several persistent organic pollutants (POPs) have been shown to disrupt the sensitive metamorphic process, resulting in dramatic changes in the animal's body and cellular organization (Gutleb et al. 2000; Gutleb et al. 1999; Gutleb et al. 2007; Soffientino et al. 2010). Polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated biphenyls (PCBs), have been shown to disrupt TH function (Boas et al. 2006; Brouwer et al. 1999; Brouwer et al. 1998; Colborn 2002; Goldey et al. 1995; Marchesini et al. 2008; Miller et al. 2009; Murk et al. 1994a; Murk et al. 1994b). The widely used brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs) and tetrabromobisphenol A (TBBPA), are also able to interfere with the TH system (Lema et al. 2008; Van der Ven et al. 2008; Zhou et al. 2001; Zhou et al. 2002). The ability of PBDEs, particularly their hydroxylated metabolites (PBDE-OH), and TBBPA to interfere with normal TH hormone transport and function is expected based on their structural similarity with TH (Freitas et al. 2011; Hamers et al. 2006). Evidence of such potential is given by the reduction of T4 levels observed in rats exposed to the PBDE technical

mixtures DE-71 and DE-79 (Zhou et al. 2001; Zhou et al. 2002), as well as in fish exposed to PBDE-47 (Lema et al. 2008). Similarly, TBBPA exposed rats showed a decrease in T4 levels and an increase in male pituitary weight (Van der Ven et al. 2008). Also the antibacterial agent Triclosan (TCS), widely used in personal care products, has been shown to reduce T4 and T3 total serum levels in rats by 43 and 89 %, respectively (Paul et al. 2010).

As THs also play a crucial role regulating metamorphosis in amphibian and flatfish species (Inui and Miwa 1985; Kanamori and Brown 1996; Klaren et al. 2008), the metamorphic process is particularly susceptible to disruption by PHAHs. Reported effects include inhibition and/or delay of tadpole metamorphosis (Balch et al. 2006; Cary Coyle and Karasov 2010), and suppression of T3 mediated tail regression (Kitamura et al. 2005). TCS exposure is also suggested to potentially cause adverse effects on amphibian metamorphosis (Veldhoen et al. 2006b).

Marine invertebrates such as echinoids (Echinodermata: Echinoidea) are known to have a TH induced metamorphosis during early life development (Chino et al. 1994; Heyland et al. 2004; Saito et al. 1998). In echinoids, T4 is regarded as the key TH inducing metamorphosis with a potency 10 times greater than T3 (Chino et al. 1994). In amphibians and flatfish, T3 is the biologically active TH (Esther Isorna 2009; KISTLER et al. 1975).

Once the planktonic echinoid larva reaches the early 8-armed pluteus stage, the formation of the sea urchin rudiment begins under the control of THs. The rudiment is a structure resulting from the fusion of the aminiotic sac (vestibule) with the hydrocoel located on the left side of the larva body next to the stomach (Cameron and Hinegardner 1974; Cameron and Hinegardner 1978; Chino et al. 1994). When the sea urchin rudiment is fully formed the larva becomes competent and metamorphosis can be completed. During the metamorphic process the larval body is completely reabsorbed by the sea urchin rudiment giving origin to a juvenile echinoid (Chino et al. 1994). In order for a larva to become competent, it is thought that a cue present in the substrate is required to induce larval settlement. Some of the substances thought to provide such cue include lipophilic inducers (*e.g.* free fatty acids) found in coralline red algae and marine microbial films (Bishop et al. 2006; Kitamura et al. 1993; Pearce and Scheibling 1991).

This study describes the development and validation of a metamorphosis bioassay using the echinoid *P. miliaris* to study the ability of POPs to disrupt TH function. In addition to being one of the few marine species for which an *in vivo* bioassay is available, the development of echinoids as model organisms for TH disruption studies has the advantage of reducing the use of vertebrates in toxicity studies (*e.g.* amphibians).

The newly developed *P. miliaris* echinoid metamorphosis assay was first validated with T4 as a metamorphosis inducer, thiourea (TU) as an inhibitor of TH synthesis (Heyland 2004) and potassium thiocyanate (KSCN) as inhibitor of iodine uptake (Manzon et al., 2001). The assay was then carried out to study the ability of an field relevant PBDE mixture, TBBPA, and TCS to interfere with the TH induced metamorphosis of the echinoid *P. miliaris*.

2. Materials and methods

2.1. Adult animals

Sea urchins (*Psammechinus miliaris*) were collected from the Eastern Scheldt (The Netherlands) and maintained in fiber glass tanks (L*W*W in cm = 200*80*30) under controlled conditions (*i.e.* temperature, photoperiod and food availability). See Anselmo et al., 2011 for further details.

2.2. Gamete collection and fertilization

Adult *P. miliaris* were dissected for collection of eggs and sperm. Eggs used in the experiments were obtained from batches with a percentage of mature eggs of at least 90%. Eggs were considered mature when they had a spherical shape and no spot/droplet was visible in the cytoplasm (Environment Canada, 1992). Sperm was considered mature if it became clearly active in artificial sea water (ASW) (Environment Canada, 1992). Fertilization took place according to Anselmo et al., 2011. Fertilization success was at least 90%, in accordance to recommendations made by Environment Canada (1992) and USEPA (2002) for bioassay validation.

ASW was prepared using Instant Ocean® synthetic salts (Spectrum Brands, Inc.) and aged under continuous aeration for at least 1 week (salinity 31±1 ‰).

2.3. Test Compounds and stock solutions

Tetrabromobisphenol A (TBBPA; CAS: 79-94-7; purity 97%; Sigma-Aldrich), and triclosan (TCS; CAS: 3380-34-5; purity ≥97%; Sigma-Aldrich) were prepared in dimethyl sulfoxide (DMSO; CAS: 67-68-5; purity 99,9%, Sigma-Aldrich) and stored in the dark at room temperature.

A polybrominated diphenyl ether (PBDE) mixture was prepared in the same concentration ratio as found in sole (*Solea solea*) collected from the Western Scheldt in 2005 (Table 1). This mixture was

selected as a model marine mixture in a polluted Dutch estuary and is currently also used for other ecotoxicological studies. BDE-28, -47, -99, 100 and 153/4 (purity ≥99%; Sigma-Aldrich) were used to prepare the mixture stock in DMSO according to the proportions of each congener described in Table 1.

Stocks of thyroxine (T4; CAS: 51-48-9; purity ≥98 %, Sigma-Aldrich) were dissolved in 1mM NaOH and then diluted with MiliQ water; thiourea (TU; CAS: 62-56-6; purity ≥99%, Sigma-Aldrich) and potassium thiocyanate (KSCN; CAS: 333-20-0; purity ≥99%, Sigma-Aldrich) were dissolved directly in ASW. Stocks were stored at -20 ºC until use.

Figure 1 presents the structures of the TH-like compounds tested in comparison to T4.

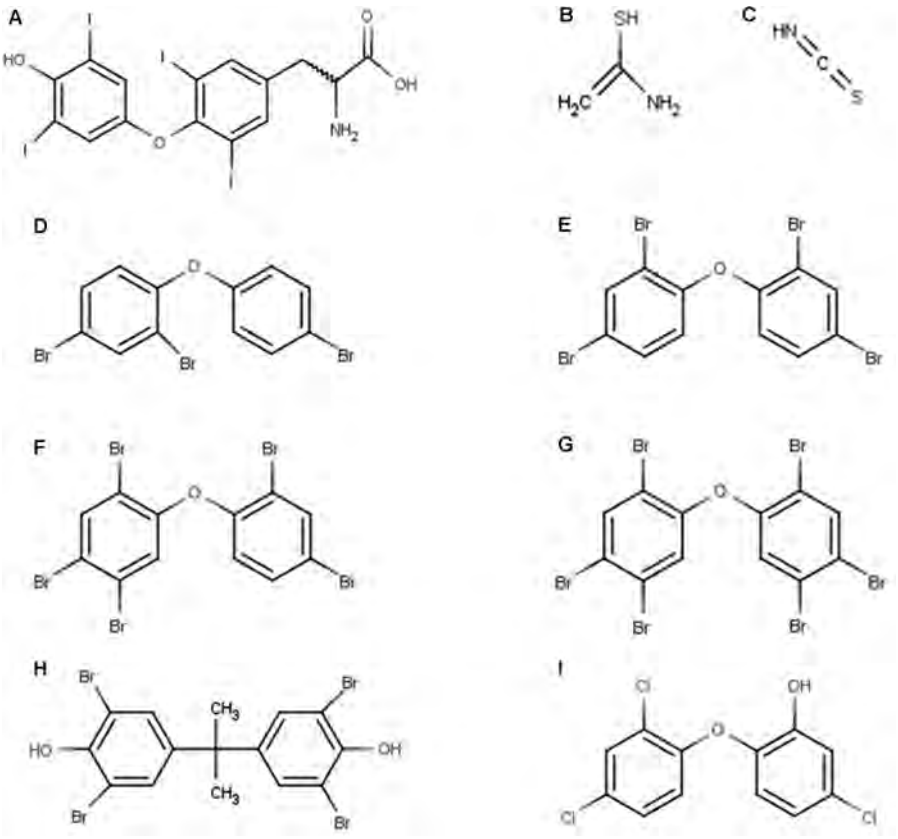

Figure 1. Chemical structures of test compounds. A - T4 (Thyroxine - CAS: 51-48-9); B - TU (Thiourea - CAS: 62-56-6); C – KSCN (Potassium thiocyanate - CAS: 333-20-0); D – BDE-28 (CAS: 41318-75-6); E - BDE 47 (CAS: 5436-43-1); F - BDE 99 (CAS: 60348-60-9); G - BDE 153/4 (CAS: 68631-49-2); H - TBBPA (CAS: 79-94-7); I - Triclosan (CAS: 3380-34-5). The structures of BDE 100 (CAS: 189084-64-8) and BDE 154 (CAS: 207122-15-4) are not represented.

Table 1. Proportion of brominated diphenyl ether (BDE) congeners in the stock solution needed to reach BDE levels in exposed *P. miliaris* larvae with the same relative BDE distribution as Sole (*Solea solea*) collected from the Western Scheldt (The Netherlands).

BDE congeners	log Kow[1)]	Relative proportion of BDE congeners in stock solutions (% mass)	Expected BDE congener proportions in the larval lipid fraction based on log Kow values (%)	BDE congener proportions in Sole (% mass)[2)]
28	5.94	29	4	4
47	6.81	56	51	48
99	7.32	12	36	36
100	7.24	3	7	7
153/4	7.86	0.2	2	2
	∑ BDEs	100	100	97

1) Log octanol-water partitioning coefficients (log Kow) based on Braekevelt et al., 2003.
2) Unpublished data.

2.4. Test method

Following fertilization, embryos were reared in ASW at a density of approximately 0.5 larvae/ml. From 2 days post-fertilization (dpf) onwards, the larvae were fed with a diet of microalgae (*Dunaliella sp.*) at densities of 1500, 2500, and 4000 cells/ml for the 4-, 6- and 8-armed pluteus stages, respectively (Kelly et al. 2000). Twice a week, 50% of the water was refreshed using a mesh as described in Anselmo et al., 2011.

At 20 dpf, the larvae reached the 8-armed pluteus stage with the sea urchin rudiment between stage J and K as described by Chino et al. (1994). A total of 15 larvae were then sampled into a 100 ml glass beaker with 40 ml of ASW spiked with the desired test concentrations (Fig. 2). Larval density was 0.4 larvae/ml at the beginning of the experiment. When DMSO was used as solvent, the final concentration was always 0.1 %. In order to provide a cue to induce larval settlement for completion of metamorphosis, test beakers were preconditioned during 3 days with 20 ml ASW containing 100 µl of a concentrated solution of *Dunaliella sp.* debris and 2 mg/ml of TetraMin. Following the preconditioning period, ASW was removed, leaving a biofilm covering the bottom of test beakers. Test concentrations were prepared in these beakers and larvae were added. For all test compounds, except for KSCN alone and the combined exposure to T4+KSCN and T4+TU, 2 independent experiments were conducted (*i.e.* A and B). Experiments were always conducted in duplicate (n=2) with 15 larvae per replicate.

Test beakers were placed in a climate room at 19±1 °C, with a photoperiod of 16 hours light: 8 hours dark throughout the entire test period at a salinity of 31±1 ‰. Feeding was done every other day using the microalgae *Dunaliella sp.* at a density of 4000 cells/ml. Twice per week, 10 ml of ASW at the desired test concentration was added to each replicate. Once the volume in the test beakers reached

80 ml half of it was removed (40 ml) and 10 ml was added again. Subsequently, water refreshments continued as described until the experiment was terminated.

The endpoints quantified were: time to completion of metamorphosis into juvenile, morphological abnormalities in juveniles, and mortality. Metamorphosis was considered completed when the entire larvae body was reabsorbed, and the experiment was terminated when 80 % of the larvae in the ASW and DMSO control groups completed metamorphosis. The DMSO control group is referred as the zero (0).

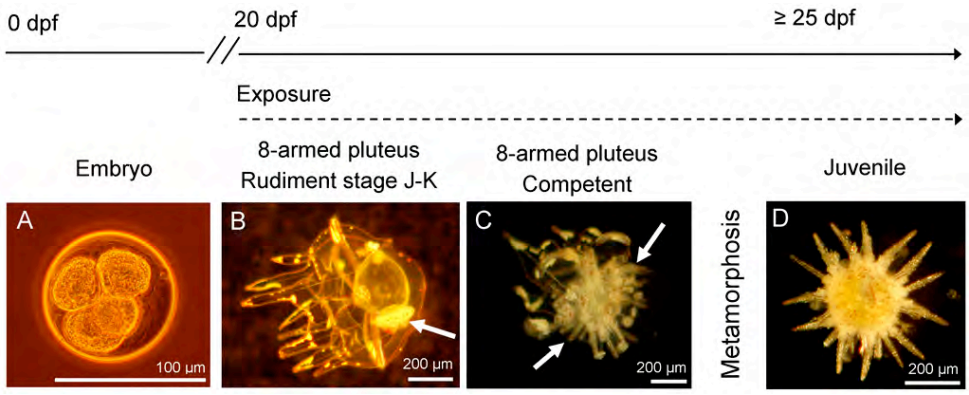

Figure 2. Echinoid metamorphosis bioassay experimental design. *P. miliaris* embryos (A) were reared at a density of 0.5 larvae/ml until reaching the 8-armed pluteus stage and rudiment stage J or K at 20 dpf (B). A total of 15 larvae per replicate (n=2) were then transferred into a 100 ml test beaker containing 40 ml of ASW spiked with the test compound at the desired concentration. Twice a week 10 ml of spiked ASW were added until the end of the experimental period. Arrow indicates the rudiment.

2.5. Statistics

Statistical analysis was performed using GraphPad Prism software (version 5). To determine EC_{50} and IC_{50} values, a sigmoidal dose-response model was fitted through the experimental data and the R^2 value is provided as an indication for the goodness of fit.

The significance of differences in % of morphological abnormalities between the control groups and treatments were determined using an one-way ANOVA followed by Dunnett's Multiple Comparison Test or a *t*-test.

3. Results

3.1. Larval development in control groups

The developed method allowed larvae to grow and develop normally with the first individuals completing metamorphosis as soon as 5 days after the start of the experiment (corresponding to 25 dpf). Although DMSO control larvae generally underwent metamorphosis slightly quicker than in the ASW control group, the percentage of metamorphosed individuals did not differ significantly at the moment when 80% of the DMSO control larvae completed metamorphosis. The incidence of morphological abnormalities in ASW or DMSO control juveniles was always <10%. The moment when 80% of the control larvae completed metamorphosis and experiments were terminated varied between 25 to 55 test days. The average duration of experiments until 80% of the control larvae completed metamorphosis was 40±12 days. However, if experiments were terminated when 50% of the control larvae completed metamorphosis the average duration was 29±12 days.

Table 2. Comparison of estimated EC_{50} or IC_{50} values for *P. miliaris* larval metamorphosis based on a metamorphosis success of 50 and 80 % in control groups as criteria to terminate experiments (for each experiment n=2, 15 larvae/replicate).

		Metamorphosis success (%)	
Test compound	Experiment	50	80
T4 [nM][1)]	A	0.14 (R^2 = 0.33)	0.09 (R^2 = 0.65)
	B	0.19 (R^2 = 0.53)	0.12 (R^2 = 0.88)
TU [mM]	A	0.08 (R^2 =0.93)	0.09 (R^2 = 0.75)
	B	0.06 (R^2 = 0.87)	0.05 (R^2 = 0.96)
KSCN [mM] [2)]	A	<1	<1
PBDE mixture [nM][1)]	A*	ND	ND
	B	1166 (R^2 = 0.94)	219 (R^2 = 0.99)
TBBPA [nM]	A*	ND	391 (R^2 = 0.84)
	B	115 (R^2 =0.92)	97 (R^2 = 0.86)
Triclosan [nM]	A*	154 (R^2 = 0.82)	581 (R^2 = 0.93)
	B	240 (R^2 = 0.80)	418 (R^2 = 0.86)

1) Estimated EC_{50} values were calculated when larvae exposed to the highest test concentration reached 50 or 80% metamorphosis success.

2) Only 1 experiment was conducted

*) Pilot test

ND) Not determined since no reliable dose response could be fitted.

3.2. Effects of T4 and TH inhibitors on metamorphosis

T4 clearly accelerated metamorphosis in a dose dependent fashion as can be seen in Fig. 3 - A, B. Larvae exposed to 1 nM T4 already started to metamorphose after test day 3 (experiment B) and 5 (experiment A). At test day 11 (experiment A) and 14 (experiment B) larvae exposed to 1 nM T4 reached 80% metamorphosis success. The corresponding EC_{50} for metamorphosis acceleration was 0.09 (R^2 = 0.65) and 0.12 nM T4 (R^2 = 0.88) for experiment A and B, respectively (Table 2). Larvae in the ASW control group started to metamorphose at test day 5 (experiment A) and 14 (experiment B), reaching ≥80% metamorphosis success only at test day 25 and 39, respectively.

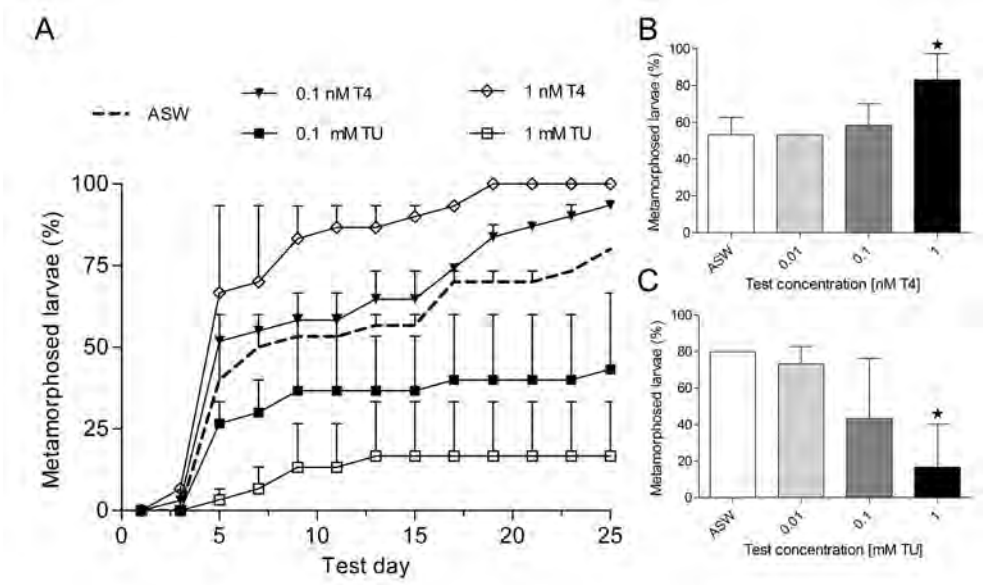

Figure 3. A - Metamorphosis of *P. miliaris* larvae exposed to T4 (Thyroxine) and TU (Thiourea). The 0.01 nM T4 and 0.01 mM TU concentrations were not included for clarity reasons; B - Percentage of metamorphosed larvae exposed to T4 at test day 25; C - Percentage of metamorphosed larvae exposed to TU at test day 25. Each value represents the mean ± SD (n=2) (experiment B). Statistical differences were determined using a *t*-test, **p* <0.05.

The TH synthesis inhibitor TU delayed metamorphosis at test concentrations ≥0.1 mM (Fig. 3 - A, C). Larvae exposed to 0.1 mM TU started to metamorphose at test day 5 (experiment A) and 18 (experiment B).When the ASW control group reached 80% metamorphosis success, at day 25 (experiment A) and 39 (experiment B), only 16±24% and 0±0% of larvae exposed to 1 nM TU completed metamorphosis, respectively. At test day 25 (experiment A) and 39 (experiment B), the corresponding IC_{50} for metamorphosis delay was 0.09 (R^2 = 0.75) and 0.05 mM TU (R^2 = 0.96), respectively (Table 2).

In another experiment, larvae were exposed to the iodine uptake inhibitor KSCN and a similar delay to TU was observed (Fig. 4 - A). When the ASW control group reached 80% metamorphosis success at test day 31, only 33±9, 23±4 and 23±4% of the larvae were able to metamorphose at 1, 5, and 10 mM KSCN, respectively (Fig. 4 - B). At test day 31, the estimated IC_{50} of 0.1 mM KSCN was unreliable since it was an order of magnitude lower than the lowest test concentration, and it was considered to be <1 mM KSCN (table 2). An experiment was also performed where larvae were exposed to a binary mixture containing the highest concentration of TU (0.1 mM) and KSCN (1 mM)

in combination with 1 nM T4 (Fig. 4 - A, C). Larvae exposed to both TU and KSCN in combination with T4 were able to complete metamorphosis as fast as larvae exposed only to 1 nM T4 (Fig. 4 - C).

No statistically significant differences in mortality or morphological abnormalities were found between controls and dose groups (data not shown).

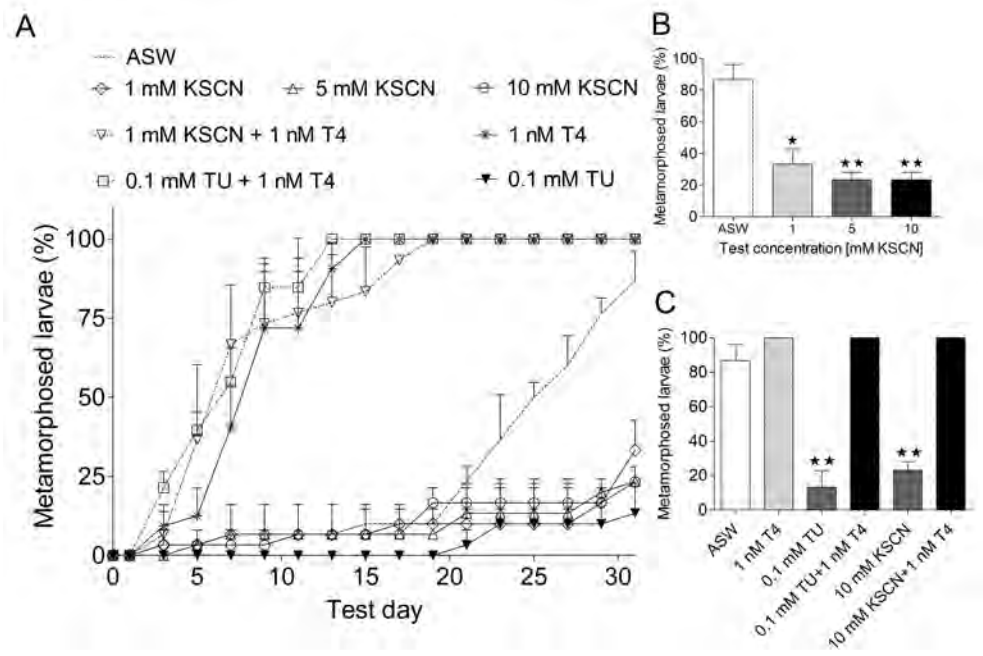

Figure 4. Metamorphosis of *P. miliaris* larvae exposed to KSCN (Potassium thiocyanate), TU (Thiourea) and T4 (Thyroxine); B - Percentage of metamorphosed larvae exposed to KSCN at test day 31; C - Percentage of metamorphosed larvae exposed to KSCN, TU and T4 at test day 31. Larvae exposed to 5 and 10 mM KSN in combination with 1 nM T4 induced similar results as 1 mM KSCN + 1 nMT4, data not shown. Each value represents the mean±SD (n=2, 15 larvsae/replicate). Statistical differences were determined using a *t*-test, **p* <0.05; ***p* <0.01.

3.3. Effects of POPs on metamorphosis

3.3.1. PBDE mixture

In the first experiment (A), exposure to ≥ 12 nM PBDE mixture accelerated metamorphosis between test days 7 and 17 compared to the DMSO control group, but by the end of the experiment this difference disappeared (data not shown). To further investigate the observed acceleration of metamorphosis, a second experiment (B) was conducted including two higher concentrations (320 and

3200 nM) of the PBDE mixture. A clear dose related acceleration of metamorphosis was observed at concentrations ≥32 nM PBDEs (Fig. 5). Larvae exposed to 3200, 320 and 32 nM PBDEs started to complete metamorphosis at test day 1, 3, and 5, respectively, while the first larvae in the DMSO control group completed metamorphosis at day 13. At test day 5, larvae exposed to 3200 nM PBDEs reached 80% metamorphosis success. The EC_{50} for acceleration of metamorphosis was 219 nM PBDEs (R^2 = 0.99) (Fig. 6). Similarly to experiment A, no dose related increase in morphological abnormalities were observed, although in all PBDEs concentrations the percentage of morphological abnormalities was higher than in the DMSO control group (Table 3). PBDEs did not affect survival in both experiments.

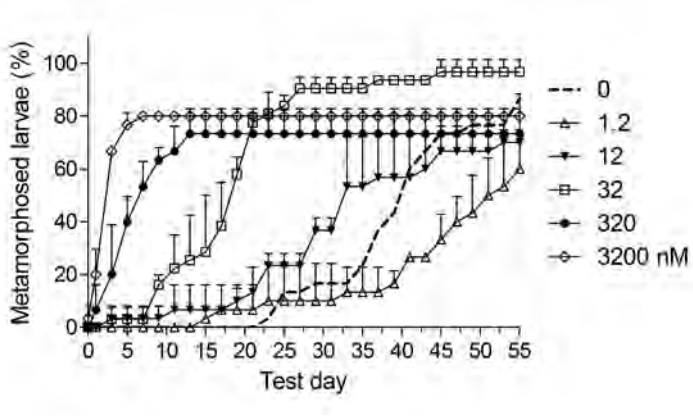

Figure 5. Metamorphosis of *P. miliaris* larvae exposed to the PBDE mixture (experiment B). Each value represents the mean ± SD (n=2, 15 larvae/replicate).

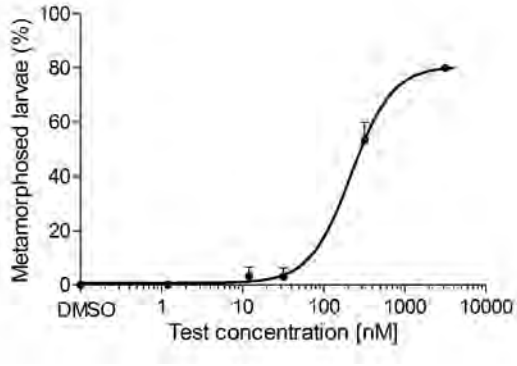

Figure 6. Effect of the PBDE mixture on *P. miliaris* metamorphosis (experiment B). Selected time point (test day 7) corresponds to the moment when 80% of larvae exposed to 3200 nM PBDEs completed metamorphosis. Each value represents the mean ± SD (n=2, 15 larvae/replicate).

Table3. Morphological abnormalities in *P. miliaris* juveniles (mean ± SD; for each experiment n=2, 15 larvae/replicate). Values presented correspond to a metamorphosis success of 50 and 80 % in control groups used as criteria to terminate experiments.

Test compound		Metamorphosis (% completed)	At test day	Concentration (nM)					
				0	**1.2**	**12**	**32**	**320**	**3200**
PBDE mixture	Experiment A	50	29	0±0	5±7	0±0	0±0	(NT)	(NT)
		80	45	3±5	3±5	3±4	11±3	(NT)	(NT)
	Experiment B	50	47	4±5	8±0	24±5	12±8	31±9*	26±9
		80	55	4±5	12±5	31±5*	12±8	31±9*	26±9
				Concentration (nM)					
				0	**7.5**	**75**	**150**	**1500**	**3000**
TBBPA	Experiment A	50	29	0±0	(NT)	(NT)	50±71	100±0***	100±0***
		80	45	3±6	(NT)	(NT)	70±14***	100±0***	100±0***
	Experiment B	50	47	3±5	10±5	37±14	63±24	70±42	100±0*
		80	55	3±5	10±5	37±14	77±24*	70±42	100±0*
				Concentration (nM)					
				0	**100**	**200**	**1000**	**5000**	
Triclosan	Experiment A	50	33	4±6	(NT)	18±2	60±57	100±0*	
		80	39	7±9	(NT)	12±3.0	44±39	100±0**	
	Experiment B	50	17	0±0	0±0	0±0	35±21*	100±0***	
		80	25	0±0	2±3	0±0	50±24*	100±0***	

Statistical analysis performed by one-way ANOVA with Dunnett's Multiple Comparison Test *p <0.05; **p <0.01; ***p <0.001. NT) - Not tested.

+

3.3.2. TBBPA

Two experiments were also conducted with TBBPA. In the first experiment (A) all test concentrations resulted in a reduction in the percentage of metamorphosed juveniles compared to the DMSO control group. Upon exposure to 3000 nM TBBPA, no larvae completed metamorphosis while no increase in mortality occurred. At 1500nM TBBPA only 17% of the larvae were able to metamorphose and all suffered from a complete or partial absence of spines and edema (Fig. 7 - D, E, G). Larvae exposed to the lowest test concentration (150 nM TBBPA) were clearly delayed in their metamorphosis, and 70±14% of the juveniles had morphological abnormalities (Table. 3).

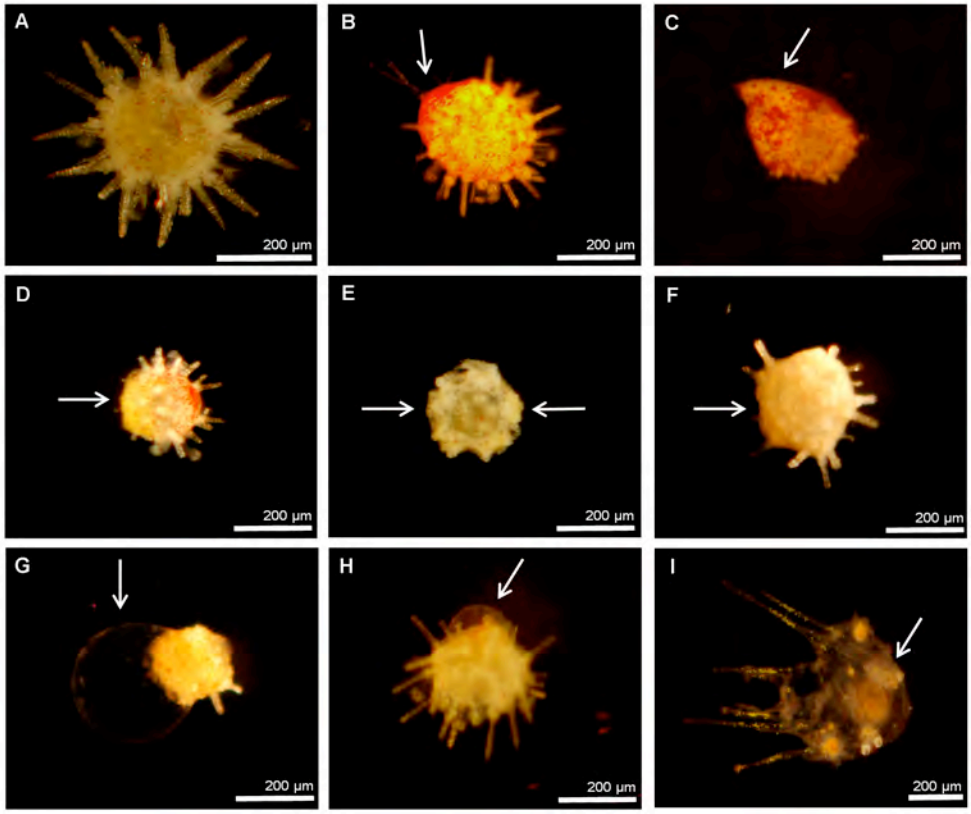

Figure 7. Normal *P. miliaris* juvenile (A) and exposed juveniles with morphological abnormalities. Larval arm rods remain attached to juvenile and incomplete reabsorption of larval body is also present (B - 320 nM, C - 3200 nM PBDE mixture). Complete or partial absence of spines (D, E - TBBPA 1500 nM; F – TCS 1000 nM). Presence of edema (G - TBBPA 1500 nM; H – TCS 1000 nM). Arrows indicate the morphological abnormality. I - Dead larvae at test day 1 exposed to 5000 nM TCS (arrow indicates the rudiment).

In the second experiment (B) two lower concentrations of 7.5 and 75 nM TBBPA were also included, and the same dose related reduction in the percentage of metamorphosed juveniles was observed (Fig. 8). At 3000 nM TBBPA almost no larvae completed metamorphosis, while at 1500, 150 and 75 nM TBBPA only 7±0, 17±24, and 67±19%, respectively, were able to metamorphose. A significant increase in morphological abnormalities (*i.e.* complete absence of spines and edema) was observed at concentrations ≥75 nM TBBPA (Table 3). The IC_{50} and EC_{50} values for metamorphosis delay (Fig. 9 - A) and morphological abnormalities in juveniles (Fig. 9 - B) were 97 nM (R^2 = 0.86) and 71 nM TBBPA (R^2 = 0.77), respectively. TBBPA did not cause a dose related increase in mortality in either of the experiments.

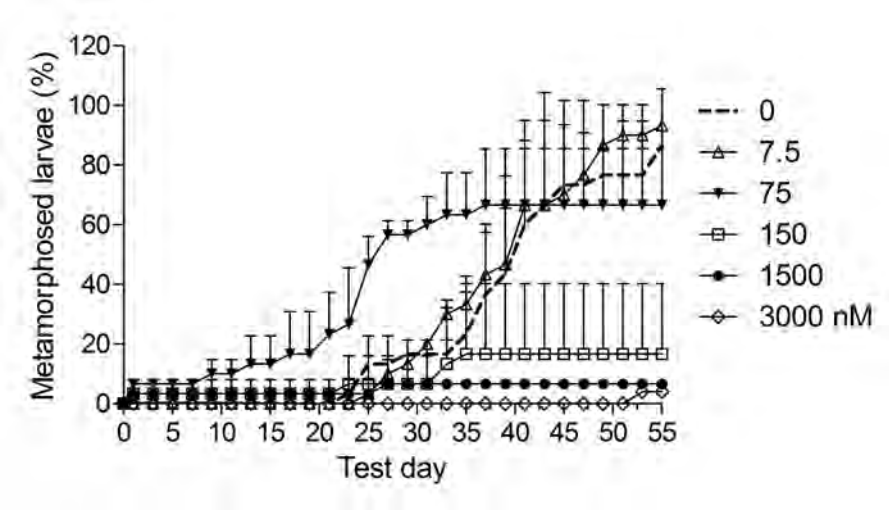

Figure 8. Metamorphosis of *P. miliaris* larvae exposed to TBBPA (experiment B). Each value represents the mean ± SD (n=2, 15 larvae/replicate).

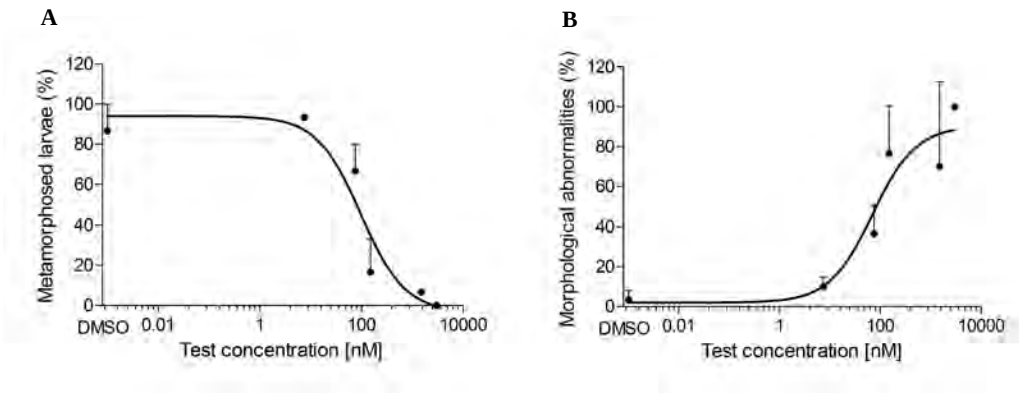

Figure 9. Effects of TBBPA on: A – *P. miliaris* larval metamorphosis. B – Juvenile morphological abnormalities. Selected time point (test day 55) corresponds to the moment when 80% of larvae in the control groups had completed metamorphosis (experiment B). Each value represents the mean ± SD (n=2, 15 larvae/replicate).

3.3.3. Triclosan

In both experiments TCS was acutely toxic at 5000 nM, leading to complete larval mortality within 24h (Fig. 7 - I). For experiment A, exposure to concentrations ≥ 200 nM TCS significantly delayed metamorphosis compared to the DMSO control group. The percentage of metamorphosed larvae at the end of the experiment was reduced to 57±14% and 41±0.8% for 200 and 1000 nM, respectively (data not shown). When a lower concentration was added in experiment B, results were similar to experiment A, with a metamorphosis success of 50±24% and 30±14% at 200 and 1000 nM TCS, respectively (Fig. 10). The IC_{50} values were identical in both experiments: 581 nM TCS (R^2 = 0.93) for experiment A (data not shown), and 418 nM (R^2 = 0.86) for experiment B (Fig. 11). In both experiments, 1000 nM TCS clearly induced occurrence of morphological abnormalities (Table 3). Abnormalities commonly observed included a total or partial absence of spines, and the presence of edema (Fig. 7 - F, H).

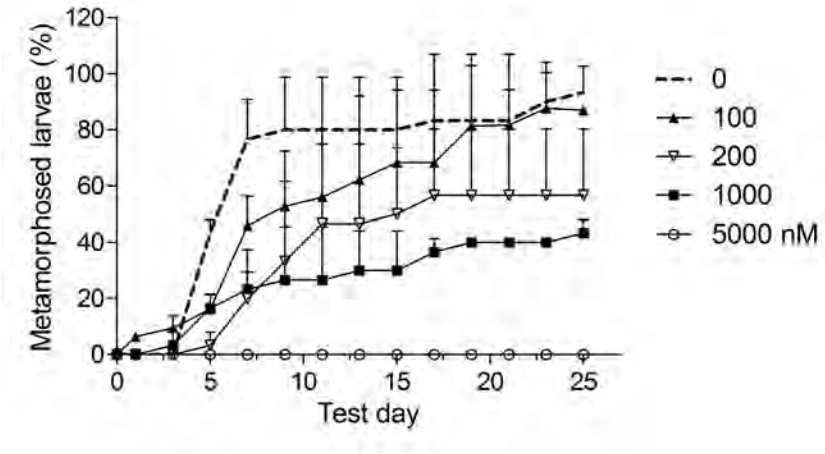

Figure 10. Metamorphosis of *P. miliaris* larvae exposed to TCS (experiment B). Each value represents the mean ± SD (n=2, 15 larvae/replicate).

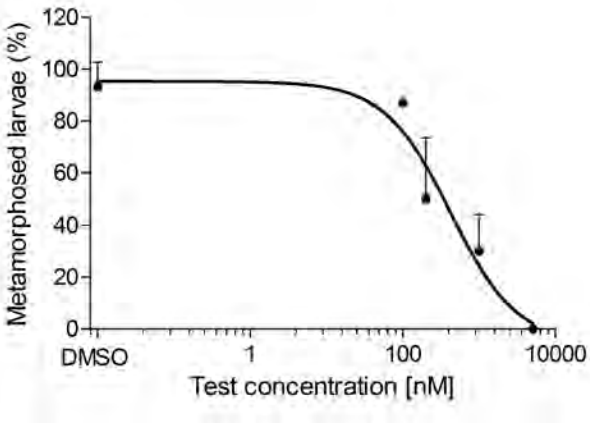

Figure 11. Metamorphosis of *P. miliaris* larvae exposed to TCS (experiment B). Selected time point (test day 25) corresponds to the moment when 80% of larvae in the control groups had completed metamorphosis (experiment B). Each value represents the mean ± SD (n=2, 15 larvae/replicate).

4. Discussion

This study describes the successful development of a small scale metamorphosis assay with the echinoid species *P. miliaris*. Exposure to T4 accelerated metamorphosis, while TU and KSCN delayed it in a dose dependent manner, demonstrating that *P. miliaris* has a TH induced metamorphosis. The PBDE mixture significantly accelerated metamorphosis, while TBBPA and TCS exposure significantly delayed it.

4.1. Larval development in control groups

For practical reasons and to prevent the risk of variations (including incidental mortality) in natural sea water quality, larvae were reared in ASW instead of natural FSW according to Anselmo et al., 2011. This proved to be effective in avoiding interferences with the metamorphic process, since larvae reared with ASW metamorphosed normally and no incidental mortality was observed.

We used 0.1% as the final DMSO test concentration since in a previous study effects of DMSO on echinoid embryo development and larval growth were only reported at 1% (Bellas et al. 2005). As expected, we did not see occurrence of morphological abnormalities or differences in survival rates between the DMSO and ASW control groups. However, DMSO control larvae metamorphosed slightly faster than those in the ASW control group. Due to this fact results are always reported in relation to the DMSO control. Slightly faster development of aquatic organisms (*i.e.* flat fish and amphibians) has been observed at 0.1% DMSO (personal communication: Edwin Foekema and Arno Gutleb), which could be due to a slight anti-bacterial effect or enhanced solubility of nutrients.

Independently of the water source used, the time required for 80% of the larvae in the control groups (*i.e.* ASW and DMSO) to complete metamorphosis ranged from 25 (TCS, experiment B) to 55 days (TBBPA and PBDE, experiment B) after the start of exposure (20 dpf). This variation between experiments could be due to the absence of a natural biofilm on the bottom of the test beakers, which is thought to act as a cue to induce larvae settlement and completion of metamorphosis (Kitamura et al. 1993). In our experiments we provided the larvae with an artificial biofilm consisting of TetraMin and *Dunaliella sp.* debris to mimic the cue present in natural conditions but avoid biological contamination with organisms (*e.g.* nematodes) even present in filtered (0.2 µM) natural sea water. Although larvae were also fed with an optimal food supply of *Dunaliella sp.*at 4000 cells/ml (Kelly et al. 2000), as the nutritional condition of larvae is also of great importance for the metamorphosis success, it is possible that the cue was not yet optimal. An option to provide the appropriate cue in a standardized way could be to maintain a biofilm-producing microbial culture based on natural sea

water in the laboratory, which can then be used to precondition test beakers prior to the start of an experiment. Alternatively, a select mixture of lipids present in such a biofilm could be provided, as they have been suggested to act as the required cue. Free fatty acids in particular seem to be responsible for such a cue, as has been shown in two sea urchins species (*i.e. Pseudocentrotus depressus* and *Anthocidaris crassispina*) (Kitamura et al. 1993). Identification and optimization of this cue is important to further standardize the echinoid metamorphosis assay and minimize variation and time to completion of metamorphosis.

Another approach to reduce the variation in the duration of experiments is to use a 50% metamorphosis success threshold instead of the 80% used in the present study, which would allow a reduction in the average duration of an experiment from 40±12 to 29±14 test days. As can be observed in tables 2 and 3, results show a similar trend and relatively small differences depending on the selected threshold of 50 or 80% metamorphosis success. This approach could also be accompanied with an upgrade of our experimental design by using triplicates instead of duplicates, as suggested for the echinoid 16-day early life stage bioassay (Anselmo et al. 2011), increasing the number of larvae from 15 to 30 per replicate while maintaining larval density or increase the number of test concentrations. This would improve the statistical power of our design and provide reliable results while significantly reducing the time required to conduct an experiment, thus making the echinoid metamorphosis assay more cost effective.

To our knowledge only 1 study used echinoid larvae at such late stages of early life development for toxicity testing (Aluigi et al. 2010). Therefore validation thresholds for the development of controls will still need to be further developed in the validation process of the assay.

4.2. Thyroid hormone induced metamorphosis of the echinoid *P. miliaris*

The acceleration of *P. miliaris* metamorphosis in our newly developed assay is in accordance with results reported for the echinoids *Hemicentrotus pulcherrinus* and *Peronella japonica*, where 1-100 nM T4, and 0.01-1 nM T4, respectively, also induced metamorphosis (Chino et al. 1994; Heyland 2004; Saito et al. 1998). As in our experiments (Fig. 4 and 5, respectively), larvae exposed to KSCN and TU showed a delay in settlement for completion of metamorphosis (Heyland 2004). TU and KSCN are known inhibitors of TH synthesis and iodine uptake, respectively, implying that the absence of sufficiently high levels of THs impairs the full development of the sea urchin rudiment and completion of the metamorphic process. In our so-called rescue experiment, exposure to T4 in combination with TU or KSCN restored the normal metamorphic process without visible adverse effects to the larvae (Fig. 5). The delay in completion of metamorphosis following exposure to TU and

KSCN suggests that *P. miliaris* larvae are able to produce their own THs, although it also has been speculated that they are able to obtain TH-like compounds from unicellular algae that they feed on (Chino et al. 1994; Heyland and Moroz 2005; Saito et al. 1998).

Our findings prove the importance of THs regulating the development of the sea urchin rudiment and completion metamorphosis from free swimming larva into a juvenile sea urchin. We exposed larvae from 20 dpf, when the sea urchin rudiment is between stages J or K, until the completion of metamorphosis since THs regulate development of the rudiment leading to the onset of metamorphosis (Chino et al. 1994). Since this window in echinoid early life development is the most sensitive to thyroid disrupting chemicals (TDCs), the invertebrate *in vivo* bioassay developed in this study is potentially suitable in the detection of TH disrupting effects.

4.3. PBDE mixture advances metamorphosis

The expected accumulation and distribution of PBDE congeners in the lipid fraction of *P. miliaris* larvae was calculated on the basis of the octanol-water partition coefficient (log K_{ow}) of each BDE congener (Braekevelt et al. 2003), and the resulting proportion in the larval lipid fraction, assuming an equilibrium state during exposure (Table 1). Our results show that exposure to the field-based PBDE mixture clearly accelerated *P. miliaris* metamorphosis in a dose dependent manner, suggesting that the PBDE mixture acts as TH agonist. As can be seen in Figure 1, PBDEs are structurally similar to THs, particularly T4 (Boas et al., 2006), and their ability to interfere with TH function has been previously reported both *in vivo* (Fernie et al. 2005); Ellis-Hutchings et al., 2006; Lema et al., 2008) and *in vitro* (Meerts et al., 2000; Marchesini et al., 2008; Crump et al., 2008; Freitas et al., 2010). *In vivo* effects of PBDEs were consistently associated with decreased levels of circulating T4 in rats (Long Evans) prenatally exposed to the technical PBDE technical mixture DE-71 (Zhou et al., 2002). The same T4 reduction was observed in American Kestrels (*Falco sparverius*) following *in ovo* and post-hatch exposure to a mixture of penta-BDE congeners (BDE-47, -99,-100, -153) (Fernie et al., 2005). Also, the TH dependent metamorphosis of *Xenopus laevis* and *Rana pipiens* tadpoles was delayed by DE-71 (Balch et al., 2006; Carey Coyle and Karasov, 2010).

In general terms the metamorphosis of echinoids is characterized by the reabsorption of the larvae body by the rudiment, which will become the juvenile sea urchin. At the highest test concentration of 3200 nM PBDEs part of the metamorphic process seemed to be advanced to such an extent that the reabsorption of the larvae body could not be effectively completed in about 25% of metamorphosed juveniles (Fig. 7 - B, C). At lower exposure concentrations, metamorphosis was still advanced but not to an extent that incomplete absorption of the larval body occurred on a large scale. Acceleration of

metamorphosis has also been shown for *Xenopus laevis* tadpoles exposed to 0.2 – 50 µg/g of the technical PCB-mixture Clophen A 50 via the food (Gutleb et al., 2007).

Acceleration in metamorphosis as we observed is not necessarily adverse or disadvantageous. In amphibians, for example, such acceleration has been observed as a response to decreasing water levels in their habitat. This adaptation provides tadpoles with a phenotypic plasticity that allows them to avoid desiccation in case their aquatic habitat dries out. However, this acceleration can have a negative impact on factors affecting post-metamorphic fitness, such as juvenile survival, time to sexual maturity and reproductive performance (Merilä 2000). In the case of PBDE exposed *P. miliaris* larvae, it seems that the acceleration of metamorphosis does not occur in the same way for all mechanisms involved, as this acceleration causes developmental morphological abnormalities in the juvenile sea urchins that still seem to have the larval features (Fig. 7 - B, C) not present in normally developed juveniles (Fig. 7 - A). However, nominal concentrations of PBDEs causing effects in the *P. miliaris* metamorphosis assay are about 2000 times higher than levels that have been reported for estuarine environments (Oros et al. 2004).

4.4. TBBPA and TCS delayed metamorphosis

Exposure of larvae to ≥ 150 nM TBBPA caused a dose dependent delay in the time to complete metamorphosis. Only the lower concentration of 75 nM TBBPA initially accelerated metamorphosis, but by the end of the experimental period a lower percentage of animals completed metamorphosis compared to the controls and the 7.5 nM TBBPA treatment (Fig. 8). In addition, the percentage of juveniles with morphological abnormalities increased (Table 2; Fig. 9 - B). The occurrence of morphological abnormalities in juveniles was as sensitive as the delay of metamorphosis. Our results suggest an antagonistic effect of TBBPA on echinoid metamorphosis. In literature, TBBPA is classified both as a potential thyroid hormone antagonist and agonist (Kitamura et al. 2005; Veldhoen et al. 2006a). This classification suggests that TBBPA is a partial agonist which at higher concentrations causes antagonistic effects. It is also possible that while at low concentrations TBBPA may act as an agonist, at higher concentrations its toxicity is the result of some other mechanism (not TH specific), which disrupts development. Without a direct measure of a TH-specific endpoint, the nature of TBBPA toxicity cannot be determined with certainty.

The biocide TCS was acutely toxic for larvae at 5000 nM. At 1000 nM TCS no acute toxicity was observed for *P. miliaris* larvae but metamorphosis was delayed (Fig. 10 and 11). As in the echinoid 16-day ELS bioassay 100% of embryos exposed to 1000 nM TCS failed to hatch (Anselmo et al., 2011), we cannot exclude that the inhibition of metamorphosis was not directly related to TH

disruption but rather to a more general toxicity mechanism. At lower concentrations (200 nM) TCS clearly delayed the metamorphosis, but after test day 19 on the percentage of metamorphosed larvae in the lowest dose group (100 nM) were almost identical to the DMSO control group. Finally, around 80% of the larvae exposed to 100 nM TCS were able to finish metamorphosis by the end of the experiment (Fig. 10). A possible explanation for this observation is a slower start of larval metamorphic process, but once they were able to do so they reached a normal metamorphosis success.

The proposed hypothesis for TCS ability to disrupt TH function is based on the similarity of its chemical structure with THs (Figure 1). This similarity means that TCS can compete with THs for its binding sites and potentially mimic or block TH function. A study conducted using the North American bullfrog (*Rana catesbeiana*) concluded that low doses of TCS (*i.e.* 0.5 nM) accelerated tadpole metamorphosis (Veldhoen et al. 2006b). In juvenile male rats TCS exposure also disrupted TH function by reducing T4 total serum concentrations (Zorrilla et al. 2009). Although these results suggest that TCS is able to disrupt TH function, there is an ongoing debate about whether TCS is a TDC or not. Fort and co-authors re-analysed some data of previous studies and concluded TCS did not interfere with the TH dependent metamorphosis of *X. laevis* (Fort et al. 2010). Because of the complexity of the TH system and feedback mechanisms, as well as the fact that TH mimicking compounds can act as agonists, antagonists, or partial antagonists, TH disrupting effects can vary depending on the level where disruption takes place. Similarly to that of tadpoles, the metamorphosis of echinoids is comprised of many different mechanisms controlling the rearrangement and reabsorption of larval tissues, development of juvenile structures and larval settlement on a substrate. As was seen in the highest PBDE exposure concentration, the acceleration of one mechanism (*i.e.* sea urchin rudiment development and larval settlement) but not the other (*i.e.* reabsorption of larval body) can result in malformed juveniles with larval structures still present (Fig. 7 - B and C). Therefore, it is advisable to study the mechanism by which TCS acts in greater detail in the future, including specific biomarkers (*e.g.* expression levels of the putative *Strongylocentrotus purpuratus* Th-receptor orthologous to vertebrate (Howard-Ashby et al., 2006) supported by *in vitro* bioassays (*e.g.* TR-Luc assay).

Similarly to the PBDE mixture, the nominal concentration of TCS capable of disrupting *P. miliaris* metamorphosis is about 2000 times higher than levels measured in water samples collected from estuaries (Fair et al. 2009). TBBPA has also been detected in estuarine and marine environments, particularly in sediment samples. However, TBBPA was absent in almost all water samples and was only detected in waste water treatment plants effluents (Morris et al. 2004; Watanabe and Sakai 2003).

To correctly determine the risk of adverse effects on larvae in those field situations it is important to determine internal effect concentrations as well as the body burdens in the field. Bioaccumulation of lipophilic POPs not only occurs via the water phase, but also to a high degree though food consumption. In addition, animals living in polluted areas such as the Western Scheldt estuary in The Netherlands will be exposed to POP mixtures composed of potential TDC. The effects of such mixtures could be tested in the sea urchin metamorphosis, as well as the prolonged early life stage test (Anselmo et al. 2011).

4.5. Potential mechanisms of thyroid hormone related metamorphosis disruption

Disruption of TH function can occur during TH synthesis, transport and metabolism (Crofton et al., 2005; Tan and Zoeller, 2007; Jugan et al., 2010). *In vitro* data suggest that PBDEs and TBBPA can compete with T4 for binding to TH transport proteins TTR (Meerts et al., 2000; Hamers et al., 2006) and TBG (Marchesini at al., 2008). Although tadpoles and sea urchin larvae do not likely possess TTR, disruption of their metamorphosis by PBDEs and TBBPA indicates that those compounds are able to disrupt TH function at a different level. Furthermore, PBDEs require bioactivation through conversion into hydroxylated metabolites to be able disrupt TH function in vertebrates. This bioactivation step also potentiates the binding of certain PBDEs to TH nuclear receptors (Freitas et al. 2011).

It is not yet determined whether sea urchin larvae have the capacity to bioactivate POPs into hydroxylated metabolites. However, adult echinoderms such as the starfish (*Asterias rubens*) have been shown to possess P450 activity (Stronkhorst et al. 2003). In general, bioactivation capability in echinoderms is expected to be lower compared to warm-blooded vertebrates, with the generation of less hydroxylated metabolites as a consequence. On the other hand, body metabolism in several cold-blooded, vertebrates including amphibians and fish, is not particularly high as well (Brett 1972), making them more comparable to echinoids. In addition, amphibian tadpoles used in the metamorphosis assay, considered the golden standard to detect TH disruption, still have their body metabolism underdeveloped compare to adults (Zeuthen 1953).

The mechanism by which THs regulate metamorphosis in echinoids may differ from vertebrates to a certain extent. T3 is regarded as the biologically active TH in vertebrates (Kistler et al. 1975), while in an echinoid species (*H. pulcherrinus)* T4 is suggested as the most potent TH inducing metamorphosis (Chino et al. 1994). However, in a different echinoid species (*Peronella japonica*), T3 and T4 were equally potent inducing metamorphosis (Saito et al. 1998). The variation in potency between T3 and T4 and the presence of deiodinase activities converting T4 into T3 should be studied in more detail. Its

understanding would have important consequences in the comprehension of TH disruption in sea urchins and the potential extrapolation of these results to other species.

In summary, a novel echinoid metamorphosis assay was developed using the marine species *P. miliaris* as test organism. To our knowledge this study is the first to evaluate the effects of POPs on the TH induced metamorphosis of echinoids. We provided evidence that PBDEs accelerated metamorphosis while TBBPA and TCS delayed the onset of *P. miliaris* metamorphosis in a dose dependent manner. This assay, together with the earlier developed prolonged ELS (Anselmo et al. 2011), the larval cellular efflux pump inhibition assay (Anselmo et al, to be submitted) and general toxicity assays (Bellas et al. 2005; Durán and Beiras 2010; Schipper et al. 2008; Warnau M 1996), demonstrates that echinoids are a multifunctional model organisms in ecotoxicological research that can be easily reared in the laboratory.

Although the echinoid metamorphosis assay still needs to be further validated, it has the potential to contribute to the reduction of amphibians used for the metamorphosis assay (OECD 231). *In vitro* bioassays, such as the TR-Luc assay (Freitas et al. 2011) or the TTR and TBG binding assays (March. et al), as well as QSAR developments could assist the hazard-based waiving of potential TH disrupting compounds based on a mechanistic insight. This combination of approaches can also assist the extrapolation from one taxonomic group to another. It has been suggested that regulatory and molecular mechanisms that govern thyroid signaling are highly conserved across vertebrates and even in several invertebrate species, allowing prediction of effects in untested species (Chino et al. 1994; Heyland and Moroz 2005; Heyland et al. 2006; Tan and Zoeller 2007). However, further efforts characterizing TH function in invertebrates are clearly required.

In the present study we demonstrated that an echinoid metamorphosis assay can be used to detect TDCs. Future work investigating TH function in echinoids is recommended to understand the mechanisms of disruption compared to vertebrates. A deeper understanding of TH function will also allow establishing more effective endpoints / biomarkers for the echinoid metamorphosis assay.

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CHAPTER 4.

Inhibition of cellular efflux pumps involved in Multi Xenobiotic Resistance (MXR) in echinoid larvae as a possible mode of action for increased ecotoxicological risk of mixtures.

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Abstract

In marine organisms the Multi Xenobiotic Resistance (MXR) mechanism via e.g. P-gp (P-glycoprotein) and MRP (multidrug resistance-associated protein) is an important first line of defense against contaminants by pumping contaminants out of the cells. If compounds would impair the MXR mechanism, this could result in increased intracellular levels of other compounds, thereby potentiating their toxicity. A calcein-AM based larval Cellular Efflux Pump Inhibition Assay (CEPIA) was developed for echinoid (*Psammechinus miliaris*) larvae and applied for several contaminants. The larval CEPIA revealed that triclosan (TCS) and the nanoparticles P-85® (P-85) were 124 and 155 times more potent inhibitors (IC_{50} 0.5±0.05 and 0.4±0.1 µM, respectively) of efflux pumps than the model inhibitor Verapamil (VER). PFOS (heptadecafluorooctane sulfonic acid) and pentachlorophenol (PCP) also were more potent than VER, 24 and 5x, respectively. Bisphenol A (BPA) and *o,p'*-dichlorodiphenyltrichloroethane (*o,p'*-DDT) inhibited efflux pumps with a potency 3x greater than VER. In a 48 hrs early life stage (ELS) bioassay with *P. miliaris*, exposure to a non-lethal concentration of the inhibitors TCS, VER, the model MRP inhibitor MK-571, the nanoparticles P-85 and the model P-gp inhibitor PSC-833, increased the toxicity of the toxic model substrate for efflux pumps vinblastine (VIN) by a factor of 2, 4, 4, 8 and 16, respectively. Our findings show that several contaminants accumulating in the marine environment inhibit cellular efflux pumps, which could potentiate toxic effects of efflux pumps substrates.

1. Introduction

Marine organisms are exposed to a wide range of potentially toxic compounds such as pollutants and biotoxins from respectively anthropogenic and natural sources (Daughton 2004; Mearns et al. 2010; Reish et al. 2000). In order to cope with these toxic compounds, freshwater and marine species have developed a defense mechanism known as the Multi Xenobiotic Resistance (MXR) mechanism (Bard 2000; Kurelec 1992). The MXR principle is comparable to the Multi Drug Resistance (MDR) mechanism first described in cancer cell lines that became resistant to anti-cancer drugs such as vinblastine (VIN) (Ambudkar et al. 1999). The key cellular entity responsible for MDR of certain cancer cells is thought to be a P-glycoprotein (P-gp) found in the membrane of resistant cells (Juliano and Ling 1976). Although P-gp pumps are the most extensively studied proteins responsible for the MDR mechanism, the multidrug resistance-associated protein family (MRP) also plays an important role conferring MDR (Smital et al. 2004; Epel et al., 2008). All these proteins belong to the ATP binding cassette (ABC) super-family and are responsible for the active efflux of a wide range of both endogenous and xenobiotic substrates (Germann 1993; Schinkel and Jonker 2003).

In the case of MXR mechanism, the key transport proteins thought to be responsible for the efflux transport in aquatic organisms also belong to the P-gp and MRP families (Bard 2000; Epel et al. 2006; 2008). Their substrates include endogenous compounds as well as xenobiotics and their metabolites (Bard 2000). The efflux activity of these pumps reduces the intracellular accumulation of endogenous metabolites and xenobiotic contamina*nts and their metabolites thus protecting exposed organisms from potentially toxic effects (Bard 2000; Litman et al. 2001). Evidence of their possible importance in protecting marine organisms from xenobiotics is provided by several studies which identified P-gp and P-gp-like activity in sponges, mussels, oysters, clams, snails, worms, crustaceans, echinoderms and fish (Kurelec 1992; Kurelec 1997; Kurelec et al. 1995; Smital et al. 2000). These pumps are highly expressed in tissues directly or indirectly involved in the metabolism and/or excretion of contaminants, for example gills, liver, kidney, intestine and hepatopancreas, but also in single sponge cells (Bard 2000; Luckenbach and Epel 2008; Smital et al. 2000). A relationship was established between increased tolerance to contaminants by marine organisms (*e.g. Monodonta turbinata*; *Mytilus galloprovincialis*) living in polluted sites and increased MXR activity (Kurelec 1997; Kurelec et al. 1996; Kurelec et al. 1995). On the other hand it has been shown that certain contaminants are able to inhibit MXR transporter activity both in vitro (Bain and Leblanc 1996; Oosterhuis et al. 2008) as well as in several aquatic organisms, thus causing the accumulation of toxic substrates (Bosnjak et al. 2009; Smital et al. 2004; Smital et al. 2000). Compounds able to interfere with MXR related transport proteins, also known as chemosensitizers, are classified in two categories:

1) competitive inhibitors, which are able to overwhelm the substrate binding capacity of pumps; and 2) non-competitive, which are able to block the ATPase activity of the pumps (Faria et al. 2011).

Echinoid embryos also possess efflux transport activity with homology to at least one P-gp and two MRP pumps (Bosnjak et al. 2009; Hamdoun et al. 2004) and have been shown to be suitable to investigate efflux transporter activity (Epel et al. 2006). Echinoids are an ecologically relevant animal group, suitable for (prolonged) early life stage and metamorphosis testing in ecotoxicology and can be easily aquacultured (Anselmo et al. 2011; Schipper et al. 2008). Therefore, the echinoid larval Cellular Efflux Pump Inhibition Assay (CEPIA) was developed to determine efflux transporter activity in the echinoid *Psammechinus miliaris* larvae exposed to several substances. The CEPIA method uses calcein-AM, a substrate of both P-gp and MRP efflux pumps (Essodaïgui et al. 1998; Holló et al. 1994; Luckenbach et al. 2008), for indirect measurement of transporter inhibition. If these pumps are inhibited or have low activity, calcein-AM accumulates in the cell where it is hydrolyzed by non-specific esterases into fluorescent calcein. In contrast to calcein-AM, calcein is not able to easily cross the cellular membrane and accumulates in the cell resulting in increased fluorescence (Essodaïgui et al. 1998).

After establishing the calcein-AM based echinoid larval CEPIA, in the present study we tested several compounds that were selected based on previous results obtained in our laboratory using MDCKII-MDR1 and Caco-2 cell lines (data not shown), namely bisphenol A (BPA); heptadecafluorooctane sulfonic acid (PFOS); *o,p'*-dichlorodiphenyltrichloroethane (*o,p'*-DDT); hexabromocyclododecane (HBCD); pentachlorophenol (PCP); triclosan (TCS) and the nanoparticles P-85® (P-85). In addition we tested the model inhibitors verapamil (VER) and PSC-833, which are well established and commonly used agents known for their capability to inhibit the up-regulated P-gp in drug resistant cell lines (Ford 1996; Tan et al. 2000; Teodori et al. 2002), and the model inhibitor MK-571 which was demonstrated to inhibit MRP in a fish hepatoma cell line (Zaja et al. 2007) as well as in sea urchin larvae (Hamdoun et al. 2004). We used VER, PSC-833 and MK-571 to investigate and characterize P-gp and MRP efflux transporter activity in *P. miliaris* larvae. Finally, we investigated the effect of efflux transporter inhibition on the toxicity of VIN which is a substrate of cellular efflux transporters such as P-gp and MRP (Evers et al. 2000; Varma et al. 2003). *P. miliaris* larvae were exposed to a dose-range of VIN in combination with the efflux transport inhibitors VER, MK-571, PSC-833, TCS or P-85.

2. Materials and methods

2.1. Adult animals

Sea urchins (*Psammechinus miliaris*) were collected from the Eastern Scheldt (The Netherlands) and maintained in fiber glass tanks (L*W*W in cm = 200*80*30) under controlled conditions (*i. e.* temperature, photoperiod and food availability) with a flow rate of 150L per day (corresponding to a renewal of half of the tank volume) at least 2 months prior to use. Sea urchins were fed *ad libitum* with freshly dissected mussels and TetraMin® (Tetra) at all times as described before (Anselmo et al. 2011).

2.2. Gamete collection and fertilization

For each experiment a single pair of adult *P. miliaris* was used to collect eggs and sperm from freshly dissected gonads. Eggs were kept in artificial sea water (prepared using Instant Ocean® synthetic salts from Spectrum Brands, Inc. and aged under continuous aeration for at least 1 week) kept at 17±1 ºC for transport. Sperm was collected "dry" and kept on ice until use. Fertilization took place within 4 hours of gamete collection. Fertilization success was at least 90% which is in accordance with optimal values of 90% as indicated by USEPA (2002) and Environment Canada (1992) for bioassay validation. Larvae were reared in ASW (artificial sea water) as this has been shown to yield reproducible good survival in contrast to filtered natural sea water. See Anselmo et al. (2011) for further details.

2.3. Echinoid larval cellular efflux pump inhibition assay (CEPIA)

The developed method was adapted from the method previously established for *Strongylocentrotus purpuratus* embryos by Hamdoun et al. (2004), and the *in vitro* method developed on MDCKII-MDR1 and Caco-2 cell lines by Georgantzopoulou and co-authors (submitted) with slight modifications. Briefly, at 18-20 hours post-fertilization (hpf) a total of 12-15 *P. miliaris* larvae at the gastrula stage were placed in each well of a 24 well plate. Stock solutions were diluted 20 times in ASW and 25 µL of each dilution was added to 225 µl of ASW present in the respective well. All concentrations were tested in triplicate. The 24 well plate was then placed on a rocking shaker (30 rpm) for 45 minutes exposure of the larvae at 19±1 ºC. Calcein-AM was then added to obtain a final concentration of 2.5 µM. Following the addition of calcein-AM the plate was covered with aluminum foil and incubated on a shaker for 40 minutes at 19±1 ºC. To capture good quality pictures to measure fluorescence, larvae were immobilized by adding 300 µl of saturated gelatin solution prepared in ASW to each well. Finally, the plate was cooled to approximately 14 ºC to solidify the gelatin in which the larvae were immobilized.

Fluorescence imaging was performed with an inverted microscope (Olympus IMT-2) equipped with mercury illumination (Olympus) at 100x magnification. Image acquisition was done using a Canon EOS 1000D and fluorescence was quantified using ImageJ 1.44 software (available at: http://rsbweb.nih.gov/ij/). Fluorescence was quantified as relative fluorescence units (RFUs) using the following equation: *RFU = Integrated density of selected larvae – (area of selected larva x mean fluorescence of background)*. Presented data correspond to the mean RFU for 8 individual larvae per well. Larvae were randomly selected. However, in every experiment there were a few larvae (1-5) located on the edge of the well plate. These larvae were not used for measurements since they interfered with fluorescence measurement/quantification due to the reflection of fluorescence on the edge of the well. Two independent sets of experiments were conducted, and each experiment was performed in triplicate (Table 1). The final concentration of solvent used in all experiments was always 0.5 % v/v DMSO, a solvent concentration that did not induce statistically significant differences compared to the ASW control group. The use of 0.5 % v/v DMSO is also according to Hamdoun et al. (2004).

Because in the echinoid 48 hrs ELS bioassay (see below) most of the exposure period (approximately 30 hrs out of a total of 48 hrs) takes place after hatching, we developed a method to measure the efflux pump inhibition in larva after hatching (at the gastrula stage) instead of the 2-cell stage embryos when the fertilization envelope still is present as done by Hamdoun et al. (2004). This implies, however, that the larvae need to be immobilized before measurement.

Table 1. Test concentrations for the echinoid (*P. miliaris*) larval cellular efflux pump inhibition assay (CEPIA)

Test compound		Test concentrations [µM]					
VER	Exp. A	1	10	25	50	100	150†
	Exp. B	1	10	25	50	100	150†
BPA	Exp. A	1	10	50	100	300	400†
	Exp. B	1	10	50	100	300	400†
TCS	Exp. A	0.1	0.3	0.5	1	2	4†
	Exp. B	0.1	0.3	0.5	1	2	4†
P-85®	Exp. A	0.1	0.3	0.5	1	2	4†
	Exp. B	0.1	0.3	0.5	1	2	4†
o,p'-DDT	Exp. A	1	5	10	20	40	80†
	Exp. B	0.2	1	2	4	20	60†
PCP	Exp. A	0.2	1	5	10	50	100†
	Exp. B	0.5	1	2	10	50	100†
PFOS	Exp. A	0.2	2	20	80	160	320†
	Exp. B	0.2	2	20	80	160	320†
HBCD	Exp. A	1	5	10	20	40	80†
	Exp. B	0.2	1	2	4	20	40

† - Acutely toxic.

2.4. Echinoid 48 hrs early life stage (ELS) bioassay

In the ELS bioassay *P. miliaris* embryos and larvae were exposed to VIN from approximately 1 hpf to 48 hpf. The experiment was performed in 6 well plates at a density of 25 embryos/ml with a total volume of 10 ml ASW per well. Larvae were kept at 19±1 ºC with a photoperiod of 16:8 (L:D). At the end of the experimental period the larvae were preserved in 4% buffered formaldehyde and a total of 100 larvae per well were scored for developmental stage, morphological abnormalities and mortality. The experiment was conducted in triplicate. In the binary exposure *P. miliaris* fertilized eggs were exposed to a single non-toxic concentration of an efflux transporter inhibitor (*i. e.* 5 µM VER, 5 µM MK-571; 5 µM PSC833; 0.25 µM TCS or 1 µM P-85) for 1 hour after which the VIN dose range was added. The 48 hrs ELS bioassay was conducted as described above. For practical reasons the animals were exposed via the water phase, as for this mechanistic study we did not try to mimic a field-realistic exposure route which would be via the food and sediment.

2.5. Test Compounds

The test compounds VER (Verapamil hydrochloride; CAS: 152-11-4; purity ≥99%), MK-571 (CAS: 115103-85-0; purity ≥ 95%), PCP (pentachlorophenol; CAS: 87-86-5; purity ≥ 98%), VIN (Vinblastine; CAS: 143-67-9; purity ≥ 95%) and TCS (Triclosan; CAS: 3380-34-5; purity ≥97%) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). PSC-833 (CAS: 121584-18-7; purity ≥ 95%) was kindly provided by Novartis (Basel, Switzerland). PFOS (heptadecafluorooctane sulfonic acid potassium salt; CAS: 2795-39-3; purity ≥98%) was obtained from Fluka (Steinheim, Germany), BPA (Bisphenol A; CAS: 80-05-7; purity ≥ 99.9 %) from Aldrich Chemical (Bornem, Belgium) and *o,p'*-DDT (*o,p'*-dichlorodiphenyltrichloroethane; CAS: 789-02-6; purity 99%) from Riedel de Haën (Seelze, Germany). HBCD (hexabromocyclododecane technical mixture) was obtained through BSEF (Bromine Science and Environmental Forum, with kind co-operation of Dr. Klaus Rothenbacher). Stock solutions of the test compounds were prepared in DMSO (CAS: 67-68-5; purity ≥99,9%) obtained from Acros-Organics (Geel, Belgium). Stock solutions used in the echinoid larval CEPIA were always 200 times more concentrated than the respective test concentrations so that the final DMSO concentration was always 0.5% v/v of the experimental volume. All stocks were stored in the dark at room temperature. The nanoparticle P-85 (Pluronic® P-85, surfacta) was obtained from BASF Corporation (Florham Park, USA) and stocks were directly prepared in ASW no longer than 48 hours before exposure. P-85 concentrations were expressed in molar (M) units based on an average molecular weight of 4600 g/mol provided by BASF. Gelatin was obtained from Merck (Darmstadt, Germany) (CAS: 9000-70-8; purity ≥99 %).

2.6. Statistics

For the calcein-AM based larval CEPIA, the inhibition concentration (IC) values were determined by fitting the available regression models in Graphpad prism 5 software and selecting the curve that gave the best fit values through the experimental data. Test concentrations that induced acute toxicity in larvae (Fig. 1 – I) were not included in the curve fitting. The accumulation of calcein was measured as RFU expressed relative to the fluorescence of the DMSO control group which was set at 100% efflux pump functioning. The maximal % inhibition of the efflux pumps was expressed for each test compound relative to the maximum inhibition (*i.e.* maximum RFU) by 100 µM VER set at 100%. The relative potency (REP) of each compound was determined as the ratio between the IC_{50} of VER and the concentration of the compound giving the same inhibition denoted VER-EQ IC_{50} (Table 2).

For the 48 hours-ELS bioassay, it was not possible to determine the 50% effect concentrations (EC_{50}) for morphological abnormalities since a dose response model could not be reliable fitted due to

the biphasic nature of the data. Data are always presented following correction for background control effects using Abbott's formula (Stark and Walthall 2003).

The significance of differences between the treatments was determined using an a Mann–Whitney U test with an acceptance level set at $p<0.05$. Statistical analysis was performed using GraphPad Prism software (version 5).

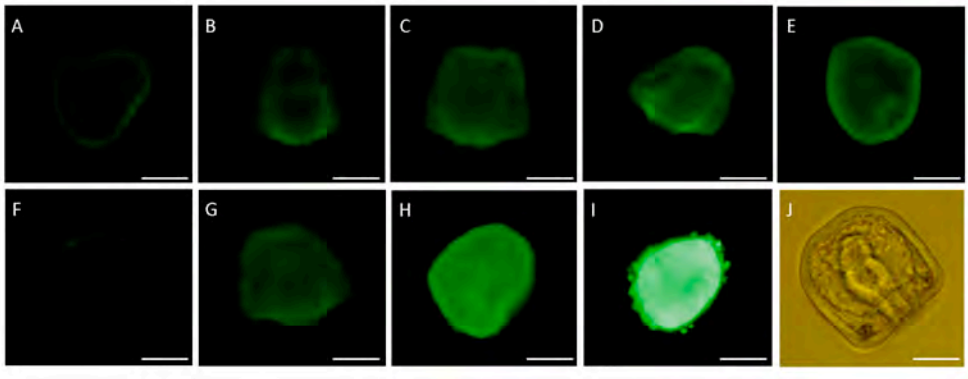

Figure 1. Accumulation of fluorescent calcein upon inhibition of cellular efflux pumps in *P. miliaris* larvae after 45 minutes of exposure to test compounds, followed by a 45 minutes incubation period with 2.5 µM C-AM. Top row - A) 1 µM; B)10 µM; C) 25 µM; D) 50 µM; E) 100 µM VER. Bottom row - F) DMSO (0.5 % v/v) G) 5 µM MK-571; H) 5 µM PSC-833; I) Larva showing signs of acute toxicity resulting in loss of body and cellular membrane integrity; J) Normal larva at blastula stage under the light microscope. Bar - 50 µm.

3. Results

3.1. Echinoid efflux pump inhibition

Exposure of *P. miliaris* larvae to the model-inhibitor VER induced a clear dose related increase in the accumulation of calcein reaching a maximum fluorescence of 291% compared to control and indicating an IC_{50} of 62±5.0 µM VER (Table 2; Fig. 2). VER was further used as the standard cellular efflux transport inhibitor to which the activities of the other compounds were related. At concentrations above 100 µM, VER was acutely toxic for larvae which was visible as a clear granulation in the larval body combined with an apparent loss of membrane integrity (Fig. 1 - I). As can be seen in Fig. 3 - A, also the model inhibitors PSC-833 and MK-571 both significantly inhibited the efflux pumps as well, but PSC-833 was the strongest inhibitor.

Table 2 shows the potency of BPA; PFOS; *o,p'*-DDT; HBCD; PCP; TCS and the nanoparticles P-85 in the calcein-AM based larval CEPIA with *P. miliaris* larva. Almost all the compounds tested showed a dose related inhibition of efflux transporter activity in *P. miliaris* larvae. The most potent compounds tested were the nanoparticles P-85 and the biocide TCS, with an IC_{50} of 0.4±0.1 and 0.5±0.05 µM, respectively. PFOS also was a potent efflux pump inhibitor with an IC_{50} of 2.6±1.2 µM.

PCP, *o,p'*-DDT and BPA also responded in the calcein-AM based larval CEPIA but with 33, 45 and 50 times lower potencies, respectively, compared to P-85 (Table 2; Fig. 4). The only exception was observed for HBCD, which caused a slight decrease in RFU values compared to control (data not shown).

Table 2. Inhibition of cellular efflux pumps in *P. miliaris* larvae (mean±SD) expressed as the conc. giving 10% (IC_{10}) or 50% (IC_{50}) inhibition relative to VER maximal inhibition at 100 µM set at 100%. Relative potencies (REP) are based on the IC_{50} of VER.

Compound	Range [µM]	IC_{10} [µM]	IC_{50} [µM]	REP	Max. response (RFU) relative to DMSO set at 100%	Max. inhibition (%) relative to VER max. set at 100 %
VER	1 - 100	6.09±1.28	62±5.0	1	291 (100 µM)	100
BPA	1 - 300	1.1±1.1	20±12.6	3	352 (300 µM)	121
TCS	0.1 - 2	0.1±0.006	0.5±0.05	124	334 (2 µM)	115
P-85	0.1 - 2	0.06±0.03	0.4±0.1	155	352 (2 µM)	121
o,p'-DDT	0.2 - 40	1.2±0.2	18±0.4	3	281 (40 µM)	97
PCP	0.2 - 40	1.1±1.0	13±8.1	5	298 (40 µM)	102
PFOS	0.2 - 160	0.2±0.02	2.6±1.2	24	362 (160 µM)	124
HBCD	0.1 - 40	> 40	> 40	—	—	—

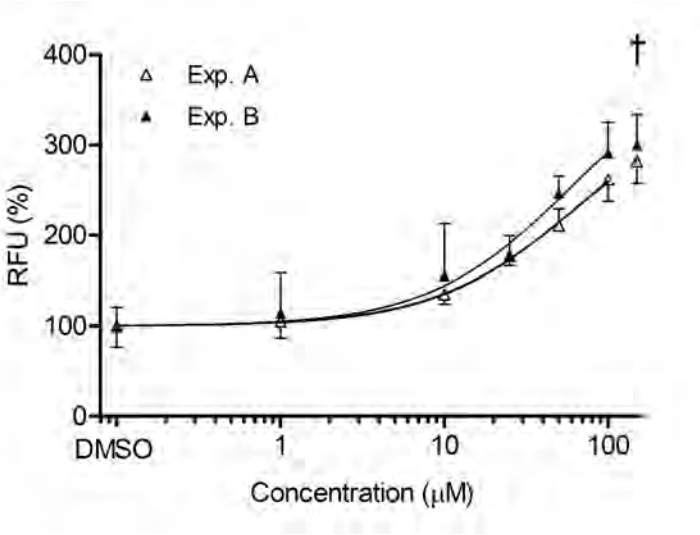

Figure 2. Accumulation of cellular fluorescent calcein as a measure of cellular efflux pump inhibition in *P. miliaris* larvae exposed to VER. Each data point represents the mean ± SD (n=3) and the DMSO control group was set at 100%. (†) Larvae showed clear signs of acute toxicity as shown in Fig. 1 – I. Experiment A and B represent 2 independent experiments conducted in triplicate.

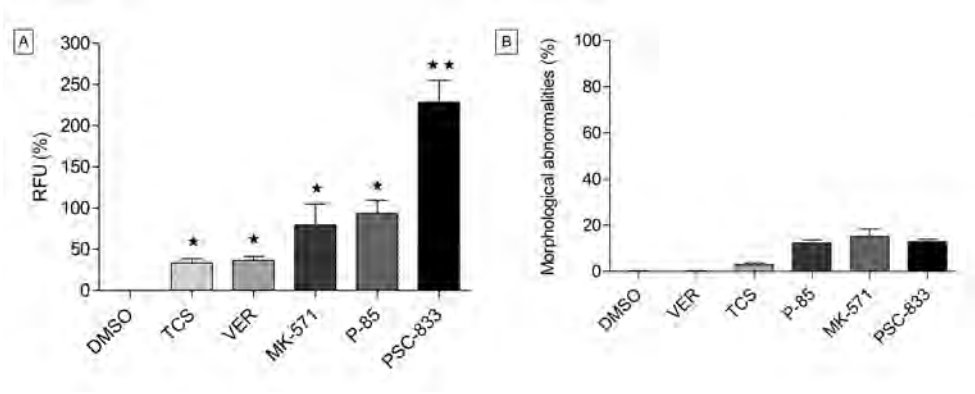

Figure 3. *P. miliaris* larvae exposed to the model MRP-inhibitor MK-571 (5 µM), the model P-gp inhibitors VER (5 µM) and PSC-833 (5 µM), the biocide TCS (0.25 µM) and the nanoparticles P-85 (1 µM). A) Accumulation of cellular fluorescent calcein as a measure of cellular efflux pump inhibition; each data point represents the mean ± SD (n=4). B) Morphological abnormalities in the 48h ELS bioassay; each data point represents the mean ± SD (n=3). *p<0.05; **p<0.01 (Mann–Whitney U test).

3.2. Mixture toxicity in the echinoid 48 hrs ELS bioassay

The toxic model P-gp substrate VIN induced a clear dose related toxic effect during *P. miliaris* early life development, with an estimated EC_{100} of 2.5 µM VIN (Table 3; Fig. 5 – A - E). Fertilized eggs exposed to concentrations ≥5 µM VIN completely failed to hatch thus embryo mortality was 100%. At 2.5 µM VIN, hatching success was normal, however, all larvae were morphologically abnormal. The lowest observed adverse effect concentration (LOAEC) for morphological abnormalities was 0.8 µM VIN.

When exposure to VIN was combined with a non-toxic concentration of selected efflux pumps inhibitors (Fig. 3), its toxicity in the *P. miliaris* 48 hour-ELS test was significantly enhanced (Table 3; Fig. 5). The greatest increase in toxicity was revealed for 5 µM PSC-833 (Fig. 5 - A), reducing the EC_{100} of VIN from 2.5 to 0.16 µM (Table 3). In addition, in combination with 5 µM PSC-833, VIN caused complete hatching failure at ≥0.63 µM instead of ≥ 5 µM VIN. In combination with 5 µM MK-571, VIN toxicity also increased resulting in an EC_{100} for morphological abnormalities of 0.63 µM VIN (Fig. 5 - B). The 100% failure of hatching success was 2x greater for the combination of VIN with PSC-833 (≥0.63 µM VIN) than with MK-571 (≥1.25 µM VIN). Exposure to VIN in combination with 1 µM P-85 resulted in an EC_{100} of 0.31 µM VIN (Fig. 5 - C) and a complete hatching failure at 0.63 µM VIN, as was the case for PSC-833. In larvae exposed to VER (Fig. 5 - D) and TCS (Fig. 5 - E), the EC_{100} of VIN decreased from 2.5 to 0.63 and 1.25 µM respectively (Table 3). A complete

impairment of hatching success occurred at ≥1.3 µM and ≥2.5 µM VIN in combination with VER or TCS, respectively.

The EC_{10} for morphological abnormalities was below the lowest test concentration of 0.02 µM VIN for all combined exposures and could therefore not be determined.

Table 3. Inhibition of efflux pumps by inhibitors (in vivo CEPIA) and toxicity of vinblastine (VIN) alone and in combination with the efflux pump inhibitors (48h ELS bioassay) at non-lethal concentrations in *P. miliaris* early life development.

Compound	In vivo CEPIA	48h ELS bioassay		
	Inhibition (RFU %) relative to VER max. set at 100% (DMSO set at 0%)	EC_{100} [VIN µM]	Factor	Max effect (%) of the first phase of the curve [VIN µM]
VIN	—	2.5	1	19±1 (0.63)
VIN + 0.25 µM TCS	11±2*	1.25	2	44±4 (0.63)
VIN + 5 µM VER	12±3*	0.63	4	31±4 (0.31)
VIN + 1 µM P-85	27±17*	0.31	8	40±3 (0.16)
VIN + 5 µM MK-571	32±11*	0.63	4	63±4 (0.31)
VIN + 5 µM PSC-833	77±18**	0.16	16	26±6 (0.08)

*p<0.05; **p<0.01 (Mann–Whitney U test).

4. Discussion

The present study describes the successful development and optimization of a echinoid larval bioassay to determine the effect of pollutants on the activity of cellular efflux pumps. A simple but important step of our method was the use of gelatin to immobilize larvae allowing focused imaging to quantify their fluorescence. This development allowed us to use echinoid larvae instead of embryos, which avoids the limitation of the fertilization envelope acting as a barrier to exposure to test compounds.

MXR is an important cellular defense mechanism of many aquatic organisms against pollutants present in the environment (Barbara Holland and Epel 1993). Echinoid embryos are no exception and e.g. the species *Strongylocentrotus purpuratus* has been shown to possess multidrug transporter efflux activity immediately after fertilization (Hamdoun et al. 2004), and gene expression of many efflux pump homologous has been identified (*e. g.* P-gp and MRPs) (Shipp and Hamdoun 2012). Schaffer et al. (2009) also suggested that *P. miliaris* embryos possess at least MRP-like efflux pump activity. Therefore, the development of bioassays, such as the calcein-AM based echinoid larval CEPIA, to assess the effects of environmentally relevant contaminants on the MXR defense mechanism is an important addition to the currently available ecotoxicological test batteries. Our results demonstrate that the calcein-AM method can also be adapted to a calcein-AM based larval CEPIA method with *P. miliaris*. With this larval CEPIA, environmentally relevant modulators of efflux pump activity can be

easily detected already after 2 hours of exposure. Furthermore, we showed that environmental contaminants such as TCS and the nanoparticles P-85 potentiate the toxicity of efflux pump substrates (*i. e.* vinblastine). These findings confirm the suggested importance of the MXR mechanism in larvae, such as of *P. miliaris*, as a first line of defense against environmental contaminants. The impairment of this mechanism could increase the ecotoxicological risk of efflux pump substrates to larval survival.

4.1. Inhibition of efflux pump activity by model compounds

The model P-gp inhibitor VER was used to validate and optimize the calcein-AM based echinoid larval CEPIA and served as reference inhibitor for further experiments. The results obtained in two independent experiments were reproducible and the variation between the two experiments only was ±10% (Table 2, Fig. 2). VER already was established as a model efflux pump inhibitor in previous studies conducted with the marine mussel *Mytilus californianus* (Stevenson et al. 2006). Interestingly, the *in vitro* IC_{50} of 55±1.2 µM VER obtained in our laboratory using MDCKII-MDR1 and Caco-2 cell lines is similar to the IC_{50} of 62±5.0 µM VER in the *P. miliaris* larval CEPIA defined in the present study.

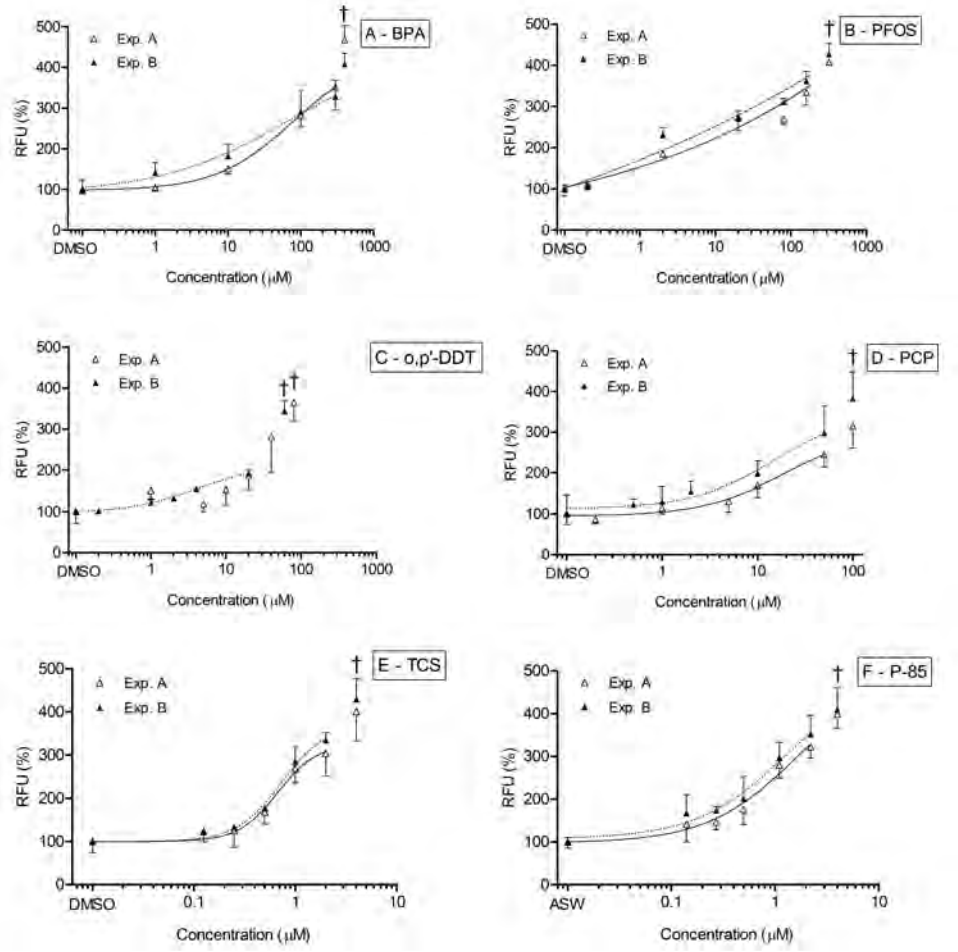

Figure 4. Accumulation of cellular fluorescent calcein as a measure of cellular efflux pump inhibition in *P. miliaris* larvae exposed to: A) BPA; B) PFOS; C) o,p'-DDT; D) PCP; E) TCS; F) P-85. Each data point represents the mean ± SD (n=3) and the DMSO and ASW control groups were set at 100%, respectively. (†) Larvae showed clear signs of acute toxicity as shown in Fig. 1 - I. Experiment A and B represent 2 independent experiments conducted in triplicate.

4.2. Inhibition of efflux pump activity by environmental contaminants

The newly developed calcein-AM based larval CEPIA was used to determine the potential to inhibit the efflux pump activity by compounds selected based on experiments conducted in our laboratory using MDCKII-MDR1 and Caco-2 cell lines (data not shown). The echinoid larval CEPIA

yielded highly reproducible results, since only minor differences were observed between the independent experiments A and B for each test compound (Fig. 4).

The most potent compounds tested in our larval assay were TCS and the nanoparticles P-85. The mechanism by which TCS interferes with efflux pumps (*e.g.* ABC family) is thought to be due to competitive inhibition since TCS is a substrate of efflux pumps in several types of bacteria (Schweizer 2001). Competition by TCS with calcein-AM for efflux transport will result in more calcein-AM being metabolized into fluorescent calcein. Given the potency of TCS in the larval CEPIA bioassay and the relatively high levels, especially in the aquatic environment, of this persistent and bioaccumulating compound and its metabolites (Fair et al. 2009; Okumura and Nishikawa 1996; Schweizer 2001), the effects of TCS and its metabolites deserve further study.

The interference of the nanoparticles P-85 with cellular efflux pumps has been shown not to be mediated by competition as substrate of efflux pumps (Batrakova et al. 2004). P-85 decreases intracellular ATP levels and inhibits ATPase activity, which may result in the inhibition of efflux pumps since they require ATP to actively efflux their substrates (Batrakova et al. 2003; Batrakova et al. 2004; Bradley et al. 1988). Another possible mechanism of inhibition can be the interference with the structure of the cell membrane which could affect the conformation and function of the efflux pumps (Batrakova et al. 2004).

Also PCP, BPA and PFOS inhibited efflux pumps but were evidently less potent than TCS and P-85. The inhibitory effect of PCP on efflux pumps activity is in accordance with previous studies conducted with mussel gills (Galgani et al. 1996), as well as with experiments conducted in our laboratory using MDCKII-MDR1 and Caco-2 cell lines (data not shown). PCP is a P-gp substrate and therefore could be a competitive inhibitor for efflux transport mediated by P-gp (Eufemia and Epel 2000). On the other hand PCP can also uncouple oxidative phosphorylation and elicit ATPase activity (Weinbach 1956), but also has been shown to inhibit intracellular ATP levels in cells (Nnodu and Whalen 2008).

In our study BPA inhibited efflux pump activity, which is also in accordance with experiments conducted in our laboratory using MDCKII-MDR1 and Caco-2 cell lines (data not shown). In another study, however, BPA reduced accumulation of calcein in BeWo cells suggesting an induction of efflux pump activity (Jin and Audus 2005). We cannot explain this difference, but as BPA is a known substrate of efflux pumps, particularly P-gp (Yoshikawa et al. 2002), we would expect competitive inhibition of BPA with calcein-AM for cellular efflux to lead to increased calcein accumulation as we observed.

PFOS could, as suggested for P-85, affect the cell membrane structure, as it has a structural similarity to fatty acids. The interference with the fluidity of a biological membrane could change its

structure and function (Hu et al. 2003), thereby possibly affecting efflux pump activity as well (Batrakova et al. 2004). The recently reported accumulation of PFOS in very distinct areas of the cell membrane which could also indicate interference with specific cellular efflux pumps (Gutleb et al., 2012).

The pesticide *o,p'*-DDT also inhibited efflux transporter activity. Similar results were reported for mussels gills exposed to the congener *p,p'*-DDT (Galgani et al. 1996). However, in experiments conducted at our laboratory with MDCKII-MDR1 and Caco-2 cell lines exposure to *o,p'*-DDT caused a slight reduction in calcein-AM accumulation (data not shown). It would be interesting to study the different DDT congeners and their DDE and DDD metabolites in more detail, as these are ubiquitous POPs that accumulate in food chains.

HBCD caused a slight, not simply dose related, reduction in the accumulation of calcein compared to control (data not shown) but HBCD did not have an effect on the calcein fluorescence itself (data not shown). The lower fluorescence suggests an induction of efflux transport activity, which might be due to an increase in mitochondrial activity, which could stimulate the activity of efflux pumps in case ATP levels would be rate limiting. This effect is in accordance with the results obtained in our laboratory using MDCKII-MDR1 and Caco-2 cell lines (data not shown). More mechanistic studies, using for example 'omics' techniques, could perhaps reveal more of the toxic action of HBCD.

4.3. Enhanced toxicity through cellular efflux inhibition

Contaminants are present in environmental compartments as a complex mixture. As we showed above some of those compounds are able to inhibit cellular efflux pumps. It is expected that the simultaneous exposure to efflux transport inhibitors and toxic substrates will result in their accumulation thus increasing chances of reaching toxic concentrations (Barbara Holland and Epel 1993; Epel et al. 2008). As a model efflux transporter substrate we used VIN since it is a widely known P-gp substrate (Mechetner and Roninson 1992; van Asperen et al. 1996). We then determined the effect of efflux transport inhibitors on the toxicity of VIN in early life development of *P. miliaris* larvae.

Besides the model P-gp inhibitor VER, we also decided to use the P-gp specific inhibitor PSC-833 and the MRP specific inhibitor MK-571. Of the three inhibitors tested in the *P. miliaris* larvae CEPIA at 5 uM, PSC-833 increased the RFU (% of control) the most, up to 229%, followed by MK-571 with an increase of 93% (Fig. 3 - A). VER also caused statistically significant increase in RFU of 37% (Fig. 3 - A). In the sea urchin *S. purpuratus* MRP mediated efflux transport was considered more

important than P-gp mediated efflux. Similarly to our findings, the sea urchin *Echinometra lucunter* also appears to show predominance of P-gp over MRP protein activity (De Souza 2010).

Exposure of *P. miliaris* larvae to VIN alone resulted in an EC_{100} of 2.5 µM VIN (Fig. 5 - A). From all the binary mixtures tested the most relevant increase in VIN toxicity was observed with 5 µM PSC-833 (16-fold) and 1 µM P-85 (8-fold) (Table 3). This is in accordance to our hypothesis as PSC-833 was the most effective inhibitor of efflux pumps in *P. miliaris* larvae (Fig. 3 - A).

The nanoparticle P-85 also increased the toxicity of VIN, as the EC_{100} was 8 fold lower compared to the EC_{100} of VIN alone (Table 3). The mechanism via which P-85 cause accumulation of efflux transporter substrates (such has VIN) is based on the inhibition of ATPase activity and ATP depletion which is required for both P-gp and MRP efflux transport (Batrakova et al. 2003; Batrakova et al. 2001). However, it cannot be excluded that inhibition of ATPase activity also affects other processes in the animals.

TCS only increased VIN toxicity by a factor of 2 (Table 3). We were particularly interested to investigate the effects of TCS on the toxicity of VIN because this compound is widely found in the aquatic environment and can reach relatively high concentrations. The toxicity mechanism of TCS in bacteria's is related to membranotropic effects, which compromise the integrity and normal function of cell membranes (Villalaı et al. 2001). Bacteria are able to become resistant to TCS expressing higher levels of efflux pumps (Mima et al. 2007). This provides evidence of the protective role of efflux pumps against TCS toxicity. However and to our knowledge, the mechanism by with TCS inhibits efflux pumps is not fully understood in vertebrates.

Interestingly the dose response curves obtained for the toxicity of VIN alone or in binary mixtures all showed a biphasic response (Fig. 5). The % of morphological abnormalities observed for the first phase of the curves for the combined exposures varies from 26±6 to 63±4%, which was always higher than the % of morphological abnormalities observed upon exposure to VIN alone (19±1%) (Table 3). Furthermore, all dose response curves of the combined exposures of VIN with the efflux pumps inhibitors, were shifted to the left on the x-axis, as were the EC_{100} concentrations of the combined exposures, thus indicating an increase in the toxicity of VIN (Fig. 5). As previously mentioned, the biphasic nature of the curve does not allow to reliably determining the EC_{50} values.

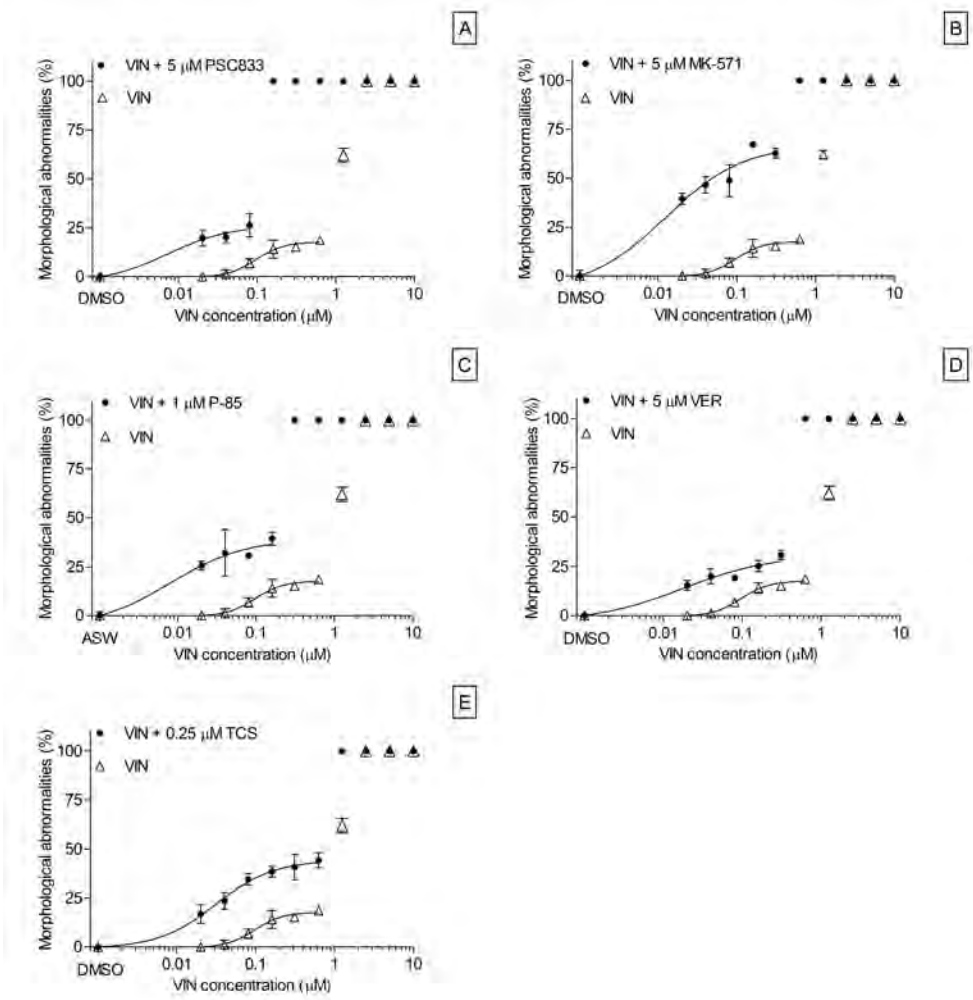

Figure 5. Morphological abnormalities in *P. miliaris* larvae exposed from 0-48 hpf to VIN alone and in combination with: A) the model MRP-inhibitor PSC-833 (5 µM); B) the model P-gP inhibitor MK-571 (5 µM); C) the nanoparticles P-85 (1µM); D) the model P-gP inhibitor VER (5 µM); E) the biocide TCS (0.25 µM). Each data point represents the mean ± SD (n=3). Dose-response curve was fitted following subtraction of the respective control containing the selected efflux pump inhibitor concentration using Abbott's formula (Stark and Walthall 2003). Due to the biphasic nature of the dose response curve, only to the "first" phase of the dose response curve was fitted.

5. Conclusion

The newly developed *P. miliaris* cellular efflux pump inhibition bioassay (larval CEPIA) allows a fast detection of inhibition of larval efflux pumps by compounds, as well as the influence thereof on the toxicity of accumulating toxic efflux pumps substrates. This is particularly relevant for studying mixture effects in field samples. Several compounds enhanced the intracellular accumulation of the fluorescent model compound calcein, including TCS, P-85, o,p'-DDT, PCP, PFOS and BPA. We showed that *P. miliaris* early life stages were much more sensitive to the toxic model efflux pump substrate VIN in combination with the anti-microbial compound TCS or the nanoparticles P-85. The new calcein-AM based echinoid larval CEPIA to measure efflux transport activity still has to be further developed, for example by characterizing the types of efflux pumps present. Interference of xenobiotics might be specific for certain types of efflux pumps and different efflux pumps have different substrates that may be affected. Further characterization of the cellular efflux in echinoid larvae, and for example fish or amphibian larvae, can reveal to what extent echinoids are suitable model organisms to study interference of xenobiotics with efflux pumps activities and potential mixture effects.

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CHAPTER 5.

Effects of a field-based mixture of persistent organic pollutants on *Psammechinus miliaris* early life development

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Based on:
To be submitted

Abstract

Persistent organic pollutants occur in the marine environment as mixtures of compounds that could influence each others effects. This study investigates the combined toxic effects of a field-based marine contaminant mixture on the prolonged early life stage (p-ELS) and thyroid hormone (TH) induced metamorphosis bioassays of the echinoid (Echinodermata) *Psammechinus miliaris*. For that purpose two field-based mixtures were tested. FM1 was composed of the following seven compounds: BDE-47 (2,2',4,4'-tetrabromodiphenyl ether); PFOS (heptadecafluorooctane sulfonic acid); PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl); PCB-126 (3,3',4,4',5-pentachlorobiphenyl); HBCD (hexabromocyclododecane); DBT (dibutyltin); TPT (triphenyltin). FM2 was the same mixture without TPT and DBT. In addition TPT and DBT also were tested alone. In the echinoid p-ELS bioassay, the FM1 significantly induced larval morphological abnormalities and delayed development in a at concentrations ≥FM1/81 (corresponding to a 81 times dilution of the highest test concentration). FM2 (without TPT and DBT) only induced morphological abnormalities at the highest concentration. TPT and, to a lesser extent, DBT alone also induced a statistically significant increase in morphological abnormalities at concentrations ≥0.2 and ≥32 µg/l, respectively. This corresponds to a TPT concentration approximately comparable to a 50 times diluted FM1 (FM1/50), while this DBT concentration is 10 times higher than that in FM1 (3 µg/l). Therefore, the addition of TPT to the FM2 would add more to the total toxicity than the addition of DBT. In the metamorphosis assay, the FM1 induced a statistically significant metamorphosis acceleration and morphological abnormalities in juveniles at concentrations ≥FM1/27 and ≥FM1/9, respectively, while FM2 did not affect metamorphosis at all. TPT and DBT alone significantly accelerated metamorphosis at ≥1.7 and ≥4 µg/l, respectively, as well as an increase in juvenile morphological abnormalities ≥0.1 and ≥32 µg/l, respectively. TPT can account for approximately 100% of the metamorphosis acceleration observed in larvae exposed to FM1. TPT is an strong inducer of the RXR (retinoic X receptor) which is known to synergize TH and retinoic acid dependent mediated mechanisms, both are crucial for early development and metamorphosis. As RXR genes are expressed in echinoids it is speculated that the strong enhancement of the toxicity of FM2 by TPT, and possibly DBT, is mediated via the RXR. Given the high environmental levels of TPT it is important to further elucidate the mechanism behind this mixture effect.

1. Introduction

Persistent organic pollutants (POPs) are ubiquitously present in the marine and estuarine environment. Due to its physicochemical properties, they tend to accumulate in sediments and biota, leading to biomagnification throughout the food web. Consequently, POPs can reach potentially toxic concentrations in especially sediment-associated organisms and organisms higher in the food chain.

POPs such as polychlorinatedbiphenyls (PCBs), brominated flame retardants, perfluorinated and alkyltin compounds, have been shown to be hazardous as individual compounds (Anselmo et al. 2011; Gutleb et al. 2007a; Gutleb et al. 2007b; Murk et al. 1994a; Murk et al. 1994b; van Ginneken et al. 2009). Although POP occur in marine and estuarine environments as complex mixtures, their combined toxicity is hardly known. The compounds in mixtures can potentially influence each other's toxicity. Mixture toxicity can be additive (equal to the summation of the toxicity of all compounds), synergistic (substantially greater than the additive toxicity) or antagonistic (significantly less than the additive toxicity of each compound) (Walker et al. 1996).

Examples of synergism include the increased toxicity of a mixture of antifouling biocides (copper pyrithione, Irgarol 1051, dichlofluanid, tolylfluanid and Sea nine 211) in echinoid embryos (*Strongylocentrotus intermedius)* compared to the summation of their individual toxicity(Wang et al. 2011), as well as the toxic effects in early life stages of Japanese killifish, *Oryzias latipes,* upon combined exposure to TBT and PCBs (Nakayama et al. 2005). In a previous study we showed that *Psammechinus miliaris* larvae were more sensitive to a toxic cellular efflux pump substrate when exposed in combination with an efflux pump inhibitor (Anselmo et al. in press). Therefore, testing environmentally relevant mixtures of POPs is of clear importance to determine and understand the effects of compounds that organisms are truly exposed to in the environment.

Echinoderms (*e.g.* echinoids) are a group of marine benthic organisms that are considered to be key-components of marine ecosystems (Sugni et al. 2007). Echinoids (Echinodermata) also are important test species in marine ecotoxicological research due to their juvenile and adult benthic lifestyle in sediments, the most contaminated compartment of marine and estuarine ecosystems. In addition, echinoids are susceptible to biomagnification because many species, including *P. miliaris*, are second or third level predators(Sugni et al. 2007). PCB concentrations measured in the sea urchin *Strongylocentrotus droebachiensis* were approximately 65 times higher relative to other invertebrate species (*i.e. Mytilus edulis*) (Bright et al. 1995). In this way, echinoids are at risk for potentially adverse effects of marine POPs accumulated in the sediment (Sugni et al. 2007). Furthermore, as egg-laying species early life development of echinoids is also potentially at risk for adverse effects due to

exposure to maternally transferred relatively high adult POP levels into the eggs (Nakayama et al. 2005).

Interestingly, echinoids are closely related to vertebrates from a phylogenetic point of view, sharing a significant degree of homology (Sea Urchin Genome Sequencing et al. 2006). Consequently, they show similarities with vertebrates in terms of physiological processes and in several hormonal pathways (Lavado et al. 2006; Porte et al. 2006). A particularly interesting similarity of echinoids with vertebrates (*i. e.* amphibians and flat fish) is a thyroid hormone (TH) induced metamorphosis (Chino et al. 1994; Klaren et al. 2008; Schreiber and Specker 1999). This makes echinoids susceptible to the effects of TH disrupting chemicals (THDCs) (Anselmo et al., submitted).

The aim of the present study was to apply the *P. miliaris* echinoid prolonged early life stage (p-ELS) (Anselmo et al. 2011) and metamorphosis bioassays previously reported (Anselmo et al., accepted with revisions). For that purpose experiments were performed with an environmentally relevant field-based mixture (FM1) of seven POPs – PBDE-47, HBCD, DBT, TPT, PFOS, PCB-153 and PCB-126 (Table 1). The FM1 tested was prepared based on the relative proportion of the most abundant POPs measured in the eggs of Common Tern (*Sterna hirundo*) that failed to hatch (Bouma et al., 1999; Heuvel-Greve van den et al., 2003; Meininger et al., 2006). In addition the FM1 was tested without TPT and DBT (FM2), and TPT and DBT were tested individually as well. We evaluated the effects of the FMs, TPT and DBT in the p-ELS and metamorphosis bioassays to assess potential mixture effects and identify the most toxic compounds present in FM1.

2. Materials and methods

2.1. Adult animals

Sea urchins (*Psammechinus miliaris*) were collected from the Eastern Scheldt (The Netherlands) and maintained in fiber glass tanks (L*W*W in cm = 200*80*30) under controlled conditions (*i.e.* temperature, photoperiod and food availability) as previously reported (Anselmo et al. 2011).

2.2. Gamete collection and fertilization

To obtain fertilized eggs, an adult male and female *P. miliaris* were dissected and eggs and sperm collected. Fertilization took place within 4 hours of gamete collection according to a procedure by Anselmo et al. (2011). Fertilization success was at least 90%, in accordance to recommendations

made by Environment Canada (1992) and USEPA (2002) for bioassay validation. Eggs and larvae were reared in artificial seawater (ASW) prepared using Instant Ocean® synthetic salts (Spectrum Brands, Inc.) and aged under continuous aeration for at least 1 week (salinity 31±1 ‰).

2.2 Prolonged Early Life Stage (p-ELS) bioassay

Fertilized eggs were transferred to glass 600 ml beakers containing 500 ml of ASW at a density of 0.5 larvae/ml, spiked with the respective test concentrations (see Table 1) plus a solvent (DMSO at 0.1% v/v) and ASW control.

Throughout the test period, water was refreshed twice a week by removing half of the exposure volume (250 ml). To prevent mechanical damage to the larvae, a PVC tube with a mesh (90 µm) at the base was inserted in the beaker to keep the larvae from being sucked, while gently siphoning water from inside the PVC tube using a vacuum pump. The beaker was then filled up to 500 ml again with new ASW at the appropriate test concentration. Experiments were conducted in duplicate (n=2). During the entire test period larval density was kept at approximately 0.5 larvae/ml by decreasing the water volume in case of toxicant induced mortality. The beakers were kept on a shaker at 30 rpm in a climate controlled room at 18±1 °C with a photoperiod of 16 hours light and 8 hours dark. From 2 days post-fertilization (dpf) on larvae were fed the microalgae *Dunaliella sp.* at densities of 1500, 2500 and 4000 cells/ml for the 4, 6 and 8-armed stage, respectively (Schipper et al. 2008). At three time points (approximately 1, 6 and 13 dpf, depending on the speed of development in control groups) a total of 20 larvae per replicate (n=2) were sampled into 10 wells of a 24 wells-plate at a density of 1 larvae/ml. The 24 wells-plates were kept at the same temperature and photoperiod as the glass beakers, but without agitation. On the day after each sampling, larvae were scored for developmental stage, morphological abnormalities and mortality.

2.3. Metamorphosis bioassay

Following fertilization, embryos were reared in 600 ml beakers containing ASW as described above until they reached the 8-armed pluteus stage. At the start of the metamorphosis experiment (15 - 20 dpf), 15 larvae at the 8-armed pluteus stage with the sea urchin rudiment between stage J and K (as described by Chino et al. 1994) were sampled into a 100 ml glass beaker with 40 ml of ASW with the respective test concentration. Exposure was in duplicate and the final DMSO concentration was 0.1% v/v. Beakers were kept without agitation at the same temperature and light conditions. Twice per week 10 ml of ASW at the desired test concentrations was added to each replicate. Once the volume in the

test beakers reached 80 ml half of it was removed (40 ml) and 10 ml was added again. Subsequently, water refreshments continued as described.

Every other day larvae were examined and the following endpoints were quantified: completion of metamorphosis, morphological abnormalities in juveniles, and mortality. Once 80% of the larvae in the ASW and DMSO controls completed metamorphosis experiments were terminated.

2.4. Chemicals

DBT (dibutyltin dichloride; CAS: 683-18-1; purity 96 %), TPT (triphenyltin chloride; CAS: 639-58-7; purity 98.6%), and PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl; CAS: 35065-27-1; analytical grade) were obtained from Sigma-Aldrich. PCB-126 (3,3',4,4',5-pentachlorobiphenyl; CAS: 57465-28-8; purity 99.2 %) was obtained from Promochem, BDE-47 (2,2',4,4'-tetrabromodiphenyl ether; CAS: 5436-43-1; purity >99 %;) from AccuStandard, PFOS (heptadecafluorooctane sulfonic acid potassium salt; CAS: 2795-39-3; purity =98 %) from Fluka and HBCD (hexabromocyclododecane technical mixture) was obtained through BSEF (Bromine Science and Environmental Forum, with kind co-operation of Dr. Klaus Rothenbacher). All stock solutions were prepared in DMSO (dimethyl sulfoxide; CAS: 67-68-5; purity 99.9 %; Acros Organics) and stored in the dark at room temperature.

2.5. Test mixtures

The choice of compounds and their relative concentration in the field-based mixture (FM1) was based on the concentrations of compounds that were increased compared to control populations in Common Tern (*Sterna hirundo*) eggs from a period 1994-1998 where the eggs failed to hatch (Bouma et al., 1999; Heuvel-Greve van den et al., 2003; Meininger et al., 2006). The ratio's between the compounds were estimated based on levels determined in the eggs in the period 1994-2007 plus additional information of the better monitored levels of compounds in eel from the same location (references in table 1). Table 1 shows the exposure concentrations of the seven compounds in the FM1 dilutions. The second field mixture 2 (FM2) is identical to FM1, but without TPT and DBT. First a dose range test was performed with the dilutions ranging from FM1 to FM1/729 in the p-ELS and from FM1 to FM1/81 in the metamorphosis bioassay (Table 1). Test concentrations of the FM1, FM2, TPT and DBT experiments were based on previous pilot tests.

Table 1. Exposure concentrations (µg/l) of the individual compounds in the field-based mixture (FM1) and FM2, which is FM1 without TPT and DBT. The mixtures are diluted in steps of 3.

Compound	FM	FM/3	FM/9	FM/27	FM/81	FM/243	FM/729
BDE-47[1]	61.78	20.59	6.86	2.29	0.76	0.25	0.08
DBT[2]	3.12	1.04	0.35	0.12	0.04	0.01	0.004
TPT[2]	10.63	3.54	1.18	0.39	0.13	0.04	0.014
PFOS[3]	404.74	134.91	44.97	14.99	5.00	1.67	0.56
PCB-153[2]	407.41	135.80	45.27	15.09	5.03	1.68	0.56
HBCD[4]	137.50	45.83	15.28	5.09	1.70	0.57	0.19
PCB-126[4]*	0.168	0.056	0.0187	0.0062	0.0021	0.0006	0.0002

[1] Van Leeuwen and De Boer, 2008; [2] Heuvel-Greve et al., 2007); [3] Kwadijk et al., 2010; [4] Morris et al., 2004; * to provide the correct TEQs, TEF of PCB 126 is 0.1

2.6. Statistics

Statistical analysis was performed using GraphPad Prism version 5. Significant differences were detected using one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test and two-way ANOVA followed by Bonferroni multiple comparisons. EC_{50} values were calculated by fitting a nonlinear regression model to the data.

The metamorphosis acceleration was calculated based on the difference between the number of days that larvae took to reach at least 80 % metamorphosis in each dose group in relation to the DMSO control, which was set as 100 %.

3. Results

3.1 Prolonged Early Life Stage (p-ELS) bioassay

In the ASW and DMSO control groups, more than 90% of the fertilized *P. miliaris* eggs hatched normally within 24 hours post-fertilization (hpf). At 2 dpf, the larvae had further developed to the 4-armed pluteus stage, at approximately 6 dpf larvae were at the 6-armed pluteus stage and around 13 dpf they reached the 8-armed pluteus stage in accordance with earlier results (Anselmo et al., 2011). No statistically significant differences were observed between the ASW and DMSO control groups for any of the endpoints measured.

Effects induced by the field-based mixtures (FM1 and FM2)

A steep dose-dependent increase in mortality and morphological abnormalities was observed for embryos and larvae exposed to the FM1 (Fig. 1- A). Fertilized eggs in the dose groups FM1 and FM1/3 completely failed to hatch. For sampling 1 (2 dpf) the dose groups ≥FM1/81 showed a statistically significant increase in the percentage of morphological abnormalities compared to the DMSO control group. For sampling 2 (8 dpf) a statistically significant increase in morphological abnormalities was already observed at the dose group FM1/243. The most prevalent morphologically abnormalities in larvae were related to abnormal skeletogenesis (*i.e.* deformed or absent arm rods). The EC_{50} was equivalent to a dose of FM1/100 (corresponding to 1% of the highest test concentration). The No Observed Adverse Effect Concentration (NOAEC) for morphological abnormalities was FM1/729.

At 8 dpf (sampling 2), a significant dose-dependent delay in development was observed at test concentrations ≥ FM1/243 (Fig. 1- B). Delayed larvae were mostly in the 4-armed pluteus stage, while larvae in the control groups were mostly in the 6-armed pluteus stage. Only the larvae exposed to the lowest test concentration, FM1/729, were not significantly delayed in their development compared to the DMSO control group. The NOAEC for delayed developments also was FM1/729. In this p-ELS experiment, the planned third sampling at 13 dpf was not performed because of a biological contamination in the ASW control by the time of the third and last sampling.

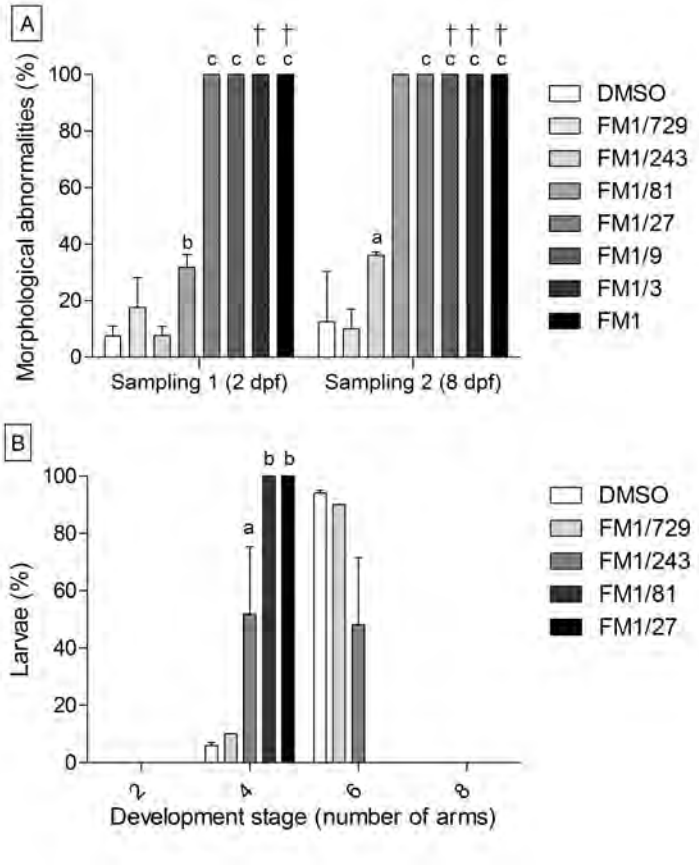

Figure 1. Prolonged *P. miliaris* ELS bioassay: A) Morphological abnormalities (%) in larvae exposed to FM1 from 0-8 dpf. Each bar represents the mean ± SD from 2 repeated observations of 20 larvae per replicate (n=2) for each sampling. (†)indicates complete mortality; B) Development stage of larvae at 8 dpf after exposure to the FM1 dilution series. a) $p<0.01$; b) $p<0.001$ c) $p<0.0001$ (two-way ANOVA with Bonferroni Multiple Comparisons Test). Dose groups higher than FM1/27 were not included since larvae were already dead at 8 dpf.

Exposure to FM2 did not cause any effect on hatching success. A statistically significant increase in morphological abnormalities only occurred at the highest concentration tested (Fig. 2). The most prevalent morphologically abnormalities in larvae were related to abnormal skeletogenesis (*i.e.* deformed or absent arm rods). The EC_{50} value was estimated to be FM2/5 and the NOAEC FM2/27.

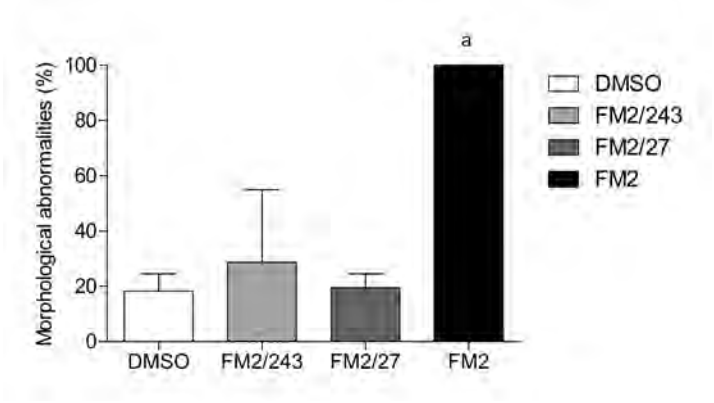

Figure 2. Morphological abnormalities (%) in larvae exposed to FM2 dilution series from 0-4 dpf in the prolonged ELS bioassay. Each bar represents the mean ± SD from 1 observation of 20 larvae per replicate (n=2) at dpf 4 . a) $p<0.001$ (two-way ANOVA with Bonferroni Multiple Comparisons Test).

Effects of TPT and DBT in the p-ELS

In a pilot experiment, fertilized eggs exposed to concentrations ≥5 µg/l TPT (equivalent to approximately ≥FM1/3) resulted in complete hatching failure. At 2 dpf, 100 % of the larvae in the lowest dose group (2 µg/l TPT) had morphological abnormalities and the experiment was terminated (data not shown).

Therefore, in the next experiment also lower TPT concentrations were tested. Again fertilized eggs exposed to ≥5 µg/l TPT completely failed to hatch (Fig. 3- A). All larvae exposed to ≥0.6 µg/l had morphological abnormalities. For sampling 1 (2 dpf) also at ≥0.2 µg/l a statistically significant induction of morphological abnormalities was observed for 30% of the larvae Similarly to what was observed for FM1, the type of morphological abnormalities in larvae exposed to TPT were related to abnormal skeletogenesis (*i.e.* deformed or absent arm rods). The EC_{50} for morphological abnormalities was 0.3 µg/l TPT. At 13 dpf, the surviving larvae exposed to 0.6 µg/l were all arrested at the 4-armed pluteus stage, while individuals in the control group or in lower dose groups had developed normally into the 8-armed pluteus stage. The NOAEC for morphological abnormalities was 0.1 µg/l TPT and for the development stage at 13 dpf 0.2 µg/l TPT.

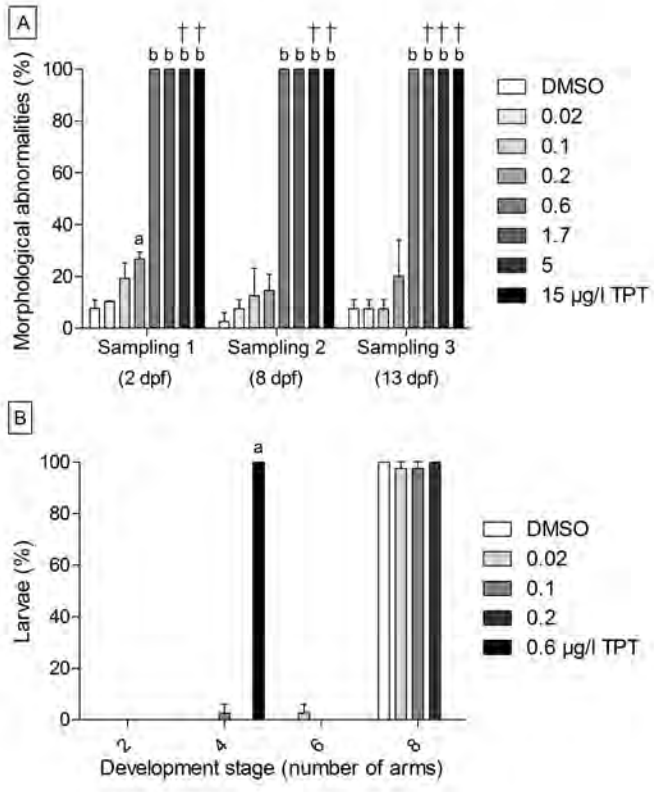

Figure 3. Prolonged *P. miliaris* ELS bioassay: A) Morphological abnormalities (%) in *P. miliaris* larvae at 13 dpf exposed to TPT from 0-13 dpf. Each bar represents the mean ± SD of 2 replicates of 20 larvae (n=2). B) Developmental stage of larvae at 13 dpf after exposure to TPT in the prolonged ELS bioassay. Dose groups above 0.6 µg/l were not included since larvae were already dead at 13 dpf. (†) indicates complete mortality; a $p<0.01$; b $p<0.001$ (two-way ANOVA with Bonferroni Multiple Comparisons Test).

Exposure to DBT also resulted in a clear dose related effect and no fertilized eggs hatched at concentrations ≥ 95 µg/l (Figure 4- A). All larvae exposed to 32 µg/l DBT were morphologically abnormal. The EC_{50} for morphological abnormalities for sampling 1, 2 and 3 was 12, 14 and 15 µg/l DBT. The NOAEC was 11 µg/l DBT. DBT also delayed larval development similarly to what was observed for TPT exposed larvae, although at an approximately 50 times higher exposure concentration. At 13 dpf, most larvae exposed to 32 µg/l DBT were still at the 4-armed (50%) or 6-armed pluteus stage (48 %), while the majority of the larvae in the control group and lower dose

groups were already at the 8-armed pluteus stage (Figure 4- B). The resulting NOAEC for delayed development was 11 µg/l DBT, again about 50 times higher than for TPT.

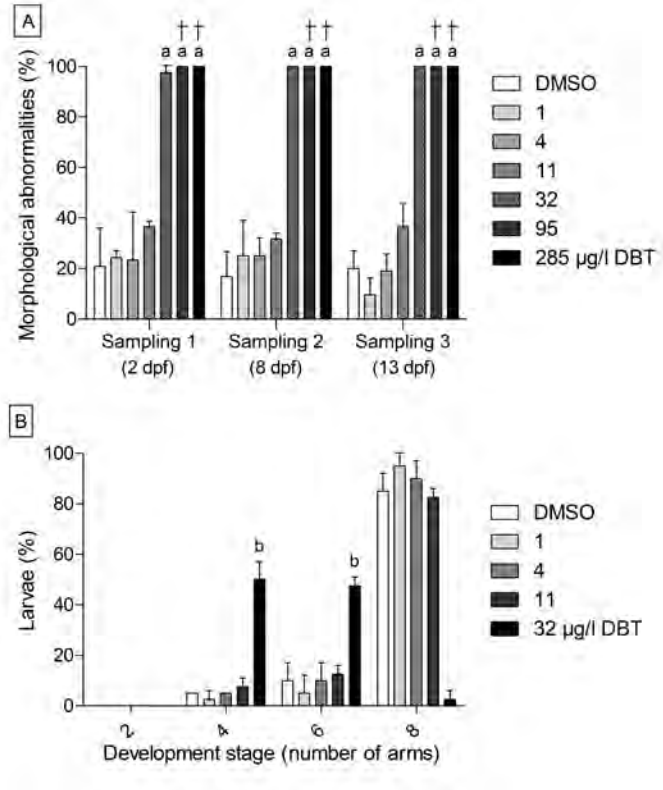

Figure 4. Prolonged *P. miliaris* ELS bioassay: A) Morphological abnormalities (%) of *P. miliaris* larvae at dpf 13 exposed to DBT from 0-13 dpf. Each bar represents the mean ± SD of two replicates of 20 larvae (n=2) . B) Development stage of larvae at 13 dpf after exposure to DBT. Dose groups above 32 µg/l were not included because larvae were dead at 13 dpf. (†) indicates complete mortality. a) $p<0.01$; b) $p<0.001$ (two-way ANOVA with Bonferroni Multiple Comparisons Test);

3.2 Metamorphosis bioassay

Larvae in the ASW and DMSO control groups developed normally, and DMSO (0.1% v/v) did not have an effect on metamorphosis success nor induced morphological abnormalities in juveniles. Experiments were terminated when 80% of the larvae in the DMSO control groups completed metamorphosis, which was at test days 31, 39, 25 and 47 days, for respectively the FM1, FM2, TPT and DBT experiments.

Effects induced by the field-based mixtures (FM1 and FM2)

Larvae exposed to FM1 showed a clear acceleration of metamorphosis and by test day 5 all larvae had completed metamorphosis at a concentration ≥FM1/27 (Figure 5- A). The larvae exposed to concentrations ≥FM1/27 showed a statistically significant acceleration of metamorphosis (Fig. 6- A). However, most larvae did not metamorphose normally and juveniles had severe morphological abnormalities (Figure 7- A). Most commonly observed abnormalities after the strongly accelerated metamorphosis were related to an incomplete reabsorption of larvae body and the presence of the skeletons of arm rods attached to the metamorphosing larvae (Fig. 8). The EC_{50} for enhanced metamorphosis was FM1/27, the EC50 for morphological abnormalities FM1/6. The NOAECs were respectively FM1/81 and FM1/27.

In contrast, exposure to FM2 did not significantly accelerate metamorphosis (Fig. 5- B) nor induced morphological abnormalities in juveniles (Fig. 7- B).

Effects of TPT and DBT on the metamorphosis

As was observed in the pilot experiment (data not shown), exposure to TPT clearly accelerated metamorphosis at concentrations ≥1.7 µg/l TPT (Figures 5- C and 6- C), similarly to what was observed in the FM1 experiment (Figures 5- A and 6- A). The 25% of the larvae in the 15 µg/l TPT groups that had completed metamorphosis at the end of the experiment, all were morphologically abnormal (Fig. 7- C). The EC_{50}s for metamorphosis acceleration and morphological abnormalities were 1 and 0.2 µg/l TPT, and the NOAECs were 0.6 and 0.02 µg/l TPT, respectively (Table 2). Similarly to FM1, the most common abnormalities resulting from the strong metamorphosis acceleration were related to an incomplete reabsorption of larval body and the presence of remaining skeletons of arm rods attached to the metamorphosing larvae (Fig. 8).

Also exposure to DBT accelerated metamorphosis in a dose dependent manner at concentrations ≥4 µg/l DBT (Figures 5 D and 6- D). The EC_{50} for metamorphosis acceleration was 60 µg/l (Table 2) and the NOAEC 1 µg/l. Already at test day 1, approximately 25% of the larvae in the 285 µg/l concentration completed metamorphosis. However, from that day on no more larvae completed metamorphosis and almost all remaining animals died (Fig. 7- D). Induction of morphological abnormalities occurred in a dose dependent manner at test concentrations ≥32 µg/l DBT. The EC_{50} value for morphological abnormalities was 96 µg/l and the NOAEC 11 µg/l DBT (Table 2).

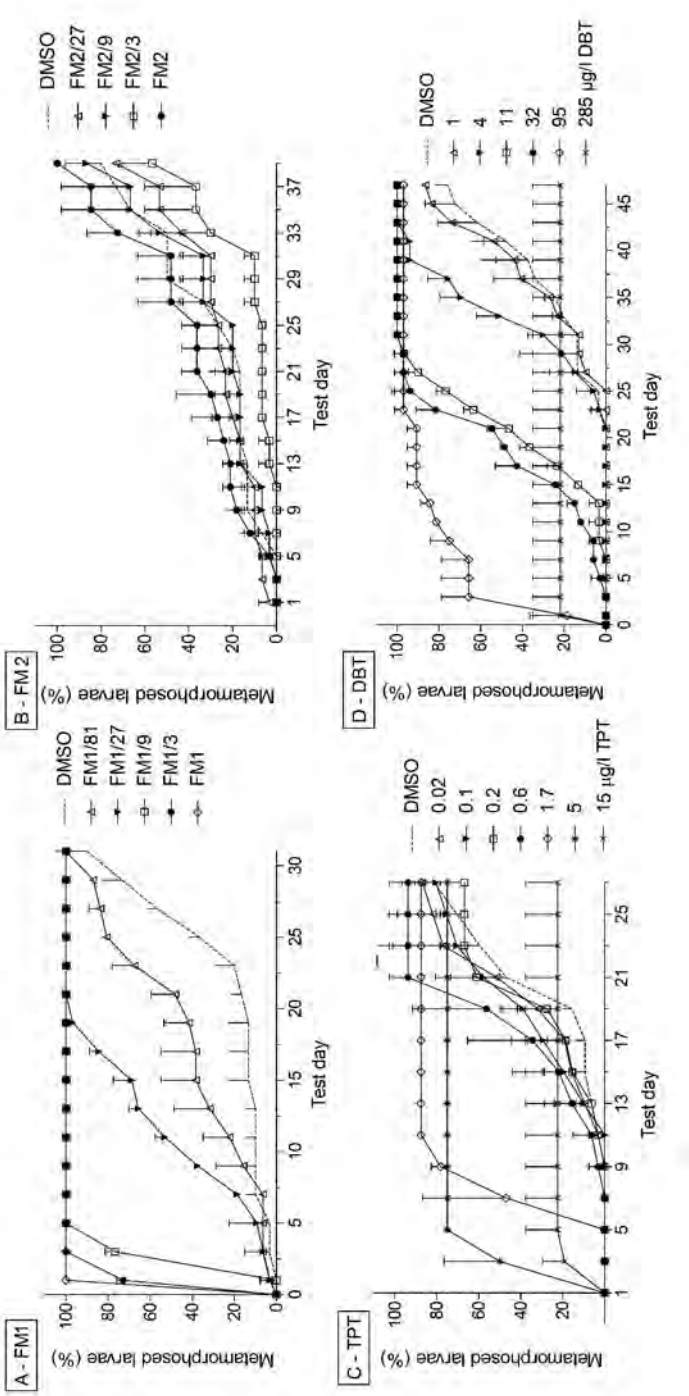

Figure 5. Metamorphosis of *P. miliaris* larvae exposed to: A) Field mixture 1 (FM1); B) Field mixture 2 (FM2); C) TPT; D) DBT. Test day 0 is at the onset of exposure when the animals are in the 8-armed pluteus stage with the sea urchin rudiment (stage J-K). Each value represents the mean ± SD (n=2).

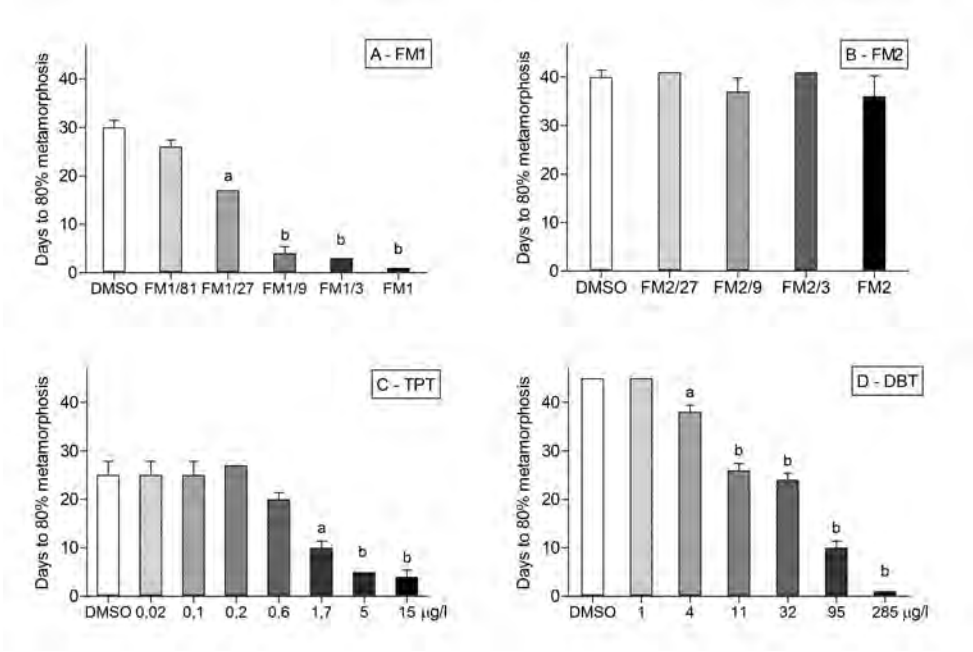

Figure 6. Metamorphosis acceleration of *P. miliaris* larvae expressed as the number days required to reach an 80% metamorphosis success. Larvae were exposed to: A) Field-based mixture 1 (FM1); B) Field mixture 2 (FM2); C) TPT; D) DBT. Each value represents the mean ± SD of 2 replicates of 15 larvae (n=2). a $p<0.01$; b $p<0.001$ (two-way ANOVA with Bonferroni Multiple Comparisons Test).

Table 3. EC_{50} (µg/l) of T4, TPT and DBT on metamorphosis acceleration in the echinoid metamorphosis bioassay, and their potencies relative to T4.

Compound	EC_{50} (µg/l)	Relative Potency
T4[1]	14	1
TPT[2]	1	14
DBT[2]	60	0,2

1) Anselmo et al., accepted with revisions.
2) This study.

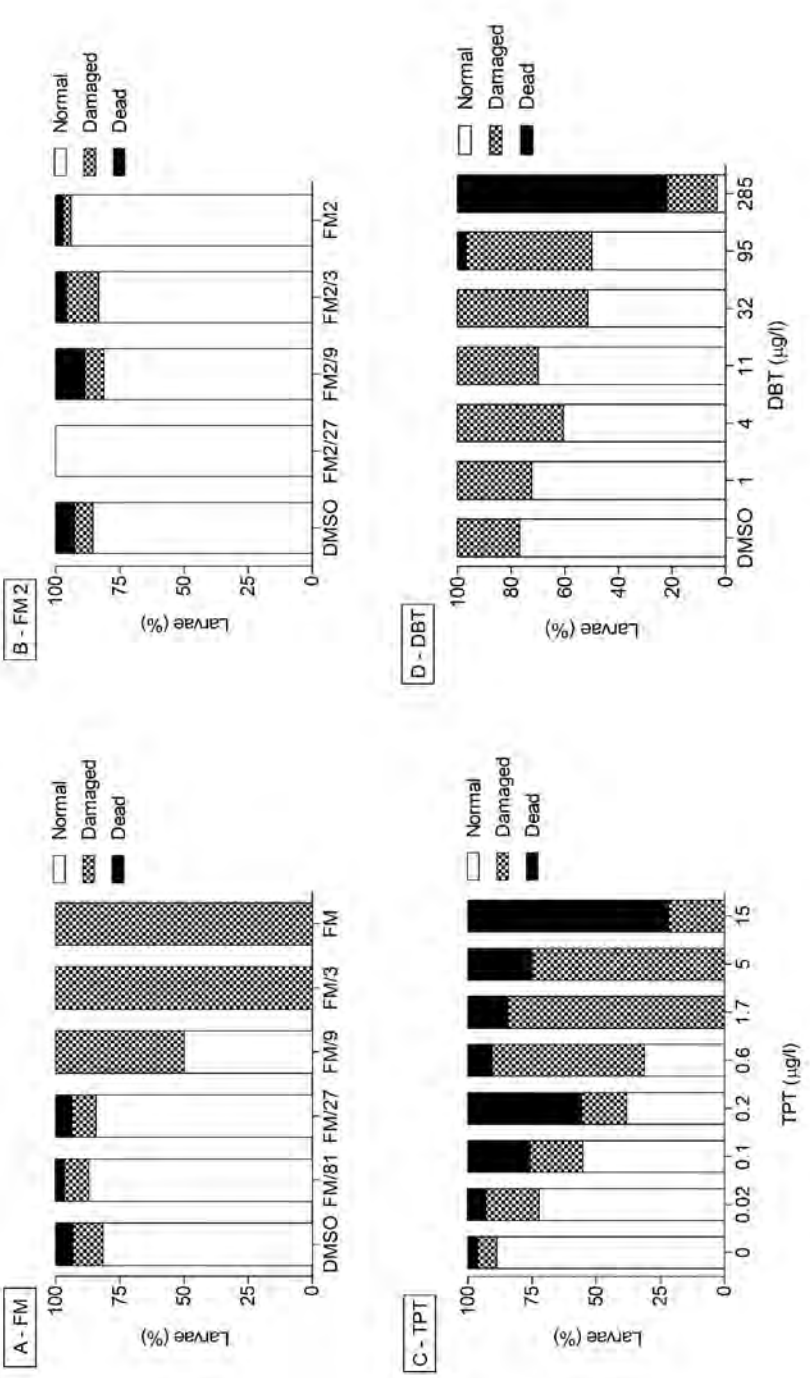

Figure 7. Percentage of normal, damaged and dead *P. miliaris* individuals at the end of the metamorphosis assays after exposure to: A) FM1; B) FM2; C) TPT; D) DBT. Each value represents the mean ± SD (n=2, 15 animals per group).

4. Discussion

In the present study we tested a field-based mixture (FM1) of POPs in the recently developed echinoid prolonged ELS (p-ELS) and metamorphosis bioassays. The FM1 consists of seven POPs that were abundant in Common Tern eggs that failed to hatch in the Terneuzen colony in the Western Scheldt in the period 1994-2007. The ratio between the POPs was estimated based on the ratios found in the Common Tern eggs plus additional information based on better monitored levels in eel (Table 1). In addition a mixture (FM2) was tested that was identical to FM1, but without TPT and DBT. These compounds were tested separately as well. Both the FM1 and the compounds TPT and DBT had strong effects on development and survival of the larvae in the p-ELS assay as well as on the duration of the metamorphosis and the morphology of the juveniles. In contrast, FM2 did not induce any effect on metamorphosis.

4.1. Effects of the alkyltin compounds

The difference in toxicity between FM1 and FM2 for the very early life development and the metamorphosis was quite dramatic. In the two highest FM1 exposure concentrations the larvae did not even hatch, while upon exposure to FM2 the animals hatched normally in all dose groups. None of the larvae exposed to FM1/81 and higher developed normally into the 8-armed pluteus stage, while in FM2 effects were only seen in the highest dose group (exposure to undiluted FM2). In the metamorphosis assay all FM2 exposed larvae developed normally, and no effects were observed on the duration of the metamorphosis and the quality of the juveniles (Fig. 6- B and 7- B). This suggests that the NOAEC for the 5 compounds in FM2 (BDE47, PFOS, PCB153, PCB126 and HBCD) is $\geq$ than their concentration in FM2 (Table 1), and levels were not high enough to induce mixture toxicity. When the alkyltin compounds TPT and DBT were tested individually the same effects occurred as observed for the complete FM1. The most extreme effects were seen for TPT, both for hatching, early development and metamorphosis. The EC_{50} for induction of malformations in the p-ELS was 40 times lower for TPT than for DBT (Table 2). TPT even was 14 times more potent in acceleration of the metamorphosis than T4 (Table 3, T4 data from Anselmo et al., accepted with revisions). DBT was 5 times less potent. The relative potency of TPT for induction of malformations in the metamorphosis assay was even much greater, with a ratio between the EC_{50}s of about 400, although the absolute EC_{50} for TPT did not really differ between the assays (0.2 an 0.3 µg/L) (Table 2). The difference in toxicity between TPT and DBT is in accordance with previous results that reported an increased frequency of morphological abnormalities in *Paracentrotus lividus* plutei larvae at DBT concentrations $\geq$ 40 µg/L

(Marin et al. 2000), while for TPT this already occurred at concentrations ≥ 1.1 µg/L (Novelli et al. 2002).

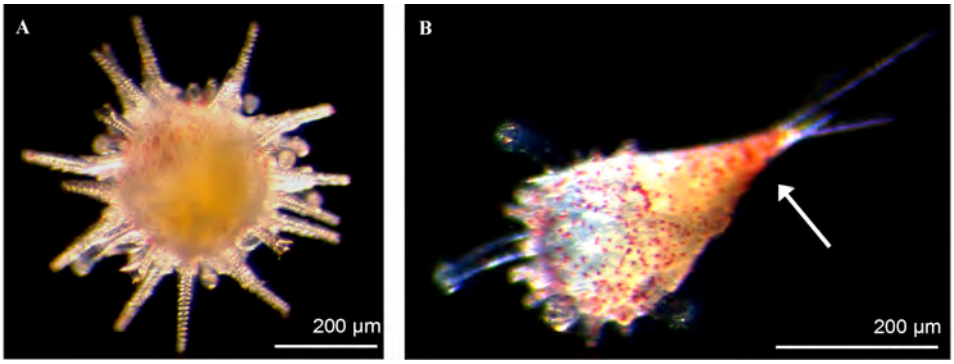

Figure 8. A) Normal P. miliaris juvenile; B) morphological abnormalities juveniles exposed to FM1 and TPT. Arrow - larval arm rods attached to juvenile and incomplete reabsorption of larval body is also present.

As in the p-ELS bioassay induction of malformations was the most sensitive endpoint, the % of malformations induced by TPT alone was compared to the % of malformations induced by FM1, with the concentration of FM1 expressed as the concentration of TPT in the mixture (Figure 9- A). The same is done for DBT (Figure 9- B). Although both TPT and DBT induced malformations in the p-ELS, Fig. 9- B shows that the concentrations of DBT alone present in the mixture would not even contribute to the toxicity of FM1 when 100 % of the animals have malformations. The contribution by TPT was much stronger, but would not contribute enough in the concentration present in FM1 to explain the major part of the toxicity. At the concentration where the FM1 already induces 100% malformations in the p-ELS (FM1/27, equivalent to 0.4 µg TPT/l), the FM1 did not induce any malformations yet (Fig. 7-A).

For the metamorphosis bioassay the same picture is shown. Figure 10- A depicts the induction of malformations and mortality by either TPT alone or the mixture FM1 expressed as the concentration of TPT in the mixture. Figure 10- B shows the same for DBT. These results strongly suggest that TPT, and possibly to a lesser extent also DBT, enhance the toxic effects of other compounds in the mixture.

Known mechanisms of action of TPT include the binding and the activation of the retinoic X receptor (RXR) (Nakanishi et al. 2005) and the peroxisome proliferator-activated receptor gamma (PPARγ) receptors (Kanayama et al. 2005). RXRs and PPARs are ligand-activated transcription

factors that coordinately regulate gene expression. They play important roles in energy balance, inflammation, and vascular biology (Plutzky 2011), all of which are very important during development as well. In vertebrates, the RXRs also play multiple roles in development and metamorphosis as common partner in the transcription regulation mediated by the nuclear receptors such as the TH receptors (TRs) and the retinoic acid receptor (RAR) (Plutzky 2011). RXR synergistically enhances the transcription regulatory effect of RAR (Chambon 2005). All-*trans*-retinoic acid (RA, a vitamin A) is the physiological ligand for RAR that forms functional dimers with the RXR. In echinoids it has been shown that RXR genes indeed are expressed during early life development (Howard-Ashby et al. 2006). RA is involved in a wide variety of processes including embryonic development, morphogenesis, survival, cell growth and differentiation, and tissue homeostasis. The importance of vitamin A is demonstrated by the fact that it is teratogenic when present in excess or deficient levels. Therefore, the induction of the RXR by alkyltin during early development, especially during TH induced metamorphosis for the metamorphosis or the presence of TH disrupting compounds could very well be the explanation of the disturbance of the metamorphosis process. These molecular mechanisms need to be further elucidated.

In a TH responsive element (TRE)-mediated reporter gene assay in GH3 cells that possess endogenous TRs and RXR, retinoic acid alone has been shown to activate the luciferase reporter as well. This demonstrates that RXR activation can result in TH-like effects (Freitas et al., submitted;). In the same cells alkyltin compounds have been shown to activate the TRE-mediated luciferase response in these cells as well (unpublished data). The alkyltins do not have a structure that resembles THs, which is supported by the fact that TPT and DBT did not bind to the TTR at all in the ANSA assay (Montano et al., 2012) that was performed with our compounds (data not shown).

Also the activation of the TR by natural THs or TH disrupting compounds will be enhanced by activation of the RXR which can form heterodimers with TRs (Wong and Shi 1995). In addition to the natural hormones that could be produced by the sea urchin larvae, also hydroxylated metabolites of PBDEs and PCBs, but not the parent compounds, have been shown to mimic THs and bind to the transport proteins TTR and TBG with even greater affinity than thyroxin (Hamers et al. 2006; Marchesini et al. 2008; Montaño et al. 2012) or induce the TH receptor (Freitas et al. 2011; Schriks et al. 2006a). In the FM mixtures tested only parent compounds were present, and it is not yet known whether echinoid larvae have enzyme activities equivalent to cytochrome P450's in vertebrates that are capable to bioactivate these compounds. Although CYP1-like (genes have been demonstrated in echinoids (Goldstone et al. 2007). In a study conducted with starfish (*Asterias rubens*) has shown that they possess P450 activity (Stronkhorst et al. 2003). However, this study was performed in adult starfish and no indication about P450 activity in larval stages was provided,

Alkyltin compounds also have been shown to activate the PPARgamma receptor, which plays a crucial role in energy metabolism and lipid metabolism, including fatty acids. Affected energy metabolism would reduce the rate of metamorphosis, fatty acids, however, have been shown to trigger metamorphosis (Kitamura et al. 1993; Takahashi et al. 2002).

From the other compounds present in FM1, HBCD is most likely to contribute to the morphological abnormalities observed. In an earlier echinoid p-ELS the EC50 for malformations was 40 µg/l HBCD technical mixture (Anselmo et al. 2011). This HBCD concentration corresponds to the concentration present in the FM1/3 dose groups. In addition indications exist that HBCD can be synergistic on TH action thereby enhancing TH dependent tail tip regression in *X. laevis* when exposed in combination with triiodothyronine (T3) (Schriks et al. 2006b). For the other compounds present in the FM1, to our knowledge no indications exist that their concentrations in the FMs will contribute to the malformations observed or the accelerated metamorphosis. Toxic TPT levels in the *P. miliaris* p-ELS and metamorphosis bioassays are relatively low compared to "worst case scenario" field concentrations. Such high TPT levels have for example been reported (~3 µgL−1) in water samples collected from a national park in the USA (Jones-Lepp et al. 2004). Furthermore, TPT concentrations in zebra mussels (Dreissena polymorpha) collected in The Netherlands ranged from 0.02 to 3.2 µg g−1 (dry weight) (Stäb et al. 1995).

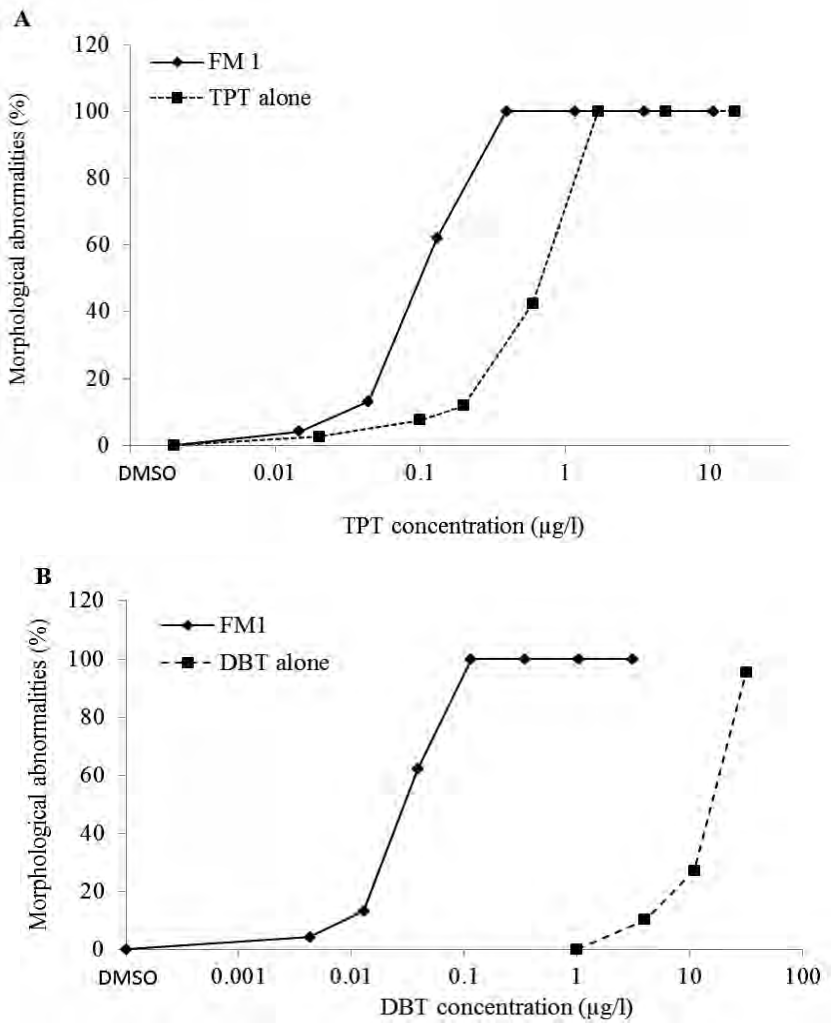

Figure 9. Percentage of malformations in the p-ELS bioassay at the end of the experimental period. A) Comparison of effects induced by the mixture FM1 (expressed as TPT concentration in the mixture, Table 1) and TPT alone; B) Comparison of effects induced by the mixture FM1 (expressed as DBT concentration in the mixture, Table 1) and DBT alone.

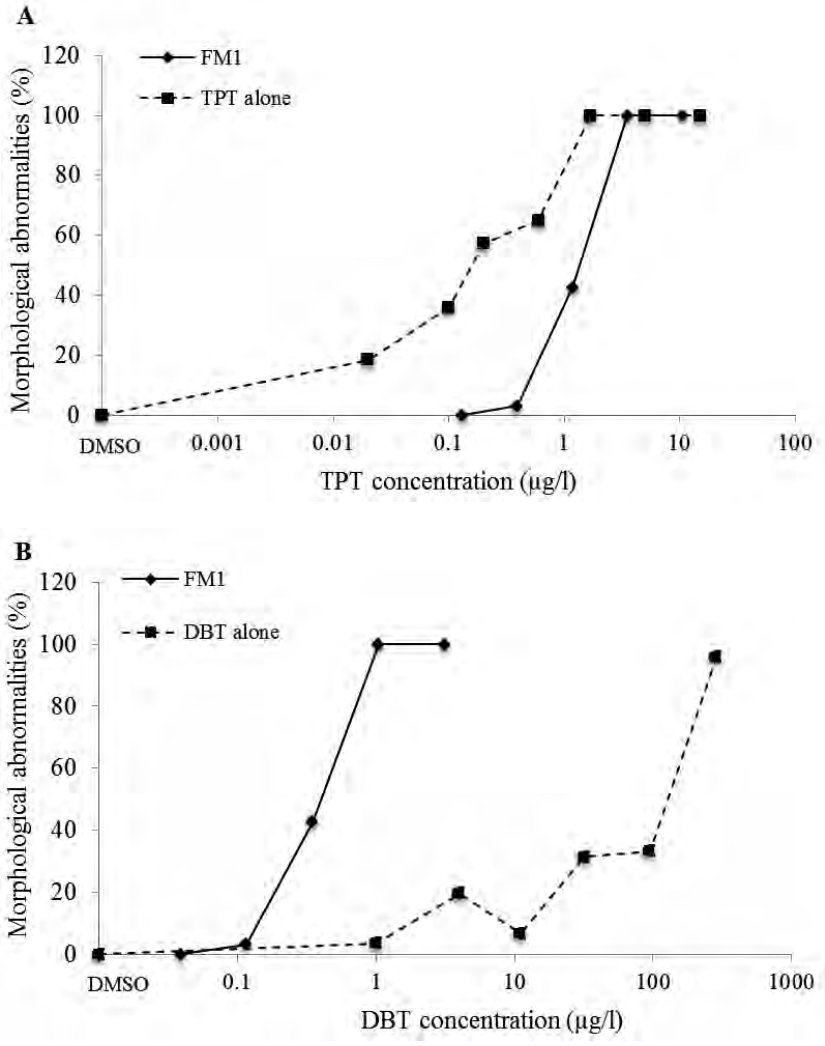

Figure 10. Percentage of malformations in metamorphosis bioassay at the end of the experimental period. A) Comparison of effects induced by the mixture FM1 (expressed as TPT concentration in the mixture, Table 1) and TPT alone; B) Comparison of effects induced by the mixture FM1 (expressed as DBT concentration in the mixture, Table 1) and DBT alone.

5. Conclusions

The present study shows that the prolonged echinoid ELS and metamorphosis bioassays are sensitive to the toxic effects of POPs and that the toxicity of mixtures can be more than the sum of the individual compounds. Especially the alkyltin TPT was very toxic for the developing larvae, inducing malformations and strongly accelerating metamorphosis. These effects could be related to their known interaction with the RXR and PPARgamma, resulting in indirect interference with the TH and vitamin A regulated processes of development and metamorphosis. These molecular toxicological processes deserve further attention as these also occur in vertebrates and currently TPT still is an ubiquitous environmental contaminant that even is present in the human food chain.

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CHAPTER 6.

General discussion

6.1 General discussion

The research presented in this PhD thesis aimed at the development and application of new bioassays covering the major developmental stages and most relevant endpoints of the early life stages of echinoids in order to assess toxic effects of persistent organic pollutants (POPs) on early development. *Psammechinus miliaris* (Echinodermata: Echinoidea) was selected as test species since this is a key species in marine ecosystems, which in its natural benthic habitat is exposed to POPs either via the sediment or via the diet. Furthermore the physiology of echinoids is highly advanced for an invertebrate, showing strong similarities with vertebrates. As POPs are mostly not acutely toxic, the current standard early life stage (ELS) bioassays with exposure periods of only 48-96 hrs may not detect the effects that occur upon a more chronic exposure. Therefore an echinoid prolonged early life stage (p-ELS) bioassay was developed plus a metamorphosis bioassays, the latter being relevant because echinoids have a thyroid hormone (TH) dependent metamorphosis. In addition an assay was developed to detect inhibition of the cellular efflux pump activity involved in the Multi Xenobiotic Resistance (MXR) mechanism which is the first line of defense against toxic compounds. The effects of individual POPs and mixtures thereof were evaluated, as well as the applicability of the echinoids as an invertebrate marine ecotoxicological animal model. This chapter discusses the content of the thesis, and presents some further perspectives for the use for these new bioassays, including their possible role as invertebrate alternatives for vertebrate in vivo toxicity testing.

Relevance of developed bioassays for marine ecotoxicology

The echinoid p-ELS bioassay (Chapter 2) covers both acute and sub-chronic effects of compounds on the early life stages of echinoids. The first sampling is performed at 2 dpf, equivalent to the exposure period in the standard ELS and detects acutely toxic compounds. An example of an acutely toxic compound was TCS since larva suffered most toxic effects already at 2 dpf. The acute toxicity of the broad-spectrum biocide TCS, which was most toxic for hatching larvae, could already be quantified at the first sampling. More sub-acute or delayed toxic effects were detected for HCBD and TBBPA, for which delayed larval development determined at the end of the experimental period (*i.e.* between 13 and 16 dpf) was the most sensitive endpoint in the *P. miliaris* p-ELS bioassay. The standard ELS would have failed to detect these sub-acute and/or delayed developmental effects, which would result in an underestimation of the hazard and risk of such POPs. This occurrence of especially delayed effects is in accordance with results from p-ELS tests performed with *Xenopus laevis* larvae (Gutleb et al. 1999; Gutleb et al. 2007) and larvae from the flatfish sole (*Solea solea*) (Foekema et al. 2008).

The metamorphosis assay (Chapter 3) covers the last phase of echinoid early life development, when larvae undergo TH induced metamorphosis (Chino et al. 1994; Heyland et al. 2004). The metamorphic process is a short but critical window in the development of echinoids, making these organisms potentially sensitive to thyroid hormone disruption. The results presented in this thesis show that T4 accelerates metamorphosis, and the standard TH synthesis inhibitor TU and the iodine uptake inhibitor KSCN delay it. The T4-like brominated flame retardant TBBPA delayed metamorphosis, which could be due to binding, but instead of activating blocking the TH nuclear receptor (TR) as an antagonist. The PBDE mixture, on the other hand, strongly accelerated metamorphosis. It is not yet known whether echinoid larvae can metabolize POPs like PBDEs into OH-PBDEs that are more potent in activating the TR (Freitas et al. 2011). In vertebrates, herbivores have, in general, an active cytochrome P450 system enabling them to metabolize toxic aromatic compounds present in plants. As echinoid larvae are "herbivores" (eating marine algae) it is to be expected that they may have some metabolizing capacity.

The extreme acceleration of the metamorphosis was accompanied by induction of morphological abnormalities in the juveniles. In the animals with a more moderate enhancement of the metamorphosis the occurrence of malformations was less. In tadpoles it has been shown that acceleration of the metamorphosis, in this case due to environmental factors (*e.g.* drought), leads to the reduction of juvenile size and fitness (Merilä 2000). As the metamorphosis test did not cover later juvenile stages, it remains to be elucidated whether accelerated metamorphosis without the occurrence of malformations is adverse for further development in echinoids.

Normal functioning of MXR mechanism is very important as a cellular defense mechanism of many aquatic organisms against the toxic effects caused by POPs (Barbara Holland and Epel 1993). Embryos of the echinoid species *Strongylocentrotus purpuratus*, have been shown to express several types of efflux pumps and possess multidrug efflux activity (Hamdoun et al. 2004). The successful optimization and application of the in vivo echinoid larval cellular efflux pump inhibition assay (CEPIA) revealed strong inhibition by some very common marine POPs such as TCS, the nanoparticles P-85, bisphenol-A (BPA), pentachlorophenol (PCP), heptadecafluorooctane sulfonic acid (PFOS) and o,p'-DDT (chapter 4). The research presented in this chapter also revealed that *P. miliaris* larvae exposed to a binary mixture containing an environmentally relevant efflux pump inhibitor (*i.e.* TCS) in combination with vinblastine, clearly increased the toxic potency of this efflux pump substrate. Some other evidence of potential mixture effects has been reported for *S. Purpuratus* embryos exposed to the model efflux pump inhibitor MK-571 leading to increased accumulation of mercury as well as an increase in its toxic potency (Bosnjak et al. 2009). It would be interesting to

further test the toxic effects of these POPs in combination with other compounds (including heavy metals) that are potential cellular efflux pump substrates.

Toxic effects of the marine POPs mixtures

The relevance of assessing the toxicity of mixtures is widely recognized by the scientific community. Nonetheless, most (eco)toxicological research is performed with single compounds. To assess the effects of mixtures is a rather challenging field of research, as the relative and absolute composition of field mixtures never is the same and contaminants present in a mixture often have different mechanisms of action. Mixture effects have been shown for inhibitors of the echinoid cellular efflux pumps (chapter 4) and for the alkyltin compounds that may act via RXR and/or peroxisome proliferator-activated receptor (PPAR) gamma receptors, indirectly influencing the TR (Chapter 5). Those mixture effects are expected to be life-stage dependent, in connection with the special relevance of such mechanisms in that life stage. As it is not feasible to test all possible mixtures on all possible life stages, it is important to elucidate molecular mechanisms of toxic action of compounds. This may be done using molecular 'omics' techniques on tissues of exposed animals. Also results from in vitro experiments can be useful to further identify the molecular mechanisms of toxic action. Insight in modes of action allows extrapolation of mixture effects to other mixtures and life stages. In combination with thorough knowledge of the most relevant molecular and biochemical processes during larval development and metamorphosis it will be possible to predict the most sensitive endpoint to test, reducing the risk of false negatives caused by choosing wrong exposure and observation periods and life stages.

It remains to be elucidated whether the current field concentrations of these and the other POPs, and field mixtures thereof, will result in toxic effects on organisms in the field. If so, the question is what the ecological implications of these effects on hatching, ELS and metamorphosis would be for echinoid populations in the field. With the new bioassays the hazard of these compounds and their mixtures was demonstrated. For a real risk assessment it is necessary to compare the internal levels in the exposed animals with those of field animals in polluted areas. For this adults can be collected and analyzed to obtain an indication of early life exposure, as POPs are transferred from the mother animal to her eggs (Foekema et al. 2012).

Possible role for echinoid bioassays in reducing the use of vertebrate species

Echinoids share several molecular and physiological commonalities with vertebrates. Strong homologies exist between the vertebrate and echinoid endocrine system. These homologies include

key enzymatic pathways involved in steroid metabolism (Lavado et al. 2006) and the induction of echinoid metamorphosis by THs as in several vertebrate species (*i.e.* amphibians and flat fish) (Chino et al. 1994; Heyland et al. 2004; Saito et al. 1998). Interestingly, RXR and PPAR also have been identified in echinoids (Vaughn, 2012)

For TH, the key question is to what extent echinoids have the same or similar TH dependent mechanisms of action as vertebrates. It is known that during amphibian (*e.g. Xenopus laevis*) early life development, the expression levels of TRs clearly increase during the premetamorphic period rendering target tissues competent to the action of TH during the metamorphic period (Furlow and Neff 2006). In echinoids, a TH receptor gene (Sp-Thr) orthologous to the vertebrate TR gene has been identified in *Strongylocentrotus purpuratus* (Howard-Ashby et al. 2006). In the present thesis, expressions levels of the Sp-Thr gene were investigated during the development from egg to the late larval stages (unpublished data). Results show that although the Sp-Thr gene was expressed during echinoid early life development, levels do not increase during the premetamorphic period as reported for the amphibian TR gene (Fig. 1- A). Furthermore, exposure of *S. purpuratus* larvae at the late 8-arms pluteus stage to T3, T4, TU and PBDEs (previously tested in chapter 2, this thesis) did not reveal any differences in Sp-Thr gene expression (Fig. 1- B).

In vertebrates, TH function is known to be mediated by nuclear TRs to which 3,5,3´-triiodo-L-thyronine (T3) binds with higher affinity than T4 (Furlow and Neff 2006; Schueler et al. 1990; Zhang and Lazar 2000). However, in the last decade a novel extracellular or nongenomic mechanism of action has been suggested to play a role in TH function (Davis et al. 2008). The existence of such an additional mechanism is supported by a cell surface receptor for iodothyronines (T4 and T3) described for a structural protein located in the plasma membrane of mammalian cells (Bergh et al. 2005). Interestingly, for echinoids T4 has been suggested to be more potent as an inducer of the metamorphosis than T3 (Chino et al. 1994).

Before echinoids can replace vertebrates as an animal model to test compounds for TH disruption it is essential to further characterize TH function in these model organisms. Particularly, to determine whether they possess a membrane receptor that binds T4 and induces metamorphosis, or only one (or more) nuclear TRs that have a higher affinity for T4 rather than T3. Until now no bioassay exists to detect interference of compounds with the vertebrate membrane receptor, only with the nuclear receptors (*e.g.* (Freitas et al. 2011). If TH function in echinoids would only be mediated by a membrane receptor, the echinoid metamorphosis assay could become a unique test for this endpoint. On the other hand, if echinoids have a functional nuclear TR, this would provide the opportunity to

use it in an intelligent testing strategy reducing the need to use vertebrate animals for testing of TH disrupting compounds.

In addition, it should also be investigated to what extent echinoid larvae can metabolize POPs such as PCBs and PBDEs into hydroxyl metabolites that have more TH-like structures than the parent compounds (Freitas et al. 2011; Schriks et al. 2006). The use of invertebrate animals such as echinoids may have clear advantages over in vitro bioassays as they possess more integrated interactions of various tissues and differentiation processes than could be present in isolated cell lines such as for example fish cell lines {Schirmer, 2006 #1146}. The echinoids could *e.g.* be used as a second step after in vitro testing of potential endocrine disrupting compounds.

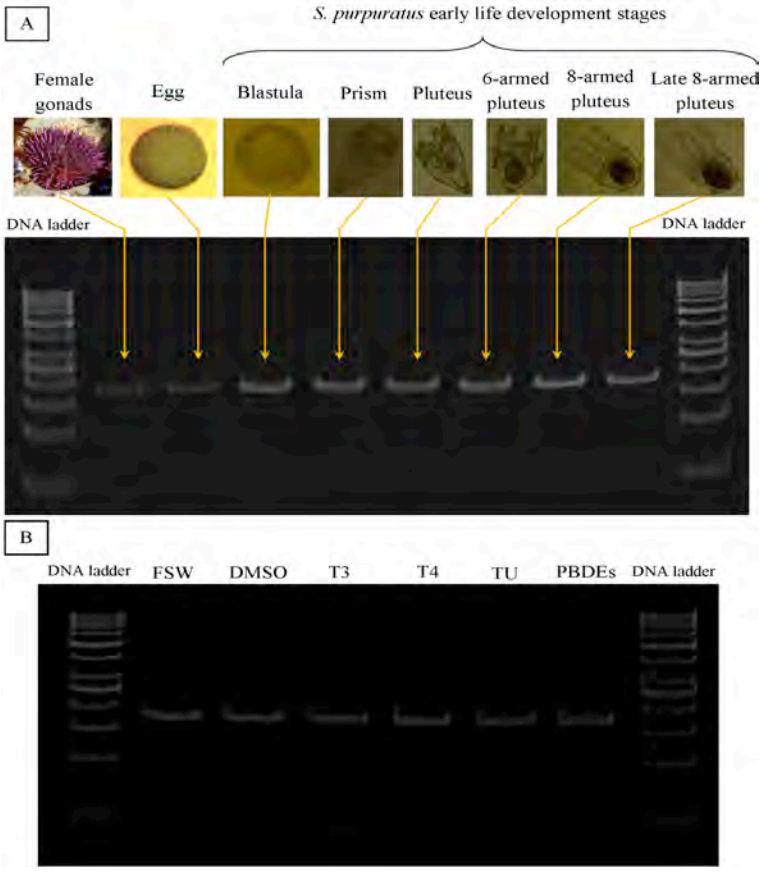

Figure 1. Acrylamide gel (8%) electrophoresis of *S. purpuratus* Thr DNA from: A) female gonads, egg and larval stages; B) late 8-armed pluteus stage larvae after 48 h exposure to T3, T4, TU and PBDEs. The molecular markers (100bp ladder) are in the right and left lanes.

6.2. Conclusions

In the present thesis three bioassays were successfully developed and applied to assess the effects of marine POPs on echinoid early life development. Results demonstrate that echinoids are a suitable marine test organism to perform early life stage tests including the phase of full metamorphosis.

The new bioassays were developed using the echinoid *P.miliaris*, but they are applicable to other echinoid species as well. Echinoids are key species in marine ecosystems including the North Sea and its estuaries, and they can be easily aquacultured in the laboratory. By manipulating the culture conditions (*i.e.* temperature, light) their spawning period can be extended to 9 months per year.

The p-ELS bioassay demonstrated that effects on the rate of larval development can occur at lower concentrations of toxic compounds than the concentrations causing the induction of malformations or mortality. This endpoint, however, is not covered in the standardized ELS echinoid bioassay. Therefore, these bioassays may seriously underestimate the toxicity of compounds that are not acutely toxic. In addition, the research presented in this thesis shows that testing single compounds can also lead to underestimation of the toxicity of environmental mixtures.

The echinoid metamorphosis also detected effects POPs during the metamorphosis of *P. miliaris* larvae. The mechanism of action is thought to be related to disruption of the TH function since T4 was able to accelerate metamorphosis similarly to effects observed for PBDEs. Furthermore, the TH synthesis inhibitor TU delayed metamorphosis in a similar way to the effects induced by TBBPA and TCS exposure.

The inhibition of cellular efflux pumps by certain POPs can compromise the MXR mechanism as a first line of defense against contaminants. Such inhibition can have serious consequences for *P. miliaris* larval survival as demonstrated for the combined exposure of vinblastine with an efflux pump inhibitor.

Overall, the outcomes of this thesis demonstrate that echinoid early life development bioassays can detect effects of marine POPs, thus representing a toxicologically relevant in vivo bioassay, and a potential ethical alternative to vertebrate animal models for (eco)toxicological research, for monitoring and for standard (eco)toxicity testing. These bioassays can be used to effectively assess the (eco)toxicological impact of POPs on the marine and estuarine environment. Marine organisms are continuously exposed to POPs which can have profound effects on population fitness by adversely affecting early life development. The implications of failing to effectively assess the risk of POP

mixtures to the marine and estuarine ecosystems can have serious environmental consequences as well as economic costs to human populations depending on this important resource.

Compounds that we revealed to be most toxic for the early life development of *P. miliaris* were:

- TPT (Chapter 5), a biocide used in agriculture as fungicide, molluscicide as well as rodent and insect repellant (Fent and Meier 1994).

- TCS (Chapters 2, 3, and 4), widely use in household hygiene products, such as toothpastes, soaps, detergents, and disinfectants (Backhaus et al. 2011).

- HBCD (Chapter 2), an additive brominated flame retardant (BFRs) applied in high impact polystyrene foams, in upholstery textiles and to a less extent in electrical equipment housings (Alaee et al. 2003).

- PBDEs (Chapter 3), are also additive BFRs used in plastics such as high impact polystyrene, in electrical and electronic equipment and textile back-coating in furniture (de Wit et al. 2010).

6.3. Future perspectives

The research described in this thesis demonstrated that echinoid bioassays for early life development are suitable tools to assess developmental effects of POPs. Nonetheless, for the further development and application of echinoids as an animal model in (eco)toxicological studies future research should aim at:

I. the characterization of the possibilities of echinoid larvae to metabolize polyhalogenated aromatic hydrocarbons, such as PCBs and PBDEs, into TH-like OH-metabolites via *e.g.* cytochrome P-450 enzymes;
II. investigating the molecular mechanisms involved TH function during echinoid early life stages and metamorphosis. This is particularly relevant to allow the comparison of TH disruption mechanisms in echinoids with those in vertebrates.
III. elucidating the molecular mechanisms involved in mixture effects to a further extent, to be able to better predict the combined toxic effects of compounds;
IV. elucidating whether accelerated metamorphosis without the occurrence of malformations is adverse for further development in echinoids, because in the echinoid metamorphosis bioassay, the development of juveniles was not followed after completion on metamorphosis;
V. elucidation of whether the effects of POPs and their mixtures on echinoid early life development described in this thesis, will adversely impact echinoid populations in the field.

Altogether the results described in the present thesis have made important contributions to the development of new bioassays for a marine organism, which can even be extended and refined to further increase their potential impact in hazard assessment of environmental pollutants in the near future.

6.4. References

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CHAPTER 7.

Summary

7. Summary

This thesis presents the development of three new bioassays for the detection of compounds disrupting the early development of echinoid larvae from hatching to metamorphosis, and the interference with cellular efflux pumps. These assays come in addition to the already existing sea urchin fertilization assay and the short term ELS assay (48 or 96 hours. This chapter summarizes the contents of the thesis.

In **Chapter 1**, background information and objectives of the thesis are presented. Firstly the risks that POPs pose to the marine environment are introduced, as well as the need to develop tools to assess toxic effects of exposure during the most sensitive period of the life cycle of organisms, their early life development. Secondly, echinoids are presented as an invertebrate marine animal model to develop early life development bioassays. Lastly, the aim of the thesis and the experimental approach chosen is outlined.

Chapter 2 describes the development of a new 16-day echinoid prolonged early life stage (p-ELS) bioassay that includes prolonged observation for the detection of possible delayed adverse effects during embryogenesis and larval development of the sea urchin *Psammechinus miliaris*. Subsequently, the newly developed bioassay was applied to study the effects of key marine POPs. Mortality, morphological abnormalities and larval development stages were quantified at specific time points during the 16-day experimental period. In contrast to amphibians and fish, *P. miliaris* early life development was not sensitive to dioxin-like toxicity in the p-ELS test. Triclosan (TCS) levels higher than 500 nM were acutely toxic during embryo development. Morphological abnormalities were induced at concentrations higher than 50 nM hexabromocyclododecane (HBCD) and 1000 nM tetrabromobisphenol A (TBBPA). Larval development was delayed above 25 nM HBCD and 500 nM TBBPA. Heptadecafluorooctane sulfonic acid (PFOS) exposure slightly accelerated larval development at 9 days post fertilization (dpf). However, the accelerated development was no longer observed at the end of the test period (16 dpf). The newly developed 16-day echinoid p-ELS bioassay proved to be sensitive to toxic effects of POPs and effects can be monitored for individual echinoid larvae. The most sensitive and dose related endpoint was the number of developmental penalty points. By manipulation of the housing conditions, the reproductive season could be extended from 3 to 9 months per year and the p-ELS experiments could be performed in artificial sea water as well.

In **Chapter 3** a metamorphosis assay was developed, using the sea urchin *P. miliaris*, to detect and quantify the potency of persistent organic pollutants (POPs) to disrupt thyroid hormone (TH) induced metamorphosis. Similar to vertebrates, echinoids have a TH induced metamorphosis, making them a potential model organisms to study TH disruption. Larvae were exposed to test compounds

from the 8-armed pluteus stage until metamorphosis completion. Thyroxine (T4) accelerated metamorphosis (EC_{50} 0.12 and 0.09 nM experiment A and B, respectively), whereas the TH synthesis inhibitor thiourea (TU) (IC_{50} 0.1 and 0.04 mM experiment A and B, respectively) or the iodine uptake inhibitor potassium thiocyanate (KSCN) delayed metamorphosis (IC_{50} <0.1 mM). Polybrominated diphenyl ethers (PBDEs) strongly accelerated metamorphosis (EC_{50} 219 nM), while TBBPA and TCS delayed it (IC_{50} 97 and 418 nM, respectively). It was concluded that echinoids are promising marine model organisms for ecotoxicological studies and further insight into TH function may contribute to reduce the use of vertebrates to study TH disruption.

Chapter 4 focusses on the interaction of POPs with the Multi Xenobiotic Resistance (MXR) mechanism, an important first line of defense against contaminants by pumping contaminants out of the cells. If compounds would impair the MXR mechanism, this could result in increased intracellular levels of other compounds, thereby potentiating their toxicity. A calcein-AM based larval cellular efflux pump inhibition assay (CEPIA) was developed for echinoid (*P. miliaris*) larvae and the effects of several contaminants in this assay were quantified. The MXR mechanism in *P. miliaris* may be mediated by the action of *e.g.* P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP) which are also present in vertebrates. The larval echinoid CEPIA revealed that TCS and the nanoparticles P-85 (P-85) were 124 and 155 times more potent inhibitors (IC_{50} 0.5 ± 0.05 and 0.4 ± 0.1 µM, respectively) of efflux pumps than the model inhibitor Verapamil (VER). PFOS (heptadecafluorooctane sulfonic acid) and pentachlorophenol also were more potent than VER, 24 and 5 times, respectively. Bisphenol A (BPA) and o,p'-dichlorodiphenyltrichloroethane (o,p'-DDT) inhibited efflux pumps with a potency 3 times greater than VER. In a 48 h early life stage bioassay with *P. miliaris*, exposure to a non-lethal concentration of the inhibitors TCS, VER, the model MRP inhibitor MK-571, the nanoparticles P-85 and the model P-gp inhibitor PSC-833, increased the toxicity of the toxic model substrate for efflux pumps vinblastine by a factor of 2, 4, 4, 8 and 16, respectively. The findings reveal that several contaminants accumulating in the marine environment can inhibit cellular efflux pumps, which may potentiate toxic effects of efflux pumps substrates.

The newly develop *P. miliaris* p-ELS and metamorphosis bioassays were applied in **Chapter 5** to investigate the effects of a field-relevant mixture of POPs. This is particularly relevant since these contaminants occur in the marine environment as mixtures of compounds that could influence each others effects. In these studies two field-based mixtures (FM) were tested. FM1 was composed of the following seven compounds: BDE-47 (2,2',4,4'-tetrabromodiphenyl ether); PFOS (heptadecafluorooctane sulfonic acid); PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl); PCB-126 (3,3',4,4',5-pentachlorobiphenyl); HBCD (hexabromocyclododecane); DBT (dibutyltin); TPT (triphenyltin). FM2 was the same mixture without TPT and DBT. In addition TPT and DBT also were

tested alone. Effects observed in the p-ELS bioassay show a significant increase in larval morphological abnormalities and delayed development at concentrations ≥FM1/81 (corresponding to a 81 times dilution of the highest test concentration). FM2 (without TPT and DBT) only induced morphological abnormalities at the highest concentration. TPT and, to a lesser extent, DBT alone also induced a statistically significant increase in morphological abnormalities at concentrations ≥0.2 and ≥32 µg/l, respectively. This corresponds to a TPT concentration approximately comparable to a 50 times diluted FM1 (FM1/50), while this DBT concentration is 10 times higher than that in FM1 (3 µg/l). Therefore, the addition of TPT to the FM2 would add more to the total toxicity than the addition of DBT. In the metamorphosis assay, FM1 induced a statistically significant metamorphosis acceleration and morphological abnormalities in juveniles at concentrations ≥FM1/27 and ≥FM1/9, respectively, while FM2 did not affect metamorphosis at all. TPT and DBT alone significantly accelerated metamorphosis at ≥1.7 and ≥4 µg/l, respectively, and caused an increase in juvenile morphological abnormalities at ≥0.1 and ≥32 µg/l, respectively. TPT can account for approximately 100% of the metamorphosis acceleration observed in larvae exposed to FM1. TPT is an strong inducer of the RXR (retinoic X receptor) which is known to synergize TH and retinoic acid dependent mediated mechanisms, both known to be crucial for early development and metamorphosis. As RXR genes are expressed in echinoids it is speculated that the strong enhancement of the toxicity of FM2 by TPT, and possibly DBT, is mediated via the RXR. Given the high environmental levels of TPT it is important to further elucidate the mechanism behind this mixture effect.

Chapter 6 discusses the relevance of developing and applying bioassays for the evaluation of toxic effects of POPs during the most sensitive developmental periods of echinoids. Acute and sub-chronic effects, disruption of TH induced metamorphosis and inhibition of the MXR defense system were detected and quantified. Compounds revealed to be most toxic for the early life development of *P. miliaris* were: TPT, a biocide used in agriculture as fungicide, molluscicide as well as rodent and insect repellant; TCS, widely use in household hygiene products, such as toothpastes, soaps, detergents, and disinfectants; HBCD, an additive brominated flame retardant (BFRs) applied in high impact polystyrene foams, in upholstery textiles and to a less extent in electrical equipment housings; and PBDEs, which also are additive BFRs used in plastics such as high impact polystyrene, in electrical and electronic equipment and textile back-coating in furniture. Furthermore, its discussed the importance to assess (eco)toxicological effects resulting from exposure to mixtures of POPs, for example with exposure to inhibitors of the MXR defense system and alkyltin compounds. These outcomes, together with the molecular and physiological commonalities between echinoids and vertebrates, open possibilities for echinoid bioassays when aiming at the reduction of vertebrate species used in toxicological studies.

CHAPTER 8.

Nederlandse samenvatting

8. Samenvatting

Dit proefschrift omschrijft de ontwikkeling van drie nieuwe in vivo bioassays (testen met dieren) met mariene ongewervelden die kunnen worden ingezet voor het detecteren van effecten van stoffen op de vroege ontwikkeling van de zee-egel larven. De drie nieuwe bioassays bestrijken de ontwikkeling van het uitkomen van de zee-egel larven tot aan de metamorfose. Eén van de assays is ontwikkeld voor het interfereren met 'cellulaire efflux pompen' waarmee toxische stoffen een cel uit kunnen worden gepompt. Deze drie nieuwe bioassays zijn een aanvulling op de reeds bestaande zee-egel ei bevruchtings assay en de kort durende 'early life stage' (ELS) assay met blootstelling gedurende 48 of 96 uur. Dit hoofdstuk beschrijft de inhoud van het proefschrift.

In **hoofdstuk 1** worden achtergrondinformatie en doelstellingen van het proefschrift uitgelegd. Als eerste zal worden ingegaan op de risico's die persistente organische verontreinigingen (POPs) kunnen vormen voor het mariene milieu, in het bijzonder via effecten op de meest gevoelige periode van de levenscyclus van organismen, namelijk de vroege ontwikkeling. Ten tweede wordt ingegaan op de geschiktheid van echinoiden, en in het bijzonder zee-egels, als ongewerveld zeedier voor het ontwerpen van bioassays voor het onderzoeken van verstoringen op de vroege ontwikkeling. Ten slotte wordt het doel van het proefschrift en de gekozen experimentele benaderingen uiteengezet.

Hoofdstuk 2 beschrijft de ontwikkeling van een 16-daagse "prolonged" ELS (p-ELS) bioassay met de zee-egel *Psammechinus miliaris.* Met deze pELS worden niet alleen de effecten op de larvale (zeer vroege) ontwikkeling bestudeerd zoals in de reeds bestaande ELS, maar worden ook uitgestelde schadelijke effecten tijdens de embryogenese en larve ontwikkeling zichtbaar. Vervolgens is deze nieuw ontwikkelde bioassay uitgevoerd met een aantal veel voorkomende mariene POPs. Sterfte, morfologische afwijkingen en de ontwikkelingssnelheid van de larves werden gekwantificeerd op specifieke tijdstippen gedurende de 16-daagse proefperiode. In tegenstelling tot amfibieën en vissen, was *P. miliaris* niet gevoelig voor de toxiciteit van dioxine-achtige stoffen in de p-ELS test. Voor Tricolsan (TCS) waren concentraties hoger dan 500nM acuut giftig voor de ontwikkeling van het embryo. Morfologische afwijkingen werden waargenomen bij concentraties hoger dan 50 nM hexabroomcyclododecaan (HBCD) en 1000 nM tetrabroombisfenol A (TBBPA). De ontwikkeling van de larven was vertraagd bij concentraties hoger dan 25 nM HBCD en 500 nM TBBPA. Na 9 dagen blootstelling aan Heptadecafluorooctane sulfonzuur (PFOS) was de ontwikkeling van de larven versneld maar aan het einde van de testperiode (na 16 dagen) was dit effect niet meer waarneembaar. De nieuw ontwikkelde 16-daagse p-ELS bioassay met zee-egel larven blijkt geschikt voor het detecteren van toxische effecten van POPs en deze effecten kunnen zelfs per individuele larve worden bekeken. Het meest gevoelige en dosis-gerelateerde eindpunt was het ontwikkelingsstadium van de

larven. De p-ELS experimenten kunnen worden uitgevoerd in kunstmatig zeewater en door manipulatie van de kweek condities kan het voorplantingsseizoen worden verlengd van 3 tot 9 maanden per jaar.

In **hoofdstuk 3** wordt de ontwikkeling beschreven van een metamorfose assay met de zee-egel *P. Miliaris* als model organisme. Net als gewervelde dieren zoals kikkers, hebben Echinoids zoals de zee-egel een schildklierhormoon (TH) gestuurde metamorfose, waardoor ze een potentieel model vormen voor het bestuderen van TH verstoring. Met de ontwikkelde assay kan de verstoring van de TH-afhankelijke metamorfose worden bestudeerd. De larve worden blootgesteld aan teststoffen vanaf de 8-armige fase tot aan het voltooien van de metamorfose. De positieve controle TH (in dit geval thyroxine, T4) versnelde de metamorfose aanzienlijk, met 6 dagen gemiddeld bij een 50% effect concentratie (EC_{50}) van 0.10 nM, terwijl de TH remmers thioureum (TU) kaliumthiocyanaat (KSCN) de metamorfose vertraagden (EC_{50} $\leq$0,1 mM). Polybroomdifenylethers (PBDE's) versnelden de metamorfose zeer sterk, bij de hoogste concentratie was de metamorfose compleet na 5 dagen (EC_{50} 219 nM). TBBPA en TCS daarentegen vertraagden de metamorfose (IC_{50} 97 en 418 nM, respectievelijk). De zee-egel metamorfose assay blijkt een veelbelovend marien model voor ecotoxicologisch onderzoek naar TH verstoring en mogelijk kan dit model in de toekomst bijdrages aan het verminderen van het gebruik van gewervelde dieren voor het onderzoek naar TH verstorende stoffen.

Hoofdstuk 4 richt zich op de interactie van POPs met het cellulaire efflux pomp (Multi Xenobiotic Resistance (MXR)) mechanisme. Dit mechanisme is een belangrijke eerste lijn verdediging tegen verontreinigingen, doordat verontreinigingen met behulp van dit mechanisme uit de cel kan worden gepompt. Wanneer het MXR mechanisme wordt geremd kan dit resulteren in hogere intracellulaire concentraties van toxische stoffen en daarmee mogelijk grotere toxiciteit. Een eenvoudig uit te voeren cellulaire efflux pomp inhibitie assay (CEPIA) was ontwikkeld voor zee-egel (*P. miliaris*) larven. Met deze assay zijn de effecten van verschillende verontreinigingen bepaald op basis van ophoping van de model stof calcein-AM dat fluoresceert wanneer het in een cel aanwezig is maar niet daarbuiten. Het MXR mechanisme in *P. miliaris* kan worden beïnvloed komt voor zover bekend binnen het hele dierenrijk voor, en voor de werking zijn vooral P-glycoproteïne (P-gp) en multidrug resistance-associated protein (MRP) verantwoordelijk. In de CEPIA assay met zee-egel larven bleken TCS en de nanodeeltjes P-85 (P-85), 124 en 155 keer sterker de efflux pompen te remmen dan de model remmer Verapamil (VER). Ook pentachloorfenol en remden de cellulaire efflux sterker, respectievelijk 5 en 24 maalen bisphenol A (BPA) en o, p'-dichlorodiphenyltrichloroethane (o, p'-DDT) 3 maal sterker. In een 48 h ELS bioassay met *P. miliaris* bleek blootstelling aan de CEPIA remmers TCS, VER, de MRP

modelremmer MK-571, de nanodeeltjes P-85 en de P-gp modelremmer PSC-833, de toxiciteit van het modelsubstraat voor efflux pompen Vinblastine 2-16 maal te versterken. De studie laat zien dat er stoffen in het mariene milieu aanwezig zijn die cellulaire efflux pompen kunnen remmen, wat mogelijk kan lijden tot een grotere toxiciteit van stoffen die substraat zijn van cellulaire efflux pompen.

In **hoofdstuk 5** werden de ontwikkelende *P. miliaris* p-ELS en de metamorfose bioassays toegepast om de effecten van een veld relevant marien POP mengsel te onderzoeken. Deze verontreinigingen komen in het mariene milieu voor als een mengsel van componenten die van invloed kunnen zijn op elkaars effect. In deze studies werden twee veld gebaseerde mengsels (FM) getest. FM1 bestond uit zeven stoffen: BDE-47 (2,2 ', 4,4'-tetrabroomdifenylether) PFOS (heptadecafluorooctane sulfonzuur), PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl) PCB-126 (3,3',4,4',5-pentachlorobiphenyl) HBCD (hexabroomcyclododecaan) DBT (dibutyltin) TPT (trifenyltin). FM2 was hetzelfde mengsel maar dan zonder TPT en DBT. Daarnaast werden TPT en DBT ook afzonderlijk getest. In de p-ELS bioassay was een significante toename in morfologische afwijkingen van de larven waarneembaar en een vertraagde ontwikkeling bij blootstelling aan een 81 maal verdunning van de stock concentratie (≥ FM1/81). Dit bleek vooral door TPT en DBT veroorzaakt omdat FM2 (zonder TPT en DBT) alleen bij blootstelling aan de onverdunde stock concentratie morfologische afwijkingen induceerde. TPT en in mindere mate ook DBT alleen induceerde een statistisch significante toename van morfologische afwijkingen bij concentraties ≥ 0,2 en ≥ 32 µg / l. Op basis hiervan kon worden berekend dat de TPT in het mengsel de meeste effecten kan verklaren in de pELS. In de metamorfose assay veroorzaakte FM1 een versnelling van de metamorfose en morfologische afwijkingen bij de jonge zee-egels bij concentraties vanaf respectievelijk FM1/27 en FM1/9. FM2 had geen invloed had op de metamorfose, maar TPT en DBT alleen wel. Op basis van de effectconcentraties van TPT en DBT kon worden berekend dat de hoeveelheid TPT in FM1 vrijwel volledig de waargenomen versnelling van de metamorfose kan verklaren. Een mogelijk verklaring hiervoor is dat TPT een sterke interactie heeft met de RXR (retinoic X receptor) waarvan bekend is dat deze een belangrijke rol speelt in de vroege ontwikkeling en metamorfose, mede in interactie met TH afhankelijke mechanismen. Gezien de sterke effecten van TPT is verder onderzoek naar het mechanisme achter de waargenomen effecten van TPT en verwante stoffen zoals DBT wenselijk.

Hoofdstuk 6 behandelt de relevantie voor het ontwikkelen en toepassen van Echinoid bioassays voor de evaluatie van de toxische effecten van POPs tijdens de vroege ontwikkeling van ongewervelden en wellicht gewervelden. Acute en subchronische effecten, evenals verstoring van TH geïnduceerde metamorfose en remming van het MXR afweersysteem werden gedetecteerd en

gekwantificeerd. Verbindingen die het meest toxisch bleken te zijn voor de vroege ontwikkeling van *P. miliaris* waren de biociden TPT en TCS, de gebromeerde vlamvertragers HBCD en PBDEs. In de CEPIA waren vooral TCS, de nanodeeltjes P-85 en PFOS sterke remmers. De onthulde toxische effecten van de geteste POPs welke grotendeels niet gevonden zouden zijn met de huidige bioassays tonen het belang van het gebruik van bioassays die ontwikkelingseffecten op langere termijn aantonen. Deze resultaten zijn niet alleen van belang voor de gebruikte mariene modelorganismen maar, vanwege de moleculaire en fysiologische overeenkomsten tussen Echinoïden en gewervelde dierenmogelijk ook voor het verminderen van het gebruik van gewervelde diersoorten voor ecotoxicologische testen door vervanging door bijvoorbeeld bioassays met zee-egels zoals gedemonstreerd in dit proefschrift.

APPENDIX.

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Author biography

Henrique Miguel Rodrigues Anselmo was born on the 12th of July 1980, in Torres Vedras, Portugal. After finishing high School in Torres Vedras in 1999 he enrolled in a Nursing study program. In 2000 Henrique decided to shift his academic career and started a Biology program at University of Azores, Portugal. During his undergraduate studies, Henrique worked at the department of Biology under the supervision of Prof. Armindo Rodrigues. With this experience he became interested in environmental toxicology.

After the completion of his undergraduate studies, Henrique decided to further pursue his academic career at the Institute for Risk Assessment Sciences (IRAS) - Utrecht University, The Netherlands by following the MSC program "Toxicology and Environmental Health". As part of his MSc studies, Henrique did his minor thesis at IRAS under the supervision of Dr. Raoul Kuiper, Dr. Rocío Fernández Cantón and Prof. Martin van de Berg, and his major thesis at the National Research Centre for Environmental Toxicology (ENTOX) - University of Queensland, Australia under the supervision of Dr. Janet Tang, Dr. Michael Bartkow and Prof. Jochem Mueller. Following the conclusion of his MSc studies, Henrique was admitted to a PhD position at the Toxicology sub-department, Wageningen University in collaboration with IMARES-WUR.

Currently, Henrique is employed as a Regulatory Affairs Manager at Wil Research - Europe, The Netherlands.

List of publications

- **Anselmo, H.M.R.**, Diwakar, J., Houtman. J., van den Berg, H., Murk, A.J. Novel echinoid metamorphosis bioassay detects thyroid hormone disrupting effects of persistent organic pollutants. Accepted with revisions in Environmental Toxicology Journal

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Overview of completed training activities

SENSE PhD Courses

o Environmental Research in Context

o Research Context Activity: Member of Young Agro - Food Sciences Group (AFSG) organizing committee (Kickoff event and Mind mapping workshop, 2010)

o Special Topics in Ecotoxicology

Other PhD and Advanced MSc Courses

o Reproductive Toxicology, Postgraduate Education in Toxicology (P.E.T.)

o Pathobiology, P.E.T.

o Epidemiology, P.E.T.

o Medical, Forensic & Regulatory Toxicology, P.E.T.

o Laboratory Animal Sciences – Theoretical part, P.E.T.

o Environmental Toxicology

o Advanced Course Guide to Scientific Artwork

Didactic Skills Training

o Supervision of five MSc theses

o Teaching assistant in the MSc courses Food Toxicology; Environmental Toxicology; and Marine Environmental Quality and Governance

External training at a foreign research institute

o Characterization of thyroid hormone function in echinoids using molecular techniques (e.g. genomics). University California, U.S.A.

Oral Presentations

o Effects of marine POPs on the early life development and metamorphosis of echinoderms. 6th SETAC World Congress, 20 - 24 May 2012, Berlin

o Effects of marine POPs on the early life development and metamorphosis of echinoderms. Dioxin 2011, 31st International Symposium on Halogenated Persistent Organic Pollutants, 21 - 25 August 2011, Brussels

o Effects of persistent organic pollutants (POPs) on the early life development and metamorphosis of echinoderms. Health and Environment Conference 2009, 28 October 2009, Luxemburg City

o Effects of the flame retardant hexabromocyclododecane (HBCD) on the early life development and metamorphosis of echinoderms. 30th Anniversary Meeting of the Netherlands Society of Toxicology, 18 June 2009, Eindhoven.

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