Eggsposed

Impact of maternally transferred POPs on fish early life development

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Thesis
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With references, with summaries in Dutch and English

Voor Gerda, Lars en Ivar

“Study to be quiet”
Izaak Walton; The Complete Angler, 1655

“Je gaat het pas zien als je het doorhebt”
Johan Cruijff
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Summary

Nederlandse samenvatting (speciaal voor niet ingewijden)

Dankwoord

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CHAPTER 1

Introduction

"The old man knew he was going far out and he left the smell of the land behind and rowed into the clean early morning smell of the ocean."

Ernest Hemmingway: The Old Man and the Sea
1.1 POPs in marine environment

Although the application and emission of major persistent organic pollutants (POPs) has been strongly reduced during the last decades, their mass production in the past in combination with their persistence make that these pollutants still are globally present in the marine environment (Moore et al., 2002).

As a result of their persistency and low water solubility (high lipophilicity), highest concentrations in the aquatic environment are found in the organic matrix of sediments and in biota, while dissolved concentrations in water are low. The major exposure route for lipophilic POPs is through food uptake (Moore et al., 2002). As a result of biomagnification the highest levels of POPs have been identified in the tissue of predators, like eel, swordfish, sharks and marine mammals (Boon et al., 2002; de Boer et al., 2010; Kannan et al., 2002; Law et al., 2006). POP concentrations are often expressed on whole tissue bases, mostly to assess the risk for human exposure by (fish) consumption (Domingo and Bocio, 2007; Dorea, 2008; Gomara et al., 2005; van Leeuwen and de Boer, 2008). Lipid-rich fish generally have higher POP tissue concentrations. However, from a toxicological point of view the lipid normalised concentrations are more relevant to assess the risk for a toxic effect on the organism (Murk et al., 1998). The lipophilicity of a compound is expressed based on the equilibrium distribution of the substance between water and octanol, usually as the logarithm of the octanol-water partitioning coefficient (log Kow). The log Kow of lipophilic POPs roughly ranges between 4 and 7 (Hansen et al., 1999; Li et al., 2008). A log Kow of 7 indicates that in an equilibrium situation the concentration of this compound in the octanol phase will be 10^7 times higher than the concentration in the water phase.

Well known lipophilic POPs that are ubiquitous in the marine environment are polychlorinated biphenyls (PCBs) and brominated flame retardants such as polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD). High volume production of PCBs started around 1930 and ceased around the 1990s. PCBs were applied especially in hydraulic fluids, electric transformers and as flame retardants (Breivik et al., 2004). They now are present in biota all over the world (Domingo and Bocio, 2007). In total 209 PCB congeners exist depending on the chlorine number and positions in the biphenyl molecule. The more chlorinated non- and mono-ortho substituted PCB congeners form the most persistent molecules (Beyer and Biziuk, 2009), and show toxic features comparable to polychlorinated dibenzo dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), with tetrachlorinated dibenzodioxin (TCDD) as most toxic representative. For that reason non- and mono-substituted PCBs are often referred to as dioxin-like PCBs. The potency of the individual congeners to cause
The highest PCB concentrations in fish were reported around the 1980s in samples from the Great lakes, e.g. rainbow trout containing 4.8 µg/g wet weight (ww) (42 µg/g lipid weight (lw)) and white sucker 3.4 µg/g ww (55 µg/g lw) of total PCBs (no data on congeners given) respectively (Niimi, 1983), and lake trout ranging from 3.5 to 13.9 µg/g ww (Mac et al., 1993). Analyses of historical monitoring data from the year 1977 to 2006 revealed that PCB concentrations in eel in the Netherlands declined relatively slow during this 30 year period with a factor 5 to 10 (de Boer et al., 2010). The maximum PCB concentration, 48 µg/g lw (Σ7 congeners), was measured in a sample from 1979. This value is comparable to values reported in rainbow trout and white sucker in the Great Lakes in 1983 (Niimi, 1983). The data-set of (de Boer et al., 2010) suggest that even higher PCB concentrations in eel tissue must have been present before 1977. In the year 2006 the PCB concentrations in eel from the same water basins ranged between 5 and 8 µg/g lw (de Boer et al., 2010). Other recent studies reported total PCB levels in Swordfish: 1.0 µg/g lw (Σ57 congeners, (van Leeuwen et al., 2007)), tuna: 1.4 µg/g lw (Σ23 congeners, (Gomara et al., 2005)), salmon: 3.2 µg/g lw (Σ19 congeners, (Svendsen et al., 2007)) Goby: 4 µg/g lw (Σ25 congeners, (Voorspoels et al., 2004), Greenland shark 5.8 µg/g lw (Molde et al., 2013), and in brown trout after a migratory starving period: 12.6 µg/g lw (Σ19 congeners, (Svendsen et al., 2007)).

The highest published levels of dioxin-like pollutants in fish include 596 pg TEQ/g lw in pike from the Baltic sea (Isosaari et al., 2006), 3764 pg TEQ /g lw in flounder from the Greenland fjord (Knutzen et al., 2003). In both cases TEF systems for mammals were applied to calculate the reported TEQ values. In
Greenland sharks, the TEQ concentrations based on the TEF system for fish were reported to range from 460 to 579 pg TEQ/g (Storelli et al., 2011).

Polybrominated diphenyl ethers (PBDEs) are flame retardants with a chemical structure comparable to PCBs, but with chlorine replaced by bromine. As with PCBs 209 PBDE congeners can exist. Market introduction of PBDEs took place about 50 years ago, and although application of PBDEs now is restricted and shifted towards the higher brominated ones, these substances are still globally present (Borghesi et al., 2009; Hites, 2004). The European Food Safety Authority recommended monitoring of the (sum)concentrations of BDE 28, 47, 99, 100, 153, 154, 183 and 209 (EFSA, 2006). In fish samples the PBDE profile is often dominated by the poorly degradable BDE 47 (Voorrips et al., 2003) (Borghesi et al., 2009; van Leeuwen and de Boer, 2008). In the Dutch eel monitoring programme PBDE 47 concentrations in eel tissue is measured since 1984. The maximum concentrations around 1.5 µg/g lw were recorded during the first years. Concentrations decreased 4 to 10 times a maximum concentration of 0.2 µg/g lw in 2006 (de Boer et al., 2010). Some of the highest PBDE levels that have been reported in fish muscle samples are 0.26 µg/g lw in salmon (Svendsen et al., 2007), 0.21 µg/g lw in bream (Isosaari et al., 2006) and 0.64 and 0.74 µg/g lw in feral carp from two Spanish rivers (Labandeira et al., 2007).

Hexabromocyclododecanes (HBCD) also are brominated flame retardants, which mainly are applied in expanded and extruded polystyrene. HBCDs have been produced and used for about three decades. They were first detected in fish at the end of the 20th century and are now ubiquitous pollutants in the environment (Covaci et al., 2006). A UN-expert group recently (Oct. 2012) recommended that HBCD be eliminated from the global market to protect human health and the environment (http://chm.pops.int). HBCDs can be found in alpha, beta and gamma diastereomers of which alpha-HBCD is the dominant (>75%) congener present in fish tissue (Covaci et al., 2006; van Leeuwen and de Boer, 2008). Extremely high concentrations of 4 to 8 µg/g lw HBCD are reported in the tissue of pike sampled near point sources (textile industries) in Sweden (Sellstrom et al., 1998). In the Western Scheldt estuary concentrations up to 1.1 µg/g lw were detected in muscle of sole (Janak et al., 2005).

A wide variety of effects on fish and other taxa have been related to these elevated POP tissue concentrations, often indicating early development as the most sensitive life stage (Hutchinson et al., 1998; McKim, 1977).
1.2 Fish early life development and effects of POPs

1.2.1 Fish reproduction and early development

The majority of the teleost fish species are ovo-parous with an iteroparous life strategy (Wooton, 1992), indication that an individual fish can produce multiple batches of eggs during a lifetime. This, in contrast to semelparous fishes that reproduce only once in their lifetime and die after spawning. Well known examples of the latter are eels and pacific salmon that undertake a long migration to the spawning grounds that are located in a completely different environment than where the fish reach maturity. Eels mature in freshwater and spawn at sea, for the pacific salmon species this is vice versa.

Although there are many exceptions, in general fish spend little energy on nursery of eggs and larvae. Especially in the marine environment most fish spawn in the pelagic zone where the eggs develop being suspended in the water column. In the freshwater, more species produce demersal eggs or eggs that attach to plants or other substrates (Wooton, 1992). Some teleost fish species are ovo-viviparous, indicating that eggs are fertilised and develop inside the mother’s body.

The reproduction success of a fish depends, as for other species, on the number of surviving offspring it can produce during its life-time. Bigger eggs have the advantages of producing bigger larvae that can take a wider range of prey, are less vulnerable to predation and better in withstanding periods of food shortage than smaller larvae. The majority of fish without parental care, however, produce small eggs in sometimes extremely high numbers (Wooton, 1992). A large number of offspring reduces the impact of predation and increases the chance of larvae encountering food and favourable conditions (Rothschild, 2000). Smaller clutch size and larger eggs are typically found in demersal spawners like salmonids, and species that show some form of parental care.

An egg is provided with all resources necessary for the development of the embryo into a larva until it is capable of external feeding. The majority of these resources is stored by the mother in the yolk during vitellogenesis. A review of this process is given by Mommsen and Walsh (1988). The protein vitellogenin is released from the liver in the blood stream and efficiently absorbed by the developing oocytes, where it is deposited in the yolk. Due to strong ion-binding properties of vitellogenin, it can act as an important vehicle for minerals to the oocyte. Vitellogenin itself consist in general of proteins and polar and neutral lipids (Johnson, 2009). Polar lipids (phospholipids) have a structural function in animal cells and are essential in cell membranes and neural tissue. Neutral lipids
are important as sources of energy and probably serve as storage place for essential fatty acids (Wiegand, 1996).

For species that do not feed during vitellogenesis like salmon and eel, the source of the lipids deposited in the eggs are the adipose reserves stored by the female fish. For other species dietary lipids can form an (additional) source.

After fertilisation, the egg chorion hardens as a protective shield allowing only limited exchange of water and molecules (Kamler, 2008). In many marine species the larvae hatch at an early stage of development. The mouth and jaws are not formed, the eye is not pigmented and a primordial finfold runs around the trunk in the median position. Most freshwater species and some marine species hatch at a further developed state, but newly hatched fish larvae are never ready to start external feeding. During the first days after hatching (the yolk-sac stage) the larvae still depend on the yolk resources. The mouth and intestine develop and enable the larvae to switch to exogenous feeding when the yolk is depleted. The duration of the yolk-sac stage differs per species. The Japanese Medaka fish can reach the free feeding state already one day after hatching (Villalobos et al., 2000), while for other species this will take several days. It has been suggested that the duration of the yolk-sac stage is related with egg size and temperature (Blaxter, 1988).

During the free-feeding stage organs and tissues develop further, and the larvae gain length and weight. At a species-dependent age, the free-feeding larvae metamorphose into a juvenile with the same appearance as the adult fish (Blaxter, 1988). This metamorphoses takes place in all teleost fish, but is most obvious in flatfish as larvae of this group undergo a drastic craniofacial remodelling (Figure 1.1), during which the symmetrical larvae with a pelagic life style change into a benthic asymmetrical juvenile (Schreiber, 2006).

1.2.2 Effects of POPs on Fish early life stages

POPs like dioxins, PCBs, PBDEs and HBCDs have the potential to cause a variety of toxic effects. From earlier research with fish larvae it is known that especially early life stages are very sensitive to the toxic action of dioxins, and dioxin-like substances such as PCDD/Fs and, and non- and mono-ortho PCBs of which the molecular structure can have a planar confirmation (Johnson et al., 1998; Walker and Peterson, 1994; Yamauchi et al., 2006). These substances are able to bind to and activate the aryl hydrocarbon (Ah) receptor, which binding has been associated with a variety of toxic responses including teratogenicity and changes in the thyroid function (Brouwer et al., 1999; Brown et al., 2004). A typical effect of dioxin-like toxicity in developing fish larvae is blue-sac disease that is characterised by pericardial and yolk-sac oedema and results in early
Some PCBs and brominated flame retardants have the potential to disrupt hormone systems. This includes the thyroid hormone system (Brouwer et al., 1999) that plays an essential role in metabolisms and differentiation during early life development, including metamorphosis. Exposure to these substances can therefore disturb the development of early life stages for a wide variety of taxa, as has been shown for echinoderms (Anselmo et al., 2012b), fish (Palace et al., 2010; Yu et al., 2011), and amphibians (Gutleb et al., 2007a; Gutleb et al., 2007b; Schriks et al., 2006).

Especially the hydroxylated (OH-) metabolites of PCBs (OH-PCBs) and PBDEs (OH-PBDEs) show a high potency for disruption of the thyroid hormone system as they have a structural similarity with thyroid hormones (Freitas et al., 2011; Zheng et al., 2012). The potency for hydroxylation of PCBs and PBDEs differs between species (Murk et al., 1994; Roberts et al., 2011). Juveniles of the common sole have been shown to metabolise PBDEs into OH-PBDEs (Munsch et al., 2010). It is however unclear during which stage of the development this ability develops. Embryos of Zebrafish did not form OH-PBDE 47 after exposure to PBDE 47 (Zheng et al., 2012).

Also for toxicants with less specific effects, the strongest individual responses to exposure are expected on early life stages (Hutchinson et al., 1998; McKim, 1977). This is believed to be related to the development of organs and tissues that can be disrupted easily, in combination with the lacking ability to compensate the impact of physiological processes affected by toxicant exposure (Crane et al., 2006). In addition, the small size and related large surface-volume ratio facilitates a faster increase of internal concentrations in fish larvae than in larger fish when exposed through the water phase.

1.2.3 Fish early life stage tests

As fish early life stages (ELS) are recognised as sensitive for toxic disruption, several test guidelines for fish ELS testing are available (OECD, 1992, 1998, 2006; USEPA, 1996). Despite this, guidance on the utilisation of marine species is scarce, especially with respect to species relevant for the West European region.

For multiple (practical, economical, and ethical) reasons the majority of the fish ELS tests that are applied are terminated just before or soon after the larvae become free-feeding, thus without covering the metamorphoses into a juvenile fish. In addition, none of the recommended test species undergoes an obvious
metamorphosis that is typical for certain taxonomical fish groups such as flatfish (Yamano, 2005). Tests with amphibians showed that developmental effects of thyroid hormone disrupting chemicals only become visual in prolonged ELS tests where metamorphosis is included (Gutleb et al., 2007a). For this reason a prolonged ELS test with a native West European marine species that undergoes obvious metamorphoses was developed and applied for the research presented in this thesis.

1.2.4 Exposure of fish early life stages to POPs in the natural environment

When the toxicity for fish larvae of a poor water soluble pollutant is being tested, most of the time a solvent/carrier is applied to maintain the water concentration of the test substance at the appropriate level. In the natural environment water concentrations of such lipophilic POPs will be very low. As the eggs and larvae of the far majority of marine fish species develop suspended in the water column, external exposure to POPs is very limited until the larvae become free feeding and might feed on contaminated food items.

Due to bioaccumulation of POPs during life, tissue concentrations can build up in the mother fish, and accumulated substances will be transferred with the yolk to the eggs (Hammerschmidt and Sandheinrich, 2005; Johnson et al., 1998; Johnston et al., 2001; Miller, 1993; Monteverdi and Di Giulio, 2000; Nyholm et al., 2008; Russell et al., 1999; Serrano et al., 2008; Wang et al., 2011; Yu et al., 2011). For POPs this results in concentrations in the eggs that on a lipid bases are comparable with the mother’s tissue (Russell et al., 1999). Via maternal transfer the developing embryo is thus exposed to relatively high levels of POPs, including those substances that may be specifically harmful during early life development. The importance of maternal transfer as exposure route has already been studied and recognised for dioxins and dioxin-like PCBs, especially in research with trout from the Great Lakes that contained extremely high PCB levels in the late 20th century (Mac et al., 1993; Niimi, 1983; Walker and Peterson, 1994). From this research it was concluded that the high concentration of PBCs contributed to the poor reproduction success of local trout population (Wilson and Tillitt, 1996).

The exposure rate of embryos and yolk-sac larvae to lipophilic POPs depends thus strongly on the concentration of these substances in the mother fish tissue, or actually in her lipids. Through bioaccumulation tissue concentrations can build up with time, and during periods of starvation the poor water soluble substances that can hardly be excreted will concentrate in the remaining tissues. This is for instance the case for fish that do not feed during spawning migration like salmon.
and eel, and has consequences for the amounts of POPs that are deposited in the eggs (Debruyne et al., 2004; Kelly et al., 2007).

1.3 The European eel, a special case

Around 1980 PCB concentrations were detected in the tissue of eel (Anguilla Anguilla) from the Netherlands that were among the highest ever recorded in fish (de Boer et al., 2010). In the same period the numbers of juveniles (glass eel) of the European eel that reach the European coasts started to decline rapidly and nowadays these are only 10% of what it used to be before the 1980’s (Dekker, 2003). A clear explanation for this extreme strong decline is not available at this moment; multiple causes are suggested, including effects of accumulated toxic pollutants.

The European eel combines different features that make it potentially vulnerable to the effects of POPs. After entering from sea as a glass eel, the eels remain in the fresh water for many years where they live in close contact with the sediment and its pore water. In the sediments the POPs accumulate to sometimes very high levels. The eel diet consisting of macro invertebrates and fish (Tesch, 2003), and their very high lipid content facilitates bioaccumulation of POPs. Eels do not reproduce during their stay in the freshwater, thus no POPs are excreted through spawning of lipid-rich eggs, which in some species results in reduced tissue concentrations (Madenjian, 2011). After spending many years in the freshwater environment the eels start a long marine migration to the spawning grounds that are believed to be located in the Sargasso Sea, at a distance of 5000 to 6000 km (Tesch, 2003). As they do not feed during migration weight loss will occur, probably leading to concentration of the POPs in the tissue lipids of the fish, conform observations in migrating pacific salmon (Debruyne et al., 2004; Kelly et al., 2011). In this case the POP concentrations in the tissue of the eel that has reached the spawning ground will exceed the concentration at the start of the migration, and these concentrations will be transferred to the eggs (Russell et al., 1999). Following this line of thinking the eel embryos will thus be exposed to the highest levels of lipophilic POPs that were ever present in the parent fish.

1.4 The common sole as test species

The common sole (Solea solea) is chosen as appropriate candidate for the development of a new fish test to study early life development including metamorphoses. It is a native European species of both ecological and economic importance, with landings ranging between 10 - 15 000 tons per year for the
North Sea (ICES FishMap Sole, http://www.ices.dk/ (accessed November 2012). Because sole is a valuable consumption fish, it is aquacultured and fertilised sole eggs were available at different moments throughout the year from the IMARES department of aquaculture.

All flatfishes (Order Pleuronectiformes) hatch as symmetrical larvae but undergo an obvious metamorphosis during early life development, that is characterised by the relocation of (in general) the left eye to the right side of the head (Figure 1.1). This metamorphosis is regulated by the thyroid hormone (Klaren et al., 2008). During the free-feeding stage the first thyroid follicles develop and the thyroid hormone tissue concentrations start to rise. The moment that the maximum thyroid hormone concentrations are reached coincides with the moment of metamorphosis (Figure 1.1). This is comparable to the metamorphosis of amphibians and sea urchins that has been shown to be easily disrupted by toxic compounds (Anselmo et al., 2012a; Gutleb et al., 2007a; Gutleb et al., 2007b). The early life development of flatfish is well studied by various researchers (Klaren et al., 2008; Martinez and Bolker, 2003; Schreiber, 2006), and the description of the different stages, including the metamorphosis is more or less standardised (Martinez and Bolker, 2003). The moment of metamorphoses marks the change from a pelagic to a benthic life style. At approximately 14°C, the eggs hatch 5-6 days after fertilisation (dpf), the larvae became free feeding around 10 dpf, and the first larvae show signs of the onset of metamorphoses around 25 dpf. Metamorphosis typically will be completed for normally developing individuals within 40 dpf. This rate of development is strongly temperature dependent. Sole reared at 18°C can complete the full development into a juvenile within 31 dpf (Palazzi et al., 2006). A full ELS developmental test with sole takes at least a 30-40 day observation period, which is still relatively short when compared with an amphibian test that requires more than 100 days to cover the full development (Gutleb et al., 2007a).
A brief description of the reproduction of Sole under natural condition is given in Rijnsdorp et al., 1992. Spawning is triggered by temperature and takes place in late winter and spring, mostly in coastal waters. The eggs are released freely in the water and due to a specific gravity that is slightly lower than that of the surrounding water remain in the upper parts of the water column. At an ambient temperature around 10°C, it takes about a week for the eggs to hatch. The newly hatched larva is poorly developed without functioning eyes, mouth and intestine (Palazzi et al., 2006). For nutrition it completely relies on the yolk-sac. The larvae live suspended in the water column and do not show activity unless externally stimulated (own observation). During absorption of the yolk-sac the organs further develop and the larvae become more active, preparing it to switch to external feeding when the yolk-sac is depleted. As the larvae remain in the water column their main natural food source consists of pelagic plankton. At a size of 5-10 mm (Amara et al., 2000), the pelagic larvae that were symmetrical until then, metamorphose into an asymmetrical juvenile flatfish with a benthic lifestyle. During this phase the juveniles enter shallow (<20 m deep) sandy or muddy coasts and estuaries, like the Western Scheldt, that serve
as nursery areas. Here, the fish remain for the next one or two years, after which they take part in spawning for the first time and spend the rest of their life in deeper waters. The relative fecundity of the common sole in the North Sea ranges between 400 and 900 eggs per gram body weight, and the estimated reproductive efficiency, being the number of 1 year old fish (recruits) per egg produced, is estimated at approximately $2.5 \cdot 10^{-6}$. There are strong indications that the number of recruits per stock is dictated by the surface of the available nursery areas (Rijnsdorp et al., 1992).

1.5 Thesis outline

The research presented in this thesis aims at the determination of the impact of maternally transferred POPs on development and survival of fish early life stages, in order to assess if this exposure route can significantly impact the development of a fish population at current environmental concentrations.

In line with this aim the following 4 research questions will be addressed in this thesis:

1. What are critical egg concentrations for PCBs, PBDEs, HBCDs and MTCS for larval development, and how do these relate to field (fish) concentrations?
2. Do the above mentioned (groups of) substances represent the major risk in case of maternal transfer, or are there indications for substantial impact of other (unknown) substances?
3. What is the influence of ecophysiological characteristics on the impact of maternally accumulated compounds transferred to the eggs on the developing larvae?
4. To what extent can the larval mortality from maternally transferred toxicants affect population development, possibly in combination with high fishing pressure?

Chapter 1, the introduction, gives some background information on the topic of the present thesis, defines the aims and research questions, and presents the outline of the thesis.

Chapter 2 presents the p-ELS test that was developed to determine the impact of lipophilic POPs on the development of fertilised sole eggs into a fully metamorphosed juvenile fish. The performance of the test is illustrated by showing the effect of the dioxin-like PCB 126.
In Chapter 3 the response to methyltriclosan was tested in the p-ELS test. Methyltriclosan is a degradation product of triclosan that has the potential to accumulate in fish tissue and disrupt the thyroid hormone system.

Chapter 4 reveals that POPs that are present in the eggs, concentrate during yolk-sac depletion to levels that can be up to 5 times higher than concentrations in the adult fish, and as such presents a possible explanation for the delayed effects that were observed in the p-ELS tests. A mathematical model (called ELS-OMEGA) based on the exciting OMEGA model (Hendriks et al., 2001) is adapted and validated with experimental results and gives insight in the development of body residues of maternally transferred POPs in fish larvae under different development scenarios.

In Chapter 5 it is shown that the effects of mixtures of POPs extracted from sole from the Western Scheldt estuary and the North Sea can be predicted based on the effect levels of individual POPs in the mixture. Dioxin-like toxicity and polar narcosis were identified as responsible for most of the observed effects, and no indications were found for synergistic effects or for a substantial contribution of unknown lipophilic substances.

Chapter 6 estimates the potential risk of maternally transferred POPs for the European eel (Anguilla Anguilla) taking into account the specific biology of the species and European field POP concentrations. Assuming a relatively high sensitivity of the eel larvae, the results indicate that it is likely that eel from specific contaminated locations experience poor larval survival.

Chapter 7 explores the relevance of early life stage mortality as could be caused by maternally transferred substances, for population development in combination with no, low or high fishing pressure. A simple population model was developed for this purpose, based on the life history of the common sole. The model was used to identify the biological features that determine the vulnerability of species for enhanced larval mortality.

Chapter 8 contains the general discussion of the presented research. It discusses the overall results and strengths and weaknesses of the research performed. Based on the insights gained species that are potentially vulnerable to the effects of maternally transferred POPs are identified and discussed. Finally, the research questions are addressed and perspectives for future research are given.
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CHAPTER 2

Prolonged ELS test with the marine flatfish sole (Solea solea) shows delayed toxic effects of previous exposure to PCB 126

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Abstract

The effect of the dioxin-like PCB 126 (3,3',4,4',5-pentachlorobiphenyl) on the early development of the marine flatfish sole (Solea solea) was tested in a newly developed early life stage (ELS) test that includes the metamorphosis of the symmetric larvae into an asymmetrical flatfish. Early life stages of sole were exposed to a concentration series of PCB 126 in seawater until 4, 8, 10 and 15 days post fertilisation (dpf). Subsequently the development of the larvae was registered under further unexposed conditions. The LC50s at the start of the free-feeding stage (12 dpf) ranged between 39 and 83 ng PCB 126/L depending on exposure duration. After the fish had completed the metamorphosis, the LC50 values ranged between 1.7 and 3.7 ng PCB 126/L for the groups exposed for 4, 8 and 10 dpf respectively. Thus exposure for only 4 days, covering only the egg stage, was sufficient to cause adverse effects during a critical developmental phase two weeks later. The internal dosages of these larvae, determined by means of an in-vitro gene reporter assay as dioxin-equivalent values (TEQ), revealed an internal lethal concentration ILC50 of 1 ng TEQ/g lipid, which is within the same order of magnitude as TEQ levels found in fish from highly polluted areas. This study indicates that ELS fish tests that are terminated shortly after the fish becomes free-feeding underestimate the toxic potential of compounds with low acute toxicity such as PCBs. Our prolonged ELS with this native marine flatfish suggests that reproductive success of fish populations at contaminated sites can be affected by persistent compounds that are accumulated by the female fish and passed on to the eggs.
2.1 Introduction

The development of an embryo into a juvenile fish is considered as the period of the fish's life that is the most sensitive to disturbances (Hutchington et al., 1998). During this early life stage critical development of organs and tissues takes place, a process which can easily be disrupted by unfavourable environmental conditions, including exposure to toxic compounds. The young stages lack the ability to compensate the impact of physiological processes affected by toxicant exposure and are therefore more sensitive to adverse effects than fully grown fish (Crane et al., 2006). Moreover, with their small body volume and relatively large surface it is expected that the internal concentration of toxic compounds will rapidly increase during exposure to contaminated water.

For this reason toxicity tests with early life stages of fish, the so-called ELS (Early Life Stage)-fish tests, are often applied for assessing the toxic potential of substances and environmental samples. Besides their sensitivity, these tests have the practical advantages over tests with older fish that they require less test volume and space. This enables the use of higher numbers of test organisms and replicates and so improves the statistical power of the test results (Wedekind et al., 2007).

The available test guidelines for fish ELS testing have their emphasis on fresh water species from temperate or tropical regions (OECD, 1992; EPA, 1996; OECD, 1998; OECD, 2006). Guidance on the utilisation of marine species is scarce, especially with respect to species relevant for the West European region. In addition, none of the test species recommended in these guidelines undergoes an obvious metamorphosis, that is typical for certain taxonomical fish groups such as flatfish and eels (Yamano, 2005) and that could be very sensitive to toxic compounds as has been shown for amphibians (Gutleb, 2006; Gutleb et al., 2007b). Therefore we investigated the applicability of the marine flatfish sole (Solea solea) as a test species in full development tests. Sole is a native European species of both ecological and economical importance. It is bred under controlled conditions for aquaculture purposes and undergoes a full metamorphosis during the development into a juvenile (see for detailed descriptions for instance: Martinez & Bolker, 2003; Schreiber, 2006).

The development from egg to larva is known to be sensitive to dioxin-like toxicity in zebrafish (Murk et al., 1996a) and amphibians (Gutleb, 1999). We used PCB 126 for studying dioxin-like toxicity on early life stages of sole. PCB 126 is a good model compound, as it is hardly metabolised and known to bind to the Ah-receptor with high potency. This binding has been associated with a
variety of toxic responses including teratogenicity, possibly induced via
disruption of vitamin A homeostasis and changes in the thyroid function
(Brouwer et al., 1995, Brown et al., 2004). The dioxin-like toxic potency of
dioxins and PCBs, is expressed as TEF, being the Toxic Equivalency Factor;
relative to that of the most potent congener 2,3,7,8-tetrachlorodibenzo-p-dioxin
(TCDD) (Safe, 1992). Since the TEF is related to a biological response, it is not
only compound-specific, but also taxon-specific. Based on available literature
Van den Berg and co-authors established TEFs for mammals, birds and fish (Van
den Berg et al.,1998), that were adopted by the World Health Organisation
(WHO). The TEF-concept assumes additivity of the dioxin-like toxicity of
compounds in a mixture, and thus enables the expression of the total dioxin-like
toxic potency of the mixture of contaminants present in an environmental
sample as Toxicity Equivalents (TEQ) of the toxicity of TCDD.

The dioxin-like toxic potency of compounds present in fish is generally assessed
in the light of human risk assessment, and therefore calculated based on the
WHO-TEF values for mammals. In vitro reporter gene assays based on Ah-
receptor activation have been developed and chemically validated for the direct
quantification of the dioxin-like potency of mixtures of contaminants in
sediments and biota (Murk et al., 1996a; 1998; Stronkhorst et al., 2002;
Hoogenboom et al., 2006; Leeuwen et al., 2007). In these in vitro reporter gene
assays the relative potency of compounds to activate the Ah-receptor is related
to that of TCDD, not the toxicity compared to that of TCDD. Although the in vitro
TEFs are not based on toxicological experiments, in practise they compare well
with the mammalian in vivo TEFs for dioxin-like compounds. The mammalian
TEF for PCB 126 is well established and consequently set at 0.1 in various
assessments including the in vitro-reporter gene systems (Safe, 1992; Ahlborg
et al., 1992; Van den Berg et al., 1998; Stronkhorst et al., 2002).

This paper describes the effects of PCB 126 on the early development of the
marine fish Solea solea in a newly developed bioassay that covers the whole
development from fertilised egg into a fully metamorphosed flatfish.

2.2 Materials and methods

2.2.1 Animals

The fertilised eggs used in this study all originated from one group of parent sole
(Solea solea). Spawning and fertilisation were temperature-induced and took
place overnight at the facilities of the commercial sole farm Solea BV in
IJmuiden, the Netherlands. The next morning, the eggs were transported to the
laboratory in Den Held in plastic bags containing seawater. During the ca. 1
hour transport period, the water temperature was maintained around 12ºC, the
temperature in the spawning tank. The eggs and larvae were kept in natural
seawater (salinity 32 ‰) collected from the Eastern Scheldt, a relatively pristine
bay of the North Sea, that is often used as a reference site in marine
ecotoxicological research in the Netherlands (e.g. Vethaak et al., 2005; Kuiper
et al., 2007).

2.2.2 Stock solutions and exposure media

Stock solutions of PCB 126 (3,3',4,4',5-pentachlorobiphenyl, purity 99.1%,
supplier Promochem) in DMSO (purity 99.9% A.C.S. spectrophotometric grade,
supplier SIGMA-Aldrich) were used to spike the seawater that was used as
exposure medium. The final DMSO concentration always was 0.1 % vv. The
stock solutions were prepared in sufficient volumes for all exposures at the start
of the first test and were stored in dark at room temperature.

2.2.3 Test procedure

Immediately after arrival at the laboratory (<12h after fertilisation), the sole
eggs were randomly divided into groups of ca. 200 eggs that were placed in
glass beakers containing 800 ml seawater spiked with the test concentrations.
The glass beakers were placed in a temperature controlled cabinet at 12-13ºC,
with a 16 hour photoperiod with dimmed light (ca. 100 LUX) per day. After 48
hours the floating fertilised eggs were collected and the non-fertilised eggs at
the bottom discarded. Out of each exposure group twenty fertilised eggs were
put together in a 100 ml glass beaker containing 80 ml of a fresh solution of the
exposure concentration. Each exposure condition was duplicated. The test
beakers were further incubated under the above described conditions.

Every other day, about 50 ml (ca. 60%) of the water in each test beaker was
pipetted off and replaced with fresh seawater at the appropriate concentration.

From the moment the first larvae had desorbed their yolk-sac and became free-
feeding, all test beakers were provided with an ad libitum amount of Artemia
nauplii (Artemia salina) on a daily basis. On an almost daily basis the dead
individuals were removed and faeces and surplus food was removed as much as
possible. Each fish that had completed the metamorphosis was removed,
narcotised in a MS222-solution (100 mg MS222 = Ethyl 3-aminonebzoate
methanesulfonic acid salt (purity 98%, ACROS Organics) and 200 mg NaHCO₃
(purity 99.0%, Fluka Chemika) in 1 l seawater) measured and checked for
malformations using a stereo microscope. While still being narcotised, the fish
was then killed and preserved in a 4%-formaldehyde solution.
Two exposure studies were performed between February and May 2007, with some differences. In the first test, the eggs and larvae were exposed until 15 days post fertilisation (15-dpf), the moment that all fish had become free-feeding. The exposure concentrations ranged from 3 to 1000 ng PCB 126/l, chosen based on test results from ELS tests with Zebra fish (Murk et al., 1996a) and tadpoles (Gutleb et al., 1999). On day 15, the surviving larvae were carefully transferred with a polyethylene pipette to another glass beaker with clean seawater and further reared unexposed until the end of the metamorphosis. During the first 19 days of this test the water temperature was maintained on 12 ± 1ºC, and on 13ºC thereafter. The fish were considered fully metamorphosed when both eyes were located at one side of the head.

Based on the results of the first experiment, the second test series was performed with lower test concentrations ranging from 0.1 to 100 ng PCB 126/l. Moreover, the exposure duration was reduced to 4, 8 or 10 days post fertilisation (dpf). The 4-dpf period covered only the egg stage, the 8-dpf exposure period covered most of the yolk-sac stage, whereas exposure of the 10-dpf group ended when the first fish became free-feeding. The water temperature was maintained at 13 ± 1ºC during the whole test period. The criterion for 'fully metamorphosed' now was not based on eye migration only, but also on the development of the dorsal fin that passes the left eye after this has reached the right side of the head.

2.2.4 Internal concentrations

To assess the actual uptake of PCB 126 into the larvae, an additional 200 eggs were exposed to a selection of test concentrations in the same way as the 4-dpf group. After two days of exposure, 150 fertilised eggs were collected from each exposure concentration and placed in 600 ml of new test medium at the appropriate test concentration, thus maintaining the ratio of 4 ml exposure media per egg. As soon as possible after hatching (<12 hr) at least 140 larvae per test concentration were collected with a polyethylene pipette and placed in clean seawater for maximal 15 minutes to rinse off exposure water before they were pipetted into a glass tube with a minimum of seawater and stored frozen.

The total wet weight per sample was about 100 mg, hardly sufficient for accurate chemical analysis of the PCB 126 concentration and expression on a lipid basis. Therefore, a sensitive in vitro reporter gene assay for dioxin-like toxic potency in H4IIE rat hepatoma cells (Murk et al., 1998) was applied for the direct quantification of the in vitro TCDD-equivalent (TEQ) levels on a lipid basis in these very small samples. This DRE-H4IIE.Luc assay (sometimes also referred to as DR CALUX) has been chemically validated before and produces a response
that is linear with the concentration of compounds with dioxin-like toxicity (Murk et al., 1998; Stronkhorst et al., 2002; Besselink et al., 2004; Hoogenboom et al., 2006; Van Leeuwen et al., 2007).

2.2.5 Calculations and statistical analysis
All statistical calculations were performed using GraphPad Prism (version 4.03, January 2005). To determine LC50-values, a sigmoid dose response curve with variable slope was fitted through the experimental data.

The significance of differences between the LC50 values of the different treatments at various moments of observation was calculated with a two-way ANOVA with Bonferroni post test. For this analysis data from both experiments were used for observations on days (dpf) 12, 15, and 20, while from the second experiments also data from dpf 10 and 56 were available. Due to this difference in the available data from both experiments, two ANOVA analyses were performed: the first included all days but excluded the data from the first experiment; the second included all experiments but excluded observations on days 10 and 56.

The relation between exposure concentrations and internal TEQ-level in the fish larvae was fitted using the same GraphPad Prism software.

2.3 Results
2.3.1 First experiment
In the first test, the sole eggs started to hatch 5 days after fertilisation. Five days later (dpf 10), the first larvae became free-feeding and on dpf 14 all larvae had reached that stage. On day 33, the first fish completed metamorphosis. The test was terminated on dpf 47 when all surviving fish had completed metamorphosis.

The development of the eggs/fish in the test beakers with clean seawater (controls) was not significantly different from that in the solvent controls (0.1% DMSO). At the end of this experiment 75% of the eggs from both controls had developed in normally metamorphosed juvenile flatfish (Figure 2.1). The mortality in the controls occurred almost exclusively before day 25.

Until day 9 no exposure related effects were observed and mortality was below 20% in all test beakers. However, within the next 5 days a high mortality rate was seen in the exposure concentrations of 32 ng PCB 126/l and above. At the moment all larvae were free-feeding, on day 15, less than 20% of the larvae
had survived these treatments, while hardly any additional mortality was observed in the 3 and 10 ng/l exposure groups.

During the following days mortality was also observed in the 3 and 10 ng/l exposure groups, even though exposure had ended. After day 22 mortality had reached >90% even in the lowest exposure concentration of 3 ng/l while in the control this was no more than 25%. At test termination (dpf 47) only two fishes survived in the exposure groups. The one in the 32 ng/l had completed metamorphoses without obvious malformations, while the fish in the 100 ng/l had developed oedema in the abdominal region and was still in the pre-metamorphose stage.

2.3.2 Second experiment

In the second experiment the development from egg into free-feeding fish was about two days faster than in the first. The first fish completed their metamorphosis 35 dpf according to the more strict criteria than those applied in the first test. The test was terminated after 56 days, when all surviving control fish had completed metamorphosis. The body length after completing metamorphosis was 8±1 mm for all animals in all treatment groups.
Figure 2.2 Mortality of sole larvae (Solea solea, n=20) that were exposed to PCB 126 via the water during 4 (top), 8 (middle) or 10 days (bottom) in the second experiment. The grey boxes indicate the period of exposure after which the animals were further reared under unexposed conditions. Presented are the mean and range of the two replicates per treatment.
The survival of the control animals was ca. 60% at completion of metamorphosis. Again, the mortality in the controls occurred mainly during the first 25 days of the test, followed by only incidental mortality later (Figure 2.2).

The timing of main mortality was the same as in the first experiment. The highest test concentrations (32 and 100 ng PCB 126/l) induced almost complete mortality of the larvae at the end of the yolk-sac/beginning of the free-feeding stage. This occurred for all exposure durations, even in the groups that were only exposed during the first 4 days. In the 3 and 10 ng PCB 126/l exposure groups mortality occurred at a later moment depending on exposure duration, but this was in all cases days after exposure had ended. When around day 35, the first fish had started the metamorphosis, other surviving fish in the 1, 3 and 10 ng PCB 126/l exposure groups that were still in the pre-metamorphosis stage, developed oedema in the abdominal region. On day 41, oedema was observed in more than 50% of the surviving pre-metamorphic fish in the 8-dpf and 10-dpf treatments at 3 ng/l and in ca. 20% of the survivors in the 4dpf group. In the 4-dpf 10 ng/l treatment this was even 100%. Remarkably, no fish with oedema were seen at the same test concentration in the 8-dpf group. At the same concentration in the 10-dpf treatments complete mortality had occurred at that moment. All fish with oedema died without any indication that the metamorphosis started. The surviving fish completed the metamorphosis without exposure-related malformations or delay.

2.3.3 Effect concentrations

The data set allowed the calculation of LC50 values between dpf 12 and 20 for all 4 exposure durations (Figure 2.3). Due to the increasing mortality in time it was not possible to calculate LC50s after day 20 for the 15-dpf group, and no data were available from day 10.

Statistical analysis revealed that treatment only affected the LC50s in a significant way at early observation. Only on day 10 the LC50 of the 4-pdf group was significantly (p<0.05) higher than other treatments. During all later observations the LC50 values of all treatment groups were statistically comparable.

The moment of observation had an extremely significant effect (p<0.0001). On day 12, the LC50 values for the various treatments ranged between 39 and 82 ng PCB 126/l. Although exposure stopped after a period of 4 to 15 days all LC50 values rapidly decreased with increasing observation time. At the end of the test all LC50 values ranged between 1.7 and 3.7 ng PCB 126/l and did not differ significantly. They were, however, significantly more than 20 times lower than the LC50 values on day 12.
2.3.4 Internal TEQ concentration

The internal TEQ-levels of the larvae after 4 days of exposure during the egg stage only, ranged from 0.59 ng TEQ/g lipid in the control animals to 33 ng TEQ/g lipid in the larvae from the 100 ng PCB 126/l group. The relation between the TEQ level in the lipids of the hatched larvae and the exposure concentration is well described ($R^2=0.998$) by the formula for a two-way exponential association (Figure 2.4):

$$
\text{TEQ} = 3563 \times (1-e^{-0.000076 \times \text{PCB}}) + 5.938 \times (1-e^{0.4247 \times \text{PCB}})
$$

Where:

- TEQ = the internal dose, expressed as ng TEQ/g lipid
- PCB = the nominal exposure concentration, expressed as ng PCB 126/l

When expressed as internal dose measured in newly hatched larvae, the internal lethal concentration for the 4 dpf-group, expressed as the ILC50 value, was 22.6 ng TEQ/g lipid (95%-confidence interval: 15.7 – 32.5) on day 12 and 1.0 ng TEQ/g lipid (95%-confidence interval: 0.6 – 1.7) on day 56 (Figure 2.5).
Figure 2.4 TCDD-equivalents (TEQs) determined with a reporter gene (DRE-H4IE.Luc) assay in lipid of newly hatched sole larvae after exposure of the eggs during 4 days to PCB 126 via the water.

Figure 2.5 Relationship between the internal TEQ concentration in lipid of newly hatched sole larvae at the moment of hatching after exposure of the eggs during 4 days to PCB 126 (two replicates per treatment), and the mortality at three time points later during the early life development of a similarly treated group (4-dpf group). On Day 12 all fish were free-feeding, on day 56 all surviving fish had completed metamorphosis.
2.4 Discussion

2.4.1 Experimental set-up

The moment of hatching and becoming free-feeding differed 1-2 days between experiments. This might be related to the 1° C difference in temperature, as the development of early life stages of sole (and other fish) is strongly temperature dependent (Imsland et al., 2003). The time required by the surviving fish to complete metamorphosis seemed longer in the second experiment, but this was only due to the application of the stricter criterion for ‘complete metamorphosis’.

As the test compound was applied in the water column, the only uptake route for the exposure periods 4 dpf and 8 dpf which ended before the fish became free-feeding, was via the water. With longer exposure conditions some indirect exposure will have occurred via PCB adsorbed to the food, the artemia nauplii. This period with an additional route of exposure lasted 1 and 5 days for the 10-dpf and 15-dpf group, respectively. However, when the LC50 values for the various exposure durations are compared (Figure 2.3) there are no indications that indirect exposure via the food affected the outcome of the tests. The differences between LC50 values are related to the exposure duration and in absolute sense strongly determined by the moment of observation.

2.4.2 Effect type and effect concentrations

At the moment that all fish were free-feeding, which is the moment that a standard ELS fish test (EPA, 1996; OECD, 1992) could have been terminated, the LC50 values ranged between 39 and 82 ng PCB 126/l. However, with a prolonged observation period the LC50-values in the sole test were revealed to be 22 times lower (1.7 to 3.7 ng PCB 126/l). Since exposure to the test substance had already stopped after 4 to 15 days, this is not due to higher body burdens. Internal concentrations will even have decreased in time due to growth dilution. The first 4 dpf appear to be the main critical period for exposure to PCB 126. The delayed effects become apparent during the first week that the fish are free-feeding and resulted in an increased mortality rate.

A substantial number of the exposed fish that survived this first critical period developed oedema in the abdominal region about 20 days later, and died before metamorphosis. Oedema is an indication of a disturbed water balance (Stouthart et al., 1998; Hill et al., 2004) and is often found in ELS fish tests with dioxin-like toxicants. It is then referred to as blue-sac disease that is characterised by pericardial and yolk-sac oedema (Stouthart et al., 1998; Zabel et al., 1995; Hill et al., 2004; Brinkworth et al., 2003). The indications of blue-sac disease develop at early larval stages or directly after the absorption of the yolk-sac. In
our tests the oedema was observed in a much later stage of the development which might, amongst other reasons, be related to the low test concentrations that we applied in combination with a long observation period.

Delayed effects of PCB exposure (including oedema) have also been described for *Xenopus laevis* tadpoles where the exposure of embryos to PCB 126, PCB 77 or PCB-mixtures during 4 dpf did not affect larval development during exposure, but resulted in increased mortality and delayed metamorphosis several weeks later (Gutleb et al., 1999; 2007a). Based on these results a prolonged FETAX (Frog Embryo Teratogenic Assay - Xenopus) was proposed for compounds without acute effects (Gutleb et al., 2007a).

The clearly delayed adverse effect of early exposure, as we found in our tests, is also not covered in the standardised ELS fish tests described in OECD 210 and 212, nor in the new embryo fish test that is currently being developed (OECD, 2006). Therefore, these tests may seriously underestimate the toxic potency of single compounds or environmental extracts without acute teratogenicity.

2.4.3 Field relevance

The 4-dpf group that was only exposed during the egg stage already showed considerable effects with effect concentrations in the same order of a magnitude as the groups that were exposed during a substantial part of the early development. This makes the reproduction success of the fish susceptible for lipophilic persistent compounds that are accumulated by the mother fish and transported to the eggs during gametogenesis (Solbakken et al., 1984; Vodicnic & Peterson, 1985).

When expressed as internal TEQ concentration in the newly hatched larvae the ILC50 after 56 days is 1.0 ng TEQ/g lipid for the 4-dpf group. This level can be compared with TEQ levels on lipid basis in adult fish to get an indication of the field relevance of these effect concentrations. The TEQ levels in field-caught fish range from 4 pg TEQ/g lipid in sardinella (Leonards et al., 2000) up to 3764 pg TEQ/g lipid in flounder from the heavily polluted Grenland fjords in Norway (Knutzen et al., 2003) on the basis of by no means an exhaustive literature review. Fourteen (20%) out of the 70 samples that we identified in our short literature search contained more than 250 pg TEQ /g lipid (Table 2.1). This is 25% of the ILC50 we determined in our prolonged sole ELS test after exposure during 4 dpf. The TEQ levels detected in the two flounder samples from the Grenland fjords even exceeded our ILC50.

It is important to realise that exposure of the sole eggs in our experiment only started at ca. 12 hours instead of immediately after fertilisation as is the case...
with eggs exposed via the lipids that the mother animal deposits in the eggs. We therefore cannot exclude that our results still underestimate the actual effects. In addition, exposure of the mother fish to toxic compounds can also affect the egg quality. She may for instance suffer from toxicant-induced reduced vitamin A levels that could result in less viable eggs as has been shown for flatfish (e.g. flounder; Besselink et al., 1998) and birds (e.g. common tern; Murk et al., 1996b).

Moreover, environmentally contaminated parent fish and consequently the eggs are exposed to complex mixtures of toxicants. Recent research with the Japanese medaka (Oryzias latipes) showed for instance that parental exposure to a combination of PCB and TBT has a more adverse effect on the embryonic development than the added effect of the individual substances (Nakayama et al., 2005).

Table 2.1 Total WHO TEQ (Van den Berg et al., 1998) levels in fish tissue that have been reported from field studies and are above 250 pg TEQ/g lipid, being 25% of the LC50 in the sole ELS test. The list is not exhaustive. Values marked with ‘*’ were calculated using the TEQ levels reported on fresh weight bases and the lipid content as reported in the same paper.

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Location</th>
<th>Lipid (%)</th>
<th>WHO PCDD/F-TEQ (pg/g WW)</th>
<th>WHO PCB-TEQ (pg/g WW)</th>
<th>total WHO TEQ (pg/g WW)</th>
<th>Total WHO TEQ (pg/g lipid)</th>
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<tr>
<td>Balthic Herring</td>
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<td>5</td>
<td>10.23</td>
<td>393*</td>
<td>R1</td>
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<td>R2</td>
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<td>1.9</td>
<td></td>
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<td>3764*</td>
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</table>

*Ref. 1 = Isosaari et al. 2006; R2 = Leonards et al., 2000; R3 = Knutzen et al, 2003
2.5 Conclusions

This study shows that the flatfish sole is a suitable marine test organism to perform early life stage tests including the phase of full metamorphosis. The sole is a test species that is relevant for the North Sea area and sole eggs are becoming more easily available since aquaculture activities are increasing worldwide.

We demonstrated that, at least for sole, the moment of observation has a significant impact on the effect concentration, even without further exposure. Exposure of only the eggs to non-acutely toxic compounds such as the dioxin-like PCB 126 resulted in delayed effects that are not covered in the standardised ELS fish tests. Therefore, these tests may seriously underestimate the toxicity of single compounds or environmental mixtures without acute toxicity.

Our study results suggest that the reproductive success of fish populations at contaminated sites can be expected to be negatively affected by (mixtures of) persistent compounds including dioxin-like compounds that are accumulated by the female fish and passed on to the eggs.

2.6 References


Eggsposed  Chapter 2


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CHAPTER 3

Critical body burden of maternally transferred methyl-triclosan for the flatfish Solea solea

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Abstract

In order to mimic exposure through maternal transfer, fertilized eggs of the marine flatfish Solea solea were exposed to a concentration series of methyltriclosan (MTCS) in water until hatching (6 days post fertilization, dpf), and were then allowed to develop further under unexposed conditions until completed metamorphoses, 40 dpf. Mortality occurred in the higher treatment levels until 20 dpf, and all surviving larvae completed metamorphoses without problems. Internal effect concentrations (IEC), reached in larvae at the end of the exposure (6 dpf), were 5.8 mg/g lipid weight (lw) and 2.1 mg/g lw for IEC50 and IEC10 respectively. These internal effect concentrations are at least 200 times higher than concentrations that due to maternally transfer can be expected in the eggs of highly exposed fish in a field situation. Our results thus do not indicate a high risk from maternally transferred MTCS for fish at the current field concentrations. However, the impact of mixtures including MTCS, the parent compound triclosan, and other persistent lipophilic substances still needs further assessment.
3.1 Introduction

Methyltriclosan (MTCS) is formed by microbiological methylation of triclosan (TCS) that is commonly used as bactericide in a wide variety of human care products. Effluents from sewage treatment plants form a major source of TCS and MTCS to the environment (Coogan et al., 2007; Coogan and La Point, 2008; Dann and Hontela, 2011; Fernandes et al., 2011), and traces have been detected in the marine environment (Andresen et al., 2007; Fernandes et al., 2011; Xie et al., 2008). MTCS is suggested to be more persistent than the parent compound (Leiker et al., 2009), and this persistence combined with a log Kow of 5.0 to 5.5 (Valo and Salkinojasalonen, 1986) gives concern about uptake in the food chain. Indeed MTCS has been detected in tissue of fresh water fish (Adolfsson-Erici et al., 2002; Balmer et al., 2004; Leiker et al., 2009), and therefore it is suggested that further research is needed, especially with regards to the bioaccumulation potential and toxicity (Dann and Hontela, 2011), and with respect to the potential of MTCS to interfere with the thyroid hormone system (Hinther et al., 2011).

A typical exposure route for bioaccumulation substances is maternal transfer. Substances like MTCS, that have accumulated in the mother’s tissues are transferred to the eggs (Russell et al., 1999) causing an early exposure of the developing embryo. Since the thyroid hormone system plays an important role during early development in many species including fish (e.g. Klaren et al., 2008; Yu et al., 2011), maternally transferred MTCS poses a potential risk for developing fish larvae. The aim of the study was to determine critical internal (no-)effect levels for MTCS in fish eggs on the survival and development. By a short exposure of fertilized eggs, maternal transfer of the contaminant to the egg was mimicked. Earlier research with PCBs and TCDD has indicated that such exposure results in comparable effects on the developing larvae as actual maternal exposure (Walker and Peterson, 1994).

As far as we know critical body burdens for MTCS have not been determined so far. The common sole (Solea solea) was chosen as test species, as during early development this species undergoes the obvious metamorphosis typical for all flatfishes. As this metamorphosis is thyroid hormone mediated (Klaren et al., 2008) it is potentially sensitive for disruption by MTCS (Hinther et al., 2011).
3.2 Materials and methods

3.2.1 Test procedure

The test was performed according to Foekema et al., 2008 where details of the test procedure are reported. In summary, fertilized eggs from Solea solea were exposed to a concentration series of MTCS (Methyltriclosan, purity 99.7%, Sigma-Aldrich) in 0.1% DMSO (Dimethylsulfoxide, purity 99.9%, A.C.S. spectrophotometric grade, supplier SIGMA-Aldrich) until full hatching 6 days post fertilization (dpf). Nominal exposure concentrations were 4, 12, 37, 111, 333 and 1000 µg MTCS/L.

Per test concentration two groups of 15 larvae were allowed to further develop until the end of the metamorphosis at 40 dpf in glass beakers containing clean seawater without DMSO or MTCS. From 9 dpf onwards, when the larvae are about to start external feeding, they were fed ad libitum with newly hatched nauplli of Artemia salina on a daily basis. Every other day, approximately 75% of the water volume in each glass beaker was replaced. On an almost daily bases mortality and development was recorded, and dead larvae, faeces and surplus food items were removed. Fish that completed metamorphosis were taken out of the test, narcotized and subsequently killed in a solution consisting of 500 mg of ethyl 3-aminonebzoate methanesulfonic acid salt (purity 98%, ACROS Organics) and 200 mg NaHCO3 (purity 99.0%, Fluka Chemika) in 1 liter seawater). The moment of completed metamorphosis, body size and observable malformations were recorded.

The experiments were approved by the Animal Care Committee of the Animal Sciences Group of Wageningen UR.

3.2.2 Chemical analysis

Those larvae that were not transferred to clean water on 6 dpf were sacrificed for chemical analysis of the lipid-normalized MTCS concentration. For that they were taken from the exposure beakers with a polyethylene pipette and transferred to clean seawater to rinse remaining exposure water, and within 15 minutes collected by filtration on a Whatman GF/C filter with known wet (seawater) weight. The filter with the larvae was weighed again and stored at -20°C until preparation for analysis. For that, the filters containing the larvae were subsequently extracted 3 times by ultrasonification with 4 ml of a mixture of 50% pentane – 50% dichloromethane (both substances picograde; LGC standards, Germany). The extract was transferred to a test tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 1 ml injection standard (PCB112, purity >99%, LGC standards) and transferred to
a vial for GC/MS analysis using an Agilent 6890 gas chromatograph (GC) coupled to an Agilent 5973 MSD quadropole mass spectrometer (MS) detector (Amstelveen, The Netherlands). Analysis was performed in electron impact (EI) mode monitoring m/z 302 and 304 for MTCS and m/z 326 and 324 for PCB112 as quantifier and qualifier ion.

3.2.3 Data analysis

Internal effect concentrations (IEC) for survival and normal development were determined by fitting the formula for a 'sigmoid dose response curve with variable slope' as incorporated in GraphPad Prism (version 4.03) to the dataset. Both the IEC₅₀ with 95%-confidence intervals as well as the IEC₁₀ for survival and development were determined from the curve.

3.3 Results and discussion

In the solvent controls more than 90% of the larvae developed into normal juvenile fish. Hatching success at the end of the exposure period (6 dpf) was not affected by any of the treatments. The newly hatched larvae in treatments 333 µg MTCS/l and 1000 µg MTCS/l however, were in poor condition and complete mortality occurred in these groups within the next two days (Figure 3.1a). In this stage no increased mortality was seen in 111 µg MTCS/l, the next lower exposure group. Between 15 and 20 dpf, when yolk had been fully absorbed, mortality increased substantially. After 20 dpf no further mortality occurred and all surviving fish developed normally, with onset of metamorphoses around 25 dpf, comparable to the controls. At the end of the test no fish were malformed and therefore effect concentrations for survival and normal development were comparable.

The ILC₅₀ and ILC₁₀ expressed as lipid normalized body burden on 6 dpf (Figure 3.1b) were 5.8 mg/g lw (95% confidence intervals 4.3 to 7.9 mg/g lw, r²=0.969) and 2.1 mg/g lw, respectively.
Figure 3.1 Survival over time after exposure during 6 days post fertilization (0-6 dpf, indicated as shaded area (A), and the internal dose (tissue concentration on 6 dpf) vs response (mortality on 40 dpf) curve (B).

The experimental setup aimed at approaching the toxic load of the eggs that could be reached as a result of transfer of MTCS accumulated in the mother’s body lipids to the eggs. Based on a fugacity model it has been reported that the ratio of the lipid normalized concentration of hydrophobic substances in eggs and maternal tissue will be close to 1 (Russell et al., 1999). Therefore the concentration of the substance in the tissue of the mother fish is an indication of the concentration in the eggs on 0 dpf. For freshwater fish species some information about tissue concentrations of MTCS is available (Adolfsson-Erici et al., 2002; Balmer et al., 2004; Leiker et al., 2009), and more references in (Dann and Hontela, 2011). The highest reported concentration of MTCS in fresh water fish tissue, 7.4 μg/g lipid, was found in carp from Las Vegas bay, USA (Leiker et al., 2009), which is 0.5% of the IEC_{10} derived for sole in this study. MTCS-levels in marine fish species have not been published yet. But, since the main source for MTCS is effluent from sewage treatment plants that in majority is discharged at fresh water systems, it is likely that levels in marine fish are (much) lower than in freshwater fish. This indicates that at actual field concentrations, the risk of adverse effects on developing fish larvae from maternally transferred MTCS is very limited, although it should be taken into account that our experiments might underestimate the toxic risks. In eggs exposed via the mother fish, the maximum MTCs concentration already is present before fertilization. Our experiments started with clean eggs and fertilization occurred under non-exposed conditions. The maximum MTCS concentrations in the eggs were only reached at the end of the exposure phase (6 dpf). Also potential effects of MTCS on the parents that may affect egg quality were not included in our experiments. Experiments with brook trout indicated
that the effects and internal effect concentrations of PCBs and TCDD were comparable for eggs that were exposed via the water phase, by injection or by maternal exposure (Walker and Peterson, 1994). However, this does not have to be true for MTCS, that can interfere with the thyroid hormone system (Hinther et al., 2011) of the parent fish. For other substances (polybrominated diphenyl ethers, PBDEs) with the potential to disrupt this hormone system, it has been shown that parental exposure can influence the levels of thyroid hormones in the eggs, resulting in decreased hatching and increased malformations (Yu et al., 2011). Finally, the effect levels we derived were based on single compounds, while in field situations fish are always exposed to cocktails of pollutants. This can give rise to mixture toxicity, e.g. when some compounds inhibit the cellular efflux pumps, an important defense mechanism against toxic compounds, as has been shown for the parent compound TCS (Anselmo et al., 2012).

We conclude that, although our results do not indicate a direct risk of MTCS for fish larvae through maternal exposure at the current field concentrations, the issues mentioned above should be taken into account and studied further. Special attention should go to the impact of mixtures of TCS and MTCS and other substances, especially those that are common in effluents from sewage treatment plants like metals and (residues of) pharmaceuticals.

3.4 References


CHAPTER 4

Toxic concentrations in fish early life stages peak at critical moment

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Abstract

During the development of an embryo into a juvenile, the physiology and behavior of a fish strongly change, affecting exposure to and uptake of environmental pollutants. Based on experimental data with sole (Solea solea) an existing bioaccumulation model was adapted and validated to calculate the development of concentrations of persistent organic pollutants in the tissue of the developing fish. Simulation revealed that toxic tissue concentrations of pollutants with \( \log K_{ow} > 5 \) peak at the moment the larvae become free-feeding, when the lipid reserves are depleted. This may explain the delayed effects observed in fish early life stage experiments with exposed eggs. In the field, eggs can be exposed through maternal transfer to adult pollutant tissue concentrations, which will increase in the larva to peak tissue concentrations exceeding those of the adult fish. The results demonstrate the risk of underestimating the effects of lipophilic persistent organic pollutants with \( \log K_{ow} > 5 \) in short term early life stage fish tests, and underline the importance of maternal transfer as an exposure route in the field situation.
4.1 Introduction

Early life stages of fish, as well as other organisms, are in general considered to be more sensitive to toxicants than fully-developed individuals from the same species (Hutchinson et al., 1998). This is related to a combination of critical developments during early life stages, the limited possibilities to compensate the impact of physiological processes affected (Crane et al., 2006), and large changes in the composition of the developing tissue that affect internal bioavailability. Early life stages of oviparous species have a unique exposure route as pollutants accumulated by the mother fish can be deposited in the egg together with lipids and proteins (Kelly et al., 2011; Russell et al., 1999). Maternal transfer is especially relevant for lipophilic persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs) and dioxins that accumulate in lipids of the mother fish.

In general, bioaccumulation/exposure models focus on the fate of pollutants in fully developed fish in a stable environment (Bodiguel et al., 2009; Eichinger et al., 2010; Veltman et al., 2005). Sometimes the impact of seasonal fluctuations and life cycles on levels in the adult fish are included as well (Ng and Gray, 2009; Sijm et al., 1992; Veltman et al., 2005), but the early life stages never are addressed as such. This, however, is an interesting phase due to the drastic biological and physiological changes that take place in the animals, sometimes in the presence of maternally transferred pollutants. More insight in the development of pollutant tissue concentration will help the interpretation of the results from standard fish early life stages (ELS) toxicity tests (EPA, 1996; OECD, 1992, 1998, 2006) in which exposure mostly takes place through the water phase only.

In the present study we measured and modeled the water and tissue concentrations of POPs during the development of an embryo into a juvenile fish. The exposure experiment was conducted with eggs of aquacultured common sole (Solea solea), an oviparous marine flatfish. The fate of a selection of PCBs with a range of lipophilicities (log $K_{ow}$ values of ranging from 5.9 to 6.8) from the technical mixture Arochlor 1254 was measured in the exposure water and larvae. Based on knowledge of the main developmental stages of fish larvae (relevant information is given below), the existing OMEGA (Optimal Modeling for EcotoxicoloGical Applications) bioaccumulation model (Hendriks et al., 2001) was adapted to calculate the development of POP-concentrations in the tissue of the developing fish. After validation with measured concentrations, different exposure scenarios were simulated using the developed ELS- OMEGA model to calculate the development of the tissue concentrations of POPs in sole larvae in time.
4.1.1 Fish developmental stages

In early life development of fish several stages can be distinguished (Blaxter, 1988; Jobling, 2002) of which three are most relevant for the present study: the egg stage, the yolk-sac stage and the free-feeding stage. The biological characteristics and possible exposure routes of those stages briefly described below, apply for the majority of oviparous fish species. The most obvious difference between species is the duration of the different stages (Blaxter, 1988), which also is temperature related. Below, the timing of the stages for our model fish *Solea solea* under experimental conditions is indicated.

At spawning, a fish egg contains all resources necessary for the development of the embryo into a larva that is capable of external feeding. The majority of these resources is stored in the yolk by the mother fish during vitellogenesis (as reviewed by (Walsh, 1988) and during this process lipophilic pollutants are transferred to the eggs as well (De Bruyn et al., 2004; Serrano et al., 2008; Daley et al., 2009; Kelly et al., 2011). For our model calculations we assume a lipid normalized maternal-to-egg transfer ratio of 1 for all persistent organic substances, conform the fugacity model (Russel et al., 1999). Immediately after spawning, the eggs are fertilized and the chorion becomes a protective shield against physical damage. The uptake and excretion of pollutants in a fertilized fish egg is low compared to uptake in newly hatched larvae (Villalobos et al., 2000; Gonzalez-Doncel et al., 2003). This probably is related to the lack of actively circulated fluids through or near the chorion and the relatively small surface area-volume ratio of a fish egg in comparison to that of a fish larva (Petersen and Kristensen, 1988). In our calculations we assume that for the egg and the larva exposure and excretion are through the water column. This is obvious for the majority of the eggs from marine fish species, including *Solea solea*, that are more or less buoyant. But even for demersal eggs it is plausible that transfer of pollutants from sediments occurs via the (pore) water phase, as is also the case with organisms that have a more intense contact with the sediment (Lu et al., 2011).

Five days post fertilization (5 dpf), the sole eggs hatch. The so-called yolk-sac larvae are not capable of external feeding. As they completely rely on the yolk for nutrition, exposure and excretion still is limited to the water phase. The exchange with water is more intense than before hatching, for reasons mentioned above and will further intensify with developing gills. During the yolk-sac stage, the mouth and intestine develops, which enables the larvae to switch from endogenous to exogenous feeding before the yolk is depleted. For the sole larvae in our experiment this critical moment takes place around 10 dpf. From then on, the larva is referred to as a free-feeding larva and food is an additional
route of exposure. As with external feeding the fish start to grow, growth
dilution of the body burden of pollutants can occur (Sijm et al., 1992). In
addition to diffusion via skin and gills, fecal egestion becomes a potential
excretion route (Paterson et al., 2010). In the following period the free-feeding
larvae progressively develop adult characteristics until they metamorphose into
the juvenile stage. For sole this metamorphosis is very obvious as the
symmetrical larvae changes into an asymmetrical flat fish. Given our
experimental conditions this metamorphosis starts around 25 dpf. The exposure
of juvenile and adult fish is not fundamentally different from that of the free-
feeding larvae, although the food choice often changes, with potential
implications for exposure levels.

4.2 Material and methods

4.2.1 Bioaccumulation experiment

Sole eggs were produced at the aquaculture department of IMARES, and
transported to the laboratory within 12 h after fertilization. The process of
handling and selection of viable eggs and the experimental conditions were as
described before (Foekema et al., 2008). The fertilized eggs were randomly
divided into groups of approximately 200 individuals that were transferred to
glass beakers containing 2 L seawater each. In total six of these glass beakers
were prepared (Table 4.1): Four beakers containing seawater spiked with 250
ng Arochlor 1254 (Monsanto, USA) per liter in 0.1% Dimethyl sulfoxide (DMSO),
and two beakers with fresh seawater representing the unexposed controls. Both
PCB and DMSO concentrations are below effect levels for the early life
development of Sole. All beakers were incubated in a climate-controlled cabinet
at 14°C with a daily period of 16 h illumination with low light intensity.

Table 4.1 Overview of the set-up of the exposure experiment. The grey fields indicate the
period that the eggs/fish were present in this replicate. Exposure level was 250 ng
Arochlor /L. At 10 dpf 25 individual larvae were transferred to smaller beakers, the rest
was sampled for chemical analyses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 dpf</th>
<th>5 dpf</th>
<th>10 dpf</th>
<th>27 dpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure-a</td>
<td>start</td>
<td>All sampled</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposure-b</td>
<td>start</td>
<td>All sampled</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposure-c</td>
<td>start</td>
<td></td>
<td>Sampled -25</td>
<td>Sampled</td>
</tr>
<tr>
<td>Exposure-d</td>
<td>start</td>
<td></td>
<td>Sampled -25</td>
<td>Sampled</td>
</tr>
<tr>
<td>Blanc-a</td>
<td>start</td>
<td>All sampled</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blanc-b</td>
<td>start</td>
<td></td>
<td>Sampled -25</td>
<td>Sampled</td>
</tr>
</tbody>
</table>
After hatching, 5 d post fertilization (5 dpf), the larvae from two beakers of the exposure group and one of the control group were sampled for chemical analyses following the procedure described below. At 10 dpf, at the end of the yolk-sac stage, 25 larvae were randomly collected from each of the remaining three beakers (one control and two exposure groups) and transferred to smaller (200 ml) glass beakers containing clean seawater for the ‘control’ group and seawater spiked at 250 ng Arochlor1254 /L for the exposed group. The remaining fish larvae from the 2 L beakers were sampled for chemical analysis. The 3 x 25 selected larvae were fed *ad libitum* daily with newly hatched *Artemia salina* nauplii. Before being fed to the exposure group, the Artemia were placed in a 250 ng Arochlor 1254 /L solution for approximately 2 h. At 27 dpf, when the first fish reached the climax of metamorphoses, all fish were collected for chemical analyses.

Between 0 and 10 dpf, while the egg/larvae were kept in the 2 L-beakers, every second day approximately 75% of the water volume was replaced with new spiked or fresh seawater. After 10 dpf, the 25 fish in the smaller beakers were transferred every 48 h with a polyethylene pipet to a new beaker that contained seawater spiked at the appropriate level. In this way, contamination of the beakers with uneaten food, microbial fouling, and feces was minimized.

The experiment was approved by the Animal Care Committee of the Animal sciences group of Wageningen UR.

### 4.2.2 Sampling for chemical analyses

Using a semi-static test set up, exponential dissipation of the PCBs from the water column with time was expected (Koponen et al., 1998; Breitholtz et al., 2006). In order to get an impression of the range of the PCB concentrations in the test beakers, water samples were collected from the freshly made exposure solution at 0 dpf to determine the initial exposure concentration. At 5, 10 and 23 dpf water samples were collected from each individual test beaker. Sampling on these days was performed just before water replacement took place and these samples were thus indicative for the minimum exposure concentration.

For the determination of the background PCB concentration, unexposed eggs were sampled and stored at -20°C. The early life stages were sampled for chemical analyses immediately after hatching (5 dpf), at the end of the yolk-sac stage (10 dpf), and at the end of the experiment (27 dpf). The larvae were collected with a polyethylene pipette and while counting, transferred for maximum 15 min to unexposed water containing a narcotizing solution (100 mg Ethyl 3-aminonebzoate methanesulfonic acid salt -MS222, purity 98%, ACROS Organics-, and 200 mg NaHCO₃ -purity 99.0%, Fluka Chemika- per 1 L
seawater). This also rinsed off the exposure water that initially came with the larvae. The narcotized larvae were collected by filtration on a pre-(wet)weighed filter (Whatman GF/C, glass microfiber filter) removing as much water as possible. After determination of the total wet weight, the filters with the larvae were stored at -20°C until chemical analyses.

Two independent samples of the spiked *Artemia nauplii* used as food source were collected by filtration, weighted, stored, and analyzed as the fish samples.

4.2.3 PCB analysis

In total, 29 PCB congeners were analyzed following an accredited in-house method. In the present study we focus on the following six PCB congeners: PCB 52, 101, 110, 118, 138 and 153. The basis behind this selection are the seven indicator PCBs (PCB 28, 52, 101, 118, 138, 153, 180) applied by the International Council for the Exploration of the Sea (ICES), minus PCB 28 and 180, which levels in the exposure water were below the analytical limit of detection. PCB 110 was included to create a series with the range log $K_{ow}$ 5.9 to 6.8. $K_{ow}$ values for the selected PCBs were taken from Hansen *et al.*, 1999 (Table 4.2).

PCB congeners as standards for chemical analysis (>99% purity) were purchased from Sigma-Aldrich, The Netherlands and CN Schmidt, The Netherlands. Pentane (picograde), dichloromethane (picograde) and isooctane (picograde) were purchased from LGC standards, Germany. Before analysis, the samples were spiked with 50 ng PCB 143 in 200 µl isooctane as internal standards and were then subsequently extracted three times by ultrasonification with 4 ml of a mixture of pentane - dichloromethane (50:50). The extract was transferred to a test tube, after which it was evaporated to dryness under a gentle stream of nitrogen. The residue was weighted to determine the lipid content of the sample and was then reconstituted in 1 ml injection standard and transferred to a vial for gas chromatography-mass spectrometry (GC/MS) analysis using a Agilent 6890 gas chromatograph (GC) coupled to an Agilent 5973 MSD quadrupole mass spectrometer (MS) detector (Amstelveen, The Netherlands) as described before. PCBs were determined using a 60 m x 0.25 mm i.d. HT8 column with a film thickness of 0.25 µm. A subsample of 1 µl was injected using split/splitless mode with an injection temperature of 290°C. Transfer line, source and quadrupole temperatures were 290, 230 and 150°C, respectively. The oven program was as follows: 90°C, hold for 3 min, then 30°C/min to 170, 1.5°C/min to 270, 30°C/min to 300°C hold for 3 min. Ionization was performed using electron impact ionization (EI) mode. PCB 52 was quantified on m/z 292 using 290 as qualifier ion, PCBs 101, 110, and 118
were quantified on m/z 326 using 324 as qualifier ion and PCBs 138 and 153 were quantified on m/z 360 using 362 as qualifier ion.

Samples sizes of exposed larvae at 5 and 10 dpf were too small for an accurate determination of the lipid content. As alternative the lipid content of these stages were determined in groups of 1225 (5 dpf) and 423 (10 dpf) unexposed larvae, which are assumed to be representative for the exposed larvae as well. Due to the limited availability of larvae no replicate samples were available for this analyses.

Table 4.2 Log K\text{ow} nominal and measured concentrations of the selected PCBs in the freshly made exposure solution (Initial) and in the exposure water sampled just before refreshment in the period dpf 0-10 and dpf 10-27 respectively. Presented are average and range of the measured concentrations, and expressed as percentages of the initial concentrations.

<table>
<thead>
<tr>
<th>ng/L</th>
<th>PCB 52</th>
<th>PCB 110</th>
<th>PCB 101</th>
<th>PCB 118</th>
<th>PCB 138</th>
<th>PCB 153</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log K\text{ow}\textsuperscript{*}</td>
<td>5.86</td>
<td>6.25</td>
<td>6.33</td>
<td>6.46</td>
<td>6.71</td>
<td>6.79</td>
</tr>
<tr>
<td>Nominal</td>
<td>14.2</td>
<td>21.7</td>
<td>25.8</td>
<td>19.2</td>
<td>18.3</td>
<td>13.3</td>
</tr>
<tr>
<td>Initial</td>
<td>14.3 (100%)</td>
<td>23.5 (100%)</td>
<td>23.8 (100%)</td>
<td>20.4 (100%)</td>
<td>17.3 (100%)</td>
<td>13.8 (100%)</td>
</tr>
<tr>
<td>N=2</td>
<td>(14.1-14.4)</td>
<td>(23.0-24.0)</td>
<td>(23.8-23.8)</td>
<td>(19.8-21.0)</td>
<td>(16.8-17.8)</td>
<td>(13.7-13.8)</td>
</tr>
<tr>
<td>Dpf 0-10</td>
<td>6.0 (42%)</td>
<td>13.5 (57%)</td>
<td>13.2 (55%)</td>
<td>13.2 (65%)</td>
<td>11.9 (69%)</td>
<td>10.2 (72%)</td>
</tr>
<tr>
<td>N=2</td>
<td>(5.2-6.8)</td>
<td>(12.7-14.3)</td>
<td>(12.4-14.0)</td>
<td>(12.6-13.8)</td>
<td>(11.8-12.0)</td>
<td>(9.3-11.1)</td>
</tr>
<tr>
<td>Dpf 10-27</td>
<td>2.6 (18%)</td>
<td>6.7 (29%)</td>
<td>6.4 (27%)</td>
<td>8.2 (40%)</td>
<td>7.3 (42%)</td>
<td>6.0 (44%)</td>
</tr>
<tr>
<td>N=4</td>
<td>(1.7-3.2)</td>
<td>(5.1-8.0)</td>
<td>(4.6-8.0)</td>
<td>(6.4-9.8)</td>
<td>(6.0-8.2)</td>
<td>(5.5-6.9)</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Log K\text{ow} values from Hansen et al., 1999

4.2.4 ELS-OMEGA bioaccumulation Model

The bioaccumulation model OMEGA (Hendriks et al., 2001) that was adjusted and applied for the present study is based on classical fugacity theory for accumulation kinetics of organic substances as a function of the octanol-water partition ratio (K\text{ow}). The OMEGA model considers the role of species weight, lipid content, and trophic level. Exchange (uptake and excretion) of the chemical between the organism and its environment via water, food, and faeces are included. The steady state tissue concentrations in adult fish in field situations, has been predicted well with the OMEGA model based on environmental concentrations (Smitkova et al., 2005).

Rather than determining the equilibrium ratios, the ordinary differential equation (ODE) provided by Hendriks et al. (2001) was used as basis to determine the
internal PCB concentrations in the egg/larvae at any given time. To this end, the ‘deSolve’ package (Soetaert et al., 2010) in R (The R Foundation for Statistical Computing) was used to solve the ODE numerically, using Euler’s method.

The OMEGA model optimized for fish early life stages will further be referred to as the ELS-OMEGA model.

For those model parameters that are assumed not to be life stage or species specific (respiration coefficient, rate exponent, body temperature correction factor, water layer diffusion resistance and lipid layer permeation resistance), the default and typical values that are presented for animals in (Hendriks et al., 2001) were applied (Table 4.4). For the water absorption-excretion coefficient ($\gamma_0$) the default value was applied after hatching at 5 dpf. The value of the egg stage $\gamma_0$ was calculated on the basis of the relative uptake of PCB 118 during the egg stage and the yolk-sac stage respectively, both covering a time frame of 5 d. PCB 118 was used for this calibration since its $K_{ow}$ was the closest to the average $K_{ow}$ of the PCBs tested. As $\gamma_0$ is typical for a species/life stage, the thus calculated value is assumed valid for all PCBs.

Parameters describing the development of body weight and lipid content were derived from experimental data.

Mortality was not included since the ELS model describes the development of an individual at non-lethal exposure levels. Biotransformation of the persistent PCBs that were used in the present study will be close to zero.

Parameters related to feeding, like food ingestion coefficient ($\gamma_1$), fraction of ingested food assimilated ($P_1$), lipid fraction of food ($P_{ch2, i-1}$) and pollutant concentration in food ($C_{i-1}$) are not applicable as long as fish are not free-feeding, and were thus set at zero until 10 dpf. After 10 dpf, $\gamma_1$ and $P_1$ were set at the default values (Hendriks et al., 2001). Lipid fraction of food ($P_{ch2, i-1}$) and PCB concentration ($C_{i-1}$) were based on the results of the chemical analyses of the food source Artemia nauplii.

For the validation of the ELS-OMEGA model and the input parameters including $\gamma_0$ based on PCB 118, the average exposure concentration of each PCB was predicted and compared to the measured range of concentrations in the exposure water. Since exposure conditions differed between dpf 0 to 10 and dpf 10 to 27, due to the application of smaller exposure beakers and the presence of food and faeces after dpf 10, the exposure concentrations for these two periods were calculated individually.
4.3 Results

4.3.1 Experimentally determined parameters

The average wet weight of a fertilized egg at the day of fertilisation (0 dpf) was 1.4 mg and that of the newly hatched larvae only 0.5 mg (5 dpf; Figure 4.1) due to the shedding of the egg chorion. The newly hatched larvae lost approximately 10% of bodyweight during the yolk-sac stage between 5 to 10 dpf. At 27 dpf, when the start of the eye migration indicated the onset of the metamorphoses in the first fish, the average body weight was approximately 2.3 mg, and the body length around 10 mm. The lipid fraction of the total fertilized eggs was 1.6%, in the larvae after leaving the eggshell this was 2.0%. At the end of the yolk-sac stage at 10 dpf, the lipid content in the larvae reached the lowest level (1.1%). At the onset of metamorphoses, 27 dpf, the average lipid content of the whole larvae had increased to 3.8%. Figure 4.1 shows the development of the wet weight and the lipid fraction of the sole larvae from 0 to 30 dpf.

![Figure 4.1 Development of body weight and lipid content during the first 30 d after fertilization (dpf) of the sole eggs. The markers represent the measured data, and the lines represent the estimated development in time that was applied for the model calculation. The measured data at 0 dpf include the egg’s chorion. For the model calculation the weight and lipid content of the embryo was set similar to that at the moment of hatching (5 dpf). The vertical lines separate the main developmental stages: E = egg stage, Y = yolk-sac larvae; F = free-feeding larvae and M = onset of metamorphose.](image-url)
Table 4.3 Average (+/- std) wet weight and lipid content of individual sole larvae and the measured PCB concentrations (n=2) in sole tissue sampled 0, 5, 10 and 23 days post fertilization (dpf) and in the spiked Artemia that was used for feeding from dpf 10 on.

<table>
<thead>
<tr>
<th></th>
<th>Wet weight</th>
<th>Lipid</th>
<th>PCB 52</th>
<th>PCB 110</th>
<th>PCB 101</th>
<th>PCB 118</th>
<th>PCB 138</th>
<th>PCB 153</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>%</td>
<td>ng/g WW</td>
<td>ng/g WW</td>
<td>ng/g WW</td>
<td>ng/g WW</td>
<td>ng/g WW</td>
<td>ng/g WW</td>
</tr>
<tr>
<td>Dpf 0</td>
<td>1.37±0.16</td>
<td>1.55</td>
<td>&lt;3.3</td>
<td>&lt;3.3</td>
<td>&lt;3.3</td>
<td>&lt;3.3</td>
<td>&lt;3.3</td>
<td>&lt;3.3</td>
</tr>
<tr>
<td>Dpf 5</td>
<td>0.52±0.11</td>
<td>2.00</td>
<td>53.7/67.9</td>
<td>78.2/98.8</td>
<td>81.6/105</td>
<td>94.1/112</td>
<td>55.2/69.9</td>
<td>49.7/64.7</td>
</tr>
<tr>
<td>Dpf 10</td>
<td>0.47±0.11</td>
<td>1.08</td>
<td>232/235</td>
<td>427/434</td>
<td>429/435</td>
<td>438/468</td>
<td>262/262</td>
<td>220/221</td>
</tr>
<tr>
<td>Dpf 27</td>
<td>2.26±0.35</td>
<td>3.8</td>
<td>144/144</td>
<td>298/306</td>
<td>328/337</td>
<td>358/370</td>
<td>258/274</td>
<td>207/221</td>
</tr>
<tr>
<td>Artemia</td>
<td>0.87</td>
<td>5.4/7.4</td>
<td>17.3/23.2</td>
<td>13.2/17.8</td>
<td>23.0/26.7</td>
<td>18.2/21.7</td>
<td>14.1/16.6</td>
<td></td>
</tr>
</tbody>
</table>

The measured PCB concentrations in the freshly prepared exposure solution ranged between 13.8 ng/L (PCB 153) and 23.8 ng/L (PCB 101), which was very close to the nominal water concentrations (Table 4.2). In the samples that were collected just before the next water replacement, the PCB concentrations were substantially lower, especially for the PCBs with a lower Kow. During the period 0 to 10 dpf, the concentration before water replacement was between 42% (PCB 52) and 72% (PCB 153) of the initial concentration as measured in the freshly prepared exposure solution. For the period 10 to 27 dpf, dissipation was faster resulting in concentrations before the next water replacement between 18% (PCB 52) and 44% (PCB 153) of the initial exposure concentration.

At the start of the exposure and in all samples from the control group, PCB concentrations in tissue were below the level of quantification (3 ng/g ww). Immediately after hatching (5 dpf) the PCB concentrations in larvae from the exposed group ranged from 50 (PCB 153) to 112 ng/g ww (PCB 118) (Table 4.3). During the next 5 d (yolk-sac stage), the strongest increase in body residue was observed, ranging from 220 ng/g ww (PCB 153) and 468 ng/g ww (PCB 118). At the end of the experiment, at 27 dpf, the tissue concentrations of the most lipophilic PCBs 138 and 153 were comparable to those of 10 dpf. For the other PCBs, the tissue concentrations had decreased compared to 10 dpf (Table 4.3).

The tissue concentrations of all seven PCBs showed a high similarity between replicate treatments: at 5 dpf, the maximum difference was 23%, at 10 and 27 dpf this was 6 and 7% respectively.

The PCB concentrations in the Artemia-naupllii that were used as food source for the exposed larvae, ranged from 6 to 24 ng/g ww (PCBs 52 and 118 respectively) with a lipid fraction of 0.87%.
4.3.2 Input parameters for the model

As the model aims at estimating the PCB levels in the developing embryo, the weight and lipid fraction during the egg stage were set equal to the values of the freshly hatched larvae at 5 dpf. Thus, it is assumed that the observed differences in weight and lipid content between the egg at 0 dpf and the freshly hatched embryo at 5 dpf were due to the loss of the chorion alone and not the result of changes of the embryo. Bioamplification of the PCBs during the egg stage (Daley et al., 2009) was therefore not covered in our calculations. The weight loss during the yolk-sac stage (5 to 10 dpf) is described by a negative biomass production coefficient ($\gamma_2$) of $-0.006$ kg$^\omega$/d. The rapidly increasing body mass after 10 dpf corresponds to $\gamma_2 = 0.002985$ kg$^\omega$/d.

The change in lipid fraction between 5 and 10 dpf when the yolk-sac is absorbed, is described as a constant decline of $-0.18%$/d. Between 10 and 27 dpf, the lipid content in the developing larvae builds up from 1.1% to 3.8%. This development is described by an exponential association with maximum value of 3.8%, assuming that the lipid content does not change after 27 dpf. For practical mathematical reasons, this development was described in the ELS-OMEGA model in four periods in which the lipid fraction increased with a constant factor: 10 to 15 dpf: +0.36%$/d; 15 to 20 dpf: +0.12%$/d; 20 to 27 dpf: +0.04%$/d; and >27 dpf: 0%$/d.

For the calculation of the egg stage $\gamma_0$, the uptake of PCB 118 during egg-stage and yolk-sac stage, both covering a 5 day period, were compared. During egg stage the uptake was 27% and 31% of that during the yolk-sac stage, for the duplicated treatments respectively. With $\gamma_0$ after hatching set at the default value of 200 kg$^\omega$/d (Hendriks et al., 2001), this corresponds to an egg stage $\gamma_0$ of 34 and 40 kg$^\omega$/d respectively (see Figure S4.1 in Annex to Chapter 4). The difference in the model outcomes calculated with egg stage $\gamma_0$ of 34 or 40 kg$^\omega$/d was less than 5%. Therefore, it was decided to use the average value of 37 kg$^\omega$/d as egg stage $\gamma_0$ in the ELS-OMEGA model.

All input values (and their origin) that were used in the ELS-OMEGA model are presented in Table 4.4.
Table 4.4 Input data used of the ELS-OMEGA model to fit the experimental data. Underlined values refer to default values as proposed in Hendriks et al., 2001.

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Symbol</th>
<th>Unit</th>
<th>Egg</th>
<th>Yolk sac</th>
<th>Free-feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td></td>
<td></td>
<td>0-5</td>
<td>5-10</td>
<td>10-27</td>
</tr>
<tr>
<td>octanol water partition ratio</td>
<td>$K_{ow}$</td>
<td>-</td>
<td>See Table 4.2</td>
<td>See Table 4.2</td>
<td>See Table 4.2</td>
</tr>
<tr>
<td>species weight</td>
<td>$W$</td>
<td>kg</td>
<td>$0.52E^6$</td>
<td>$0.52-0.47E^6$</td>
<td>$0.47-2.26E^6$</td>
</tr>
<tr>
<td>lipid fraction of organism</td>
<td>Pch2</td>
<td>-</td>
<td>0.02</td>
<td>0.02-0.011</td>
<td>0.011-0.038</td>
</tr>
<tr>
<td>lipid fraction of food</td>
<td>Pch2,i-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0087</td>
</tr>
<tr>
<td>concentration in organism</td>
<td>Ci</td>
<td>mg/kg</td>
<td>See Table 4.3</td>
<td>See Table 4.3</td>
<td>See Table 4.3</td>
</tr>
<tr>
<td>concentration in food</td>
<td>Ci-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>concentration in water</td>
<td>$C_{0,w}$</td>
<td>mg/l</td>
<td>estimated</td>
<td>estimated</td>
<td>estimated</td>
</tr>
<tr>
<td>biomass production coefficient</td>
<td>$y_2$</td>
<td>kg/d</td>
<td>0</td>
<td>$-0.0060$</td>
<td>0.002985</td>
</tr>
<tr>
<td>mortality rate constant</td>
<td>$k_4$</td>
<td>/d</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>food ingestion coefficient</td>
<td>$\gamma_1$</td>
<td>kg/d</td>
<td>0</td>
<td>0</td>
<td>0.005</td>
</tr>
<tr>
<td>fraction of ingested food assimilated</td>
<td>$P_1$</td>
<td>kg/kg</td>
<td>-</td>
<td>-</td>
<td>0.8</td>
</tr>
<tr>
<td>water absorption-excretion coefficient</td>
<td>$\gamma_0$</td>
<td>kg/d</td>
<td>37</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>respiration coefficient</td>
<td>$y_3$</td>
<td>kg/d</td>
<td>0.0024</td>
<td>0.0024</td>
<td>0.0024</td>
</tr>
<tr>
<td>rate exponent</td>
<td>K</td>
<td>-</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>temperature correction factor</td>
<td>$\rho T:C$</td>
<td>kg/kg</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>water layer diffusion resistance</td>
<td>$\phi_{h2o,0}$</td>
<td>d/kg</td>
<td>0.00280</td>
<td>0.00280</td>
<td>0.00280</td>
</tr>
<tr>
<td>water layer diffusion resistance</td>
<td>$\phi_{h2o,1}$</td>
<td>d/kg</td>
<td>0.00001</td>
<td>0.00001</td>
<td>0.00001</td>
</tr>
<tr>
<td>lipid layer permeation resistance</td>
<td>$\phi_{ch2,i}$</td>
<td>d/kg</td>
<td>68</td>
<td>68</td>
<td>68</td>
</tr>
</tbody>
</table>

4.3.3 Modelled and measured PCB concentrations

The PCB water concentrations for the period 10 to 27 dpf that were calculated by the ELS-OMEGA model based on the measured concentrations in the larvae were within the chemically analysed range PCB-water concentrations (Figure 4.2). Interestingly, the predicted water concentration of the PCB with the lowest $K_{ow}$ (PCB 52) was on the upper side of the measured range and the more hydrophobic PCBs 138 and 153 were on the lower side. This relation with $K_{ow}$ was stronger for the exposure period 0 to 10 dpf. Here, the predicted exposure concentrations for the moderately hydrophobic PCBs 101, 110, and 118 were well within the range determined by the chemical analyses of the water samples. The predicted concentration of the more hydrophobic PCBs 138 and 153 only were 85% and 89% of the lowest measured concentration, whereas for the least hydrophobic PCB 52, the estimated exposure concentration exceeded the maximum measured concentration with 48%.
Figure 4.2 shows the development of the PCB concentrations in the tissue of the developing fish as calculated by the ELS-OMEGA model, based on the measured tissue concentrations. The PCB-uptake during the egg stage (0 to 5 dpf) is limited for the embryo that is protected by the chorion. After hatching, the larvae have a higher surface/volume ratio, which facilitates faster uptake of the PCBs from the water. This results in a maximum tissue concentration reached at 10 dpf. Once the fish starts to feed externally, it increases rapidly in weight, and growth dilution plays an important role in the further development of the tissue concentration. The model calculates that the PCB tissue concentration reaches an equilibrium around 25 dpf. The levels of the less lipophilic PCBs decrease more relative to the concentration at 10 dpf (on a w/w-basis) than the levels of the more lipophilic PCBs. For PCB 52, this is 72% of the concentration at 10 dpf and for PCB 153 this is 99%.

When expressed on lipid base, the PCB concentration increase during the yolk-sac stage (5-10 dpf) even is steeper as the result of the depletion of lipid reserves. After 10 dpf, when the fish starts feeding, the lipid content builds up again, so the lipid normalized concentrations decline fast. Around 15 dpf, when in the model the final lipid fraction is approached, the lipid normalized PCB concentration stabilizes at a concentration that is about four times lower than the maximum concentration at 10 dpf.
Figure 4.3 With ELS-OMEGA predicted (lines) and measured (markers) tissue PCB concentrations in developing early life stages of Sole. Solid markers/lines refer to concentrations on wet weight basis, open markers/dotted lines refer to concentrations on lipid basis. The vertical lines separate the main developmental stages: E = egg stage, Y = yolk-sac larvae; F = free-feeding larvae.
4.4 Discussion

The OMEGA model for bioaccumulation of POPs in adult fish, was refined to provide an ELS-OMEGA model for calculation of the POP tissue concentrations during the development of fish eggs, via yolk-sac larvae into a free-feeding juvenile. Application of this model and validation with PCB-exposed sole eggs and larvae revealed that the internal levels rise to reach a peak at the end of the yolk-sac stage. Below the developed ELS-OMEGA model is discussed and subsequently a number of different realistic exposure scenarios are modelled and discussed.

4.4.1 Model validation

Validation of the ELS-OMEGA model was performed by comparing the external exposure concentrations that were calculated based on measured tissue concentrations with measured external exposure concentrations. This sequence is opposite to the more standard approach in bioaccumulation modelling where water- and/or food concentrations are used to try to predict the tissue concentrations. We choose this approach being aware of the fact that it is impossible to maintain a stable exposure concentration of PCBs in semi-static tests (Breitholtz et al., 2006; Rufli et al., 1998) due to exponential dissipation of the PCBs from the water column with time (Koponen et al., 1998; Breitholtz and Wollenberger, 2003).

The measured tissue concentrations were very similar between the independent replicates (Table 4.3) and can thus be considered to give a more reliable representation than the measured water concentrations that indicated strong dissipation between refreshments. Most of the predicted water concentrations fell within, or were close to the measured ranges of the water concentrations (Figure 4.6). An exception was the less lipophilic PCB 52 water concentration during the egg and yolk-sac stage (dpf 0 – 10) that was predicted twice as high as what was measured. This could indicate that the uptake of this less lipophilic PCB during these early stages is more efficient than assumed by the ELS-OMEGA model especially during the yolk-sac stage, since the tissue concentration at the moment of hatching seems overestimated.

4.4.2 Model parameter values and sensitivity

In the ELS-OMEGA model, the parameter values applied for respiration coefficient, rate exponent, water layer diffusion resistance, and lipid layer permeation resistance were for all developmental stages kept constant at the default values proposed by Hendriks (Hendriks et al., 2001). Although one could
argue if some of these values might change during the early life development, the impact of these parameters on the model outcome was limited (no more than 5% difference) as long as they were maintained within the ranges given by Hendriks et al. (2001).

Based on the experimental data for PCB 118, the water exchange rate during the egg stage, expressed as the water absorption-excretion coefficient $\gamma_0$ in the model, was calculated to be 17-20% (34-40 kg$^\text{w}$/d) of the default value that was applied after hatching (200 kg$^\text{w}$/d) (Hendriks et al., 2001). This relative difference between egg and larval stage is in accordance with observations with zebra fish early life stages, where uptake rate constants for PAHs with log $K_{ow}$ 4.5 to 6.1 during the egg stage were 16 to 20% of those after hatching (Lu et al., 2011). The impact, however, of the egg stage $\gamma_0$ on the outcome of the ELS-OMEGA model for sole is limited, as it only affects a short, 5 d period.

The model outcome is mostly depending on (changes in) the lipid content of the organisms. The lipid content we found for the freshly hatched larvae (2% of wet weight) is in accordance with the 1.9% (20.3% of dry weight) reported for the closely related species *Solea senegalesis* (Mourente et al., 1996). In the same study the total lipid fraction during the yolk-sac stage was reduced with 33%, while in our study this was 45%. Our juvenile sole consisted of 3.8% lipid at 27 dpf. As this value is on the high side of the 1% to 3.5% that is presented in literature for adult sole muscle tissue (Baeyens et al., 2003 and Bordajandi et al., 2006 respectively), we assume in the model that our 3.8% is representative for a fully developed sole. This is somewhat higher than the lipid content of three total fish samples composed of juvenile sole from the Western Scheldt estuary (2.2, 2.9 and 3.3%, average 2.8%; unpublished data IMARES, Netherlands, 2005, 2006), which could be related to the life in captivity without food limitation.

The exposure scenarios presented below, were also performed with alternative lipid dynamics (egg 1.9%; end yolk-sac 1.3%; juvenile 2.8%; Figure 4.4) based on the data presented above. This does not fundamentally change the outcome, but shows how the relative height of the exposure peak and the relative depletion of the lipid are related. Results are presented as Figures S4.2 and S4.3 in Annex to Chapter 4.
4.4.3 Exposure scenarios

The peak in PCB tissue concentrations at the end of the yolk-sac stage, shortly before the onset of growth dilution because of external feeding, is most pronounced when lipid normalized. Which from a toxicological perspective is most relevant for lipophilic bioaccumulating toxic pollutants (Murk et al., 1998). The lipid normalized peak occurred with all PCBs tested with a relative height that correlated positively with the $K_{ow}$. This indicates that the peak will be absent with less lipophilic substances. The ELS-OMEGA model was applied to study the relative peak height reached for three hypothetical POPs with a log $K_{ow}$ value of 5, 6 or 7, according to three exposure scenarios that are relevant in practice: a fish ELS test scenario with uncontaminated eggs and continuous waterborne exposure, a steady state field situation where the full life cycle takes place in the same environment, and a field situation reflecting migration to a pristine environment where the maternally exposed eggs are spawned and develop. The first scenario is representative for ELS tests with water borne exposure (OECD, 1998, 1992, 2006; EPA, 1996), and the first 10 days reflects tests that are terminated at the end of the yolk-sac stage (OECD 1998, 2006).
For all scenario calculations, the ELS-OMEGA model parameter settings were applied as presented in Table 4.4. In order to facilitate the comparison between the POPs with different log $K_{ow}$ the calculated tissue concentrations are expressed relative to the lipid based equilibrium concentration that is reached at 50 dpf.

The ELS test scenario (Figure 4.5) reveals a much faster uptake in the egg phase and elimination during the larval phase of substances with log $K_{ow}$ value of 5, compared to log $K_{ow}$ value of 7, even preventing concentration in the depleting lipids during the yolk-sac stage. Therefore, in ELS tests a peak in lipid normalized POP concentrations at the end of the yolk-sac stage is only to be expected with substances with log $K_{ow} > 5$. In the sole ELS test this peak concentration of compounds with Log $K_{ow}$ of 6 or 7 can be 2 to 3 times higher than the (adult) equilibrium tissue concentration (Figure 4.5). Also in the steady state field scenario, POPs with log $K_{ow}$ of 5 do not develop a lipid normalized concentration peak (Figure 4.6) while more lipophilic compounds do develop a peak of maximally 2.5 and 4.5 times the adult equilibrium level. When using the field-based lipid dynamics the relative peak height would be 1.9 and 2.9. As the model uncertainty was not quantified in the present study, the log $K_{ow}$ value at which POP concentrations start to peak could not statistically significant be determined.

Figure 4.5 Predicted lipid normalised tissue concentrations in developing fish (sole) larvae of POPs with log $K_{ow}$ values of 5, 6 and 7 during the first 50 d post fertilization under continuous water borne exposure (ELS-test scenario). The vertical lines separate the main developmental stages: $E =$ egg stage, $Y =$ yolk-sac larvae; $F =$ free-feeding larvae.

ELS test scenario

- log Kow = 7
- log Kow = 6
- log Kow = 5
In the scenario where the mother fish migrates to a spawning area with pristine water and food, a concentration peak before free-feeding is only formed for the most lipophilic POPs with log $K_{ow}$ of 6.5 or more (Figure 4.6). POPs with log $K_{ow}$ up to 6 are almost completely eliminated at the end of the yolk-sac stage.

4.4.4 Implications for ecotoxicological risk assessment and research

Our data confirm the observations of other researchers (Villalobos et al., 2000, Gonzales-Doncel, 2003) that the exposure to water borne substances of a fish embryo inside the egg is limited compared to the post hatch situation. In toxicity tests in which eggs are exposed via the water, this results in a serious underestimation of the exposure of the developing embryo during the egg stage. The possible toxic effects on this development actually are not fully tested in such an ELS assay. In addition to exposing the mother animal, this could be overcome by exposure via nano-injection of the egg (Villalobos et al., 2000a; Nassef et al., 2010) or dechorination of the egg prior to exposure (Villalobos et al., 2000b). In any case, the lipid normalized tissue concentration of lipophilic pollutants (Log $K_{ow} > 5$) will peak at the end of the yolk-sac stage. This could induce a toxicity peak in prolonged ELS fish tests at or just after the beginning of the free-feeding stage as was observed in Chapter 2 (Foekema et al., 2008). This critical moment only is covered in tests that are continued at least a few days after the fish are free-feeding (OECD, 1992; EPA, 1996). However, for.

Figure 4.6 Predicted tissue concentrations in developing fish (sole) larvae of POPs with log $K_{ow}$ values of 5, 6 and 7 during the first 50 d post fertilization given two field relevant scenarios. Steady state scenario: the full life cycle takes place under constant exposure conditions. Migration scenario: development of maternally exposed eggs takes place in a pristine environment. The vertical lines separate the main developmental stages: $E =$ egg stage, $Y =$ yolk-sac larvae; $F =$ free-feeding larvae. Concentrations on wet weight are included in supplementary data Figure C.
practical and ethical/legal reasons, the majority of ELS fish tests are terminated (just) before the end of the yolk-sac stage (OECD 1998, 2006). Our data indicate that this is valid for testing substances with log $K_{ow} < 5$. But when more hydrophobic substances or (mixtures of) unknown substances as present in effluents or sediments (Hollert et al., 2003; Kosmehl et al., 2006), are being tested, the effects of lipophilic substances will be seriously underestimated.

Our scenarios reveal that maternally transferred high lipophilic POPs ($\log K_{ow} > 6.5$) are hardly excreted, and reach peak concentrations at the end of the yolk-sac stage, the critical moment when the larvae has to switch to external feeding. Current levels of dioxin-like and other teratogenic pollutants may therefore cause serious adverse effects on developing fish at realistic maternal concentrations (Foekema et al., 2008). The amount of maternally transferred POPs to the eggs can be estimated based on the lipid normalized maternal concentrations (Russel et al., 1999). By applying the ELS-OMEGA model the peak exposure concentration for the larvae can be calculated for species specific lipid dynamics and local (exposure) circumstances. Our data indicate that the lipid normalized peak concentration of substances with $\log K_{ow} > 5$ at the end of the yolk-sac stage may exceed 2 to 4 times the concentration in the spawning parent fish. The latter concentration could also be greatly enhanced in species with reproductive migration without feeding like salmon and eel. The greatly increased (lipid normalized) POP concentrations in the maternal tissue and eggs (Debruyn et al., 2004; Van Ginneken et al., 2009; Kelly et al., 2011) in combination with the further concentration in the developing larvae as shown in the present study, could result in extreme body burdens during critical stages of larval development with serious consequences for larval survival, even when eggs and larvae can develop in pristine areas.

4.5 References


Bordajandi, L. R., Martin, I., Abad, E., Rivera, J., Gonzalez, M. J. 2006. Organochlorine compounds (PCBs, PCDDs and PCDFs) in seafish and seafood from the Spanish Atlantic southwest coast. Chemosphere, 64, (9), 1450-1457.


Figure S4.1: Influence of the water absorption excretion coefficient (Y0) on the uptake of PCB 118 in the egg relative to that of the yolk sac larvae with Y0=200 Kg/d. Measured PCB 118 concentrations in eggs was 29% (27 and 31% respectively) of that in the larvae. This corresponds with a Y0 of 37 Kg/d during egg stage. This value was used for all calculations in the ELS-OMEGA model.
Figure S4.2: Development of tissue POP concentrations with field-based lipid dynamics following the ELS test scenario. The modelling results with experimental lipid dynamics is presented as figure 4.5 in Chapter 4.

Figure S4.3: Development of tissue POP concentrations with field-based lipid dynamics (see fig S2) following the Steady state or Migration field scenarios. The modelling results with experimental lipid dynamics is presented as figure 4.6 in Chapter 4.
CHAPTER 5

The risks of maternally transferred organic substances for fish early life development

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Abstract

The present study investigates the likelihood that early life development of fish from contaminated areas is affected by maternally transferred persistent organic substances (POPs). The common sole (Solea solea) was used as model species and maternal transfer was mimicked by exposing fertilised eggs via the water until hatching. The newly hatched larvae were allowed to develop further under unexposed conditions until the end of the metamorphosis. Effects on the larvae were determined for the dioxin-like PCB 126, the technical PCB-mixture Aroclor 1254, PBDEs and HBCDs, for an artificial mixture of PCBs and PBDEs, and for ‘field mixtures’ extracted from sole from the North Sea and the contaminated Western Scheldt estuary. Effect levels were expressed as maximum tissue concentrations in the newly hatched larvae at the end of the exposure period. Exposure to PCBs, PBDEs and the artificial and field mixtures caused mortality that started to occur shortly after the larvae became free-feeding (10 days post fertilization) and continued to increase until the onset of metamorphoses, 15 days later. The effects induced by the field mixtures correlated well with the ΣPCB concentrations in the tissue of the exposed larvae. No indications were found for synergistic effects or for substantial contribution of other (unknown) substances in the field mixtures. HBCD did not induce toxic effects. POP levels in sole from the Western Scheldt estuary are about 20 times lower than the larval tissue concentration that produced 50% early life stage mortality. Levels in North Sea sole are an order of a magnitude lower. At more heavily contaminated sites negative effect of PCBs, especially of those with dioxin-like toxicity can be expected.
5.1 Introduction

Although bulk production and use has been stopped for persistent organic pollutants (POPs), compounds like polychlorinated biphenyls (PCBs), brominated flame retardants (polybrominated diphenyl ethers PBDEs, hexabromocyclododecane HBCDs), and chlorinated pesticides (dichloro-diphenyl-trichloroethane DDT, Lindane, Dieldrin, etc) still are present in the environment (de Boer et al., 2010; Maes et al., 2008; Voorspoels et al., 2004a). As a result of the low water solubility/high lipophilicity in combination with high persistence these substances concentrate in sediments and accumulate in biota, including fish. During oogenesis, POPs that were accumulated in female lipid tissue are transferred to the eggs (Miller, 1993; Monteverdi and Di Giulio, 2000; Nyholm et al., 2008; Serrano et al., 2008), and can cause negative effects on the developing off-spring after fertilisation (Tietge et al., 1998; Olsson et al., 1999; Nakayama et al., 2005; Ishibashi et al., 2006).

Due to a homogeneous distribution of the POPs over the lipids in the female tissue, the lipid normalised concentration in the eggs will be comparable to the mothers tissue (Russell et al., 1999). Thus, the POP concentration in the tissue of the mother fish represents the minimum toxic pressure for the developing offspring. For lipophilic substances (log K_{ow}>5), the lipid normalised concentration will further increase in the developing larvae when the lipids stored in the yolk are depleted. This results in an exposure peak to these substances at the end of the yolk-sac stage that is the highest to be expected during the life time of the fish (Chapter 4; Foekema et al., 2012). Compared to fully developed fish, larvae are relatively sensitive to toxicants (Hutchinson et al., 1998) as a consequence of the critical development of organs and tissues during this life phase.

In an earlier study we determined that in a prolonged Early Life Stage (pELS) test with the common sole (Solea solea) the effect concentrations of dioxin-like compounds in the larvae were in the same order of magnitude as levels found in tissue of fish from highly contaminated areas. This implies that in these areas maternal transfer of such compounds can negatively affect the larval development (Foekema et al., 2008). In addition to dioxin-like compounds also other POPs are found in fish from polluted field locations, and the present paper assesses the effect of well-known field-relevant lipophilic POPs (PCBs, PBDEs, and HBCDs) that will be maternally transferred, on early life development of Solea solea. These POPs have, among others, been identified as potential endocrine disrupters (Zimmer et al., 2011), including the thyroid hormone system (Brouwer et al., 1999; Noyes et al., 2011; Palace et al., 2010; Schriks et
which plays an essential role in early development and (flat)fish metamorphosis (Klaren et al., 2008).

The common sole (Solea solea) was chosen as model species for this study as it is available from aquaculture which ensures almost year round availability of fertilized eggs. Also, sole is a common marine species along the North Sea coast, that uses the contaminated Western Scheldt estuary (Baeyens et al., 2003; De Vijver et al., 2003; Roosens et al., 2008; Voorspoels et al., 2004a; Voorspoels et al., 2004b; Voorspoels et al., 2003) as nursery area and feeding ground until first reproduction (Rijnsdorp et al., 1992). Further advantage of using sole is that, as a flatfish, it undergoes an obvious thyroid hormone mediated metamorphosis (Klaren et al., 2008) and thus is potentially sensitive to thyroid hormone disruption as has been described for other species (Noyes et al., 2011; Palace et al., 2010; Schriks et al., 2006; Yu et al., 2011; Gutleb et al., 1999). Last but not least, an optimized protocol is available for testing the impact of substances on the full development from a fertilized egg into a metamorphosed flat fish (Foekema et al., 2008).

In this study first the effect concentrations are determined for the selected POPs PCBs, PBDEs and HBCD, and secondly, a test with an artificial mixture of PCBs and PBDEs is performed in order to determine possible mixture effects. Finally, sole eggs are exposed to two ‘field mixtures’ of organic lipophilic pollutants extracted from tissue of sole from the North Sea and the Western Scheldt estuary. Invertebrates and fish from the Western Scheldt estuary are known to be contaminated with PCBs and brominated flame retardants (Baeyens et al., 2003; De Vijver et al., 2003; Roosens et al., 2008; Voorspoels et al., 2004a; Voorspoels et al., 2004b; Voorspoels et al., 2003) but also with other substances as perfluorooctane sulfonic acid (PFOS; De Vijver et al., 2003) and organo-chlorine pesticides (OCPs; Voorspoels et al., 2004a). The comparison of the effects of the field mixtures with those induced by the artificial mixture, composed of only PCBs and PBDEs, indicates to what extent other (unknown) substances present in the cocktail of POPs extracted from the fish contribute to an effect on the developing larvae.

In summary, the present study aims to assess the likelihood that maternally transferred POPs negatively affect early life development of fish from contaminated areas with special focus on PCBs, HBCD and PBDEs, and (other) POPs present in sole from the Western Scheldt estuary.
5.2 Materials and methods

5.2.1 Test organisms and seawater

Fertilized Sole eggs were produced at IMARES (location IJmuiden, the Netherlands) where a group of mature male and female soles is kept together in large spawning tanks. Spawning and fertilization was temperature-induced and took place overnight at water temperatures around 12 ºC. The following morning, the fertilized eggs were transported in plastic bags with oxygen saturated seawater to the IMARES laboratory in Den Helder, the Netherlands, were the tests were performed. Together with the eggs, artificial seawater (Instant Ocean Aquarium Salt in demineralized water, salinity ~35‰) from the spawning tank was transported to the laboratory. Here, the water was stored in a container were it was continuously aerated and circulated through a particle filter and UV-disinfection system. This water was used for the start of the tests. During the test the artificial seawater was gradually replaced with natural seawater (salinity ~32 ‰) collected from the Eastern Scheldt, a relatively pristine bay of the North Sea, often used as a reference site in marine ecotoxicological research in the Netherlands (e.g. Kuiper et al., 2007; Foekema et al., 2008; Foekema et al., 2012).

For the tests presented in this paper three batches of eggs were used, one for the tests with the PCB mixture Arochlor 1254, PCB 126 and HBCD, and two others for testing the PBDE and the artificial and field mixtures.

5.2.2 Sole ELS test

The sole prolonged early life stage (pELS) test was performed according to Foekema et al., 2008, with some slight modifications to improve test performance or to simplify the method.

Immediately after delivery at the laboratory, fertilized eggs were transferred to 5 L glass beakers and placed in a temperature controlled (15°C) room. The beakers were left untouched for about one hour after which non-fertilized/dead eggs had sunk to the bottom of the beaker. Exposure started approximately 12 hours after fertilization by transferring 500 fertilized eggs in approximately 50-100 ml seawater to the glass ‘exposure beakers’ containing 2 L seawater with the desired exposure concentration.

The exposure beakers were covered with individual glass lids and kept in a temperature controlled cabinet at 14.5 ±0.5°C, with a 16 hour photoperiod (ca. 100 LUX). During the following 5 days, about 60-75% of the water volume was replaced every other day with freshly spiked seawater. Dead eggs were removed
daily. The first larvae hatched around 5 days post fertilization (dpf). At 6 dpf, from each exposure group, two times 15 larvae were randomly selected and transferred to two smaller glass beakers containing 80 ml of clean seawater. This was done by means of a polyethylene pipette, with as little exposure water as possible. The larvae were then allowed to develop further under similar, yet unexposed conditions. Every other day, approximately 75% of the water volume in each glass beaker was replaced with fresh seawater, and almost daily, feces, surplus food items, and where appropriate dead larvae were removed with a polyethylene pipette. From 9 dpf onwards, when pigmentation of the eyes indicates that the larvae are about to start external feeding, they were fed ad libitum with newly hatched nauplli of *Artemia salina* on a daily basis.

The development of the fish was recorded following the stages described for the development from fertilized egg to a fully metamorphosed summer flounder (*Paralichthys dentatus*) (Martinez and Bolker, 2003). At the given test conditions sole metamorphosis (stage G) starts around 25 dpf, and is completed (stage I) around 40 dpf. Fish that completed metamorphoses were taken out of the test, narcotized and subsequently killed in a strong MS222-solution (500 mg of ethyl 3-aminonbenzoate methanesulfonic acid salt, purity 98%, ACROS Organics, and 200 mg NaHCO₃, purity 99.0%, Fluka Chemika, in 1 liter seawater). Moment of completed metamorphosis, body length, and morphological deviations, if any, were recorded.

All experiments were approved by the Animal Experimentation Board of Wageningen UR.

5.2.3 Chemicals, stock solutions and exposure concentrations

The technical PCB mixture Arochlor 1254 (Monsanto, USA) was used to prepare the stock solutions for the treatment that will be further referred to as ARO. The contribution of the different PCB congeners in this mixture was chemically analyzed (Table S4.2 in Annex to Chapter 5). The PBDE mixture was composed of standards of five different congeners: BDE 28, 47, 99, 100 and 153 (Accustandard, USA, purity 99.3-100%). The rationale behind the chosen composition (Table S5.2 in Annex to Chapter 5) was that exposure of the eggs will result in internal mixture of the individual congeners with a composition that is comparable to what is found in fish tissue from the Western Scheldt estuary. The HBCD and PCB 126 that were used in this study were obtained from Albermarle and Promochem respectively. Some background information on the substances selected for this study is given in the supplementary data, including the molecular weights and the log $K_{ow}$ values that were used for calculations (Table S5.1 in Annex to Chapter 5).
For each exposure concentration a 1000x concentrated stock solution was prepared in dimethyl sulfoxide (DMSO, purity 99.9% A.C.S. spectrophotometric grade, supplier SIGMA-Aldrich), so that addition of 1 ml of this stock solution in 1 liter seawater produced the appropriate exposure concentration in 0.1% DMSO. In every test series triplicate solvent controls and seawater controls were included.

The stock solution for the test with the artificial mixture was prepared by combining Arochlor-1254, PCB 126 and the PBDE mixture, in such proportions that, based on accumulation kinetics as a function of the octanol-water partition ratio ($K_{ow}$), will result in a composition that is comparable to what is found in tissue of fish from the Western Scheldt estuary.

Tests with PCBs, PBDEs and HBCD were performed twice in duplicate. The exposure concentrations (Table 5.1) were determined based on a range finding tests (data not shown). The field mixtures only were tested once, due to the limited amount of fish extract available.

PBDEs, the artificial mixture (Mix), and the field mixtures were tested in five concentrations, while for arochlor (ARO), PCB 126 (P126) and HBCD only three concentrations could be tested due to the limited number of available eggs.

Table 5.1 Nominal exposure concentrations of PCB 126, ∑PCBs (Arochlor 1254), PBDEs and HBCD that were used in the tests with the individual substances.

<table>
<thead>
<tr>
<th>Code</th>
<th>Unit (µg/L)</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>P126</td>
<td>PCB 126</td>
<td>0.003</td>
<td>0.01</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARO</td>
<td>∑ 25 PCBs</td>
<td>2.1</td>
<td>7</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBDE</td>
<td>∑ 5 PBDEs</td>
<td>0.4</td>
<td>1.1</td>
<td>3.3</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>HBCD</td>
<td>∑ HBCD</td>
<td>25</td>
<td>80</td>
<td>250</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.2.4 Extraction of field mixtures from field samples

Sole from the Western Scheldt estuary was collected by beam trawl fishing at two locations near the places Terneuzen (WS1) and Hansweert (WS2), in the autumn of the year 2006. From each location, about 60 individual Sole with a length between 10 and 20 cm, (1 to 2 years of age) were collected to compose a sample of about 1 kg wet weight (ww). A sample of sole from the North Sea (NS) with a length between 25 and 40 cm was obtained from commercial fishermen. Filets were used to compose a sample of about 3 kg ww. Spawn that was present in female fish from the North Sea sample was collected separately (NS-spawn).
The samples were freeze-dried, ground, mixed with dried Na₂SO₄ and in portions extracted in a Soxhlet extractor with n-pentane and dichloromethane (1:1). The extract was concentrated in a rotary evaporator with iso-octane as a keeper. Lipid was removed on an Al₂O₃ column eluted with n-pentane. For a more detailed method description see De Boer & Hagel, 1994.

A sub sample of all extracts was used for chemical analyses of the concentrations of PCBs, HBCDs, and PBDEs. The residues were evaporated to almost dryness and dissolved in DMSO to be used for exposure in the p-ELS test. Because of the limited volume, the extract of the NS spawn was only used for chemical analyses and was not used in the p-ELS test.

### 5.2.5 Chemical analysis of larvae

All larvae that were not included for observation of the development in the P-ELS test were sampled for chemical analysis at the end of the exposure period (6 dpf). They were transferred with a polyethylene pipette from the exposure beakers to clean seawater to rinse remaining exposure water, and within 15 minutes collected by filtration on a Whatman GF/C filter with known wet (seawater) weight. The filter with the larvae was weighed again and stored at -20°C. To prepare for analysis the filters with the larvae were subsequently extracted three times with 4 ml of a mixture of pentane : dichloromethane (1:1, both picograde LGC standards, Germany) assisted with ultra-sonification. The extracts were combined in a test tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 1 ml injection standard (PCB112, purity >99%, LGC standards; 13C-α, β,γ HBCD (purity >99%, Wellington, >99%), and BDE58 (Wellington, >99%) and transferred to a vial for GC/MS analysis of 25 PCBs, PCB 126, total HBCD and 5 PBDEs using an Agilent 6890 gas chromatograph (GC) coupled to an Agilent 5973 MSD quadropole mass spectrometer (MS) detector (Amstelveen, The Netherlands).

### 5.2.6 Expression of results, calculations and statistical analysis

Since all test substances were very lipophilic (log Kow >5; (Hansen et al., 1999; Li et al., 2008) (see Table S5.2 in Annex to Chapter 5), the analyzed tissue concentrations were expressed on lipid weight (lw) (Murk et al., 1998). Survival and development were plotted against this internal dose to estimate the internal EC50 and LC50 (IEC50 and ILC50), using GraphPad Prism (version 4.03, January 2005).

Based on the IEC50 values for the individual chemical groups the toxic contributions were quantified as Toxic Units (TU = tissue concentration / IEC50) for substances found in the larvae exposed to the artificial and field mixtures.
The total toxicity of the compounds found in the larvae exposed to the mixtures was then expressed as the $\Sigma$TU. Since the TU are calculated as fraction of the IEC50 value 50% effect can be expected at $\Sigma$TU =1. This approach follows the concept of concentration addition and can be regarded as a conservative method to calculate the toxicity of mixtures (Backhaus and Faust, 2012).

5.3 Results

5.3.1 General test performance

Hatching success was normal for cultured sole, about 50% in all test series, and not related to the exposures. After hatching the average survival in the seawater controls in all test series, was 87% (73-100%, n=9) at the end of the test (40 dpf). In the DMSO controls this was slightly higher (average 91%, range 80-100%, n=9) without being significantly different. Around 25 dpf the first fish reached the onset of metamorphoses, and all surviving fish in the controls had completed the metamorphoses without problems at 40 dpf.

Table 5.2 Summary of the test results with PCB 126, Aroclor, PBDEs and HBCD. Presented are the range of tissue concentrations in the larvae from the different exposure groups at the end of the exposure (6 dpf), and characteristics of the internal-dose vs response curves on 40 dpf (IEC50 with 95% confidence intervals and R square).

<table>
<thead>
<tr>
<th></th>
<th>Mortality</th>
<th>Disrupted development</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB 126</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae 6 dpf</td>
<td>4.3-52</td>
<td>4.3-52</td>
</tr>
<tr>
<td>(range µg/g lw)</td>
<td>1.3 (0.20 to 8.36)</td>
<td>&lt;1.3</td>
</tr>
<tr>
<td>IEC50 (µg/g lw)</td>
<td>0.76</td>
<td>No fit</td>
</tr>
<tr>
<td>$R^2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\sum 25$ PCBs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae 6 dpf</td>
<td>320-502</td>
<td>320-502</td>
</tr>
<tr>
<td>(range µg/g lw)</td>
<td>374 (330 to 424)</td>
<td>341 (328 to 356)</td>
</tr>
<tr>
<td>IEC50 (µg/g lw)</td>
<td>0.88</td>
<td>0.98</td>
</tr>
<tr>
<td>$R^2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\sum 5$ PBDEs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae 6 dpf</td>
<td>81 - 1950</td>
<td>81 - 1950</td>
</tr>
<tr>
<td>(range µg/g lw)</td>
<td>1777 (1717 to 1839)</td>
<td>1636 (1570 to 1704)</td>
</tr>
<tr>
<td>IEC50 (µg/g lw)</td>
<td>0.99</td>
<td>0.98</td>
</tr>
<tr>
<td>$R^2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBCD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae 6 dpf</td>
<td>2280-12400</td>
<td>2280-12400</td>
</tr>
<tr>
<td>(range µg/g lw)</td>
<td>&gt;12400</td>
<td>&gt;12400</td>
</tr>
<tr>
<td>IEC50 (µg/g lw)</td>
<td>No fit</td>
<td>No fit</td>
</tr>
<tr>
<td>$R^2$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3.2 Effects of selected POPs

The concentrations of the selected substances in tissue of the larvae at the end of the exposure period (6 dpf), clearly reflected the exposure concentration (Figure 5.1). The ARO (ΣPCB), and PBDE concentrations seemed to approach a plateau at higher exposure levels. Concentrations were below the limit of detection in larvae from non-exposed controls and reached relatively high concentrations at the highest exposure levels: 52, 502, 1950 and 12400 µg/g lw for PCB 126, ΣPCBs (Arochlor), ΣPBDEs and HBCD respectively (Table 5.2).

The larvae that had been exposed to PCB 126, Arochlor, and PBDEs, showed the first indications of increased mortality around 10 dpf, the end of the yolk-sac / start of the free-feeding stage (Figure 5.2, left column). The timing of the increased mortality was comparable for all these substances, clearly dose related, and continued at least until the fish reached the onset of the metamorphoses (25-30 dpf). Only in individual cases mortality occurred after metamorphoses had started. Complete mortality was observed in the highest exposure level of PCB 126 (0.03 µg/L; P126-C3). In the lower exposure levels (0.01 and 0.003 µg PCB 126/L) overall survival was 10 and 25% respectively, and less than half of these survivors completed metamorphoses. At all exposure levels of PCB 126, some larvae developed oedema before dying.

All larvae in the highest exposure level of Arochlor (21 µg/L; ARO-C3) died during the observation period. At the lower exposure levels survival was higher, and in ARO-C1 (2.1 µg/L) 70% of the larvae survived the 40 day test period. The majority (86%) of these larvae successfully completed metamorphoses. In the intermediate ARO-C2 27% of the larvae survived, but only 7% had completed metamorphoses at 40 dpf.

Survival and development of the larvae at exposure levels up to 3.3 µg PBDE/L (PBDE-C1 to C3) was comparable with the controls. In PBDE-C4 (10 µg/L) survival on dpf 40 was 67%, and in PBDE-C5 (30 µg/L) this was 36%. Only in PBDE-C5 some of the surviving larvae were arrested in an early developmental stage, and in total 27% of the hatched larvae developed in normally metamorphosed juveniles within 40 dpf. HBCD did not induce any mortality or adverse effects.
Figure 5.1: Concentrations of POPs in the tissue of exposed larvae plotted against nominal exposure concentrations. Presented concentrations at 6 dpf were based on chemical analyses of the larvae. Concentrations at 10 dpf (the end of the yolk-sac stage), and at 25 dpf (the onset of the metamorphosis) were calculated by the ELS-OMEGA model (Chapter 4) with the 6 dpf data as input. Note that the Y-axis for the HBCD data is on a log scale.

Figure 5.2 (right) shows the internal-dose-response curves for PBDE and Arochlor induced mortality and disrupted development. The effect concentration for mortality (ILC50) for PBDEs was 1777 µg/g lw, and for Arochlor 374 µg ΣPCBs/g lw. Disrupted development was defined as the fraction of the fish that did not develop into a normal juvenile fish, including the fish that died. Differences between IEC50 values for disrupted development and mortality were less than 10% and not statistically significant.

As it was not possible to prepare a full dose response curve for PCB 126 due to high mortality already at the lower exposure levels, the ILC50 of 1.3 µg/g lw that was estimated for this substance (Figure 5.2, Table 5.2) is less reliable.
Figure 5.2 Survival over time (left column) after exposure during 6 days post fertilization (0 – 6 dpf, indicated as grey area) to the substances indicated in the heading of the graphs, and (right column) related internal dose vs response curves on 40 dpf for mortality and disrupted development including mortality. For exposure concentrations see Table 5.1.
5.3.3 POP concentrations in field samples

The POP concentrations in the original fish samples from the two locations in the Western Scheldt estuary were comparable, with average values of 16, 0.13 and 0.15 µg/g lw for PCBs, PBDEs and HBCDs respectively. Levels in the North Sea fish sample were at least 10 times lower (Table 5.3). In all samples PCB 153 and 138, and PBDE 47 and 100 were the dominant substances within their chemical groups. Details about individual congener levels are presented in Table S4.4 in Annex to Chapter 5.

The lipid normalized concentrations of the POPs in the filet sample (composed of male and female fish) and the spawn of the North Sea sole were highly comparable (Figure 5.3).

Table 5.3 Chemically determined concentrations of PCBs, PBDEs and HBCD, in the tissue of Sole that were sampled at two locations in the Western Scheldt estuary (WS1 and WS2), and one sample from the North Sea (NS) that were used to create the field mixtures for exposure tests. The spawn that was present in fish from the NS sample was analyzed separately (NS spawn) and was not used in the exposure tests. For details about individual congeners see supplementary data

<table>
<thead>
<tr>
<th>µg/g lw</th>
<th>WS1</th>
<th>WS2</th>
<th>NS</th>
<th>NS spawn</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB 126</td>
<td>0.0014</td>
<td>0.0004</td>
<td>0.0017</td>
<td>0.0003</td>
</tr>
<tr>
<td>∑ 25 PCBs</td>
<td>13.8</td>
<td>19.2</td>
<td>1.51</td>
<td>1.80</td>
</tr>
<tr>
<td>∑ 5 PBDEs</td>
<td>0.09</td>
<td>0.18</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>HBCD total</td>
<td>0.18</td>
<td>0.13</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Figure 5.3 Relation between lipid normalized concentrations of individual PCBs and PBDEs in fish muscle and spawn in the sole sample from the North Sea (NS).
5.3.4 Effects of mixtures

Chemical analyses confirmed exposure-related tissue concentrations of PCBs and PBDEs in the larvae that were exposed to the artificial and field mixtures (Table 5.4). The highest concentrations were reached in the artificial mixture (Mix) and WS2 treatments. In all larvae the $\sum$PBDE concentration was only 2 to 4% of the $\sum$PCB concentration, and PBDE 47 was the most dominant PBDE in all samples. In the larvae exposed to the field mixtures (NS, WS1 and WS2) the dominant PCBs were PCB 138, 153, 110, and 101, whereas in the tissue of the Mix larvae PCBs 110, 101 and 118 formed the dominant congeners. PCB 126 was only detected in the larvae from the two highest Mix treatments. More details about concentrations of individual congeners can be found in table S5.5 in Annex to Chapter 5. In general, congeners with a high lipophyly (high $K_{ow}$) were present in relative higher concentrations in the exposed larvae than in the mixtures that were used for exposure.

Figure 5.4 Survival over time (left) after exposure during 6 days post fertilization (0 – 6 dpf, indicated as grey area) to the artificial mixture of PCBs and PBDEs (mix) and to field mixtures of POPs extracts from sole tissue the North sea (NS) and two field locations in the Western Scheldt estuary (WS1 and WS2). Cx indicate dilution steps of 3.
For all exposures to mixtures, survival of the developing larvae was clearly dose-related (Figure 5.4). The highest mortality was observed in the highest exposure levels of Mix and WS2. The development of the mortality over time was comparable in all tests; in most cases it started at the end of the yolk sac stage, thus a few days after the exposure was terminated, and continued until the onset of the metamorphoses starting around 25 dpf. Some of the larvae developed oedema before dying.

5.4 Discussion

Egg stage exposure to PCB 126, Arochlor and PBDE followed by unexposed further development resulted in increased mortality of the developing sole after the end of the yolk-sac stage. Although some exposed larvae were arrested in an early developmental stage and/or developed oedema, effect concentrations for mortality and development (including mortality) did not significantly differ. Exposure to high levels of HBCD did not lead to effects. Exposure to dilutions of an artificial mixture of PCBs and PBDEs, and to a field mixture of POPs extracted from sole filet from the Western Scheldt estuary and to a lesser extent the North Sea, induced comparable effects as exposure to PCBs and PBDEs.

5.4.1 General test performance

With the current test protocol about 90% of the larvae transferred to the smaller beakers on 6 dpf developed normally in clean seawater (reference) as well as in the solvent control (0.1% DMSO). This is a significant improvement compared to the first version of this assay where this was only about 70% (Chapter 2; Foekema et al., 2008). The main improvement was achieved by the use of the artificial seawater from the tank where spawning and fertilization took place during the first days of the test, followed by gradual replacement with natural seawater. In the earlier version of the protocol the fertilized eggs were immediately transferred to the natural seawater. Apparently the embryos are sensitive to abrupt changes in water characteristics. Disinfection of the seawater with UV before use further added to a good survival in the control groups.

Hatching success was not affected by any of the treatments in this study. Under the given test conditions sole larvae deplete the (lipid) resources in the yolk around dpf 10, which results in a peak in internal exposure for substances with log $K_{ow}>5$ (Chapter 4; Foekema et al., 2012). Indeed, mortality rates increased beyond this moment, as was earlier reported in a similar experiment with the dioxin-like PCB 126 (Chapter 2; Foekema et al., 2008). The far majority of the surviving larvae completed metamorphoses without observable malformations. For this reason the differences between lethal and non-lethal effect
concentrations were very small. For calculation of the toxic units the more robust mortality data were preferred over the development data.

5.4.2 Exposure conditions and effect concentrations

The aim of the experimental approach was to simulate maternal transfer of bioaccumulated substances, by a short term exposure of the eggs. For that reason the exposure concentrations used in the tests are much higher than water concentrations in even the highest contaminated field situation, and exposure was terminated after hatching. For PBDEs and Arochlor the relation between nominal water concentration and tissue concentration in the exposed larvae was not linear as would be expected assuming equilibrium between external and internal concentrations. The most likely explanation for this is that the short exposure time was not sufficient to reach equilibrium in the higher exposure levels, which is supported by reports that uptake rate during the egg stage is limited (Villalobos et al., 2000; Gonzalez-Doncel et al., 2003).

The reliability of the estimated ILC50 for PCB 126 is low since the lowest tested concentrations already caused 76% mortality. The ILC50 for PCB 126 of 1.3 µg/g lw (95% confidence intervals 0.2-8.4; Table 4.2) that was estimated in this study, is high compared to earlier work in a more or less comparable experiment with PCB 126 where a ILC50 of 1 ng TCDD-TEQ/g lw was determined using an in-vitro reporter gene assay (Foekema et al., 2008). Applying the WHO TCDD-TEF value of 0.1 for PCB 126 (Van den Berg et al., 2006), this TCDD TEQ would correspond to a PCB 126 concentration of 10 ng/g lw, which is below the lower 95%-confidence limit that was calculated in the present study. This suggests that the IE50 value of PCB 126 from the present study is likely to be an underestimation of the real toxicity of PCB 126.

The timing of the observed effects, with mortality starting around the end of the yolk-sac stage (10 dpf) was comparable for all substances and mixtures tested, and comparable to earlier observations (Foekema et al., 2008). Most of the larvae that did not survive died before the onset of the metamorphosis that started around 25 dpf.

At high concentrations, accumulation of organic pollutants in biological membranes can affect cell integrity (vanWezel et al., 1996; Qin et al., 2010; Escher and Hermens, 2002; Escher and Schwarzenbach, 2002). This type of effect is referred to as narcosis or baseline toxicity and can be regarded as the minimum toxicity of a substance. Lethal body burdens of non-polar chemicals without specific toxicity are comparable and additive on molecular basis (vanWezel et al., 1996; Kipka and Di Toro, 2009). When the tissue concentration in the larvae at 6 dpf from our experiments is plotted on a
molecular basis against the observed survival (Figure 5.6 left), the high toxicity of PCB 126, and the low toxicity of HBCD are obvious. The low toxicity of HBCD can be related to the log $K_{ow}$ of 5.6, which is low compared to the other POPs tested. Based on the ELS-OMEGA model (Chapter 4; Foekema et al., 2012) it can be predicted that the HBCD tissue concentration at the end of the yolk-sac stage (10 dpf) will be only 0.3% of what was present at 6 dpf (Figure 5.5). For more lipophilic substances, with log $K_{ow}$ above 6.3, further concentration can be expected in the same period as is illustrated for PCB 126 (log $K_{ow}$ 6.6; Figure 5.5). Thus, at the end of the yolk sac stage, when mortality starts to occur in the other treatments, the fish in the HBCD treatments had almost completely excreted the HBCD. This may explain why in this treatment no effects occurred around that critical period.

Figure 5.5 Predicted concentration of PCB 126 (log $K_{ow}$ 6.6) and HBCD (log $K_{ow}$ 5.6) in the tissue of developing sole in our tests as modeled with the ELS-OMEGA model (Foekema et al., 2012). Time points on the x-axis indicate the moment of hatching/end of exposure (6 dpf), the end of the yolk-sac stage (10 dpf), and the onset of metamorphosis (25 dpf). Tissue concentration is presented relative to the concentration at 6 dpf.
Chapter 5 Eggsposed

Figure 5.6 Survival at 40 dpf plotted against the tissue concentration on molecular basis as measured in newly hatched larvae at 6 dpf (left), against the modeled concentration at 10 dpf, at the end of the yolk sac stage (right).

The tissue concentration at 10 dpf therefore is a better predictor of the mortality than the concentration at 6 dpf. The survival data indeed show a better correlation with the calculated molecular tissue concentrations at 10 dpf (Figure 5.6 right) than with the concentrations at 6 dpf (Figure 5.6 left), mainly due to a shift of the HBCD data. PCB 126 is still more toxic than what would be expected based on narcosis alone, which is explained by its specific dioxin-like activity. When the PCB 126 dataset is excluded for this reason, a dose-response curve can be fitted through the remaining data points for 10 dpf with $R^2=0.34$ and an ILC50 of 1.4 mmol/kg lw (95% confidence interval 0.7 to 2.7). Using the 6 dpf data the correlation is very poor with $R^2=0.01$.

The toxicity of the PBDEs is less than would be expected compared to the other compounds in Figure 5.6 right, probably due to the absence of dioxin-like substances in this mixture that was composed of analytical standards. When PCB 126 and PBDEs are excluded an ILC50 of 0.71 mmol/kg lw (95% confidence interval 0.48-1.1) can be calculated with $R^2=0.63$. This value is low compared to other studies, that were brought together by (Kipka and Di Toro, 2009) and that revealed critical body burdens for different fish species to range from 36 ($Jordinella floridae$) to 300 ($Alburnus alburnus$) mmol/kg lw. This also indicates that the effects we observed were not only due to baseline toxicity, which is likely since from the technical PCB mixture Arochlor, and the field extracts, especially those from the Western Scheldt, dioxin-like toxicity can be expected (Sanctorum et al., 2011; Sanctorum et al., 2007). However, also for the PBDEs, without dioxin-like toxicity, the critical body burdens were low (>60% mortality at 4 mmol/kg lw) compared to other fish studies reviewed by Kipka and Di Toro (2009). These studies however, were based on experiments with fully developed
fish, thus without covering the critical period at the end of the yolk-sac stage as in our tests.

As baseline toxicity is the result of accumulation of substances in the biological membranes, the observed effects are actually related to the concentration of these substances in the relevant lipids in these membranes (Escher and Hermens, 2002; Escher and Schwarzenbach, 2002). Body burdens are often (also in our study) expressed on total lipids, but to relate the sensitivity of organisms the amount of membrane (polar) lipids compared to neutral lipids should be known. Neutral lipids mainly serve as energy storage pool which is being used after hatching in response of increased energy demand (Kamler, 2008). Upon lipid mobilization neutral lipids were depleted 3 times faster than polar lipids in larvae of the Senegal Sole (Solea senegalensis) (Mourente and Vazquez, 1996). Hence, at the end of the yolk-sac stage the contribution of the neutral lipids to the total lipid content reaches a minimum, and as a consequence POPs that are hardly excreted due to their high lipophilicity (Foekema et al., 2012) are concentrated in the polar lipids, including bio-membranes. This can thus be regarded as a worst case situation for the development of baseline toxicity, which might explain the low critical body burdens, expressed on total lipid basis, in our study compared to studies with fully developed fish.

Indications for effects of HBCD and PBDEs on the thyroid hormone dependent metamorphosis were not observed. Although the potency of PBDEs and HBCDs to disrupt thyroid hormone mediated metamorphosis has been shown in tests with echinoderms (Anselmo et al., 2012) and amphibians (Schriks et al., 2006). The ELS-OMEGA calculations predict that at 25 dpf, the onset of the metamorphoses, lipid normalized tissue concentrations of the POPs will be only fractions of those at 6 dpf (Figure 5.5) (ranging between 3.7% for ∑PBDEs and less than 0.01% for HBCD, Figure 5.1). This suggests that in our test, tissue concentrations were too low to affect the thyroid hormone system at the moment of metamorphoses. Apparently the relatively high concentrations between 6 and 10 dpf did not result in irreversible disruption of the thyroid hormone system, or other delayed adverse effects. In the tests reported by (Anselmo et al., 2012) and (Schriks et al., 2006) exposure continued during the metamorphoses.
Table 5.4 Concentrations (in µg/g lipid) of PCBs and PBDE’s, and translation of these values into toxic units (TU) in of newly hatched sole larvae after exposure for 6 days to a concentration series of the artificial mixture of PCBs and BDEs (Mix), and to field mixtures extracted from sole tissue from the North Sea (NS) and Western Scheldt estuary (WS1 and WS2) respectively. The last three rows show the concentrations in the original fish samples that were used to prepare these extracts. Cx indicate dilution steps of 3.

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<th>toxic units (TU)</th>
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The limited contribution of specific toxicity to the overall larval toxicity is confirmed by the observation that the concentration addition concept based on toxic units successfully describes the toxicity of the artificial mixture (Figure 5.7). In the tissue concentrations expressed as toxic unit (TU) PCBs were most dominant, including PCB 126 (Table 5.4). PBDE concentrations were too low to add to the overall toxicity of the mixtures present in the fish larvae at 6 dpf. This was also the case for the mixture that was extracted from North Sea sole, which indicates that also within this more complex mixture of organic pollutants PCBs were the dominant toxic substances. On average also the mortality of the larvae exposed to the WS extracts corresponds with the Toxic Units of the PCBs. In all mixtures (artificial and field) the PCB accounted for >99% of the $\Sigma$TU, while the impact of PBDEs and HBCDs was never significant. Also no indications were found of impact of additionally, not chemically analyzed substances (Voorspoels et al., 2004a). The $\Sigma$TU in the sole that were sampled from the field were 0.04 TU for the Western Scheldt estuary, and 0.004 TU for the North Sea.
5.4.3 Implication for wild Sole populations

Our comparison between the concentration of PCBs and PBDEs in filet and spawn of the North Sea sole confirms observations by other researchers (e.g. reviewed by Russell et al., 1999) that POPs present in the tissue of the mother fish are in general transferred to the eggs with a lipid normalized 1:1 ratio. Therefore the lipid normalized tissue concentration in the mother fish is a good descriptor of the POP concentrations in the eggs.

The Western Scheldt is considered to be one of the most contaminated estuaries of the North Sea with main pollution sources in the heavily industrialized area upstream along the river Scheldt (De Vijver et al., 2003; Voorspoels et al., 2004a; Sanctorum et al., 2011; Sanctorum et al., 2007). When the concentrations of PCBs, PBDEs and HBCD measured in the sole from the Western Scheldt estuary are translated into $\Sigma TU$ levels for sole early life development, these do not exceed 0.05 (Table 5.4). In order to cause 50% mortality of the developing off-spring, the $\Sigma TU$ needs to be 20 times higher (Figure 5.7). In the sole sampled from the North Sea the $\Sigma TU$ was one order of a magnitude lower (Table 5.4). Higher PCB levels in sole tissue have been reported from the Mediterranean with a maximum concentration of 85 µg/g lw ($\Sigma 31$ PCBs) (Dierking et al., 2009). This translates to $\Sigma TU=0.23$, and is comparable to the concentration in larvae from treatment WS1, where 33% mortality was observed.

For several reasons it can be argued that our estimated effect concentrations underestimate the actual risk. Our experiments started with clean healthy eggs from parental fish that were not affected by toxic compounds, and exposure to the test substances commenced about 12 hours after spawning and fertilization. As a consequence the first 12 hours of the development of the embryo occurred under non-exposed conditions, and once the exposure started the internal concentration can be expected to have built up gradually. In a natural situation where maternal transfer is involved, potentially harmful substances will be present in the egg from the beginning. In our experiments only lipophilic POPs were ‘transferred’ to the eggs. In nature also metabolites of these POPs (Montano et al., 2013) will be deposited in the eggs, together with other substances that are known to be able to interfere with early life development, as for instance methylmercury (Latif et al., 2010) or tributyltin oxide (Nakayama et al., 2005).

Thus, although our data indicate that in a relatively contaminated estuary like the Western Scheldt the direct impact of maternally transferred POPs on early life development is limited, impact of the POPs on the whole reproduction process cannot be ruled out.
Figure 5.7 Larval mortality at the end of the tests with the artificial mixture (Mix) and the field mixtures (NS, WS1 and WS2) plotted against the tissue concentrations of PCBs, PBDEs and HBCDs on dpf 6 expressed as total toxic units. The crossing of the dotted lines indicates the point where theoretically 1 Toxic unit should result in 50% larval mortality. 'NS' and 'WS' on the x-axis indicate the TU concentration that was present in the sole sampled from the North Sea and Western Scheldt estuary respectively.

5.5 References


Mourente, G., Vazquez, R., 1996. Changes in the content of total lipid, lipid classes and their fatty acids of developing eggs and unfed larvae of the Senegal sole, Solen senegalensis Kaup. Fish Physiology and Biochemistry 15, 221-235.


Annex to Chapter 5

Table S5.1: Octanol-water partitioning coefficients (log $K_{ow}$) (Hansen et al., 1999; Li et al., 2008) and molecular weights (www.lentech.com) of the compounds that were applied for the calculations in this paper.

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Table S5.2: Composition of the PCB mixture (Arochlor 1254) and the PBDE mixture that was composed of 5 pure PBDE standards. Both mixtures were used for the tests.

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Table S5.3: Composition of the artificial mixture and the natural mixtures that were used for the tests. The artificial mixture was composed of the technical PCB mixture Arochlor 1254, and standards of PCB126 and the 5 PBDEs. The natural mixtures were extracted from fish from the Westerschelde estuary (WS) and the North Sea (NS).

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</tr>
<tr>
<td>HBCD</td>
<td>0%</td>
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### Artificial mixture vs. Natural mixtures

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<th>WS1</th>
<th>WS2</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Sigma 25) PCB</td>
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<td>98.4%</td>
<td>98.4%</td>
</tr>
<tr>
<td>PCB126</td>
<td>0.02%</td>
<td>0.01%</td>
<td>0.002%</td>
<td>0.11%</td>
</tr>
<tr>
<td>(\Sigma 5) PBDEs</td>
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<td>0.6%</td>
<td>0.9%</td>
<td>0.8%</td>
</tr>
<tr>
<td>HBCD</td>
<td>0.0%</td>
<td>1.3%</td>
<td>0.7%</td>
<td>0.7%</td>
</tr>
<tr>
<td>Totaal%</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
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</table>
Table S5.4: Concentrations of PCB, PBDEs and HBCDs in the tissue of the sole (filet) sampled from the Westerschelde estuary and the North Sea (filet and spawn). The filet samples were used to prepare the natural mixtures WS1 and WS2. 'nd': below level of detection.

<table>
<thead>
<tr>
<th>Code</th>
<th>Westerschelde estuary</th>
<th>North Sea</th>
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<td>Hansweert</td>
<td>Filet</td>
</tr>
<tr>
<td></td>
<td>WS1</td>
<td>WS1</td>
<td>WS2</td>
</tr>
<tr>
<td></td>
<td>ng/g lw</td>
<td>%</td>
<td>ng/g lw</td>
</tr>
<tr>
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<tr>
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<td>180</td>
</tr>
<tr>
<td>PCB-49</td>
<td>200</td>
<td>1.4%</td>
<td>290</td>
</tr>
<tr>
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<td>280</td>
<td>2.0%</td>
<td>390</td>
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<tr>
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<td>0.9%</td>
<td>150</td>
</tr>
<tr>
<td>PCB-87</td>
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<td>1.4%</td>
<td>260</td>
</tr>
<tr>
<td>PCB-97</td>
<td>150</td>
<td>1.1%</td>
<td>170</td>
</tr>
<tr>
<td>PCB-101</td>
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<td>8.0%</td>
<td>1300</td>
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<tr>
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<td>53</td>
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<td>PCB-138</td>
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<tr>
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<td>430</td>
</tr>
<tr>
<td>PCB-153</td>
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<td>6100</td>
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<tr>
<td>PCB-156</td>
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<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>PCB-170</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<td>PCB-194</td>
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<td>nd</td>
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<td>PCB-126</td>
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</table>

\[ \Sigma \text{PCB} \] 13795 100% 19162 100% 1508 100% 1799 100%
### Table S5.5: Concentrations of PCBs and PBDEs in the tissue of the sole larvae on 6 dpf, at the end of being exposed to natural mixtures WS1 and WS2 and the composed mixture Mix. Presented are the data from the highest exposure concentration.

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<th>North Sea</th>
<th>Spawn</th>
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<td>WS1</td>
<td>WS2</td>
<td>WS2</td>
</tr>
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<td>ng/g lw</td>
<td>%</td>
<td>ng/g lw</td>
<td>%</td>
</tr>
<tr>
<td>PBDE 28</td>
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<td>11</td>
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</tr>
<tr>
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<td>80</td>
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</tr>
<tr>
<td>PBDE 99</td>
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<td>6.2%</td>
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<tr>
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<tr>
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<tr>
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<th>%</th>
<th>Mix-C5 ng/g lw</th>
<th>%</th>
<th>NS-C3 ng/g lw</th>
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<td>ng/g lw</td>
<td>%</td>
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<td>99.7%</td>
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</tr>
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</table>
CHAPTER 6

Is the reproduction success of European Eel affected by maternally transferred dioxin-like compounds?

E.M. Foekema\textsuperscript{a}, M. Kotterman\textsuperscript{b}, P. de Vries\textsuperscript{c}, A.J. Murk\textsuperscript{d}

\textsuperscript{a} IMARES, Department Experimental Ecology
\textsuperscript{b} IMARES, Department Fish
\textsuperscript{c} IMARES, Department Maritime
\textsuperscript{d} Wageningen University, Division of Toxicology
Abstract

Reported levels of dioxin-like compounds accumulated in European eel (*Anguilla anguilla*) were used to perform a risk assessment for eel larval survival, taking into account a modelled amplification of tissue concentrations with a factor of 1.33 or 2 during spawning migration. The calculated levels of dioxin-like compounds finally deposited in the eggs were compared to the internal effect concentrations for survival of early life stages of European eel as estimated from a sensitivity distribution based on literature data. As no toxicity data are available for eel larvae, it was assumed that they belong to the 5% or 1% most sensitive teleost fish species.

A risk assessment was performed for direct effect (lethality) of maternally transferred dioxin-like compounds for the developing embryos and larvae. Given concentrations of dioxin-like pollutants, and following the worst case scenarios, it can be expected that larvae of eel from highly contaminated locations in The Netherlands and Belgium will experience more than 50% mortality due to maternally transferred dioxin-like toxicants. Potential effects of other compounds or effects on the migration, condition and fertility of the parental animals were not taken into account. It is important to further study the overall impact of toxicants on the reproduction success of the European eel, as this may have been underestimated until now.
6.1 Introduction

The numbers of juveniles (glass eel) of the European eel (*Anguilla Anguilla*) that reach the European coasts to populate the fresh water bodies, has dropped to a level below 10% of what it used to be before the 1980’s (Dekker, 2003). This decline gives rise to worries that the European eel may be on the edge of extinction. Over-exploitation of glass and mature eel, global changes in currents and environmental conditions, parasite infections, loss of habitat, and the presence of migration barriers such as dams and hydropower cyclones are mentioned as possible causes, and also the presence of anthropogenic toxicants in the eel's environment is suggested to contribute (Palstra *et al.*, 2006; Geeraerts *et al.*, 2011; Brusle, 1991; Robinet and Feunteun, 2002). Due to the specific life history and eco-physiological challenges, eel could be higher exposed and more sensitive to the adverse effects of environmental pollutants than other fish species. After entering from sea as a glass eel, the eels remain in the fresh water for many years where they live in and near the, sometimes highly contaminated, sediment. With increasing body length, their diet shifts from macro invertebrates to fish, turning them into relative top predators (Tesch, 2003). The high lipid content of eel (10-30%) facilitates bioaccumulation of especially lipophilic substances, and chemical analysis confirm the presence of mixtures of PCBs, dioxins, brominated flame retardants, PAHs, organo-chlorine pesticides, TBT and perfluorinated compounds in eels (Kwadijk *et al.*, 2010; Macgregor *et al.*, 2010; Maes *et al.*, 2008; Mariottini *et al.*, 2006; McHugh *et al.*, 2010; Roosens *et al.*, 2010; Stachel *et al.*, 2007; Tapie *et al.*, 2011; de Boer *et al.*, 2010; Geeraerts *et al.*, 2011). This fed the idea that the observed collapse of the eel populations might, at least partly, be related to toxicological effects (Palstra *et al.*, 2006; Geeraerts *et al.*, 2011; Brusle, 1991; Robinet and Feunteun, 2002), for instance by reducing the chance for successful migration to the spawning grounds. After their stay in the freshwater environment as 'yellow eels', eels undergo a metamorphosis (silvering), during which the body is prepared for the long migration in marine water to the spawning grounds, most likely located in the Sargasso Sea, at 5000-6000 km distance (Tesch, 2003). As the fish, that now are referred to as 'silver eels', do not feed after silvering, they use their bodily resources as fuel during migration, for the sexual maturation, and to produce the eggs including the lipid-rich yolk. This makes efficient lipid storage before silverying essential for successful migration and reproduction. Eel monitoring programs in the Netherlands and Belgium from the last 15 years show a decline in the lipid content which has been suggested to be an effect of PCB exposure (Belpaire *et al.*, 2009). Indeed, several environmental contaminants including PCBs, dioxins and some pesticides, have been shown to
interfere with fat accumulation and energy consumption (Robinet and Feunteun, 2002; van Ginneken et al., 2009).

For pacific salmon it has been shown that POPs accumulated in the tissue of the fish concentrate during spawning migration (Debruyn et al., 2004; Kelly et al., 2011). For eel this only has been shown experimentally during a simulated migration of 4 weeks (Ginneken et al., 2009). The increased POP concentrations could result in adverse effects on survival and sexual maturation of the eel, as such compounds have been shown to interfere with steroidogenesis in vitro (Montano et al., 2011; Zimmer et al., 2011), and with reproduction in fish (Daouk et al., 2011). In addition, female eel will deposit their lipids in the eggs which could then affect the developing larvae (Johnson et al., 1998; Latif et al., 2001; Nakayama et al., 2005; Yu et al., 2011). Lipophilic substances tend to evenly distribute over the lipids in the mother fish, including the gonads and hence the lipid normalised POP concentrations of the maternal tissues are close to those in the eggs (Russell et al., 1999).

During development of the early life stages the yolk-POP concentrations increase and peak when yolk lipids are depleted, shortly before the larvae become free feeding (Foekema et al., 2012). This implies that during larval development, tissue POP concentrations become higher than the concentrations in the adult mother fish, even if the eggs/larvae can develop under relative pristine conditions such as in the Sargasso Sea for the European eels. Embryonic and larval life stages are more sensitive to toxicants than fully developed individuals of the same species (Hutchinson et al., 1998) due to critical processes taking place such as development of tissues and the endocrine system.

Experiments with early life stages of the marine flatfish Solea solea that were exposed as eggs to mixtures of lipophilic POPs extracted from adult sole from a heavily contaminated estuary, indicated that mainly dioxin-like PCBs were responsible for the observed mortality (Chapter 5). Based on these findings, and the information that fish early life stages in general are very sensitive to dioxin-like toxicity (King-Heiden et al., 2012; Elonen et al., 1998; Foekema et al., 2008 –Chapter 2–), we here focus on the risk that dioxin-like compounds may pose to the early life stages of eel. The substances known to be mainly responsible for dioxin-like toxicity, PCDDs, PCDFs and some PCBs (Van den Berg et al., 1998; Van den Berg et al., 2006) still are ubiquitous in the environment. Their high lipophilicity (log Kow >6) facilitates bio accumulation, maternal transfer, and the development of a relative high internal peak concentration in the larval tissue at the end of the yolk sac stage (Foekema et al., 2012–Chapter 4–).
In the present paper we investigate to what extent the larval survival of European eel could be negatively affected by maternally transferred dioxin-like compounds. For this purpose, we collected and calculated the following information:

1. Lipid-normalised concentrations of dioxin-like compounds in European eel tissue.
2. Development of concentrations of POPs with different log \( K_{ow} \) in eel tissue during migration. For this, information about weight loss and changes in tissue composition during simulated migration in a laboratory set-up (Palstra and van den Thillart, 2010; van Ginneken et al., 2007; van Ginneken and Maes, 2005) was used to calculate the concentration of dioxin-like compounds in the lipids during migration using a mathematical model for the distribution of POPs in aquatic organisms (Hendriks et al., 2001).
3. Estimated sensitivity of eel early life stages. As successful larval development of the European eel in captivity still is not possible, no exposure studies can be performed to study the sensitivity of eel larvae to dioxin-like compounds. Therefore, the sensitivity of eel larvae for dioxin-like compounds was estimated based on the sensitivity distribution of larvae of other teleost fish species as published in Steevens et al., 2005.

Based on this information a risk assessment was performed for larval survival as consequence of maternal concentrations of dioxin-like compounds.

6.2 Data collection and calculations

6.2.1 Concentrations in European eel

A non-exhaustive literature search was performed to collect data about concentrations of dioxin-like pollutants (PCDDs, PCDFs, and PCBs) in European eel during the last decade. For the Netherlands also unpublished information was included from eel chemical monitoring programs performed by our own institute IMARES (previously RIVO). Most monitoring results of eel contaminant levels are reported on wet weight basis to be able to assess risks for human consumption, and often no indication of the lipid level is given. For our application only lipid-based tissue concentrations are useable, and therefore our datasets were limited to these data that were reported on lipid-base, or where reported lipid levels enable lipid-based normalisation. Furthermore, only datasets were included that were based on chemical analyses of the 12 dioxin-like PCBs (4 non-ortho and 8
mono-ortho) and 17 PCDDs/PCDFs as recommended by the WHO for the calculation of dioxin-like toxic potency.

The levels of individual congeners can be expressed as TCDD equivalency (TEQ) by multiplication of the concentration with Toxic Equivalency Factors (TEF). Based on differences in the sensitivity of taxonomic groups TEF-systems have been proposed for mammals, fish, and birds (Van den Berg et al., 1998). In 2005, the TEF system for mammals was revised (Van den Berg et al., 2006). The choice of the TEF-system to use has consequences for the resulting TEQ. As the far majority of the research on the presence of dioxin-like toxicity in fish focuses on the risk for human consumption, results are normally expressed using the WHO-TEF system for mammals as proposed in 1998 ((Van den Berg et al., 1998) and/or revised in 2005 (Van den Berg et al., 2006). The TEQ values for mammals however, overestimate the contribution of mon-ortho PCBs to the overall toxicity for fish. Therefore, in our study the toxicity of the measured compounds was expressed as TEQ by using the WHO Toxic Equivalence Factors (TEF) for fish (Van den Berg et al., 1998).

Information about dioxin-like toxicants in eel tissue for seven European countries is presented in Table 6.1. The most extended datasets were found for Belgium and the Netherlands both covering about 40 sites including heavily contaminated and relatively clean locations. The three included Norwegian locations are all highly contaminated (Grenland fjord), and the dataset for Germany originates from 10 locations in one river (Elbe). The dataset for Ireland, Poland and Portugal covered 7, 3 and 1 location(s) respectively, without indications that these sites were selected for their background contamination levels.

Table 6.1 Concentrations (median and range) of dioxin-like toxicants (in pg/g lw) in eel tissue from different locations in European countries expressed as Dioxin Toxic Equivalents (TEQ) calculated from tissue concentrations applying WHO1998 Toxic Equivalence factors (TEFs) for fish and mammals respectively. Presented are the averages and range of the reported values per location per country, sorted from low to high mammalian TEF-based TEQ values. The calculations are based on 12 WHO PCBs (4 non ortho and 8 mono-ortho) and 17 WHO PCDD/Fs

<table>
<thead>
<tr>
<th>Country</th>
<th>Period</th>
<th>n locations</th>
<th>TEQ1998fish</th>
<th>TEQ1998mammals</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Portugal</td>
<td>2009</td>
<td>1</td>
<td>no data</td>
<td>4.7</td>
<td>Nunes et al 2011</td>
</tr>
<tr>
<td>Poland</td>
<td>2000-2008</td>
<td>3</td>
<td>no data</td>
<td>10.0 (2.5-31.4)</td>
<td>Szlinder-Richert 2010</td>
</tr>
<tr>
<td>Ireland</td>
<td>2005-2007</td>
<td>7</td>
<td>3.3 (1.2-24.1)</td>
<td>15.6 (1.7-23.6)</td>
<td>Mchugh et al 2010</td>
</tr>
<tr>
<td>Norway</td>
<td>2000-2001</td>
<td>3</td>
<td>no data</td>
<td>60.7 (53.4-170)</td>
<td>Knutzen et al 2003</td>
</tr>
<tr>
<td>Belgium</td>
<td>2000-2007</td>
<td>38</td>
<td>11.3 (2.3-1565)</td>
<td>103 (12.3-5093)</td>
<td>Geeraets 2011</td>
</tr>
<tr>
<td>Germany</td>
<td>2002-2005</td>
<td>10</td>
<td>no data</td>
<td>122 (15.2-289)</td>
<td>Stachel et al 2007</td>
</tr>
<tr>
<td>Netherlands</td>
<td>2004-2011</td>
<td>42</td>
<td>20.4 (1.5-285)</td>
<td>120 (12.6-352)</td>
<td>IMARES unpubl data</td>
</tr>
</tbody>
</table>
The dioxin-like toxicity of all datasets was reported as TEQ applying the mammalian TEF system from 1998. Although not directly relevant for our calculations these data are included in Table 5.1 for rating the relative rank of pollution. It shows that the lowest TEQ levels reported are from the locations in Portugal, Poland, and Ireland, and that eel with the highest levels originate from Belgium, Germany, and the Netherlands. Detailed information on the underlying chemical dataset that allowed calculation of TEQs for fish was only available for Ireland, Belgium and the Netherlands. On average the TEF system for mammals resulted in 5 to 9 times higher TEQ levels than the TEF system for fish, but the ranking of the countries did not differ (Table 6.1).

6.2.2 Development of tissue POP levels during migration

The bioaccumulation model OMEGA (Hendriks et al., 2001) was applied in the present study to estimate the developments of POP concentrations in tissue of migrating silver eel. This model is based on classical fugacity theory for accumulation kinetics of organic substances as a function of the octanol-water partition ratio (K_{ow}), and considers the role of species weight, lipid content and trophic level. Exchange (uptake and excretion) of the POP between the organism and its environment via water, food and faeces are included. The OMEGA model has been shown to well-predict steady state tissue concentrations in adult fish in field situations based on environmental concentrations (Veltman et al., 2005; Smitkova et al., 2005). Rather than determining the equilibrium ratios, the ordinary differential equation (ODE) provided by Hendriks and colleagues (Hendriks et al., 2001) was used as basis to determine the POP concentrations in the eel's tissue in time. To this end, the ‘deSolve’ package (Soetaert et al., 2010) in R (The R Foundation for Statistical Computing, Vienna) was used to solve the ODE numerically, using Euler's method, as proven successful before to calculate the development of PCB levels in sole larvae (Foekema et al., 2012-Chapter 4-).

For the model parameters that are assumed not to be species specific (respiration coefficient, rate exponent, body temperature correction factor, water layer diffusion resistance and lipid layer permeation resistance) the default and typical values presented in Hendriks et al., 2001 were applied (Table 5.2). Food intake (y1) was set at zero, as migrating eels do not feed. Mortality rate (k4) was set at zero since the model describes the development in an individual eel until spawning. As the major part of the migration takes place in a more or less pristine oceanic environment, the water concentration of POPs (C0,W) also was set at zero. The POP concentration in the tissue of the eel at the start of the migration was set at 1, so that the model outcome can be interpreted as the fraction of this concentration.
The remaining parameters for a standard silver eel were set at a body weight of 1 kg (W) and a lipid content of 25% (Pch2i = 0.25). We assumed that migration over 5500 km will take normally 180 days (Palstra and van den Thillart, 2010), but at optimal swimming speed this distance could be covered in 100 days (van Ginneken et al., 2005). During simulated migration experiments in swim tunnels with a continuous flow of fresh water covering the same distance in 180 days, an average loss of body weight of 19.7% was determined (van Ginneken et al., 2005). Later experiments from the same research group showed that the costs of transport are 20% higher when similar experiments are performed in seawater (Palstra et al., 2008). As almost the entire migration of the eel takes place in seawater, we assumed an average total weight loss of 25%. For a worst case situation we assume 50% weight loss during migration. The composition of the tissues does not change during the simulated migration, indicating that fat, protein and carbohydrate were metabolized in the same proportion (van Ginneken et al., 2005). Therefore, in our calculations, the lipid fraction was assumed not to change during migration.

The OMEGA model was used to predict the development of the POP concentration in the eel's tissue for four migration scenarios. Two scenarios assume 25% weight loss during 180 (scenario 1) and 100 days (scenario 2) of migration, and two scenarios assuming maximum (50%) weight loss during 180 (scenario 3) and 100 days (scenario 4) of migration (Table 6.2).

The developments of the tissue concentrations are calculated for POPs with a log Kow of 4, 5, 6, and 7. As can be seen in Figure 6.1, model simulations indicate that tissue concentrations of POPs with a log Kow of 5 or less decrease during the migration. The removal mechanism involved is passive excretion through the water phase which is more effective for more water soluble substances. For POPs with log Kow 5 the model predicts a loss of approximately 50% of the initial concentration by the time the eel reaches the spawning grounds. Substances with log Kow of 4 and below can be expected to be completely washed out by then. Concentrations of POPs with log Kow values of 6 and higher are predicted to further magnify due to weight loss. Assuming there is no excretion or metabolism of a compound during migration, the tissue concentration at the end of the migration period can increase to 133% (Figure 6.1) and 200% of the initial tissue concentration for 25% and 50% weight loss respectively.

Changing the migration duration from 180 days into 100 days assuming optimal swimming speed (van Ginneken et al., 2005), does not affect the magnification of substances with log Kow 6 and above, as long as the total weight loss at the end of migration is not changed (Figure 6.2). Only the excretion of less lipophilic compounds (log Kow <6) is less with a shorter migration duration.
All included substances with dioxin-like toxicity have a log $K_{ow}$ above 6, a value where the OMEGA model predicts that the maximum magnification will occur to either 133% or 200% assuming respectively average (25%) and extreme (50%) weight loss. Since the lipid fraction of the tissue does not change during migration (van Ginneken et al., 2005), these magnification rates are valid for both the lipid normalised as well as wet weight-based tissue concentrations.

Table 6.2 Input data used of the OMEGA model to describe the development of POP concentrations in eel tissue during migration. Four different scenarios are modelled: 1: 25% weight loss during 180 d migration; 2: 25% weight loss during 100 d migration; 3: 50% weight loss during 180 d migration; 4: 50% weight loss during 100 d migration. Underlined values refer to default values as proposed by Hendriks et al., 2001.

<table>
<thead>
<tr>
<th>Description</th>
<th>Symbol</th>
<th>Unit</th>
<th>Scenario 1</th>
<th>Scenario 2</th>
<th>Scenario 3</th>
<th>Scenario 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Migration duration</td>
<td>$d$</td>
<td>days</td>
<td>180</td>
<td>100</td>
<td>180</td>
<td>100</td>
</tr>
<tr>
<td>species weight start</td>
<td>$W_{\text{start}}$</td>
<td>kg</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Species weight end</td>
<td>$W_{\text{end}}$</td>
<td>kg</td>
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<td>0.75</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>lipid fraction of organism</td>
<td>$P_{\text{ch2i}}$</td>
<td>-</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>POP concentration in organism</td>
<td>$C_{\text{i}}$</td>
<td>mg/kg ww</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>POP concentration in water</td>
<td>$C_{\text{0,w}}$</td>
<td>mg/l</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>biomass production coefficient</td>
<td>$y_{2}$</td>
<td>kg/d</td>
<td>-0.0015</td>
<td>-0.0027</td>
<td>-0.0065</td>
<td>-0.0117</td>
</tr>
<tr>
<td>mortality rate constant</td>
<td>$k_{4}$</td>
<td>/d</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>water absorption-excretion coefficient</td>
<td>$y_{0}$</td>
<td>kg/d</td>
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<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>respiration coefficient</td>
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<td>kg/d</td>
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<td>0.0024</td>
<td>0.0024</td>
<td>0.0024</td>
</tr>
<tr>
<td>rate exponent</td>
<td>$K$</td>
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<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>temperature correction factor</td>
<td>$q_{T:C}$</td>
<td>/d</td>
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<td>1</td>
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<td>water layer diffusion resistance</td>
<td>$\rho_{h2o,0}$</td>
<td>d/kg</td>
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<tr>
<td>water layer diffusion resistance</td>
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<td>0.00001</td>
<td>0.00001</td>
</tr>
<tr>
<td>lipid layer permeation resistance</td>
<td>$\rho_{\text{ch2},i}$</td>
<td>d/kg</td>
<td>68</td>
<td>68</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>food related parameters (not relevant for migrating eels)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>food ingestion coefficient</td>
<td>$y_{1}$</td>
<td>kg/d</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>lipid fraction of food</td>
<td>$P_{\text{ch2},i-1}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>POP concentration in food</td>
<td>$C_{\text{i-1}}$</td>
<td>mg/kg ww</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>fraction of ingested food assimilated</td>
<td>$P_{1}$</td>
<td>kg/kg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 6.1 Development of the concentration of POPs with different log $K_{ow}$ (legend) in the tissue of a silver eel during a 180 d migration period and limited (25%) weight loss (scenario 1), calculated with the OMEGA model. The maximum bioamplification factor in this situation (1.33) is indicated by the horizontal dotted line.

Figure 6.2 The relative concentration of POPs in eel tissue after spawning migration calculated with the OMEGA model for compounds with different log $K_{ow}$, expressed as the fraction of the concentration present at the start of the migration. Scenarios are: migration duration of 100 and 180 days, and a low (25%) or high (50%) loss of body weight. The shaded area indicates the log $K_{ow}$ range of the dioxine-like compounds considered.
6.2.3 Estimating the sensitivity of eel early life stages

The sensitivity of eel larvae is estimated based on the distribution of the sensitivities of other teleost fish species as determined in 12 laboratory tests with TCDD, and 1 test with TCDD-like toxicants following egg exposure via water, injection or parental exposure and observation of the developing larvae at least until the end of the yolk sac stage (Steevens et al., 2005). From this distribution Steevens and colleagues derived egg concentrations below which 90% (Hazard Concentration 10; HC10), 95%, or 99% (HC1) of the teleost fish species larvae do not exceed the median 50% internal lethal concentration (ILC50) or the no/lowest observed internal (INOEC/ILOEC) concentration respectively (Table 6.3). The difference between ILC50 and INOEC/ILOEC were not statistically significantly under the exposure conditions applied. For our worst-case approach we applied the 99% values (HC1), thus assuming that eel larvae belong to the 1% most sensitive teleost fish species.

The dataset assembled by Steevens et al., 2005 shows a positive correlation between the sensitivity of the species and the lipid content (1.7-8%) of the eggs. This is likely to be due to magnification of the lipophilic toxicant concentrations during depletion of the yolk lipids. The greater the lipid content, the greater the magnification can become. For example, in lean eggs (2% lipid) from Solea solea the increase in POP concentration at the end of the yolk-sac stage was a factor 2 to 3 (Foekema et al., 2012), while for the more lipid rich (8% lipids) eggs of Oncorhynchus tshawytscha this was around a factor 5 (Daley et al., 2012). As the sensitivity of larvae for dioxin-like compounds will mainly depend on the internal concentration reached, larvae of fish species with higher lipid content will be more vulnerable given the same POP-concentration in the egg yolk. Eel produce eggs with a relatively high lipid content (4.8-5.3% for A. japonica derived from Furuuita et al., 2006; 5-7.5% for A. Anguilla, (personal comment M. Kotterman IMARES) This justifies the assumption that eel larvae belong to the more sensitive larvae among fish species.

Table 6.3 Dioxin-equivalent (TEQ) effect concentrations in fish eggs below which 99% (HC1), 95% (HC5) or 90% (HC10) of the teleost fish species are expected to experience 50% larval mortality or less (<ILC50), or no adverse effects on larval survival (<INOEC/ILOEC), with 95% confidence limits. Data from Steevens et al., 2005.

<table>
<thead>
<tr>
<th>Species effect level</th>
<th>pg TEQ/g lw in egg</th>
</tr>
</thead>
<tbody>
<tr>
<td>99% &lt;ILC50 (HC1)</td>
<td>58 (0.3 - 382)</td>
</tr>
<tr>
<td>95% &lt;ILC50 (HC5)</td>
<td>386 (11.7 - 1430)</td>
</tr>
<tr>
<td>90% &lt;ILC50 (HC10)</td>
<td>909 (58.3 - 2640)</td>
</tr>
<tr>
<td>99% &lt;INOEC/ILOEC (HC1)</td>
<td>57 (15 – 201)</td>
</tr>
<tr>
<td>95% &lt;INOEC/ILOEC (HC5)</td>
<td>321 (88 – 1050)</td>
</tr>
<tr>
<td>90% &lt;INOEC/ILOEC (HC10)</td>
<td>699 (199 – 2220)</td>
</tr>
</tbody>
</table>
Chapter 6 Eggsposed

Observations of survival time of European eel embryos in relation to dioxin-like toxicity also suggested a high sensitivity with reduced survival already at 4 pg/g ww in the gonads of the parent fish (Palstra et al., 2006). However, these values were derived under sub-optimal conditions and all embryos, including the non-exposed died before hatching.

6.3 Risk assessment

To indicate theoretical tissue concentration in the female eel at the start of the migration that will result in 50% larval mortality, the critical TEQ-level in eggs were divided by 1.33 or 2.0 being the maximum magnification factor that may be expected during migration to the spawning grounds with 25% and 50% weight loss respectively. In the worst case scenario assuming that European eels are among the 1% most sensitive fish species, and the female eel loses half her body weight during migration, 50% larval mortality due to maternally transferred dioxin-like toxicants can be expected for tissue concentrations in silvering eel of 28.5 pg TEQ/g lw. At 29% of the sampled sites in the Netherlands and 21% of the Belgium sites these levels are exceeded (Figure 6.3A), sometimes even 10-50 fold. All samples from Ireland are below this critical level.

Assuming a lower weight loss of 25%, the critical tissue concentration increases to 43 pg TEQ/g lw. Obviously, all Irish locations are below this critical level. But at 11% of the locations in Belgium and 14% in the Netherlands also these levels are exceeded and according to this scenario more than 50% larval mortality may be expected for eggs from eel coming from these locations. At the most polluted locations in the Netherlands and Belgium this critical tissue concentration is exceeded 6 to 35 times. Even if we assume that eel larvae are amongst the 5% of the most sensitive fish species these critical levels for 50% larval mortality are exceeded at specific locations in the Netherlands and Belgium. Given the fact that historical POP levels in eel tissue from the Netherlands were 3 to 20 times higher in the year 1977 compared to 2006 (de Boer et al., 2010) these calculations indicate a serious risk for the larval survival of dioxin-like compounds in eel that manage to reach the spawning ground in the Sargasso sea.

Figure 6.3B shows the TEQs for the same eel samples from Ireland, Belgium and the Netherlands plus a number of other countries, now calculated with the mammalian TEF system (Van den Berg et al., 1998). Compared to the TEQs\textsubscript{fish} the TEQs\textsubscript{mammalian} are higher since in the mammalian TEF system the mono-ortho PCBs are assigned higher TEF values and these compounds are relatively
abundant in aquatic species. Based on the TEQ(mammalian), the majority of eel sampled in Germany, Norway, Belgium and The Netherlands would exceed the critical tissue values. This reveals that it is very important to use the relevant TEF system, and also suggests that the TEQs in eel pose a serious risk to mammalian consumers.

In this study we only focus on the potential effects of dioxin-like substances, based on the indications that fish early life stages are especially sensitive to this group of compounds. However, many other POPs also have been shown to be accumulated in eel, including other compounds such as DDT/DDE and PBDEs (de Boer et al., 2010) with a log $K_{ow}$ of 6 or more. This means that these compounds too will be further concentrated during migration. For known accumulated POPs with lower log $K_{ow}$ values, such as HCH and HBCD, the body burdens are predicted to decrease during migration (Figure 6.2), and during larval development until free feeding as has been shown before (Foekema et al., 2012). The fate of other compounds is harder to predict. For example, perfluorinated compounds such as perfluorooctanesulfonate (PFOS) have been shown in high concentrations in eel tissue (Kwadijk et al., 2010). With both hydrophilic and hydrophobic physical properties PFOS behaves like fatty acids and therefore are not accumulated in lipids but in blood and cell membranes. These compounds are extremely persistent and have been shown to be maternally transferred also to fish eggs and to cause adverse effects in developing larvae (Wang et al., 2011). An important group of compounds that also have not been considered here are the hydroxylated POP-metabolites. Their effects on developing embryos can only be tested by exposing the mother fish to the parent compounds. A recent meta-analysis has shown that these metabolites still do occur at effect levels in several species, including humans that are exposed to POPs via the marine food chain (Montano et al., 2013).

Our risk assessment only was performed for direct effect (lethality) of maternally transferred dioxin-like compounds on the developing embryo and larvae. The potential impact of the presence of other toxicants including their metabolites was not included.

Potential effects of accumulated compounds and their metabolites on the migration, condition and fertility of the parental animals were not taken into account. It is important to further study the overall impact of toxicants on the reproduction success of the European eel, as this may have been underestimated until now.
Figure 6.3 Levels of total WHO-TEQ (pg/g lw) in European eel different countries, calculated from chemical analyses of PCDDs, PCDFs and PCBs applying WHO Toxic Equivalence Factors (TEFs) for fish (A) and mammals (B). Presented are average values per sampled location (dots) and median value per country (line). The dotted lines indicate estimated levels at which 50% larval mortality may be expected due to maternally transferred TEQs for 95% (HC5) or 99% (HC5) of the teleost fish species, taking into account migration amplification due to average (25%, avg Wloss) or maximum weight loss (50%, max Wloss).
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CHAPTER 7

The potential impact of reduced larval survival on fish population development

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Abstract

The potential impact of (toxicant induced) early life stage mortality on the population development of sole (Solea solea) was explored by application of an age structured matrix model. Sole was chosen for this exercise since the life history is well known and it is applied as model species in ecotoxicological research. The model is used to explore the population response to a combination of larval mortality and fishing-related mortality of mature fish. The results indicate that the impact of larval mortality that occurs before metamorphosis is very low, even in combination with high fishing pressure. This is the result of the combination of the high fecundity and the fact that the larval mortality occurs before the moment when the number of recruits is limited by the carrying capacity of the nursery areas. Mortality that occurs after the nursery areas are populated will have a more pronounced impact on population development. The results further imply that population development of pelagic fish species that do not concentrate in nursery areas and species with low fecundity is more vulnerable for disturbance through mortality of early life stages.
7.1 Introduction

When present in high enough concentrations in the tissue of adult fish, maternally transferred organic substances will have a negative impact on the survival and development of the larvae into juveniles and eventually adult fish. Our experiments, performed with the common Sole (Solea solea) as model species, show an increased mortality rate before the onset of the metamorphosis (Chapter 2 and 5). Due to bio amplification of lipophilic pollutants such as dioxins and PCBs, fish larvae will experience the highest exposure and therefore the strongest acute toxicity at the end of the yolk-sac stage.

Translation from impact on specific life stages to ecological relevant endpoints is not straightforward, as the most sensitive endpoint is not necessarily the most important for population development (Forbes et al., 2010). Forbes and co-workers argue that this makes evolutionary sense as it dampens variation in population growth rates. Hence, to be able to determine the relevance of a toxic effect on early life stages at population level, it is important to know the population dynamics of the species.

Survival rates of fish early life stages in nature are generally low, at least in the case of oviparous species without parental care like sole and most other commercially exploited species. This low survival rate is compensated by the production of great numbers of eggs. Large changes in fecundity can be compensated by changes in mortality or survival rate that are often density dependent (Rothschild, 2000).

An analysis of datasets of 104 exploited marine species revealed that the population resilience at low adult abundance varies strongly between species. Many species exhibited strong compensatory dynamics with increasing reproduction at low adult densities (Keith and Hutchings, 2012). One of the mechanisms behind the compensation is described as the concentration hypothesis: Species that make a shift from a pelagic to a benthic habitat during the early development, experience an immediate concentration of their habitat from three into two dimensions, which could lead to density dependent mortality. The stronger this effect, the better the population can compensate for changes in population size (Beverton, 1995). The maximum number of recruits that can be sustained in a certain habitat is limited, and therefore a limited number of adults can produce sufficient amounts of eggs. Data analyses confirmed this hypothesis, revealing low compensation rates for fully pelagic species like mackerel and sardine, and intermediate compensation for gadoid species like cod and saithe of which many juveniles life close to the seabed. The highest compensation rates were found for flatfishes like sole and plaice of which...
the larvae become demersal after metamorphosis and the adults retain a benthic lifestyle (Iles and Beverton, 2000).

For sole, this concentration effect is enforced by the fact that suitable nursery areas are limited because of the need to meet specific characteristics with respect to water depth, substrate particle size etc. (Rijnsdorp et al., 1992). As a result, the surface area of the three dimensional pelagic habitat in which hatching and larval development takes place is only partly available as nursery area after metamorphoses. Clear correlations were found between the numbers of 1-year old recruits that are produced by a sole population and the surface area of the estuaries that serve as nursery ecosystems (Rijnsdorp et al., 1992).

Table 7.1 Biological characteristics of sole that were used for the matrix model. Data estimated from ICES Fishmaps (2012a) unless indicates otherwise. # Weight (W) calculated from length (L) applying condition factor of 0.9 (Rijnsdorp et al.,2004); *(Rijnsdorp et al., 1992).

<table>
<thead>
<tr>
<th>Age</th>
<th>Stage</th>
<th>L(cm)</th>
<th>W(g)#</th>
<th>Fecundity (eggs/g)</th>
<th>Carrying capacity of biotope (individuals)</th>
<th>Natural mortality (fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Egg/larva</td>
<td>20</td>
<td>108</td>
<td>0</td>
<td>108</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>Juvenile</td>
<td>25</td>
<td>72</td>
<td>0</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>Adult</td>
<td>27</td>
<td>141</td>
<td>800</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>Adult</td>
<td>30</td>
<td>177</td>
<td>800</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>Adult</td>
<td>31</td>
<td>243</td>
<td>800</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>Adult</td>
<td>33</td>
<td>268</td>
<td>800</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>6</td>
<td>Adult</td>
<td>35</td>
<td>323</td>
<td>800</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>7</td>
<td>Adult</td>
<td>35</td>
<td>386</td>
<td>800</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>8</td>
<td>Adult</td>
<td>35</td>
<td>386</td>
<td>800</td>
<td>-</td>
<td>0.1</td>
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<tr>
<td>9</td>
<td>Adult</td>
<td>35</td>
<td>386</td>
<td>800</td>
<td>-</td>
<td>0.1</td>
</tr>
</tbody>
</table>

An undisturbed fish population will produce a surplus of eggs, which increases the chance of larvae encountering food and favourable conditions (Rothschild, 2000), and maximises the exploitation of the nursery areas. The impact of larval mortality on the development of such a population is limited, as long as this mortality appears before the fish enter the nursery areas and does not reduce the number of survivors below the number that can be supported by this nursery ecosystem.

When overexploitation of a fish population would reduce the number of reproducing individuals (the spawning stock biomass, SSB) to a level where recruitment is no longer limited by the carrying capacity of the nursery ecosystem, a stronger effect of larval mortality is to be expected. In extreme
situations this could be simply because the remaining SSB is not enough to produce sufficient numbers of eggs. In addition, the reproduction of fish populations could decline stronger than predicted only based on the population density (Kramer et al., 2009; Keith and Hutchings, 2012) when the density of mates is too low to ensure participation of all mature animals in reproduction (Rowe and Hutchings, 2003), a so-called 'Allee effect' (Kramer et al., 2009).

The ecotoxicological experiments presented in chapters 2 and 5 of this thesis, show that the impact on sole larvae of maternally transferred lipophilic toxicants as studied, appears early in the development. For sole this is before the onset of the metamorphosis. Thus, before the fish colonise the nursery areas where density dependent processes become effective (Rijnsdorp et al., 1992). Potential effects that might develop in a later stage were not considered. In order to explore the potential impact of this toxicant induced early life stage mortality on the population development of sole, a matrix population model was developed based on the life history of sole in the North Sea. The model was kept as simple as possible and does not take into account different factors known to be important in the recruitment of fish, such as ambient water temperature (Fassler et al., 2011; Rijnsdorp et al., 1992), food availability (Nash and Dickey-Collas, 2005), currents and predation, or Allee effects (Rowe and Hutchings, 2003; Kramer et al., 2009). The results must therefore be regarded as purely indicative for the relevancy of (toxicity induced) early life mortality for sole population development.

7.2 Model description

The model describes the numerical development of a sole population per age class, with a maximum age of 9 years (Figure 7.1). Although sole can live up to 40 years individuals older than 10 years nowadays are scarce as a result of high fishing pressure (ICES FishMap, 2012a). At age 2 (‘Age-2’ in Figure 7.1 all individuals mature. Adults annually produce a number of eggs (‘Age-0’) based on the age specific body weight and a general fecundity of 800 eggs per gram female fish (Table 7.1) (ICES 2012a; Rijnsdorp et al., 1992). A 1:1 sex ratio is assumed; hence for the calculation of the population fecundity adult biomass is multiplied by 0.5 to model egg production.

Of all eggs entering the ‘Age-0’ box, 50% are not viable, for instance because they are not fertilised or of poor quality. The 50% hatching success of the spawned eggs is normal in sole aquaculture where maturation, spawning and fertilisation are achieved under more or less natural conditions (pers. comm. A. Kamstra IMARES). If toxicity related mortality (‘Tmort’) occurs, a fraction of the
hatched larvae will not further develop. The remaining larvae have the potential to develop into juveniles that are assumed not to suffer from additional toxicity. However, the number that can be transferred to the ‘Age-1’ box is limited by the carrying capacity of the nursery ecosystem, and the surplus is assumed to die. The carrying capacity of the nursery ecosystem hence sets the maximum number of individuals in the population of age 1 and older. For North Sea sole the geometric mean of the recruitment level was determined at approximately 94 million individuals (Rijnsdorp et al., 1992), for our model this was rounded off at 100 million individuals, and set as ceiling for the number of ‘Age-0’ individuals that can develop into ‘Age-1’ juveniles. From Age-1 onwards, natural yearly mortality was set at 10%, conform the average value that is applied by ICES for sole stock management advices (ICES, 2011). After Age-1 the survival can be further reduced by fishing related mortality (‘Fmort’). The fishing mortality of sole in the North Sea that was used as default value in the model was 30%, being the estimated average fishing mortality in 2011 (ICES, 2012).

Figure 7.1 Overview of the matrix model that was used for the calculations. The grey arrows indicate the external conditions that affect the survival.

\[ A = \text{Age-0} \times 0.5 \times (1 - Tmort) \]

If \( A > \) carrying capacity, then \( A = \) carrying capacity

\[ B = \text{Age-n} \times (1 - (Nmort + Fmort)) \]

\[ C = \text{Age-n} \times 0.5 \times \text{Weight-n} \times \text{Fecundity} \]

Age-n = number of individuals of age-n
Weight-n = weight of individuals of age-n
Nmort = fraction natural mortality
Tmort = fraction mortality caused by toxicity
Fmort = fraction mortality caused by fishing
Different scenarios were run to explore the response of the modelled sole population to changes in (fishing related) mortality of adults, and (toxicity related) mortality of larvae. In addition the impact of changes in the fecundity and the carrying capacity on these model responses was explored. The model describes the development of a sole population during a 50 year period, expressed as development of the biomass of adult fish ($\sum$Age-2 to 9; spawning stock biomass, SSB), and the number of Age-1 recruits.

7.3 Results

In the default situation with a fishing mortality of 30%, no toxicity related mortality, and a carrying capacity of 100 million recruits, the population reached equilibrium at 31 717 tonnes SSB (Figure 7.2). This is close to the 35 000 to 46 700 tonnes that is estimated as the SSB of sole in the North Sea in 2011 and 2012 respectively (ICES, 2012), indicating that despite the simplicity of the model, the order of magnitude of the outcome corroborates with other estimates. Applying a natural mortality of 5 and 0%, instead of the default 10% leads to equilibrium SSBs of 41 987 and 55 472 tonnes respectively.

When, starting from the equilibrium default situation the fishing mortality is changed, new equilibrium SSB levels develop (Figure 7.2). These equilibria logically show a negative correlation with fishing mortality as a consequence of the mortality being a fixed fraction of the population. Increasing fishing mortality above 90% in combination with 10% natural mortality, leads to immediate extinction of the modelled population. Also changes in the carrying capacity for maximum numbers Age-1 recruits result in new equilibrium values of SSB. These are positively correlated with the carrying capacity of the nursery ecosystem as this carrying capacity sets the maximum number of survivors to Age 1.

The impact of larval mortality was explored for populations that were in equilibrium in the absence of fishing mortality, and with 30% and 80% fishing mortality respectively. In all of these situations the population development was very insensitive to the effects of larval mortality (Figure 7.3). Without fishing pressure only 99.999% mortality resulted in a population decline. In combination with fishing mortality of 30% or more, population development is affected when larval mortality is more than 99.995%, thus almost complete and then leads to extinction of the population. Even with 80% fishing mortality only a larval mortality of more than 99.9% contributes to population decline.

It can be recognised that the impact of toxicity increases with increasing fishing mortality. However, these differences are of no significance.
Figure 7.2 Modelled development of the sole population with yearly fishing mortality rates ranging from 0 to 80%. The bold line indicates an estimated realistic mortality rate of 30%.

This insensitivity of the population for even extremely high early life mortality is the resultant of the large numbers of surplus eggs that are produced by the population, relative to the available niches for the juveniles. This is still the case when the population density is strongly reduced by extreme fishing pressure causing 80% yearly mortality. Figure 7.4 presents the modelled relationship between the population density and the numbers of eggs and larvae, with and without additional toxicity induced mortality. According to the model parameters, less than 1 tonnes of SSB of sole, actually 0.0004% of the estimated current SSB of 30 000 tonnes, is sufficient to produce the number of larvae required to fill the carrying capacity of the nursery ecosystem, in situations without toxic pressure. Even when less than 1% of these larvae do survive, still less than 10 tonnes of SSB is sufficient to produce the amount of larvae that allows maximum population growth, and thus to maintain SSB.

When the SSB drops below this critical level, for example for a population where extremely over exploitation resulted in an unrealistic low SSB of only 0.02 tonnes, the model predicts recovery within 11 years for a further undisturbed population. In combination with additional 99% larval mortality, for instance due to toxicity, it takes 5 years longer to recover in the absence of fishing mortality (Figure 7.5 left). When the fecundity would be reduced to only 20 eggs/g body weight (Figure 7.5 right), the recovery time of the further undisturbed population increases only slightly, but the impact of larval mortality then becomes more evident.
Figure 7.3 Impact of different levels (legend) of larval mortality in combination with 0%, 30% and 80% yearly fishing mortality on the development of the modelled sole population. Note the large differences between the y-axes.
Figure 7.4 Schematic presentation of the relation between the spawning stock biomass (SSB) and the estimated numbers of eggs and larvae with and without (toxicity related) larval mortality. The horizontal dotted line indicates the maximum number of juveniles that can be supported by the nursery ecosystem (carrying capacity). The bold line indicates the number of juveniles that can survive when experiencing 99% toxic mortality before density dependent mortality occurs. Fecundity data for sole were used. The dotted vertical line indicates the estimated current SSB for sole.

Figure 7.5 Impact of (toxicity related) mortality (legend) of early life stages on the recovery time of an overfished population, after fishing has stopped. Left using default sole fecundity (800 eggs/g bw); and right with reduced fecundity (20 eggs/g bw).
7.4 Discussion

The model outcomes suggest that sole populations are able to compensate high levels of larval mortality that occurs before the nursery areas are colonised. This is related to the high degree of concentration of individuals that takes place when the pelagic sole larvae change to a demersal lifestyle while entering the nursery grounds, of which the carrying capacity is limited by the surface area. Compared to other fish species the concentration effect is relatively large for flatfish (Iles and Beverton, 2000). Species of which the whole life cycle takes place in the pelagic, like mackerel, sardine, and anchovies can be assumed to be more sensitive to early life stage mortality. Whereas the degree of concentration for gadoid species like cod and saithe forms an intermediate between flatfish and pelagic species (Iles and Beverton, 2000). For herring, also a pelagic fish, but with demersal eggs, the situation might be different. At high population density, the number of produced eggs can be limited by availability of suitable substrates needed by this species for egg deposition. Once the carrying capacity of these substrates is reached a further increase of the population will not result in more compensation for early life stage mortality.

The maternally transferred lipophilic toxicants that we studied caused mortality at an early stage of development. The impact of this type of larval mortality on our modelled sole population only became apparent at very low population levels. It is questionable if such a population would be able to sustain, even without impact of toxicants. Our model overestimates the recovery potential of the population since it assumes that all surviving larvae will be able to reach the nursery grounds, which in reality is not the case (Bolle et al., 2009).

Besides this, the number of viable eggs produced by a female is not affected by so called Allee effects in our model, although that can be expected at low population densities (Rowe and Hutchings, 2003; Kramer et al., 2009; Keith and Hutchings, 2012). The combination of these simplifications in the model makes that only one single adult fish would be able to produce sufficient eggs to start a sustainable population.

In addition factors like water temperature (Fassler et al., 2011; Rijnsdorp et al., 1992), food availability (Nash and Dickey-Collas, 2005), predation (Nash and Geffen, 2012), that have large impact on the recruitment process are not included in our model. Therefore, the outcome should only be considered as purely explorative for the potential role of (toxicity related) larval mortality on fish population development. Despite this, the general conclusion that sole is protected against a high level of toxic effects on early life stages by the combination of high fecundity and the concentration of the older larvae in the
nursery areas seems valid. These findings are in line with the outcome of a literature review that compensatory interactions and hence buffering of toxicant effects on the dynamics of density limited populations are likely (Forbes et al., 2001). Pelagic species can be expected to be more sensitive as here larval mortality is not compensated by concentration in nursery areas. This would especially be the case for species with relatively low fecundity.

7.5 Conclusions

The model results indicate that a high fecundity in combination with density dependent regulation of the maximum numbers of recruits, protects sole and other (flatfish) species with comparable reproductive biology, against the impact of (toxicant induced) high early life stage mortality. At least as long as this mortality occurs before density dependent processes take place, which may be expected with the type of toxic effects that are associated with maternally transferred substances.

Population development of pelagic species and of species with low fecundity can be considered more sensitive to the impact of early life stage mortality.

7.6 References


ICES (2011) WGNSSK REPORT 2011 Sec 10 Sole in Subarea IV


"You did not kill the fish only to keep alive and to sell for food, he thought. You killed him for pride and because you are a fisherman. You loved him when he was alive and you loved him after. If you love him, it is not a sin to kill him. Or is it more?"

*Ernest Hemmingway: The Old Man and the Sea*
8.1 Introduction

The overall aim of the research presented in this thesis was to investigate whether and how maternal transfer of lipophilic POPs, that are ubiquitous in the marine environment, can affect the development and survival of fish larvae. And if so, to assess if this could result in reduced population development taking into account fishing as major pressure for fish populations. The focus was on lipophilic POPs that can cause dioxin-like toxicity or that are known to disrupt the thyroid hormone system. Both effect types are relevant for early life development. In addition, mixtures of lipophilic POPs were tested as they are accumulated in fish tissue.

In the study, fish early life stage tests were performed with exposure during the egg stage only, followed by observation until metamorphosis into juveniles. Results clearly show delayed mortality days after exposure had stopped, when the fish already are free-feeding. Crucial in this aspect is the peak in exposure that develops for highly lipophilic pollutants at the end of the yolk-sac stage. The height of this peak is the resultant of a combination of the lipophilicity of the pollutant and the utilisation of the yolk lipids by the developing larvae. Dioxin-like toxicity and baseline toxicity are the most likely toxicological mechanisms causing the observed effects. The test set up, with the common sole (*Solea solea*) as model species, did not provide indications for disruption of the thyroid hormone mediated metamorphosis. The effects of mixtures of lipophilic POPs extracted from the tissue of fish collected at contaminated field locations could be explained based on the individual pollutants present. No indications were found for synergistic mixture effects or for a substantial role of other lipophilic POPs in the mixtures than the PCBs and the brominated flame retardants that were subject of this study. Based on this research it is estimated that the levels of lipophilic POPs currently present in sole from the relatively contaminated Western Scheldt estuary pose little risk ($\Sigma$TU=0.05; Chapter 5) for causing larval mortality related to maternal transfer of these pollutants. For other species including European eel, seriously reduced larval survival can be expected when the mother fish originates from highly contaminated locations. A simplified population model was applied to explore to what extent reduced larval survival may result in effects on population development of the common sole, in combination with and without (high) fishing pressure. Due to the high fecundity and the density dependent survival of juvenile stages that occurs after metamorphosis, the development of a sole population is insensitive to even very high larval mortality. Species that combine a low fecundity with density independent mortality of early life stages will be most sensitive to the type of mortality that can be expected as a result of maternal transferred lipophilic POPs.
This chapter discusses the results presented in this thesis. First the results obtained in the current p-ELS test are discussed, also in comparison with the results that would (not) be observed in standard fish ELS tests with a shorter observation period. Next, the comparability of the chosen experimental set-up with the effects of maternally transferred POPs is discussed followed by the potential consequences of ELS mortality for population development of fish species. The characteristics that determine the vulnerability of a fish population are indicated plus some potentially sensitive fish species based on the most relevant characteristics identified in this thesis. Finally, in a concluding paragraph the research questions are addressed and future perspectives are discussed.

8.2 Observed effects in the prolonged ELS test

8.2.1 Timing of effects revealed with the p-ELS

With exception of HBCD, that induced no effects at any of the test concentrations, all compounds tested caused larval mortality that started a few days after yolk-sac absorption and continued in most cases until the onset of metamorphosis. The dose-related delayed mortality resulted from egg exposure only and occurred during further development of the larvae under unexposed conditions. Actually, mortality continued when the larvae already were free feeding and tissue concentrations of the toxicants already were declining due to growth dilution. The timing of the effects are highly reproducible (Chapters 2 and 5). At the lower test concentrations, this mortality occurred without initial indications for negative effects. Figure 8.1 shows the development in tissue concentrations of POPs depending on their log $K_{ow}$ in developing sole larvae exposed during the egg stage (6 days) in the p-ELS tests. The concentrations are as calculated with the ELS-OMEGA model (Chapter 4) and expressed relative to the concentration at 6 dpf. Pollutants with a log $K_{ow}$ of 6 and above are hardly excreted during the utilisation of the yolk lipids resulting in an exposure peak at the end of the yolk-sac stage. As a result the threshold level for toxicity can then be exceeded which can explain the timing of the observed mortality a few hours to days later (depending on the exposure concentration). Interestingly, similar timing of effects occurred after exposure to MTCS for which the ELS-OMEGA model (Figure 8.1) predicts no peak in toxicity at the end of the yolk-sac stage as this substance has a log $K_{ow} < 5.5$ (Valo and Salkinojasalonen, 1986). This suggests that MTCS may have some interaction with tissue proteins thus reducing the passive diffusion based on lipophilicity. For TCS it has been shown that it blocks the cellular efflux pumps (Anselmo et al., 2012b), thereby
enhancing internal concentrations. It is not yet known whether the TCS metabolite MTCS also exerts this mechanism.

In all our tests the effects became more pronounced with longer observation time. This continued until the onset of the metamorphosis, days after the exposure had stopped. Early test termination would thus result in an underestimation of the impact of the exposure because the longer observation period results in lower effect concentrations. The observation period needed can be determined based on knowledge of the basic biology of the test organisms. In this case the substantial ecophysiological changes they undergo during the early life stage development resulting in bio-amplification of the tissue concentrations. The ideal ecotoxicological test setup should therefore not be defined in time, but be terminated after the main developments have ‘stabilised’.

For practical and ethical reasons standard ELS-test protocols are in general restricted in observation time (Figure 8.2). For instance, the critical moment in the p-ELS test is not included in short term fish early life stage tests that for
practical reasons are terminated before the larvae become free feeding (OECD, 1998), or even before hatching (OECD, 2006). As this risk is recognised it is advised to perform what is called a full early-life-stage test (OECD guideline 210) when substances with log $K_{ow} > 4$ are being tested (OECD, 1998). However, OECD guideline 210, and the comparable EPA guideline OPPTS 850.1400 (USEPA, 1996) prescribe that this test continuous at least until the control fish are free feeding. Although in both guidelines recommendations are made for longer observation, this still leaves possibilities that delayed observable effects will be missed, and the toxicity of a substance is underestimated.

Insight in the mechanisms that determine the fate of the test compound in the tissue of the exposed organism through modelling (Chapter 4) helps to determine the minimal test duration based on the known characteristics of the substances to be tested. When mixtures with unknown composition are tested, like industrial effluents or extracts of tissues or contaminated sediments (Hallare et al., 2005; Rocha et al., 2011; Zielke et al., 2011) the observation period should best last until metamorphosis, to prevent underestimation of the risk posed by the most lipophilic substances (Log $K_{ow} > 6$).

8.2.2 (No) effects on thyroid hormone dependent metamorphosis

When starting this research it was expected that exposure to compounds like HBCD, PBDEs, and MTCS that are known for their potential to affect the thyroid hormone system (Anselmo et al., 2012a; Hinther et al., 2011; Legler, 2008; Palace et al., 2010; Yu et al., 2011), would result in disturbance of the sole's metamorphosis. It is thyroid hormone mediated (Klaren et al., 2008), and has been shown to respond on manipulated thyroid hormone levels (Okada et al., 2005; Tagawa and Aritaki, 2005). However, none of the tests showed dose related effects on the onset or duration of the metamorphosis as has been shown for frogs or sea urchins (Gutleb et al., 1999; Anselmo et al., 2012a). Measurements of PCBs as model compounds and calculations with the ELS-OMEGA model revealed that at the onset of metamorphosis residue tissue concentrations only are a fraction of what they were earlier (Figure 8.1). Apparently, tissue concentrations were too low at the moment of metamorphoses to disturb the process.

It is likely that pollutants like PBDEs and MTCS that affect the thyroid mediated metamorphosis in other species under continuous exposure (Anselmo et al., 2012a), are also able to disturb the metamorphoses of flatfish when tissue concentrations are above a critical level when the organism becomes competent to start the metamorphosis. This tissue concentration must then be supported by external exposure. Maternal transfer of these pollutants on its own can never
result in such tissue concentrations during the metamorphosis without causing mortality in an earlier stage of development, as was shown by the results presented in Chapter 5, and indicated by the calculations with the ELS-OMEGA model. In field situation, external additional exposure after hatching could occur when larvae develop in an area with contaminated food.

8.2.3 Dioxin-like toxicity

The sole larvae appeared relatively sensitive for dioxin-like toxicity. Compared to the dataset compiled by (Steevens et al., 2005) consisting of 13 records covering 10 fish species with internal LC50 (ILC50) values ranging from 0.5 to 150 ng TEQ/g lw in eggs, sole belongs to the more sensitive species (ILC50 1 ng TEQ/g lw; Chapter 2).

A comparison of ELS test results did not reveal significant differences between TCCD ILC50 values for trout eggs that were exposed via the water phase, by nano-injection, or eggs that were produced by mother fish that were exposed during more than three months before spawning (Walker and Peterson, 1994; Johnson et al., 1998). This indicates that the TCDD exposure of the mother fish did not add to the effect of the TCDD that was deposited in the eggs. For various reasons this is not remarkable. First TCDD is hardly metabolised, which excludes parental transfer of metabolites as a factor. Due to its high teratogenicity, the direct effects on the early life stages overshadow the more subtle effects that could be caused by an affected parental condition. And finally, the effects of TCDD occur at a moment far after the tissue concentration via egg exposure is build up. This implies that the high comparability between the results of test with water exposed eggs and eggs from exposed parent fish that were found for TCDD does not necessarily apply for other pollutants.

It cannot be excluded that TCDD has an impact through epigenetic effects (King Heiden et al., 2009). However, the observation periods in the tests mentioned above (Figure 8.2), as well as in the p-ELS test were too short to be able to reveal these.

8.2.4 Baseline toxicity

Although it is difficult to separate the contribution of dioxin-like toxicity and baseline toxicity in the mortality caused by the PCBs and the mixtures, the data suggests that the sole p-ELS test is also relatively sensitive for base-line toxicity compared to other fish tests not restricted to early life stages (4 mmol/kg lw (Chapter 5) vs 36-300 mmol/kg lw (Kipka and Di Toro, 2009). This could indicate a special sensitivity to this type of toxicity for fish early life stages. This might be explained by the fast depletion of neutral lipids during the yolk-sac
stage as energy source (Kamler, 2008), compared to the polar lipids that have a more structural function, for instance in cell membranes. Upon lipid mobilization neutral lipids were depleted three times faster than polar lipids in larvae of the Senegale Sole (Solea senegalensis; Mourente and Vazquez, 1996). This will result in bioamplification of the lipophilic pollutants in the cell membrane lipids that are the target lipids for baseline toxicity (Escher and Hermens, 2002; Escher and Schwarzenbach, 2002). Although it does not show when tissue concentrations are normalised on total lipid content, the relative stronger concentration of lipophilic pollutants in the cell membranes could further enhance the impact of the toxicity peak at the end of the yolk-sac stage. Indeed, it has been recognised that models that in general are able to predict critical body burdens for a variety of aquatic species within acceptable ranges (Kipka and Di Toro, 2009), tend to underestimate the sensitivity of fish early life stages (Barron et al., 2004). The presence of specific toxic effects (Barron et al., 2004), or biotransformation of the mother compounds (Mathew et al., 2008) are mentioned as explanation for the deviation of the model prediction and experimental results. In both these studies that were performed with crude oils and early life stages of pacific herring (Carls et al., 1999) and pink salmon (Heintz et al., 1999), tissue concentrations were expressed on wet weight basis, and changes in the lipid content of the developing larvae were not determined. Also in the model calculations (Kipka and Di Toro, 2009) tissue lipid concentrations are assumed to be stable during larval development. Hence, although the presented explanations can very well be valid for a complex mixture as oil, it is likely that the changing lipid content and lipid composition that occurs during yolk absorption forms an additional factor explaining the relatively high sensitivity of fish early life stages to organic lipophilic compounds.

8.3 Simulation of maternal transfer in the p-ELS fish test

The p-ELS test procedure developed for this research intends to mimic, to a certain extent, maternal transfer of lipophilic POPs in order to study their effects on the development of an embryo into a juvenile fish. The metamorphosis into a juvenile fish was included in the test design as this process is thyroid hormone regulated and therefore potentially sensitive to thyroid hormone disrupting compounds (Okada et al., 2005; Tagawa and Aritaki, 2005).

The p-ELS revealed toxic effects that would have been missed with more standard ELS tests observing until free feeding. However, notwithstanding its advantages, also the p-ELS test cannot fully cover all effects of maternally transferred POPs on developing larvae.
The most reliable way to study the impact of maternally transferred pollutants on the developing offspring is by exposure of the parent fish; long term when effects on egg quality are to be included. This however, was not feasible given the resources available for this research. As alternative the possibilities of in-ovo injection (González-Doncel et al., 2003; Gonzalez-Doncel et al., 2003) were explored. But this extremely labour intensive procedure resulted in too high embryo mortality, probably related to a combination of the small egg size (max. 1 mm) and the very resistant chorion. Therefore it was decided to expose the eggs via the water until the moment of hatching, realising that this implied that the exposure situation in the test differs from real maternal exposure at least with respect to:

- The time needed to reach the desired internal POP concentrations in the egg.
- The absence of parental POP metabolites.
- The absence of impact of parental condition on egg quality.
- Non detectable epigenetic effects

These topics and their implications for the results of the p-ELS test are briefly discussed below.

8.3.1 Time needed to reach the desired internal POP concentrations in the egg

Figure 8.2 schematically shows the development of the lipid normalised POP concentration in an adult fish, in the produced eggs, and in the subsequent life stages. It is assumed that the POP concentration is transferred 1:1 from the mother’s tissue to the eggs (Russell et al., 1999). As can be seen the concentration peaks at the end of the yolk-sac stage, then declines due to growth dilution when the larva starts external feeding (Chapter 4). Finally the POP concentration gradually increases due to bioaccumulation/-magnification as the fish grows older.

Eggs that are produced by exposed female fish contain the POPs already before spawning and fertilisation. The p-ELS test starts with clean eggs, in which POP concentrations only start to build up after fertilisation. Exposure of early embryonic stages is thus low in the water exposed eggs compared to eggs produced by exposed parents. Hence, effects on fertilisation or very early embryonic development are not included in the p-ELS test.
During waterborne exposure the embryo is protected to a certain extent by the presence of the chorion, which limits exchange with the environment and so reduces the exposure of the embryo (González-Doncel et al., 2003; Gonzalez-Doncel et al., 2003). This could be overcome by enzymatic dechorionation of the eggs (Mizell and Romig, 1997; Usenko et al., 2011). Comparative studies show effects at lower water concentrations for dechorionated eggs (Villalobos et al., 2000), or for eggs that were exposed before fertilisation and consequent chorion hardening (González-Doncel et al., 2003; Gonzalez-Doncel et al., 2003). In our test set-up, effect concentrations were based on chemical analyses of the larvae immediately after hatching. The impact of the protective chorion thus did not affect the effect concentrations that were expressed as tissue concentration of the embryo.

Waterborne exposure resulted in a clear dose related larval accumulation of the lipophilic pollutants that were subject of the experiments presented in this thesis, MTCS, PCBs, PBDEs, and HBCDs. However, we have clear indications that
waterborne exposure of eggs with intact chorion does not work with surface 
active compounds. Despite extremely high exposure levels of 
perfluorooctanesulfonate (PFOS) during the egg stage, no effects on the 
developing sole larvae were recorded (unpublished data), while in a study with 
parental exposure to the same compound clear effects on the developing 
offspring of zebrafish were observed (Wang et al., 2011). It is to be expected 
that the surface active PFOS concentrates on/in the surface of the egg shell 
during exposure without penetrating the chorion. For this type of pollutants 
actual maternal transfer is necessary to deliver it inside the egg.

8.3.2 Absence of parental POP metabolites

In the p-ELS test eggs were exposed only to the parent compound. With this, it 
is ignored that some POPs can be transformed by fish into often more toxic 
metabolites that will also be deposited in the eggs bound to the deposited 
proteins. This is the case for halogenated phenolic compounds (HPCs) including 
the metabolites of PCBs (OH-PCBs) and PBDEs (OH-PBDEs). These metabolites 
are present in plasma of many organisms, including fish, at concentrations that 
exceed no effect levels in in-vitro assays (Montano et al., 2013). HPCs show a 
structural resemblance to thyroid hormones, and are in this way actively 
transported to the eggs. There, OH-PBDEs can induce developmental arrest in a 
concentration-dependent manner as was shown after exposure of dechorinated 
eggs of zebra fish (Usenko et al., 2012). Maternal transfer will probably be an 
important exposure route for these metabolites.

Juveniles of the common sole have been shown to form hydroxylated 
metabolites after PBDE exposure (Munschý et al., 2010), it is not yet known in 
what phase of development this ability occurs. Exposure of zebrafish embryos to 
PBDE47 did not result in formation of OH-PBDE47 (Zheng et al., 2012). Although 
species specific differences cannot be excluded (Roberts et al., 2011), these 
results might indicate that the ability for hydroxylation in fish develops 
somewhere between hatching and the juvenile stage. As biotransformation is a 
mechanism to protect an organism against the accumulation of poorly water 
soluble (natural) substances in their diet, it seems reasonable to assume that 
this ability will develop when the larvae starts external feeding. In the natural 
situation hydroxylated POPs are already present in the egg before fertilisation 
via maternal transfer (Figure 8.2, dotted line). In the p-ELS test, the metabolites 
only can be present after the larva itself has developed the capability for 
biotransformation of the mother compound, thus during the free-feeding stage. 
As a consequence the p-ELS test underestimates the potential impact of these 
metabolites compared to the natural situation.
8.3.3 Impact of parental condition on egg quality

Thyroid hormones (T3 and T4) are deposited in eggs by the mother fish (Tagawa et al., 1990; Yamano, 2005). Altered thyroid hormone levels in parent fish can be reflected in the eggs, as shown after parental exposure of zebra fish with PBDEs (Yu et al., 2011). Since thyroid hormones are deposited in considerable amounts in fish eggs a critical role in early development of the embryo can be expected (Tagawa et al., 1990). Administration of thyroid hormones to eggs or newly hatched larvae sometimes results in better survival and development (Yamano, 2005). The effects of hydroxylated POP metabolites are mostly related to the blocking of the TH transport proteins and blocking or activating TH receptors (Montano et al., 2013). Lowering of TH levels will only become a problem when the reduction is extreme, as otherwise the strong feedback mechanisms in the TH homeostasis is able to compensate. For example in medaka eggs that contained only 10% of the normal total TH levels due to chemical repression of the thyroid hormone production by the mother fish, no effects on hatching, survival and development was observed (Tagawa and Hirano, 1991). The level of the active thyroid hormone (T3) probably still was high enough, but at that time this could not be analysed separately.

Finally, impact of exposure of parents on ovary histology (Daouk et al., 2011; Mochida et al., 2010), sperm quality (King Heiden et al., 2009; Dietrich et al., 2010) or behaviour that could affect reproduction success are not included in our p-ELS test set-up.

8.3.4 Not detectable epigenetic effects

Toxic compounds can induce effects that only become visible later in life, e.g. when certain functions become relevant such as reproduction (Vandegehuchte and Janssen, 2011). Early life exposure even can lead to effects on the next generation as has been shown with zebra fish that were exposed during the first 7 weeks of their life to TCDD (King Heiden et al., 2009). The second generation that could only have been exposed as gametes, showed increased mortality, reduced egg production, and reduced fertility. This suggests an transgenerational epigenetic effect (Youngson and Whitelaw, 2008) of the parental exposure. Epigenetic effects are the result of changes in gene function without changing the DNA sequence, with DNA methylation as the most studied phenomenon. Evidence for epigenetic effects on among others fish, has been reported for pollutants as Benzo(a)pyrene, mercury, tributyltin, triphenyltin, zinc, cadmium, oestradiol and HBCD (reviewed in Vandegehuchte and Janssen, 2011. In some cases these alterations have been shown to be inheritable to the next generation (Vandegehuchte et al., 2010). In these situations the overall
impact of such a pollutant would be underestimated when based only on observation of development until metamorphosis of larvae exposed as eggs.

Some situations are described where chronic exposure has resulted in resistance of a population for a specific stressor. This was shown for fish populations from field locations with high concentrations of Ah-receptor agonists such as dioxin-like PCB and PAHs. Apparently the negative effects from these AhR active compounds were so strong that there was a fitness/evolutionary advantage for losing the activity of the physiologically relevant AhR. The developed resistance to the effects of these pollutants was genetic and transmitted to at least the F2 generation (Wirgin and Waldman, 2004; Wirgin et al., 2011).

8.4 Characteristics that determine sensitivity

Specific characteristics can make a species more vulnerable to the effect of maternally transferred POPs than others that share the same environment. This is the resultant of differences in toxicokinetic, toxicodynamic and population dynamic characteristics. The following paragraphs discuss these characteristics, illustrated with some examples.

8.4.1 Toxicokinetic and toxicodynamic characteristics

Due to biomagnification maximum POP concentrations are to be expected in the tissue of top predators and long living species (Moore et al., 2002), this implies that female top predatory fish deposit the highest POP loads in their eggs. As lipid normalised concentrations of POPs in the tissue of the mother fish will in general be comparable with her eggs (Chapter 5; Russell et al., 1999), it is the lipid normalised POP concentration that determines the exposure of the eggs. This means that lipid-rich fish species that have high POP-levels per g of fillet (as is relevant for human consumption fish) could transfer lower POP loads to their eggs than lean fish (such as e.g. cod) with lower POP levels per g of fillet but high levels per g of lipid.

Some fish species like eels and salmon undertake a spawning migration, during which they cease feeding. As the fish loses weight during migration, POPs accumulated in the tissue will concentrate. Bio-amplification factors of 2 to 8 in gonads of pacific salmon (Debruyn et al., 2004), and of 2 to 5 for sockeye salmon (Ewald et al., 1998) have been established. Compared to these values, the amplification factor calculated for European eel of 1.3 to 2 (Chapter 6) is relatively low. This is related to the rather unique fact that the tissue composition of the eel does not change during migration. Lipids, protein and carbohydrates are utilised as energy source in the same proportion as they are
present in the tissue (van Ginneken et al., 2005). Similar use of bodily tissues during migration has been reported for some reef fish. But in most fish species proteins are conserved, resulting in a clear decrease of the lipid content of the tissue (Stobutzki, 1997). In all cases migration can be expected to result in higher POP concentrations in the eggs.

Once the POPs have been deposited in the egg, they can affect the developing larvae. The sensitivity of the larvae, and thus the impact of the transferred POPs can vary between species. This is due to differences in biochemistry and (eco)physiology between species. It is this interspecies variation that forms the bases of the species sensitivity distribution (SSD) theory that is applied to estimate the critical concentration of a toxicant for causing effects on the community level (Posthuma et al., 2010). Normally these SSDs are based on external exposure concentrations (eg. Selck et al., 2002; Smit et al., 2009). This implies that interspecies variation in these datasets is not only determined by variation in the toxicological response of the organism once the toxicant reaches the target organs, but also by variation in internal exposure that can depend on the biological/physiological characteristics of the species. By expressing the effects on internal tissue concentrations during the exposure peak, the latter source of variation is excluded. In the SSD as produced by Steevens et al. (2005) the concentration in the yolk was used as measure of exposure, as was the case in this thesis when determining the critical tissue concentration of TCDD for fish early life stages (Chapter 6). Still, the variation in sensitivity between species (expressed as ILC50) ranges from 0.5 to 150 ng TCDD/g lw. Although already expressed on lipid bases, the effect concentrations shows a strong correlation (p<0.0001) with the egg’s lipid content (Figure 8.3). The lower effect concentrations of larvae from eggs with higher lipid content is likely to be related with stronger bio-amplification of the TCDD at the end of the yolk-sac stage (Chapter 4; Daley et al., 2012) when the majority of the neutral lipids is used as energy source. The larvae that hatch from eggs with high lipid content can thus be considered more sensitive, as they are higher exposed, to the impact of maternally transferred lipophilic POPs. It is interesting to note that zebra fish, a standard species in fish ELS tests, shows the lowest sensitivity to TCDD in this dataset (Elonen et al., 1998; Steevens et al., 2005), which is in accordance with the lowest lipid content (1.7%) for zebra fish of all the eggs included.
Finally, as shown by the modelled scenarios in Chapter 4, also the environment where spawning and larval development take place influences the height of the toxicity peak that will develop, as well as the duration and rate of exposure. When spawned in a pristine environment the concentration of less lipophilic POPs (log $K_{ow}$<5) rapidly decreases after hatching, and once the larvae reach the free feeding phase growth dilution will reduce the tissue concentration of more lipophilic POPs (log $K_{ow}$>5). POP exposures will be more persistent in the tissue of the larvae when development takes place in a contaminated environment and exposure through food continuous.

8.4.2 Population dynamic characteristics

In Chapter 7, factors determining the sensitivity of a fish population to the effects that could be caused by maternally transferred POPs are explored with the common sole as an example. Sole turned out to be quite insensitive to reduced larval survival by the combination of a high fecundity and substantial density dependent mortality of early life stages, despite the relatively sensitivity of the larvae.

Not surprisingly, population development of species with a low fecundity turned out to be most sensitive to increased ELS mortality. Further, if density dependent mortality would occur before the mortality due to maternally transferred substances, this also would make a population more sensitive.
Maternally transferred lipophilic POPs however as we tested, resulted in larval mortality in an early stage of the development, and thus before most density dependent mechanisms become effective, that are often related to increased predation pressure or competition for food and/or space.

8.5 Identification of vulnerable species

With the insight in the critical characteristics presented above it is possible to predict the vulnerability of species for the effects of maternally transferred POPs.

The most vulnerable species combine:

1. A position high in the food chain or a narrow food choice of relatively highly exposed food items facilitating biomagnification of POPs.
2. A period of starvation prior to spawning, for instance during spawning migration, resulting in bio-amplification of accumulated POPs.
3. Eggs with high lipid content, resulting in strong bio-amplification of POPs at the end of the yolk-sac stage.
4. A low fecundity.
5. Density dependent mortality or concentration of early life stages after increased ELS mortality due to maternally transferred substances.

As an example, some of these characteristics are listed in Table 7.1 for selected marine fish species. This is not an exhaustive overview but an impression based on readily available data on fecundity of fish species collected by Mertz and Myers (1996). Only a limited number of orders are included, sometimes with only a single species per order. The dataset, for instance does not include large predatory fish like tuna and swordfish, known for high bioaccumulation of POPs (Kannan et al., 2002; Storelli et al., 2008).

Within this dataset the lowest fecundity is found in the order of Squaliformes (sharks). In combination with a diet that mainly consists of fish and higher organisms this makes this order theoretically vulnerable for effects of maternally transferred POPs. Salmonids also have a low fecundity. As facultative predators, their place in the food chain is in general lower than sharks, but many salmonid species undertake a spawning migration without feeding. The only other order in this dataset that undertakes such a migration is the Anguilliformes. The estimation of the vulnerability on the individual level is described for the European eel in Chapter 6. In the following paragraphs an attempt is made to determine the sensitivity for effects on a population level of this species, as well as for salmonids and sharks.
Table 8.1 Some characteristics that determine the vulnerability of fish species for the effects of maternally transferred POPs. Presented are the range (min-max) in fecundity based on the available data for that order, the species with the lowest fecundity per order, the main diet of that species and whether this species undertakes spawning migration without feeding. Fecundity data from Mertz and Myers (1996) and expressed per individual fish year. Data for Anguilliformes were taken from MacNamara and McCarthy (2012), as fecundity per fish and divided by 10 in order to calculate fecundity per year assuming a 10 year lifespan for eels. The fecundity data for Lophiiformes and Squaliformes consisted only of a single species. Diet information from sources as indicated.

<table>
<thead>
<tr>
<th>Order</th>
<th>range fecundity $x10^3$/ind/yr</th>
<th>Species with lowest fecundity</th>
<th>Diet</th>
<th>Migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lophiiformes</td>
<td>1320</td>
<td>Lophius piscatorius (Monkfish)</td>
<td>Fish (Laurenson and Priede, 2005)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Benthos polychaetes (Schuckel et al., 2012)</td>
<td>No</td>
</tr>
<tr>
<td>Pleuronectiformes</td>
<td>80-2500</td>
<td>Pleuronectes platessa (Plaice)</td>
<td>Fish (Helser and Alade, 2012)</td>
<td>No</td>
</tr>
<tr>
<td>Gadiformes</td>
<td>79-9500</td>
<td>Merluccius productus (Pacific hake)</td>
<td>Opportunistic macrofauna – fish</td>
<td>No</td>
</tr>
<tr>
<td>Anguilliformes</td>
<td>70-3480</td>
<td>Anguilla anguilla (European eel)</td>
<td>Zooplankton (Bacha and Amara, 2012)</td>
<td>Yes</td>
</tr>
<tr>
<td>Clupeiformes</td>
<td>25-335</td>
<td>Engraulis sp (Anchovy)</td>
<td>Zooplankton (Eliasen et al., 2011)</td>
<td>No</td>
</tr>
<tr>
<td>Perciformes</td>
<td>15-1000</td>
<td>Ammodytes marinus (Sandeel)</td>
<td>Opportunistic zooplankton- fish</td>
<td>No</td>
</tr>
<tr>
<td>Salmoniformes</td>
<td>1.5-40</td>
<td>Oncorhynchus gorbuscha (Pink salmon)</td>
<td>Nielsen, 2004</td>
<td>Yes</td>
</tr>
<tr>
<td>Squaliformes</td>
<td>0.003</td>
<td>Squalus acantbias (Spiny dogfish)</td>
<td>Fish (Dunn et al., 2013)</td>
<td>No</td>
</tr>
</tbody>
</table>

8.5.1 European eel

The European eel (*Anguilla Anguilla*) is often mentioned as a species sensitive to the effect of organic contaminants (Belpaire et al., 2009; Clevestam et al., 2011; Geeraerts and Belpaire, 2010; Geeraerts et al., 2011; Palstra et al., 2006; Robinet and Feunteun, 2002; van Ginneken et al., 2009). This is primarily based on the relatively high contents of POPs that can be detected in the lipid rich tissue of the eel in comparison with other species from the same area, and the typical life history making this species potentially vulnerable to accumulating substances. For this reason an estimation of the chance that larvae of the European eel will be negatively affected by maternally transferred POPs was performed in Chapter 6. We assumed a high sensitivity of the larvae to dioxin like compounds based on a high lipid content of the eggs, and calculated a reasonable amplification of the accumulated POPs during spawning migration.
The results indicate that 50% larval mortality is likely for eel from highly contaminated locations, due to maternally transferred dioxin-like pollutants (Chapter 6).

Since only limited knowledge is available about the reproductive biology of the European eel it is unclear to what extent the eel population is able to compensate for increased larval mortality. Eels have a very high fecundity ranging from 1600-3600 eggs/g body weight (MacNamara and McCarthy, 2012). However, it must be taken into account that eel most likely is a semelparous species, indicating that it only spawns once in a lifetime (Palstra and van den Thillart, 2010). Assuming an average life span of 10 year, the fecundity ranges between 160 and 360 eggs/g body weight per year. This is lower than for instance sole, that as fish with an iteroparous life strategy produces very year about 800 eggs/g body weight (Rijnsdorp et al., 1992).

Based on modelling studies it is estimated that it will take the larvae 1 to 3 years to reach the European coast (Righton et al., 2012). High mortality, due to natural causes can be expected to occur during this migration. Whether also density related mortality occurs is unclear. It seems reasonable to assume that some concentration of the glass eels occurs when they leave the ‘infinite’ ocean to enter the fresh water environment. This concentration thus takes place long after the moment that larval mortality due to maternal transferred POPs would occur. The relative high fecundity in combination with concentration of early life stages may result in not too high vulnerability of the population development for reduced larval survival. But whether in addition to high glass eel fishing mortality (Dekker, 2004) also density dependent natural mortality occurs is unknown.

Chapter 6 indicated that a strong negative impact of maternally transferred dioxin-like POPs can be expected for eels from highly contaminated locations. Effects of other pollutants like polyfluorinated compounds (PFCs), and halogenated phenolic compounds (HPCs) were not included in this study as they have completely different toxicokinetics than the lipophilic POPs. PFCs however, have been detected in high concentrations in eel tissue (Kwadijk et al., 2010), and can cause effects by maternal transfer (Wang et al., 2011). HPCs including OH-PCBs and OH-PBDEs have been identified in the blood of fish at levels that exceed effect concentrations in in-vitro assays, and can also be maternally transferred (Montano et al., 2013 in press)). In addition, early life exposure to dioxins could result in transgenerational epigenetic effects (King Heiden et al., 2009). Finally, toxic substances can have adverse effects on juvenile and adult fish, especially eel migration and reproduction could be negatively affected by for reduced fitness of the parent fish (Belpaire et al., 2009; Cleestam et al.,
The European eel that inhabits the European and North African waters form one population that all share the same spawning grounds (Als et al., 2011). As indicated in Chapter 6, a part of this population probably experiences reduced larval mortality due to maternally transferred dioxin-like POPs, but the exact proportion of the impact of these and other pollutants on the population remains unclear. It is therefore uncertain whether pollutants contribute to the decline of the eel population that has been observed since the 1980 (Dekker, 2003).

8.5.2 Salmonids

The impact of POPs on the condition and reproduction of salmonids has been studied for a long time. In the past the majority of these studies focussed on the North American Great Lakes and indications were found for reduced reproduction as a consequence of bioaccumulated dioxin-like POPs (Giesy et al., 2002).

A substantial part of the salmonid species is anadromous, thus migrate from salt to freshwater to spawn. The Atlantic salmon (Salmo salar) returns to sea after spawning, but all pacific salmon species (Oncorhynchus sp.) are semelparous and die at the spawning grounds. As in this semelparous life strategy it is not essential to save energy for the migration back to sea, energy and thus lipid resources are strongly depleted when spawning grounds are reached (Wootton, 1992). This results in relatively high bio-amplification factors in the fish. For sockeye salmon (Oncorhynchus nerka) reported bio-amplification factors range from 2 to 12 (Ewald et al., 1998; Debruyn et al., 2004; Kelly et al., 2007) which correlates positively with the migration distance (Debruyn et al., 2004; Kelly et al., 2007). Although it is expected that in an equilibrium situation the lipid normalised concentrations of POPs in tissue and eggs are comparable (Russell et al., 1999), concentrations were 50 to 20% lower in eggs than in tissue of salmon that had reached the spawning grounds. Possibly the redistribution of especially the more lipophilic substances during the migration is not fast enough to reach an equilibrium between bodily tissues including the eggs (Kelly et al., 2007). Hence, a 1 to 1 translation from tissue to egg after spawning migration, most likely overestimates the concentrations in the eggs. Nonetheless chemical analyses revealed that the concentration of dioxin-like pollutants in the gonads of the sockeye salmon still concentrate more than 3 times during migration (Debruyn et al., 2004; Kelly et al., 2007).

Salmonids produce relatively large eggs with high lipid contents, ranging from 6.5 to 8.6% for brook trout, lake trout, rainbow trout and Chinook salmon...
Eggs posed Chapter 8

(Johnson et al., 1998; Walker and Peterson, 1994; Ballestrazzi et al., 2003; Daley et al., 2012). These large lipid rich eggs supply the developing embryos during the long egg and yolk-sac stages that can last up to several months (Milner et al., 2003). Especially after hatching, yolk lipids are applied as energy source for the developing larvae. Eggs with an initial lipid content of 8.6% produced larvae with a lipid content of 1.2% at the end of their yolk-sac stage 168 days post fertilisation. During this period POPs with log Kow >5.8 showed a 5 fold bio-amplification (Daley et al., 2012). Since dioxin-like POPs in fish all have a log Kow above 5.8, the lipid normalised concentrations of dioxin-like compounds at the end of the yolk-sac stage can be more than 15 times higher than in the maternal concentration at the start of the spawning migration. Measured concentrations in roe from salmon returning to the Frazer river in some cases exceeded the threshold level above which salmonid larval mortality can be expected (Kelly et al., 2011).

Salmonids have a relatively low fecundity (Table 7.1) which makes them potentially sensitive for increased larval mortality. The populations are regulated by density-dependent mortality that takes place typically during the early stages of free-feeding life after fry emerge from spawning gravels (Elliott, 1989) and display aggressive, territorial behaviour (Milner et al., 2003). Larval mortality due to maternally transferred POPs would occur during the same period, thus reducing the pressure of competition for space. It is to be expected that in a healthy population a surplus of larvae is produced so larval mortality due to toxic compounds will mostly relieve the competition. However, when salmon stocks are minimised due to overexploitation or migration barriers the impact of reduced larval mortality may become relevant.

8.5.3 Greenland shark, the worst case scenario?

The highest levels of dioxin-like pollutants in tissues of shark species were reported for the Greenland shark (Somniosus microcephalus) and ranged from 460 to 579 pg TEQ/g lw based on the TEF system for fish (Storelli et al., 2011). These tissue concentrations in adult fish fall within the range where at least 50% larval mortality may be expected for 5 to 10% of all teleost fish species (Chapter 6). No other fish species from the Atlantic ocean (17-88 pg TEQ/g lw), other top predator fish like swordfish (9 pg/g lw) or dolphin species from the Mediterranean (42-270 pg TEQ/g lw) have such high tissue concentrations of dioxin-like pollutants (Storelli et al., 2011).

Also other POP concentrations are extremely high in Greenland shark tissue. PCB concentrations in liver and muscle have been reported to range from 990 to 10 000 ng/g lw (Strid et al., 2007), while mean concentrations of DDT and
chlordane pesticides in livers of 7159 and 1815 ng/g lw respectively have been reported (Fisk et al., 2002). Sum PBDE concentrations range between 7-200 ng/g lipid, with means of 35 and 41 ng/g lw for liver and muscle respectively (Strid et al., 2010). The POP concentrations in Greenland sharks are amongst the highest reported for an Arctic fish and are in the range of top Arctic marine predators (MacNeil et al., 2012). Lipid normalised concentrations of PCBs, DDT and other chlordane pesticides in plasma of Greenland sharks were even higher than the values reported for plasma of polar bears and white whales from the same region (Svalbard, Norway; Molde et al., 2013). It reflects the diet of the Greenland shark that for substantial part consists of marine mammals (Yano et al., 2007; Fisk et al., 2002; Leclerc et al., 2012).

Relatively little is known about the biology of Greenland sharks (Leclerc et al., 2012). The species is found from the North Atlantic Ocean to the Arctic Ocean, but given their tolerance for extremely cold water (up to -2ºC) and great water depths (>2000 m) the distribution range is potentially unlimited throughout the deep sea (MacNeil et al., 2012). Greenland sharks are considered to have a low growth rate, and females mature only at a length above 450 cm (Yano et al., 2007). As many shark species, Greenland sharks are ovoviviparous; the eggs are fertilised and hatch inside the female’s body. After absorption of the yolk-sac, the young are believed to feed on non-developed eggs and each other. In one bearing up to 10 young are born (Yano et al., 2007) as fully developed juveniles with a length probably ranging from 35 to 100 cm (MacNeil et al., 2012).

As the developing embryo/larva rely on their yolk for resources, the development of a toxic peak at the end of the yolk-sac stage is likely. Unlike oviparous fish, the shark eggs/larvae do not develop in relatively clean seawater. Hence, passive excretion that would prevent the development of a toxic peak for POPs with log Kow <6, is much lower for the ovo-vivi-parous sharks than for ovo-parous fish. Not only will this result in a higher bio-amplification factor at the end of the yolk-sac stage, but in addition it is likely that brominated flame retardants like HBCD and PBDEs with the potential to disrupt the thyroid hormone system maintain relatively high levels during the full early life development, whereas in e.g. sole larvae the concentrations of these substances rapidly decline. In addition, after yolk absorption the young sharks do not feed on less polluted zooplankton as most other fish, but first use undeveloped eggs and each other as food source. Given the tissue concentrations of the Greenland shark this must be the most contaminated food source that can be found.
The additional impact of these adverse ecophysiological parameters on the development and survival of the young is not known, but it adds to the estimated 50% larval mortality from the dioxin-like toxicants only, as explained above. Here we assume that the shark embryos have a comparable sensitivity for dioxin-like compounds as other fish species. However, being a member of another infraclass of fish, the *Chondrichthyes*, for which the effects of dioxin-like compounds on the early life development has not been studied yet, it is not known how the sensitivity relates to that of the well-studied fish species. There are indications that the vitamin A and E systems of Greenland sharks are affected by high levels of POPs (Molde *et al.*, 2013), which could result in reduced quality of the eggs, and thus add additional pressure on the developing larvae.

As Greenland sharks still are relatively abundant (Leclerc *et al.*, 2012) it is apparently able to compensate for this toxic pressure. Favourable is that the species experiences relatively little fishing pressure, as the flesh is toxic for mammals for reasons that are not completely clear, but are most likely related to specific natural metabolites (MacNeil *et al.*, 2012). Only for the production of oil from the sharks liver some extensive fisheries took place during the first half of the 20th century. Nowadays fishing mortality is limited to accidental by-catches. There is, however, concern about the impact of global warming for this cold water species. Even if the warming of their habitat does not directly affect the physiology of the species, it could lead to increasing fishing pressure with the retraction of the ice cover resulting in northward migration of commercial fish stocks. With its slow growing life-history, *S. microcephalus* may not be able to cope with increasing losses through by-catches (MacNeil *et al.*, 2012) in combination with reduced survival of early life stages due to the high exposure to POPs.

### 8.6 Conclusions

To conclude the answers to the research questions posed in Chapter 1 are briefly summarised below.

1. What are critical egg concentrations for PCBs, PBDEs, HBCDs and MTCS for larval development, and how do these relate to field (fish) concentrations?

Critical egg concentrations were determined for all substances except for HBCD that did not cause observable effects in our test. With exception of dioxin like substances at highly contaminated locations, the reported tissue concentrations in fish are lower than the critical effect concentrations for larval survival.
2. Do the above mentioned (groups of) substances represent the major risk in case of maternal transfer, or are there indications for substantial impact of other (unknown) substances?

The highest risk is revealed for dioxin-like substances and very lipophilic substances that can induce narcosis. No indications were found that other lipophilic POPs extracted from Dutch sole substantially contribute to the reduced larval survival due to maternally transferred POPs.

3. What is the influence of ecophysiological characteristics on the impact of maternally accumulated compounds transferred to the eggs on the developing larvae?

Both spawning migration and high lipid content of eggs contribute to bio-amplification of lipophilic compounds to levels in the larvae that are higher than what they will ever reach during adult life. Through depletion of the yolk lipids maternally transferred lipophilic POPs with a log $K_{ow}$ around 6 and above concentrate in the remaining lipids, resulting to higher available concentrations and higher concentrations in cell membranes. As a result larval mortality starts at the end of the yolk sac stage and commences until the onset of metamorphosis. Due to growth dilution and passive excretion concentrations of maternally transferred substances have then reached such low tissue concentrations that the metamorphosis is not affected.

4. To what extent can the larval mortality from maternally transferred toxicants affect population development, possibly in combination with high fishing pressure?

The combination of high fecundity and density dependent survival of early life stages make most fish species capable to compensate for large variations in early life mortality. Since the effects of the maternally transferred substances studied occur in a stage when these density dependent mechanisms are not yet acting, the impact is limited, even in combination with extreme fishing pressure. Species with low fecundity and less density dependent regulation of population growth can be considered potentially vulnerable. It cannot be excluded that effects of maternally transferred substances that act at a later moment in life will have more impact on the population development.

8.7 Future perspectives

This thesis revealed the effects of maternally transferred dioxin-like compounds on the survival of fish larvae between hatching and metamorphosis. For some
fish species, a significant effect on larval survival is to be expected. To be able to assess the full risk of POPs accumulated in the tissues of these fish, the effects on other important life cycle parameters need to be assessed. In particular effects on adult survival during spawning migration, and on fertility and egg quality are important to study. Also toxic effects of POPs that were not included in this study because their maternal transfer cannot be mimicked via water exposure deserve further attention. For example PFCs and OH-POPs should either be injected in the developing egg or exposure should take place via the mother animal. Exposure via the mother animal also offers the advantage of including effects on egg quality and on the very early embryonic development in the egg.

Further, it is important to investigate if the larvae that develop without obvious adverse effects in normal juveniles after early life exposure to POPs, do not experience negative effects at a later moment for instance through trans generational epigenetic effects (Vandegehuchte and Janssen, 2011; Wirgin and Waldman, 2004; Youngson and Whitelaw, 2008). To predict such delayed effects biomarker techniques could be applied that already can indicate sub-lethal changes in an early stage. Focus of such studies could be effects that reduce reproductive performance, hormone disruption, behavioural changes (Mennigen et al., 2011; Soffker and Tyler, 2012) or age of maturation (Hutchings et al., 2012).

The sensitivity of sharks, especially the Greenland shark, deserves more attention. Little is known about the sensitivity of early life stages of shark species, and more specifically on ovoviviparous species. Further, the insights gained in the characteristics what makes species vulnerable to maternally transferred substances can be applied to other orders and species that were not included in the exploration presented above. New potentially vulnerable species can be identified and for targeted species, population modelling can be applied to identify the most critical life phase for population development. Given the high potency of most fish populations to compensate for high early life mortality through high fecundity and density dependent survival mechanisms of the early life stages, population development probably will be more affected by toxic substances that impair the condition or reproductive efficiency of mature fish. For such species, it is recommended to focus future research on adult fish, including impact of age of maturation (Hutchings et al., 2012), hormone disruption, behavioural changes (Mennigen et al., 2011; Soffker and Tyler, 2012), and (transgenerational) epigenetic effects (Vandegehuchte and Janssen, 2011; Wirgin and Waldman, 2004; Youngson and Whitelaw, 2008).
Besides toxicants, additional stressors should also be included in the assessment of the sensitivity of species, as an organism will always experience multiple stressors. Relevant additional stressors for fish can for instance be fishing pressure, global warming, ocean acidification, loss of specific habitat like spawning ground or nursery areas, or the ability to reach these, and competition with invasive species may make species more vulnerable to toxicological effects that would be far less relevant in otherwise less impacted conditions.

8.8 References


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Summary

“He stopped for a moment and looked back and saw in the reflection from the street light the great tail of the fish standing up well behind the skiff’s tern.”

Ernest Hemmingway: The Old man and the Sea
Persistent organic pollutants (POP), with well-known representatives as polychlorinated biphenyls (PCBs), dioxins, and brominated flame retardants as polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecanes (HBCD), are still globally present in the marine environment, despite the substantial reduction of application and emission that was achieved during the last decades. Apart from their persistency these compounds share low water solubility and a high lipophilicity which make that the highest concentrations in the aquatic environment are found in the organic matrix of sediments and in biota. Dissolved water concentrations are low. Hence, intake of contaminated food items forms the major source for POPs exposure of aquatic organisms, and through biomagnification the highest concentrations can be found in the tissue of top predators. POPs have the potency to cause a variety of toxic effects, among which endocrine disruption and teratogenic effects that especially apply to early life stages. As the early life stages of most fish species develop suspended in the water column, exposure to POPs may be considered relatively low, at least until the larvae start feeding after yolk absorption. However, POPs accumulated in the tissue of the mother are transferred to the eggs. The research presented in this thesis aims at the determination of the impact of such maternally transferred POPs on development and survival of fish early life stages, in order to assess if this exposure route can significantly impact the development of a fish population at current environmental concentrations, especially in combination with high fishing pressure.

For this purpose a bioassay was developed with the common sole (Solea solea). The advantages for this research of this new bioassay above standard fish early life stage (ELS) tests are that sole is a native West European species that as all flatfishes undergoes an obvious metamorphosis. The test set-up includes this metamorphosis that is thyroid hormone mediated and therefore expected to be easily disrupted by POPs, based on research with amphibians. The prolonged Early Life Stage test (p-ELS) with sole is presented in chapter 2. Early life stages were exposed to a concentration series of the dioxin-like PCB 126 (3,3',4,4',5-pentachlorobiphenyl) in seawater until 4, 8, 10 and 15 days post fertilisation (dpf). Subsequently the development of the larvae was registered under further unexposed conditions. The LC50s at the start of the free-feeding stage (12 dpf) ranged between 39 and 83 ng PCB 126/l depending on exposure duration. After the fish had completed the metamorphosis, the LC50 values ranged between 1.7 and 3.7 ng PCB 126/l for the groups exposed for 4, 8 and 10 dpf respectively. Thus exposure for only 4 days, covering only the egg stage, was sufficient to cause adverse effects during a critical developmental phase two weeks later. This study indicates that ELS fish tests that are terminated shortly after the fish becomes free-feeding underestimate the toxic potential of compounds with low acute toxicity such as PCBs. The internal dosages of these larvae at the end of
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the exposure, determined by means of an in-vitro gene reporter assays as dioxin-equivalent values (TEQ), revealed an internal lethal concentration, ILC50 of 1 ng TEQ/g lipid, which is within the same order of magnitude as TEQ levels found in fish from highly polluted areas. This suggests that larval survival of fish populations at contaminated sites can be affected by persistent compounds that are accumulated by the female fish and passed on to the eggs.

Based on these first results the p-ELS test procedure was improved to reach a better control performance. The exposure period was terminated when all larvae had hatched (6 dpf), this in order to mimic exposure through maternal transfer as good as possible without exposing parent fish or manipulation the eggs. In a second test (Chapter 3) the eggs were exposed to a concentration series of methyltriclosan (MTCS), a metabolite of triclosan (TCS) that is commonly used as bactericide in a wide variety of human care products. MTCS and TCS are discharged with waste water, bioaccumulate in fish tissue, and are known to have the potency to disrupt the thyroid hormone system. Mortality occurred in the higher treatment levels until 20 dpf. Indications for thyroid hormone disruption were not observed; all surviving larvae completed metamorphoses without problems. Internal effect concentrations, reached in larvae at the end of the exposure (6 dpf), were 5.8 mg/g lipid weight (lw) and 2.1 mg/g lw for ILC50 and ILC10 respectively. These internal effect concentrations are at least 200 times higher than concentrations that due to maternally transfer can be expected in the eggs of highly exposed fish in a field situation. Our results thus do not indicate a high risk from maternally transferred MTCS for fish at the current field concentrations.

In order to get more insight in the fate of the POPs in the larvae, in Chapter 4 the existing bioaccumulation model OMEGA was adjusted for sole early life stages and validated with experimental data with PCBs. This study revealed, that tissue concentrations of compounds with log $K_{ow}$ >6, peak in the tissues of developing sole at the end of the yolk-sac stage, when lipid reserves are depleted. As a result, just before the larvae become free feeding, the peak tissue concentrations of the pollutants in the larvae exceed that of the adult fish. This also explains at least partly, the delayed effects that were observed in Chapter 2 (and 5).

Chapter 5 assesses the likelihood that early life development of fish from contaminated areas is affected by maternally transferred POPs. Following the p-ELS test protocol, effects on sole larvae were determined for the dioxin-like PCB 126, the technical PCB-mixture Arochlor 1254, PBDEs and HBCDs, for an artificial mixture of PCBs and PBDEs, and for ‘field mixtures’ extracted from sole collected from the North Sea and in the contaminated Western Scheldt estuary.
As was earlier observed with PCB126 and MTCS, exposure to PCBs, PBDEs and the artificial and field mixtures caused mortality that started to occur shortly after the larvae became free-feeding and continued to increase until the onset of metamorphoses. The effects induced by the field mixtures correlated well with the $\sum$PCB concentrations in the tissue of the exposed larvae. No indications were found for synergistic effects or for substantial contribution of other (unknown) substances in the field mixtures. HBCD did not induce toxic effects. POP levels in sole from Western Scheldt estuary are about 20 times lower than the ILC50, the larval tissue concentration that produced 50% early life stage mortality. Levels in North Sea sole are an order of a magnitude lower.

Chapter 6 describes a risk assessment for toxicant induced larval survival for European eel (Anguilla anguilla). Eels are considered sensitive for the effect of POPs that can accumulate to high levels in their lipid rich tissue. During spawning migration without feeding high lipophilic dioxin-like POPs in the eel’s tissue were estimated to increase 1.33 or 2 fold, due to weight loss. As no toxicity data are available for eel larvae, the critical egg concentrations for larval survival was estimated from a sensitivity distribution based on literature data of other species. It was assumed that eel larvae belong to the 5% or 1% most sensitive teleost fish species. Given concentrations of dioxin-like pollutants as reported for European eel, and following the worst case scenarios with respect to sensitivity of the larvae and bio amplification during migration, it can be expected that larvae of eel from highly contaminated locations in The Netherlands and Belgium will experience more than 50% mortality due to maternally transferred dioxin-like toxicants.

Chapter 7 explores the potential impact of (toxicant induced) early life stage mortality on the population development of sole by application of a simple age structured matrix model. The model is used to explore the population response to a combination of (toxicant induced) larval mortality and fishing-related mortality of mature fish. The results indicate that the impact of larval mortality that occurs before metamorphosis is very low, even in population subject to high fishing pressure. This is the result of the combination of a high fecundity and the fact that the larval mortality occurs before the moment when the number of recruits is limited by the carrying capacity of the nursery areas. When colonising the nursery areas the, until than pelagic sole larval metamorphose into flatfishes with a benthic life style. The individuals hence concentrate from the three dimensional pelagic environment to a two dimensional benthic environment, which caused density dependent mortality. This concentration of early life stages is typical for flatfish. Mortality that occurs after the nursery areas are populated will have a more pronounced impact on population development. The results further imply that population development of pelagic
fish species that do not concentrate in nursery areas, and species with low fecundity is more vulnerable for disturbance through mortality of early life stages.

Chapter 8 synthesises and discusses the outcome of the research. It is stressed that short term fish tests, often covering only the embryonic development, will underestimate the real risk of lipophilic substances. Toxicity of these substances will peak after yolk sac absorption when these tests have already been ended. When the characteristics of the test substance are known this risk is predictable with for instance the ELS-OMEGA model. However, especially when mixtures of unknown composition (effluents, sediment extracts) are being tested one must realise that the contribution of lipophilic substances may be underestimated in test that are terminated before, or too soon after the fish larvae are free feeding.

The absence of effects on metamorphosis in our P-ELS test is explained by the prediction of the ELS-OMEGA model that the POPs concentrations in the larvae, had reached too low concentrations at the moment of metamorphoses to disrupt the thyroid hormone system. This was due to passive excretion (for substances with log $K_{ow} < 6$) and growth dilution.

It must be realised that the experimental set-up that was followed to mimic the effects of maternally transferred POPs does not include potential effects of maternally transferred metabolites of these POPs that can be formed by the parent fish and that are often more toxic than the mother compounds. Also effects of the mother’s condition on the quality of the eggs and epigenetic effects were not included. This implies that the results of the tests as performed in some cases might underestimate the actual effects of these substances.

The species most vulnerable to the effects of maternally transferred POPs share a high exposure, low fecundity and the absence of density dependent mortality of early life stages. According to these criteria sharks and especially the Greenland shark (*Somniosus microcephalus*) that is highly exposed to POPs can be considered as highly vulnerable. It is therefore recommended to investigate the actual sensitivity of this species, in order to get more insight in the potential vulnerability of the populations.
Nederlandse samenvatting
(speciaal voor niet-ingewijden)
De hoeveelheden slecht afbreekbare organische verontreinigende stoffen ('persistent organic pollutants' kortweg POPs) die in het milieu terecht komen zijn de afgelopen decennia sterk teruggebracht. Vanwege de grote hoeveelheden die in het verleden zijn geproduceerd en hun slechte afbreekbaarheid zijn deze stoffen echter nog steeds in het milieu aanwezig. Bekende voorbeelden hiervan zijn PCBs en dioxines. Omdaat zij beter in vet oplossen dan in water hopen deze stoffen zich op in organismen en via stapeling in de gehele voedselketen. POPs die zijn opgehoopt in het lichaam van een vrouwelijke vis worden aan haar eieren doorgegeven via het vet in de dooier. De eieren zijn dus al verontreinigd als zij het moederlichaam verlaten.

Het onderzoek beschreven in dit proefschrift is gericht op de effecten van deze aan eieren doorgegeven POPs op de ontwikkeling van jonge vis. Onderzocht is welke type effecten kunnen optreden, en of deze effecten ook te verwachten zijn bij de concentraties zoals die in vissen in Nederland en elders aanwezig zijn.

Uit de omvangrijke groep POPs zijn voor dit onderzoek stoffen gekozen die veel worden aangetroffen in het mariene milieu. Hieruit zijn stoffen geselecteerd die dioxine-achtige giftigheid vertonen of het schildklierhormoonsysteem kunnen verstoren. Dioxines en dioxine-achtige stoffen veroorzaken ernstige effecten bij ontwikkelende vislarven, zoals oedeem en misvormingen. Het schildklierhormoon speelt een rol bij verschillende onderdelen van de ontwikkeling van vislarven, maar is vooral belangrijk bij de metamorfose van de larve naar een juveniele vis. Dit is ook het geval bij de metamorfose van een kikkervisje in een kikker, waarbij is aangetoond dat verstoring van het schildklierhormoonsysteem de metamorfose kan verstoren, met misvormingen tot gevolg.

Om deze effecten te onderzoeken is een test ontwikkeld met de platvis tong. Dit is hiervoor een geschikte testsoort, omdat tong net als alle platvissen een duidelijke metamorfose ondergaat. Hierbij verandert de tot dan toe symmetrische larve in een asymmetrische platvis, perfect aangepast voor een leven op de zeebodem. Bovendien is tong een belangrijke commerciële vissoort voor de Noordzee, en zijn bevruchte eieren beschikbaar uit aquacultuur. Voor het onderzoek zijn geen moederdieren aan POPs blootgesteld. De eieren werden zo snel mogelijk na de bevruchting aan de teststof blootgesteld. Na 5 tot 6 dagen kwamen de eieren uit, en in de meeste testen werd op dat moment de blootstelling aan de teststof beëindigd. De pas uitgekomen larven bezitten geen ogen, mond of ingewanden en zijn voor hun voedingstof volledig aangewezen op de dooier. Deze periode wordt aangeduid als het 'dooierzak stadium' en duurt ca. 5 dagen. In dit stadium wordt het grootste deel van de dooier gebruikt en ontwikkelt de larve onder andere organen, ogen, een mond en ingewanden,

Het eerste experiment (Hoofdstuk 2) werd uitgevoerd met PCB-126, een stof waarvan dioxine-achtige giftigheid verwacht mag worden. De eieren werden via het water blootgesteld gedurende 4, 8, 10 en 15 dagen. Opvallend was dat in alle gevallen sterfte van de larven pas zichtbaar werd nadat de larven de dooierzak hadden verbruikt en overgingen op externe voeding, ca. 10 dagen na de bevruchting. In sommige gevallen was dit dus lang nadat de blootstelling was beëindigd. In alle gevallen bleef de sterfte toenemen totdat het moment van metamorfose bereikt was. Vrijwel alle overlevende larven doorliepen de metamorfose zonder problemen.

De volgende testen (Hoofdstuk 3) zijn uitgevoerd met het potentieel schildklierhormoonverstorende "methyltriclosan". Hoewel van deze stof dus een heel ander effect verwacht werd dan van PCB 126, waren de resultaten vergelijkbaar. Wederom vond de sterfte vooral plaats nadat de dooierzak was geabsorbeerd en de overlevende larven voltooiden de metamorfose zonder problemen.

Om beter te begrijpen hoe deze effecten tot stand komen wordt in Hoofdstuk 4 een rekenkundig model gepresenteerd dat het verloop van de concentratie van POPs in een zich ontwikkelende vislarve beschrijft. Hiervoor is het al bestaande OMEGA model aangepast op basis van experimenten waarin het gehalte van PCBs in tonglarven werd gevolgd. De resultaten laten zien dat de slecht in water oplosbare POPs die in de dooier aanwezig zijn de vislarven nauwelijks verlaten. Naarmate de dooier meer geabsorbeerd wordt neemt dus de concentratie van deze POPs toe, totdat het maximum bereikt wordt aan het eind van het dooierzakstadium. Dit verklaart waarom de effecten pas na dat moment zichtbaar worden. Het model laat ook zien dat de POP concentraties voornamelijk door verdunning dalen zodra het visje begint te eten en toeneemt in gewicht. Wanneer de larven klaar zijn voor de metamorfose zijn de gehalten zo laag geworden dat er geen effecten meer zullen optreden.

In Hoofdstuk 5 wordt het effect van andere POPs onderzocht die ook veel in het mariene milieu aanwezig zijn. Het betreft PCBs en gebromeerde brandvertragers. Naast het effect van de individuele stoffen is ook het effect van
mengsels van stoffen onderzocht. Hiervoor werd niet alleen een mengsel in het laboratorium gemaakt, maar werden ook extracten gemaakt van tong afkomstig uit de verontreinigde Westerschelde en de Noordzee. Zeker van de extracten van de vis uit de Westerschelde mag verwacht worden dat zij meer stoffen bevatten dan alleen PCBs en gebromeerde vlamvertragers.

De meeste stoffen en mengsels lieten weer hetzelfde type effect zien: sterfte na het dooierzak stadium en geen effect op de metamorfose. De waargenomen effecten van de mengsels konden worden voorspeld aan de hand van de concentraties aan PCBs. De sterfte van de vislarven na het dooierzak stadium is waarschijnlijk het gevolg van de ophoping van deze stoffen in (het vet van) de celmembranen, waardoor deze hun functie verliezen. Dit type effect wordt als basis toxiciteit beschouwd en kan worden veroorzaakt door alle POPs die makkelijk in vet oplossen. Stoffen met dioxine-achtige werking zijn vele malen giftiger. De resultaten uit hoofdstuk 5 laten verder zien dat de POP gehalten van tong uit de Westerschelde veel lager zijn dan de gehalten waarbij doorgifte aan de eieren tot effecten op de larven zal leiden. In tong uit de Noordzee zijn deze gehalten nog 10 maal lager. Elders in Europa zijn wel locaties bekend waar verwacht kan worden dat de voortplanting van de vissen daar verstoord is. Het betreft dan wel zwaar verontreinigde gebieden.

Hoofdstuk 6 richt zich op de Europese aal, de paling. In vergelijking tot vóór 1980 zijn de aantallen jonge alen (glasaal) die jaarlijks vanuit zee het zoete water intrekken met ruim 90% afgenomen. In Hoofdstuk 6 wordt onderzocht of het mogelijk is dat deze afname (deels) veroorzaakt wordt door in de eieren aanwezige POPs. De aal is potentieel gevoelig omdat deze vis slechts eenmaal in zijn leven voortplant en daarvoor lange tijd heeft gehad om potentieel giftige stoffen op te hopen in het vetrijke weefsel. Omdat de aal niet eet tijdens de ruim 5000 km lange, maanden durende migratie naar de Sargasso zee zal gewichtsverlies optreden. Op basis van elders uitgevoerde zwemproeven met alen wordt geschat dat door dit gewichtsverlies de concentratie van slecht wateroplosbare POPs in het aalweefsel met 33 tot 100% toeneemt voordat de POPs aan de eieren worden doorgegeven. Omdat aal (nog) niet gekweekt kan worden, kan niet worden vastgesteld hoe gevoelig aallarven voor POPs zijn. Deze gevoeligheid is daarom geschat op basis van gegevens van andere vissoorten. Wanneer wordt aangenomen dat een aal(larve) tot de meest gevoelige vissoorten behoort, dan zijn de gehalten van dioxine-achtige POPs zoals aanwezig in alen uit verontreinigde locaties in Nederland en België hoog genoeg om sterfte onder de larven te veroorzaken.

In de vorige hoofdstukken is vastgesteld dat larvale sterfte kan voorkomen bij vissen van ernstig vervuilde locaties. Hoofdstuk 7 onderzoekt of dergelijke
sterfte van invloed kan zijn op de ontwikkeling van een vispopulatie, en welke soortspecifieke eigenschappen daarbij een rol spelen. Het effect zou groter kunnen zijn als een populatie ook nog zwaar (over)bevist wordt. Het rekenmodel dat hiervoor werd opgesteld op basis van de biologie van de tong, laat zien dat juist platvispopulaties goed bestand zijn tegen larvale sterfte. Dit hangt samen met de grote hoeveelheden eieren die geproduceerd worden (enkele honderden per gram lichaamsgewicht) en het gegeven dat de jonge visjes zich na de metamorfose op de bodem van de opgroeigebieden verzamelen. Doordat de ruimte in de opgroeigebieden beperkt is, vindt hier hoe dan ook grote sterfte plaats onder de pas gemetamorfoseerde visjes. Door gifstoffen veroorzaakte sterfte onder de larven heeft daardoor weinig invloed. Dit blijft zelfs het geval bij extreme visserijdruk. Uit deze analyse blijkt dat populaties van soorten die weinig eieren produceren en waarbij geen dichtheidsafhankelijk sterfte onder de jongen optreedt het meest kwetsbaar zijn voor de effecten van larvale sterfte en visserijdruk.

In Hoofdstuk 8 worden de bevindingen uit de voorgaande hoofdstukken gecombineerd om tot algemene conclusies te komen en aanbevelingen voor verder onderzoek te formuleren. Omdat duidelijk is dat de grootste effecten van POPs op vislarven tot uiting komen aan het eind van het dooierzakstadium, wordt in laboratoriumtesten die eerder worden beëindigd de giftigheid onderschat. Dit is vooral een risico als de giftigheid moet worden bepaald van (mengsels met) onbekende stoffen, zoals mogelijk aanwezig in afvalwater of sedimenten. Ook wordt besproken waarom de resultaten in dit proefschrift waarschijnlijk een onderschatting zijn van de effecten die kunnen optreden wanneer de moedervis vóór het leggen van de eieren aan de POPs zou zijn blootgesteld. In dit geval zijn de gifstoffen immers al in het ei aanwezig voordat de bevruchting plaatsvindt. Bovendien worden ook door de moedervis aangemaakte afbraakproducten van gifstoffen (metabolieten) aan de eieren doorgegeven. Daarnaast kan de kwaliteit van de eieren minder zijn wanneer de moedervis zelf last ondervindt van de POPs.

Uit een vergelijking van verschillende vissoorten lijkt de Groenlandse haai bijzonder kwetsbaar voor de effecten van door de moedervis aan de jongen doorgegeven POPs. Vanwege hun plaats aan de top van de voedselketen bevat het weefsel van deze haaien hoge POP concentraties, die aan de eieren worden doorgegeven. Bovendien verblijven de jongen gedurende hun hele ontwikkeling tot juveniel in de moedervis. Na het verteren van de dooierzak voeden zij zich met de niet ontwikkelde eieren en mogelijk met andere jongen. De blootstelling blijft daardoor hoog, zeker in vergelijking met vissen waarvan de ontwikkeling in relatief schoon water plaatsvindt, met relatief schoon voedsel. Bovendien is het aantal jongen per worp beperkt, waardoor het onwaarschijnlijk is dat
vroegtijdige sterfte van een deel van de jongen door minder dichtheidsafhankelijke sterfte wordt gecompenseerd. Nader onderzoek naar de effecten van POPs op de voortplanting van de Groenlandse haai wordt daarom aanbevolen.
'Keep the blanket around you', the boy said.
'You’ll not fish without eating while I’m alive'.
'Then live a long time and take care of yourself', the old man said.

*Ernest Hemmingway: The Old Man and the Sea*
In 2006 besloot ik om dit promotietraject te starten. Het is nu duidelijk dat ik de
tijd, aandacht en energie die nodig waren om deze klus naast mijn normale werk
te voltooien ernstig heb onderschat. De knelpunten die daardoor ontstonden
konden alleen worden opgelost door (vaak ongevraagd) een beroep te doen op
het begrip en de loyaliteit van de mensen in mijn directe omgeving.

Gerda, mijn lief, jij leverde zonder twijfel het grootste offer. Dat ik vaak 's
avonds en in de weekenden op het lab was, was misschien nog het minst erg.
Maar zeker de laatste 2 jaar waren mijn gedachten ook als ik wel thuis was,
vaak niet bij de zaken die we eigenlijk samen zouden moeten doen. Zonder jou
was dit proefschrift er misschien ook wel gekomen. Maar zeker ten koste
van meer dan een proefschrift ooit waard kan zijn. Deze promotie is daarmee
onze gezamenlijke prestatie. Wat zal ik trots zijn als ik tijdens de ceremonie jou,
geflankeerd door onze prachtige zonen Lars en Ivar op de eerste rij zie zitten. Ik
draag dit proefschrift aan jullie op, maar eigenlijk verdien jij het om als co-
auteur vermeld te worden. En dat staat dan nog los van je bijdrage aan de
vormgeving van de omslag van het proefschrift.

De weg die uiteindelijk naar dit proefschrift heeft geleid begint in 1987 toen ik
door het toenmalig afdelingshoofd Willem Christiaan (Pim) de Kock als
veldmedewerker ecologisch onderzoek werd aangenomen bij TNO in Den Helder.
Zijn eigenzinnige kijk op de wereld heb ik altijd erg gewaardeerd. Ik had me er
daarom op verheugd om dit proefschrift straks aan Pim te kunnen aanbieden.
Helaas overleed hij onverwacht in de week dat ik het akkoord van de
leescommissie ontving.

Na mij aanstelling werd Martin Scholten al snel hoofd van de onderzoeksgroep.
Martin, door het vertrouwen en de ruimte die je me gaf in het werk en de
persoonlijke manier waarop je mij coachte, heb je een enorme bijdrage geleverd
aan mijn ontwikkeling. Dit betrof niet alleen praktische zaken als projectleiding
en rapportage, maar zeker ook het besef dat je met goed teamwork en een
gezamenlijk doel het meeste kunt bereiken. In 2001 bepleitte jij met succes bij
de directie van TNO dat ik, hoewel 'slechts' opgeleid op HBO niveau, op
academisch niveau functioneerde en als zodanig beloond diende te worden. Ik
was destijds al erg vereerd met deze erkenning, maar zou pas jaren later
beseffen hoe bijzonder dat werkelijk was.

Mede gestimuleerd door Martin overwoog ik rond diezelfde periode al om een
promotietraject te starten. Omdat toen echter ook Lars en later Ivar werden
geboren, is dit plan niet doorgezet en verdween deze ambitie naar de
achtergrond van mijn volle leven. Het werd echter weer aangewakkerd door de
inspirerende gesprekken met mijn nieuwe IMARES collega Charlotte Deerenberg tijdens een lange autorit naar Zeeland in september 2006.

Niet lang daarna bezocht ik voor de eerste keer de Wageningen Universiteit om mijn plannen te bespreken met Tinka Murk, mijn latere promotor. Tinka, je wees me er toen direct al op dat mijn ideeën in elk geval nooit binnen één promotietraject verwezenlijkt zouden kunnen worden en adviseerde mij om de ambitie sterk in te perken. Zonder dat advies was mijn proefschrift nooit gereed gekomen. Ook wil ik je danken voor de effectieve training in het schrijven van wetenschappelijke publicaties, en voor je vertrouwen en bemoedigende woorden waarmee je me door de dipjes heen trok als de dubbele baan even teveel werd. Vanaf het begin inspireren onze gesprekken tot nieuwe ideeën en ik weet zeker dat we in de toekomst nog veel mooi werk samen zullen verzetten.

Vanaf 2007 was ik dus echt begonnen aan mijn promotieonderzoek. Omdat hiervoor slechts zeer beperkte ruimte was (binnen het EU-project ‘Modelkey’), was ik voor de uitvoering van de langdurige en arbeidsintensieve experimenten sterk afhankelijk van de inzet van vrijwilligers en studenten. Dear Henrique, Elisa, Astrid, Maria, Merlijn, Mekuria, and Bastiaan, your contributions were crucial for the research presented in this thesis. Apart from the practical work that you did, I really enjoyed your presence and sharing thoughts about science, art, politics, life, and the effect of swearing on the quality of rice. Thanks for that. I hope that you will be able to develop the personal career that fulfils your dreams.

Ewout Blom en Jan van der Heul wil ik danken voor het leveren van de tongeieren, en voor de adviezen die zij samen met Andries Kamstra gaven over de verzorging van de eieren en de larven. Mede hierdoor kon de p-ELS bioassay behoorlijk snel worden ontwikkeld.

Hans van den Berg toonde zich een vakman door in monsters van slechts enkele milligrammen tonglarven toch betrouwbare TEQ gehalten te kunnen bepalen. Christiaan Kwadijk, en de analisten van het chemisch lab van IMARES in IJmuiden leverden vergelijkbare prestaties door dezelfde monsters chemisch te analyseren. Allemaal hartelijk bedankt, en excuses voor al die keren dat ik toch nog een keer een extra analyse wilde of de meetgegevens, eigenwijs als ik ben, nog een keer extra ging doorrekenen. Michiel Kotterman dank ik voor de adviezen over het gedrag van PCBs, en de inspirerende discussies over al dan niet proefschrift gerelateerde zaken.

Naast deze mensen die vrij direct betrokken waren bij mijn onderzoek, zijn er nog veel meer collega’s waar ik veel aan heb gehad. Al mijn collega’s op locatie
Ambachtsweg dank ik voor hun interesse in mijn werk en de bereidwilligheid om altijd bij te springen als dat nodig was, maar bovenal voor het begrip voor het feit dat ik me het laatste jaar wat afzonderde om zaken af te kunnen ronden. Ik wil hierbij Klaas Kaag en Andrea Sneekes met name noemen die het meeste last hebben gehad van mijn verminderde betrokkenheid omdat onze werkvelden sterk overlappen. Zij pakten zonder morren op wat ik liet liggen of had laten vallen.

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De opmaak van dit proefschrift is mij uit handen genomen door een collega die niet met naam genoemd wil worden, maar die ik daarvoor toch heel hartelijk wil bedanken. Liesbeth, je weet wel wie ik bedoel.

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Mijn familie en vrienden waren op de achtergrond belangrijk door met interesse te informeren naar de voortgang en de bevindingen van het onderzoek. Ik ben er bovendien van overtuigd dat elk onderzoek erbij geab staat wanneer de relevantie van de resultaten met niet-wetenschappers wordt bediscussieerd. En dat kun je natuurlijk nergens beter doen dan met vrienden of familie onder het genot van een drankje, met vismaten aan het water, of met je volleybalteam in een kantine.

Helemaal aan de wieg van dit alles staan natuurlijk mijn ouders. Pap en mam, jullie hebben jullie kinderen altijd gestimuleerd en geholpen om zichzelf te ontwikkelen, met de vrijheid om daarin zelf de richting te bepalen. De belangstelling voor het onderwaterleven was bij mij al jong aanwezig en ik kreeg
alle ruimte om dit met aquaria, netten en hengels uit te bouwen. De kliederzooi
die dat met mijn experimenteerdrang regelmatig opleverde, leidde wel eens tot
gemopper maar nooit tot een verbod. Deze ‘praktijkervaring’, in combinatie met
de bij de opvoeding meegegeven instelling dat je (bijna) alles kunt als je maar
hard genoeg je best doet ("Kan Niet ligt op het kerkhof, en Wil Niet ligt
ernaast."), is een goede opmaat gebleken voor mijn professionele loopbaan.
Hierdoor ben ik nu al meer dan 25 jaar werkzaam in een vakgebied waarin
werken vaak niet als werk voelt. Mij promotie is hiervan de bekroning die ook op
jullie afstraalt.

Als je zoals ik bijna 50 jaar oud bent, is het onmogelijk om in een dankwoord,
dat nu eigenlijk al te lang geworden is, volledig te zijn. Daarom wil ik tenslotte
iedereen bedanken die niet met name genoemd is, maar wel in mijn gedachten
is nu ik dit schrijf. Bedankt voor de bijdrage die jullie op welke wijze dan ook
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Publications
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CERTIFICATE

The Netherlands Research School for the Socio-Economic and Natural Sciences of the Environment (SENSE), declares that

Edwin Matheus Foekema

born on 7 November 1963 in Wormerveer, The Netherlands

has successfully fulfilled all requirements of the Educational Programme of SENSE.

Wageningen, 4 October 2013

the Chairman of the SENSE board
Prof. dr. Rik Leemans

the SENSE Director of Education
Dr. Ad van Dommelen
The SENSE Research School declares that Mr. Edwin Foekema has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 40 ECTS, including the following activities:

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- Higher-tier Aquatic Risk Assessment of pesticides and biocides, SETAC short course
- Mixture toxicity, SETAC short course
- Good laboratory practices (GLP)
- Effective time management
- Personal effectiveness

Management and Didactic Skills Training
- Co-organisation of the symposium Nabehandeling van RWZI-effluent? Ja, natuurlijk! Praktijkervaringen met de Waterharmonica, Kaatsheuvel, 2012
- Supervision of nine MSc theses

Oral Presentations
- The impact of environmental contaminants on early life development of Sole: first results and future plans. GDR IMOPHYS annual meeting, 5-6 July 2007, Nantes, France
- Towards a tool to assess the actual impact of accumulated substances on fish reproduction. SETAC Europe 18th annual meeting, 25-29 May 2008, Warsaw, Poland
- Factors associated with effects of pollutants on fish. Workshop on the effects of oil-spill on Pelagic Ecosystems, 28-29 January 2008, Bergen, Norway
- A tiered approach to assess impact by chemicals on ecological status. Final MODELKEY Conference: How to assess the impact of key pollutants in aquatic ecosystems, 30 November - 2 December 2009, Leipzig, Germany

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