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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 a LD50 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.

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, 1998, 2006; EPA, 1996). 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 and Bolker, 2003; Schreiber, 2006).

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The development from egg to larva is known to be sensitive to dioxin-like toxicity in zebrafish (Murk et al., 1996a) and amphibians (Gutleb et al., 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; Van 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 practice 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. Materials and methods

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 Helder in plastic bags containing seawater. During the ca. 1 h transport period, the water temperature was maintained around 12 °C, the water 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. 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% v/v. 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.

2.3. Test procedure

Immediately after arrival at the laboratory (<12 h 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 12–13 °C, with a 16 h photoperiod with dimmed light (ca. 100 lx) per day. After 48 h the floating fertilised eggs were collected and the non-fertilised eggs at the bottom discarded. Out of each exposure group 20 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-sollution (100 mg MS222 = Ethyl 3-aminonebzoate methanesulfonic acid salt (purity 98%, ACROS Organics) and 200 mg $NaHCO_3$ (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 ± 1 °C, and on 13 °C there after. 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 ± 1 °C 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.

2.4. 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 2 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 h) at least 140 larvae per test concentration were collected with a polyethylene pipette and placed in clean seawater for maximal 15 min 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 very 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).

2.5. 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.

3. Results

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 (Fig. 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.

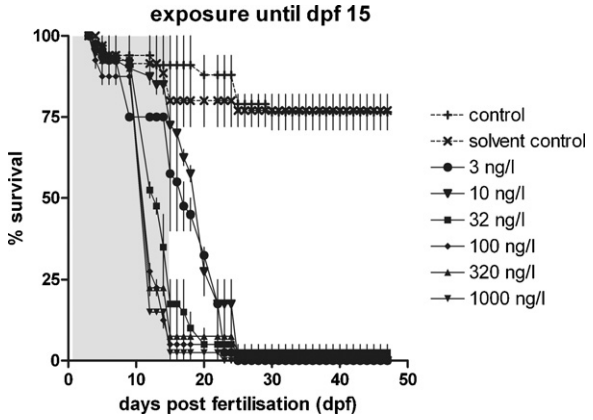

Fig. 1. Survival of sole larvae (*Solea solea*, $n = 20$) that were exposed to PCB 126 via the water for 15 days in the first test. The grey boxes indicate the period of exposure after which the animals were further reared under unexposed conditions. Presented are the mean and range of the two replicates per treatment.

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.

3.2. Second experiment

In the second experiment the development from egg into free-feeding fish was about 2 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.

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 (Fig. 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 4 dpf 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.

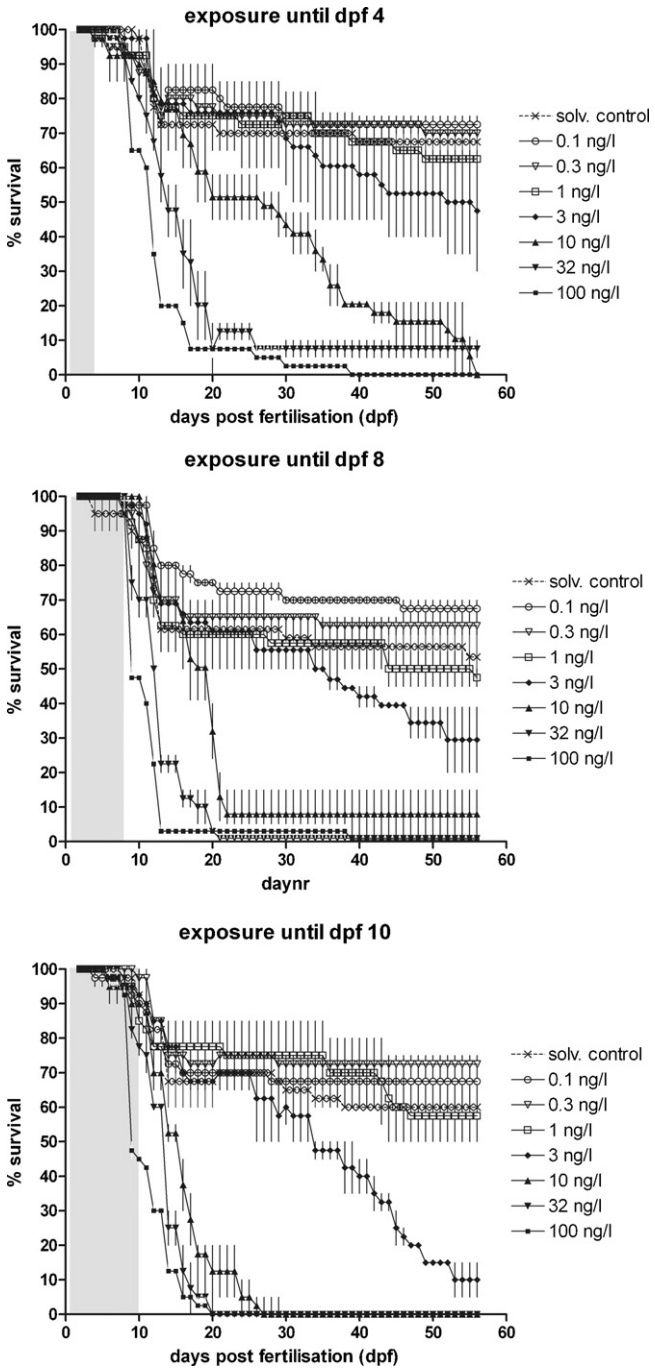

Fig. 2. Mortality of sole larvae (*Solea solea*, $n = 20$) that were exposed to PCB 126 via the water during 4 (top), 8 (middle) or 10 days (bottom) in the second experiment. The grey boxes indicate the period of exposure after which the animals were further reared under unexposed conditions. Presented are the mean and range of the two replicates per treatment.

3.3. Effect concentrations

The data set allowed the calculation of LC50 values between dpf 12 and 20 for all 4 exposure durations (Fig. 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

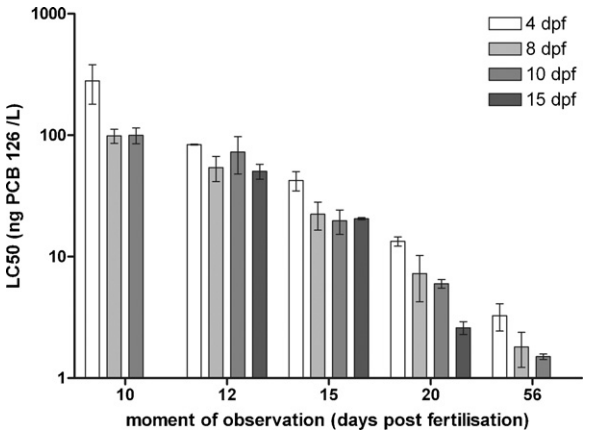

Fig. 3. The LC50 (ng PCB 126/l) for early life stages of sole with increasing observation duration exposed during 4 (covering only egg stage), 8, 10 (covering egg and yolk sac stage) and 15 days post fertilisation (dpf). Presented are the mean and range of the two replicates per treatment. The dataset of the 15 dpf exposure group did not allow the calculation of an LC50 before Day 12 and after Day 20 see text.

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 4–15 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.

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

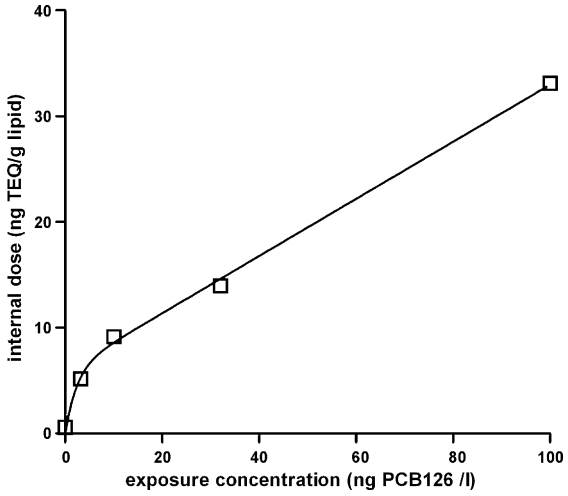

Fig. 4. TCDD-equivalents (TEQs) determined with a reporter gene (DRE-H4IIE.Luc) assay in lipid of newly hatched sole larvae after exposure of the eggs to PCB 126 via the water for 4 days.

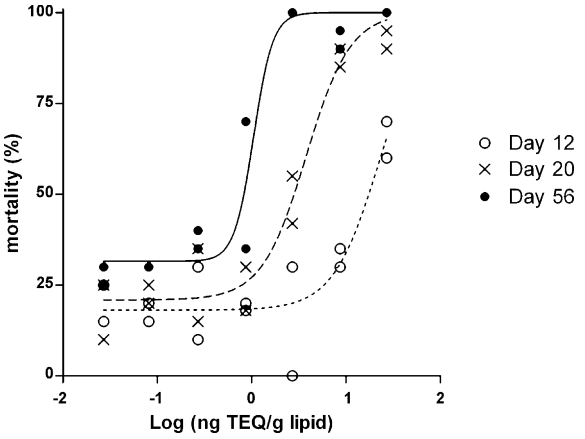

Fig. 5. Relationship between the internal TEQ concentration in lipid of newly hatched sole larvae at the moment of hatching after exposure of the eggs to PCB 126 (two replicates per treatment) for 4 days, and the mortality at three time points later during the early life development of a similarly treated group (4 dpf group). On Day 12 all fish were free-feeding, on day 56 all surviving fish had completed metamorphosis.

($R^2 = 0.998$) by the formula for a two-way exponential association (Fig. 4):

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

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 LC50 values for the 4 dpf-group 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 (Fig. 5).

4. Discussion

4.1. Experimental setup

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 (Fig. 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.

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 (1.7–3.7 ng PCB 126/l). Since exposure to the test substance had already stopped after 4–15 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.

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 and Peterson, 1985).

When expressed as internal TEQ concentration in the newly hatched larvae the LC50 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 14 (20%) out of the 70 samples that we identified in our short literature search contained more than 250 pg TEQ/g lipid (Table 1). This is 25% of the LC50 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 LC50.

It is important to realise that exposure of the sole eggs in our experiment only started at ca. 12 h instead of immediately after fertilisation as is the case with eggs exposed via the lipids that the

Table 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.

Fish species	Location	Lipids (%)	WHO PCDD/F-TEQ (pg/g W/W)	WHO PCB-TEQ (pg/g W/W)	Total WHO TEQ (pg/g W/W)	Total WHO TEQ (pg/g lipid)	Reference[a]
Baltic Herring	Baltic sea					257	R1
Pike-perch	Nw. Merwede	0.9				267	R2
Pike-perch	Lek, Hagestein	1.0				272	R2
Pike-perch	Waal	1.3				282	R2
Sea bass	France	3.6				307	R2
Perch	Baltic sea	2.6	5.23	5	10.23	393*	R1
Whitefish	Baltic sea	2.6	7.07	3.75	10.82	416*	R1
Herring	Grenland fjords	2.3			12.1	524*	R3
Bonito	Italy	1.4				565	R2
Pike	Baltic sea	0.5	1.31	1.67	2.98	596*	R1
Cod	Grenland fjords	0.29			1.9	655*	R3
Sea trout	Grenland fjords	1.93			12.9	668*	R3
Flounder	Grenland fjords	0.26			3.4	1308*	R3
Flounder	Grenland fjords	0.89			33.5	3764*	R3

The list is not exhaustive. Values marked with '*' were calculated using the TEQ levels reported on fresh weight basis and the lipid content as reported in the same paper.
[a] R1 = Isosaari et al. (2006); R2 = Leonards et al. (2000); R3 = Knutzen et al. (2003).

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 laptipes*) 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).

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 world wide.

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.

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