Toxicological Efects of Aroclor 1254 on the Embryonic Development of the Olive Flounder *Paralichthys olivaceus*

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Abstract

We investigated the toxicological effects of Aroclor 1254 on the fertilized eggs, embryos and larvae of the olive flounder *Paralichthys olivaceus*. The survival rate and hatching success of the embryos decreased significantly in treated groups in an Aroclor 1254-dose-dependent manner. Significant differences were found at ≥ 5 µg/L Aroclor 1254 compared to the control group. Hatching success occurred at ≤ 10 µg/L Aroclor 1254, which was not significantly different to the control. Embryo malformation increased significantly at ≥ 1 µg/L, and included yolk-sac and tail-flexure abnormalities. There was a significant decrease in the survival rate of the larvae at ≥ 5 µg/L, which was accompanied by the malformations described above. Notably, concentrations as low as 1 µg/L caused a significant increase in abnormalities in the larvae, including incidences of multi-focal hemorrhages, pericardial and yolk-sac edema, inhibition of swim bladder inflation and severe developmental delay. The responses to Aroclor 1254-induced toxicity were generally similar among fertilized eggs, embryos and larvae from three separate flounder hatcheries: Cheju Island, Yeosu and Chungnam, South Korea. These results indicate the high acute toxicity of Arolcor 1254 concentrations of which as low as 1 µg/L in olive flounder larvae can affect unhatched embryos. To conclude, the average LC_{50} values for Aroclor 1254 in the embryos and larvae were 50.92 and 3.08 µg/L, respectively. Additionally, the average EC_{50} values, based on the rate of damage were 14.72 and 5.61 µg/L, respectively.

Key words: Aroclor 1254, Olive flounder, *Paralichthys olivaceus*, Early-life-stage toxicity

Introduction

Polychlorinated biphenyls (PCBs) are considered to be important environmental pollutants worldwide, despite their use being restricted in most countries since the 1970s. Due to their high persistence and strong lipophilic properties, PCBs have bioaccumulated in aquatic food webs. PCB mixtures have very low solubility in water but high solubility in oils and low-polarity organic solvents. Even though the water solubility of Aroclor 1254 is only 2.7 µg/L, PCBs are classed as probable human carcinogens by the Environmental Protection Agency (EPA), based on substantial evidence of cancer in animals (Walker et al., 1994). Despite the decrease in PCB levels dur-

ing the last 30 years, they remain a major contaminant in fish (Bignert et al, 1998).

The early life-stage toxicity test is generally considered by aquatic toxicologists to be the most useful test for risk assessment. The embryonic period begins with fertilization or union of the gametes, and is characterized by an endogenous food source (yolk), finally ending at hatching. The larval period begins with egg hatching and lasts until the appearance of a full complement of fin rays and spines. This period is divided into two phases; the initial yolk-sac larva or alevin period, which begins at hatching and ends after complete absorption of the

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yolk, resulting in juvenile (Jones et al., 1978).

Aroclor 1254 is a PCB mixture that is acutely toxic to aquatic animals (Hansen et al., 1971; Schimmel et al., 1974). Mac et al. (1993) reported a strong correlation between total egg mortality and total PCB concentration in the eggs. It has been suggested that PCBs in the Great Lakes (USA) were responsible for the significant mortality of the early life stages of the lake trout, *Salvelinus namaycush*. Niimi (1983) suggested that exposure to contaminated water, sediments and maternal transfer of PCBs to the eggs resulted in toxicity of early fish life stages. PCBs are a class of environmental contaminant known to reduce hatching success and larval survival in fish (Matta et al., 1997). Notably, PCBs are over 30-fold more toxic to fry of the sheepshead minnow, *Cyprinodon variegates*, than to juveniles or adults (Matta et al., 1997). Most fry form sheepshead minnow eggs containing ≥ 7 μg/g Aroclor 1254 died within the first week following hatching (Schimmel et al., 1974).

Previous studies have investigated the ecotoxicological responses of fish from a single location. Such narrow studies cannot account for geographical or physiological patterns of adaptation that can occur in the natural environment (Hallare et al., 2005). For example, in South Korea, different fish stocks are used at different aquaculture facilities, including Cheju Island, Yeosu, and Chungnam. The condition of the mother fish differs according to the aquaculture farm she comes from and hence, the quality of eggs received in the laboratory differs, depending on where they are sourced. Accordingly, this study compared the responses of the olive flounder from different regions of Korea, as this has important implications for monitoring environmental conditions using the olive flounder as an indictor species. The main purpose of this study was to investigate the effects of sub-lethal Aroclor 1254 exposure on survival and hatching, as well as on embryo and larvae deformities and survival of the olive flounder.

Materials and Methods

Experimental animals and water conditions

Fertilized olive flounder eggs were obtained from hatcheries in Cheju Island, Yeosu and Chungnam, South Korea. The water quality parameters measured for the bioassays conducted were as follows; pH, 8.10 ± 0.2; salinity, 32.70 ± 0.4 ‰; dissolved oxygen, 6.74 ± 0.84 mg/L; chemical oxygen demand, 1.52 ± 0.008 mg/L. All experiments were carried out separately for each region, but at a single test location. All experiments were conducted at a seawater temperature of 20 ± 0.5°C under a 12-h light/ 12-h dark cycle, over either a 36-h or 72-h period. Other pertinent test conditions for the acute toxicity tests are summarized in Table 1.

Chemicals

Aroclor 1254 was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Five solutions (1, 5, 10, 20, and 40 μg/L) were prepared by diluting the stock solution. The stock solution was made up in seawater that had been filtered through a grade GF/C, Whatman filter (Maidstone, UK) and ultraviolet-treated.

Embryo toxicity test

The eggs obtained from each regional hatchery were approximately at the 8-10 h post-fertilization (blastula) stage. They were transferred directly into cleaned 500 mL glass beakers containing 1, 5, 10, 20, or 40 μg/L Aroclor 1254 for static exposure. Each Aroclor 1254 concentration (containing 50 embryos), and the controls were carried out in triplicate. Embryonic development was monitored every 3 h, up to an endpoint at 40 h, or until hatching was complete. The bea-

Table 1. Test conditions for the acute toxicity tests with olive flounder *Paralichthys olivaceus* embryos and larvae

Test types	Conditions
Toxicity test type	Static acute with water renewal
Duration (h)	40 (embryos)/72 (larvae)
End points	Survival rates, malformation rates, hatching rates
Light quality and photoperiod	Ambient laboratory light (12L:12D)
Water for exposure	Filtered and UV-treated
Test chamber size (mL)	500
Test solution volume (mL)	300
Renewal of test solution (h)	Every 24
Age of test animals at start of exposure (h)	Less than 10 h after fertilized (8-10)
No. of embryos and larvae per chamber	50 (embryos)/50 (larvae)
No. of replicates per concentration	3
Feeding regime	Not required
Test concentrations	5ea + control
Test acceptability criterion	90% or greater hatching in control

L, light; D, dark.

ker contents were replaced every 24 h throughout the toxicity test (Table 1). At each time interval, embryo mortality, hatching success and abnormalities were noted using a microscope (×400) connected to a camera. Embryos were considered dead when any part of the embryo turned opaque or white. Preliminary Aroclor 1254 exposure experiments identified embryonic malformations such as irregularities in the egg membrane (chorion), yolk sac atrophy, and abnormal tail flexure.

Larvae toxicity test

Newly hatched larvae were exposed to identical Aroclor 1254 or to control conditions (Table 1). The larvae were monitored every 6 h for up to 72 h. Larvae were considered dead when there was no visible heartbeat or body movement. The survival rate was recorded throughout the experiment and abnormalities were quantified at the end of the experiment. The specific abnormalities monitored included yolk-sac edema or deformation, erosion of the fins, spinal cord curvature, and abnormal tail flexure.

Statistical analysis

Percentage data were log transformed to approximate a normal distribution, and treatment groups or hatchery locations were compared to one another using a one- or two-way analysis of variance (ANOVA). If the ANOVA was significant ($P < 0.05$), a Dunnett's multiple comparison test was used to identify means that were similar and those that were significantly different.

Results and Discussion

Embryo toxicity of Aroclor 1254

In the seawater controls and for each Aroclor 1254 concentration, survival of olive flounder embryos from all three Korean hatcheries was generally similar. Specifically, survival was above 90% in the controls and in the 1 μg/L groups, but was significantly lower in the higher Aroclor 1254 concentration groups. The combined results from the three sites suggested a linear relationship between embryo survival and Aroclor 1254 concentration (Fig. 1), with 50.92 μg/L (upper, 85.66; lower, 37.71) being the predicted concentration for 50% mortality (LC_{50}). The results from this study confirm that the early life stages of the olive flounder are sensitive to low concentrations of Aroclor 1254; exposure reduces the survival of embryos and negatively affects their development (Fig. 1). However, the responses were generally similar in fish raised form stocks form different regions of Korea ($P > 0.05$). This lack of variability among populations may reflect their migratory patterns and widespread distribution. These results suggest that the
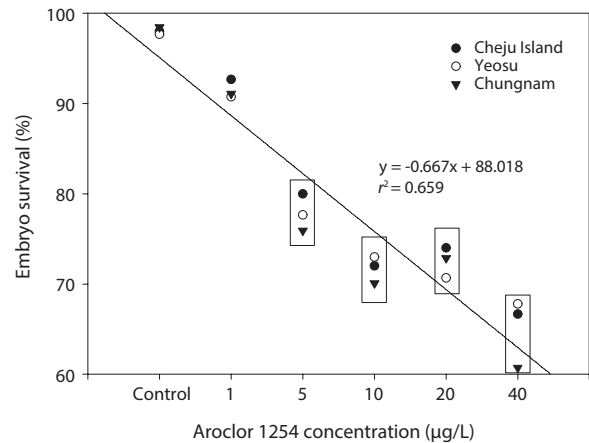

Fig. 1. Linear relationship shown between embryos survival rate and Aroclor 1254 concentration in the olive flounder *P. olivaceus* collected from three different hatcheries and then exposed in the laboratory to various Aroclor 1254 concentrations. □ indicates significant differences between control groups and exposure groups ($P < 0.05$).

olive flounder is a good indicator species for monitoring environmental conditions.

In the flag fish, *Jordanella floridae*, 5 μg/L of Aroclor 1248 caused significant mortality of embryos (Nebeker et al., 1974), whereas in the sheepshead minnow, 10 μg/L Aroclor 1254 had little effect on survival (Schimmel et al., 1974). However, in rainbow trout *Oncorhynchus mykiss* embryos, exposure to 5 mg/L PCB caused 55.2% mortality (Matta et al., 1997). In this study, exposure of olive flounder embryos to Aroclor 1254 concentrations above 5 μg/L led to significantly reduced survival rates (Fig. 1). Additional studies have suggested PCBs present in lake trout eggs may explain the observed mortality during the early life stages (Mac et al, 1985, 1993; Monod, 1985).

Our results showed no significant difference ($P < 0.05$) in the proportion of damaged embryos from three different flounder hatcheries (Fig. 2). The percentage of damaged embryos increased in a linear fashion with Aroclor 1254 concentration, and the Aroclor 1254 EC_{50} (50% embryo damage) was 14.72 μg/L (upper, 25.30; lower, 6.28) (Fig. 2). At 5 μg/L, 45-61% of embryos were damaged which differs from the Japanese medaka, *Oryzias latipes*, where 99% embryos were damaged after exposure to PCB 126 (0.8 μg/L) and 68.8% were damaged after exposure to PCB 77 (1.6 μg/L) (Kim and Copper, 1999). This suggests that Aroclor 1254 is less toxic than PCB 126 in embryonic fish, or that embryo of the seawater fishes such as the olive flounder are less sensitive to PCBs than freshwater species.

Hatching rates for embryos from all three locations were similar, especially for embryos in the seawater controls and Aroclor 1254 concentrations of ≤5 μg/L (Fig. 3). The combined results calculated using mean values showed a linear relationship: hatching rate reduced significantly with increas-
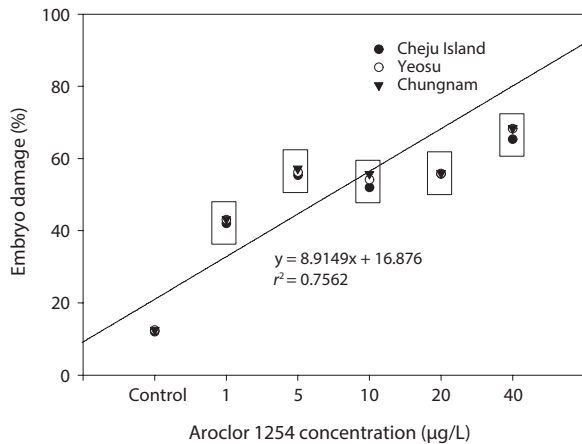
Fig. 2. Linear relationship shown between embryo damage rate and Aroclor 1254 concentration in the olive flounder *P. olivaceus* collected from three different hatcheries and then exposed in the laboratory to various Aroclor 1254 concentrations. □ indicates significant differences between control groups and exposure groups (*P*<0.05).
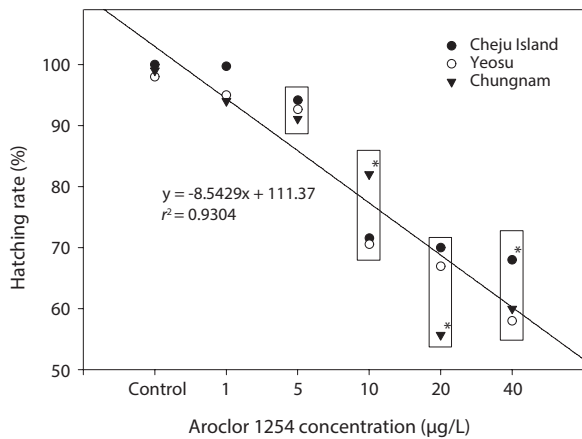

Fig. 3. Linear relationship shown between hatching rate and Aroclor 1254 concentration in the olive flounder *P. olivaceus* collected from three different hatcheries and then exposed in the laboratory to various Aroclor 1254 concentrations. *Superscripted letters indicate a significant difference within a same concentration of Aroclor 1254 (*P*<0.05). □ indicates significant differences between control groups and exposure groups (*P*<0.05).

ing Aroclor 1254 concentrations (Fig. 3). As a comparison, the egg hatching rate of the fathead minnow, *Pimephales promelas*, was reduced relative to the control when eggs were exposed to 7.7 µg/L Aroclor 1254 (Nebeker et al., 1974), and the hatching rate of the sheepshead minnow was significantly at 10 µg/L Aroclor 1254, relative to the control (Schimmel et al., 1974). Half-hatched embryos have also been observed in other fish species exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and PCB congeners (Guiney et al, 1990). However, Hansen et al. (1974) found that fertilization success of sheepshead minnow eggs was not affected by Aroclor 1254

concentrations as high as 201 µg/g. Our results suggest that the embryos of the olive flounder have similar Aroclor 1254 sensitivity as the eggs of some freshwater species.

In this study, control embryos 10 h post-fertilization were normal (Fig. 4A). However, eggs exposed to Aroclor 1254 exhibited developmental abnormalities, including blastoderm coverage of only half the yolk sphere, with a darkened outer layer and an irregular outer circle (Fig. 4B). At 20 h post-fertilization, control embryos in seawater developed normally with the usual optic cup development (Fig. 4C). In contrast, 40 µg/L Aroclor 1254-treated embryos had either no or abnormal optic cup formation (Fig. 4D). At 30 h post-fertilization, skin melanophores and an eye lens appeared in the control embryos (Fig. 4E), but these features were absent in the 40 µg/L Aroclor 1254-treated embryos (Fig. 4F). Other apparent defects resulting from Aroclor 1254 exposure were an abnormal tail flexure and a morphologically irregular or atrophied yolk-sac (Fig. 4F). In this study, the most common morphological defects observed in the olive flounder embryos following Aroclor 1254 exposure were edema (atrophy) and hemorrhage in the yolk-sac (Fig. 4). The lake trout had similar malformations in response to other organic and inorganic pollutants, which are characterized by leakiness of the endothelial vessels supplying the yolk sac as a result of cardiovascular dysfunction (Guiney et al., 1990).

Larvae toxicity of Aroclor 1254

Survival of olive flounder larvae decreased significantly with increasing Aroclor 1254 concentration (Fig. 5); the LC_{50} value was 3.08 µg/L (upper, 3.38; lower, 2.78). Other studies have shown that PCB congeners in particular cause early life stage mortality in other fish species, where 3.7 and 8.7 µg/g H_XCB in the Chinook salmon *Oncorhynchus tschawytscha* sac-fry, and lake trout larvae, respectively, caused 100% mortality (Broyles and Noveck, 1979). In the presence of PCB congeners, larval mortality was associated with hemorrhages and fluid accumulation beneath the yolk sac epithelial membrane, resembling blue-sac disease (Spitsbergen et al., 1991; Walker et al., 1994, 1996). The cardiovascular system appears to be the location of TCDD-induced blue-sac disease (Spitsbergen et al., 1991), leading to lake trout and rainbow trout *Oncorhynchus mykiss* early life-stage mortality (Walker et al., 1994, 1996). However, Kim and Copper (1999) reported that PCB77 (1.6 µg/mL) caused 100% mortality but no hemorrhages in larvae of Japanese medaka.

In our study, the percentage of larvae displaying developmental defects was similar in embryos collected from all three sites (Fig. 6). There were no larval abnormalities in the controls. The malformations observed can be classed as relatively unspecific responses to a number of pollutants (Strmac and Braunbeck, 1999). The percentage of larval abnormalities increased in accordance with Aroclor 1254 concentration (Fig. 6) and the Aroclor 1254 EC_{50} was 5.61 µg/L (upper, 17.08;
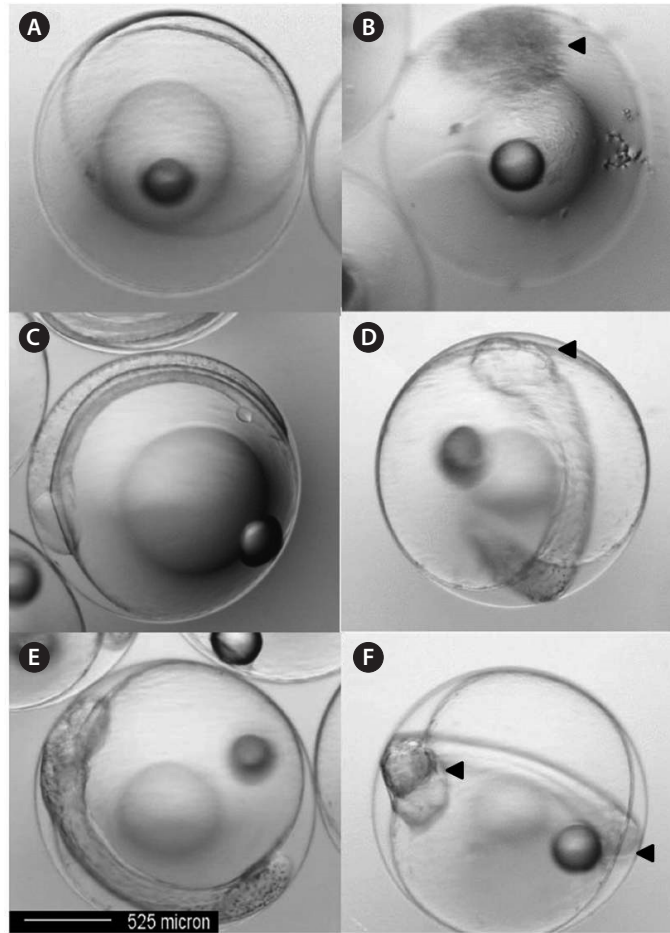
Fig. 4. *Paralithys olivaceus* eggs exposed to 0 μg/L groups (A, C, E) and 40 μg/L Aroclor 1254 concentration groups (B, D, E). (A, C, E) Normal egg or embryo of control group. (B) Darkening of outer layer of egg and irregular egg membrane (12 h after exposure). (D) No optic cup formation (24 h after exposure). (F) Abnormal head region and undeveloped tail (36 h after exposure). The black arrows point out the abnormalities of egg or embryo exposed to Aroclor 1254.
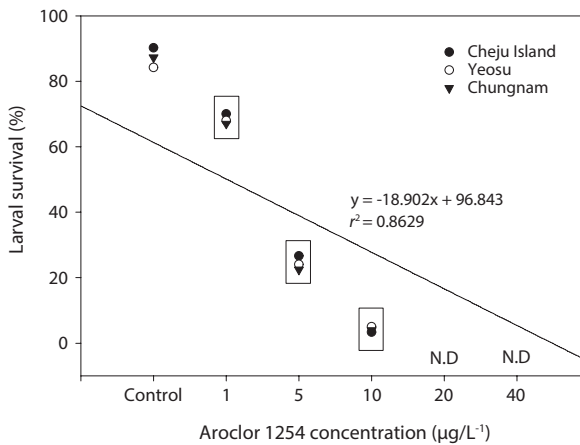

Fig. 5. Linear relationship shown between larval survival rate and Aroclor 1254 concentration in the olive flounder *Paralithys olivaceus* collected from three different hatcheries and then exposed in the laboratory to various Aroclor 1254 concentrations. N.D, no data because of 100% mortality in 20 and 40 μg/L groups. □ indicates significant differences between control groups and exposure groups ($P < 0.05$).
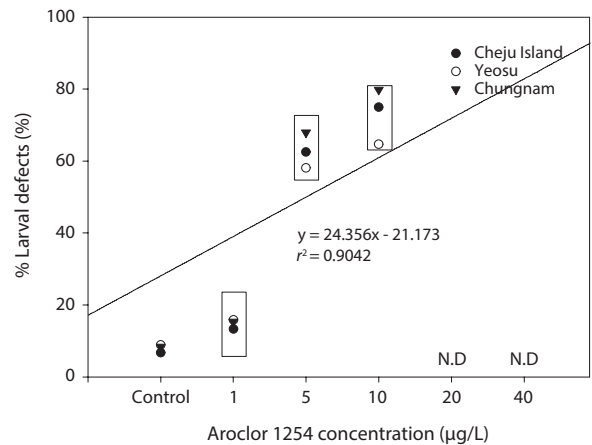

Fig. 6. Linear relationship shown between larval defects rate and Aroclor 1254 concentration in the olive flounder *Paralithys olivaceus* collected from three different hatcheries and then exposed in the laboratory to various Aroclor 1254 concentrations. N.D, no data because of 100% mortality in 20 and 40 μg/L Aroclor 1254. □ indicates significant differences between control groups and exposure groups ($P < 0.05$).
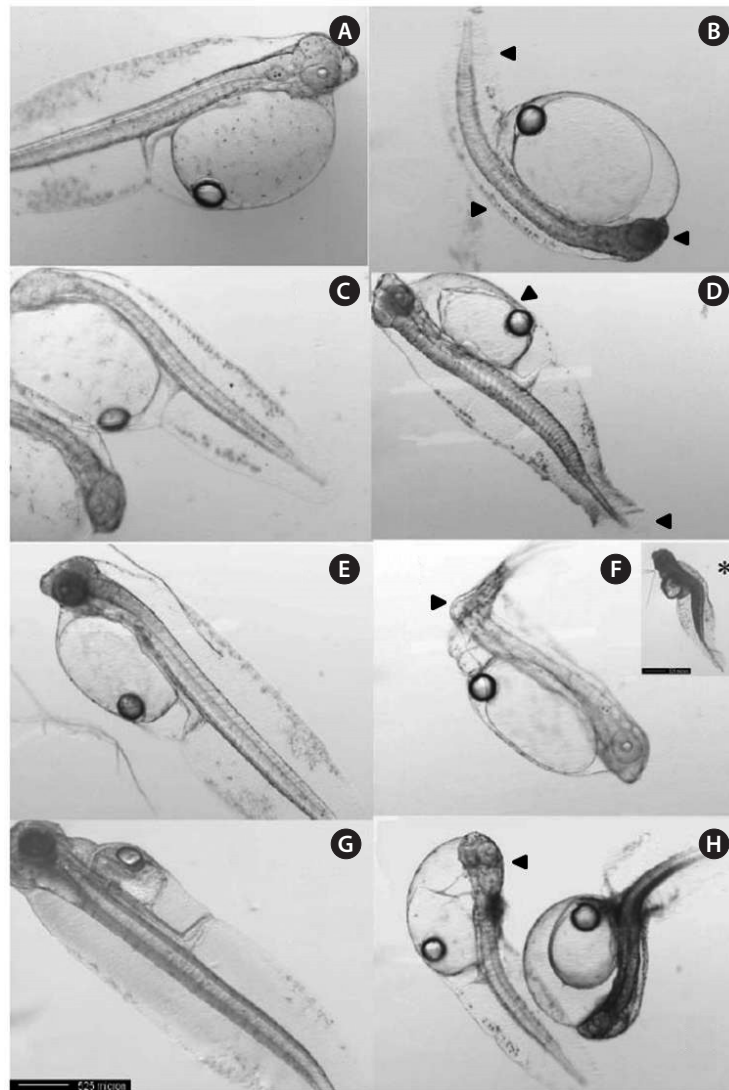
Fig. 7. *Paralithys olivaceus* embryo-larvae exposed to 0 μg/L groups (A, C, E, G) and 40 μg/L Aroclor 1254 concentration (B, D, F, H). (A, C, E, G) Newly hatched larvae of control group (normal). (B) Tail abnormality and erosion of dorsal and ventral fins and darkened head (12 h after exposure). (D) Caudal-fin erosion and atrophy of the yolk (24 h after exposure). (F) Circumflex caudal-born. *Inset is the extreme spinal curvature (48 h after exposure). (H) Abnormal portion of head, darkening of body and yolk-sac edema; Disability of development (72 h after exposure). The black arrows point out the abnormalities of larvae exposed to Aroclor 1254.

Table 2. Total length of olive flounder *Paralichthys olivaceus* larvae exposed to various Aroclor 1254 concentrations

Aroclor 1254 Concentration (μg/L)	Duration time of exposure (hours)				
	0	**6**	**12**	**18**	**24**
Control	2.49 ± 0.16	3.11 ± 0.35	3.14 ± 0.56	3.45 ± 0.27	4.47 ± 0.30
1	2.49 ± 0.16	2.61 ± 0.46	2.90 ± 0.13	2.91 ± 0.22*	3.54 ± 0.19*
5	2.49 ± 0.16	2.71 ± 0.22	2.73 ± 0.15*	3.27 ± 0.88*	3.33 ± 0.12*
10	2.49 ± 0.16	2.59 ± 0.69	2.78 ± 0.17*	2.93 ± 0.17*	3.25 ± 0.63*
20	2.49 ± 0.16	2.95 ± 0.74	2.99 ± 0.22	3.22 ± 0.89*	3.30 ± 0.93*
40	2.49 ± 0.16	2.62 ± 0.67	2.88 ± 0.30	3.25 ± 0.17*	3.23 ± 0.23*

All values represent the length ± S.E.M (mm) for larvae.
*Superscripted letters indicate significant difference between the control and exposed groups ($P < 0.05$).

lower, 4.41). For larvae exposed to 1 μg/L, approximately 21% exhibited lateral curvature or spinal cord deformation (Fig. 7B shows larvae after 12-h exposure). Many of the surviving larvae displayed abnormal tail flexure and this became more pronounced with increased exposure (Fig. 7F and H show larvae after 48-h and 72-h exposure). All Aroclor 1254-treated groups exhibited ventral and caudal fin erosion, vertebral deformity, and abnormality of the yolk-sac (Fig. 7D, F, H). The incidence of gross lesions in larva exposed to Aroclor 1254 was similar to previous reports (Wilson and Tillitt, 1996), where larvae with craniofacial deformities, yolk-sac edema, and hemorrhaging was affected by 8.8 μg/kg total PCBs (Aroclor 1248, 1254, and 1260). Pericardial edema, yolk sac edema and hemorrhage in the liver rudiment occurred in the Japanese medaka after exposure to TCDD (2.5-12.3 pg/mL) and PCB 126 (0.05-0.8 μg/L). Aroclor 1254 produces similar toxicity effects to TCDD (Zable et al., 1995), polychlorinated dibenzodioxins and polychlorinated dibenzofurans in olive flounder larvae (Guiney et al, 1990). These effects, which are indistinguishable from blue-sac disease, start to appear about 12 h prior to hatching. They include yolk sac, pericardial and meningeal edema, craniofacial malformations, circulatory disruption, arrested growth and development, and ultimately death (Spitsbergen et al., 1991; Walker et al., 1994).

Black et al. (1988) reported that winter flounder larvae *Pseudopleuronectes americanus* from New Bedford Harbor (MA, USA), which contained high levels of PCBs, were significantly shorter and weighted less than those from Fox Island (British Columbia, Canada). These data suggest that water-borne PCBs might have contributed to growth retardation. In this study, newly hatched larvae had a total length (TL) of 2.40-2.53 mm. The TL of larvae was significantly reduced by Aroclor 1254 exposure (Table. 2). This confirms that Aroclor 1254 is highly toxic to the olive flounder larvae. There was a dose-dependent increase in toxic effects, and a similar type and sequence of lesions developed in the embryos and newly hatched larvae after exposure to Aroclor 1254.

We confirmed that olive flounder embryos were more tolerant than larvae of exposure to Aroclor 1254 and that malformations due to Aroclor 1254 were more common in the larvae than embryos. These results support the hypothesis of a protective role for the egg membrane against water-borne Aroclor 1254 (Noor et al., 1986).

Conclusions

This study highlights the potential susceptibility of the larval-stage compared to the embryo stage and stresses the importance of maintaining good water quality during production. It also demonstrates that the larval stage of the olive flounder would be a good indicator organism for Aroclor 1254 contamination in coastal waters, and that the response recorded may be typical for other contaminants (Fraysse et al., 2006). Finally, exposure to Arolcor 1254 concentrations as low as 1 μg/L disrupted early developmental stages of the olive flounder, with higher toxicity in the larvae than in the developing, unhatched embryos. To conclude, this study highlights the importance of testing different life stages and recording a range of endpoints when assessing the potential effects of contaminants on fish populations.

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