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A standard ecotoxicological bioassay using early life stages of the marine fish *Psetta maxima*⋆

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Abstract – Fish tests must be developed for both regulatory and conservation reasons, as such testing forms an indispensable component of integrated toxicity testing strategy for the marine environment. To standardise a fish embryo test, the acute toxicity of metals and polycyclic aromatic hydrocarbons (PAHs) to early life stages (ELS) of turbot (*Psetta maxima*) was studied. Embryos were exposed to dilutions of cooper, cadmium, mercury, fluoranthene, phenanthrene, pyrene and naphthalene in darkness and under visible light. Hatching success, yolk sac alterations, pericardial edema, skeletal deformities and mortality were observed. The effective concentrations (EC_{50}, EC_{10},) no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) were determined at the end of the bioassays. Exposure to metals and PAHs in ecologically relevant concentrations, either in darkness or under artificial light, caused significant lethal and sublethal effects in turbot, such as alterations in yolk sac, pericardial edema and skeletal abnormalities. According to the 96 h EC_{50}, the ranking of acute toxicity for metals was respectively: 47.4 (46.8–59.2) μg L^{-1} Cu; 51.5 (20.9–91.7) μg L^{-1} Cd, and 83.2 (62.3–125) μg L^{-1} Hg. For PAHs, acute toxicities were 5.2 (4.3–6) μg L^{-1} pyrene, 12.3 (6.4–12.7) μg L^{-1} fluoranthene, 52.2 (30.4–82.7) μg L^{-1} phenanthrene, and 142 (55–228) μg L^{-1} naphthalene. Pyrene was consistently the most toxic PAH. In addition, light exposures were performed and photo-enhanced toxicity was found only for fluoranthene and pyrene. Turbot embryos were found to be more tolerant to metals and PAHs than were hatched larvae. These results suggest that, considering the short duration of the ELS turbot test and its high sensitivity, it is suitable for use as a standard test for marine fish.

Key words: PAH / Metal bioavaibility / Acute toxicity / Embryo / Larva / Bioassay / Fish

Résumé – Des tests de toxicité doivent être développés sur les poissons en vue de la conservation et la régulation de l'environnement marin. Afin de standardiser un test sur des embryons de poisson, la toxicité de métaux et d'hydrocarbures aromatiques polycycliques sur les premiers stades de développement (ELS) est étudiée chez le turbot (*Psetta maxima*). Les embryons sont exposés à des solutions de cuivre, cadmium, mercure, fluoranthène, phénanthrène, pyrène et naphthalène en obscurité et à la lumière. Les éclosions, les altérations du sac vitellin, oedèmes du péricarde, déformations du squelette et mortalités sont étudiés. Les concentrations effectives (EC_{50}, EC_{10}), ou non (NOEC) et les concentrations minimums provoquant un effet (LOEC) sont déterminées à la fin des essais biologiques. Les expositions aux métaux et hydrocarbures aromatiques en concentrations écologiquement plausibles, soit en obscurité, soit en lumière artificielle, causent des effets létaux et sublétaux significatifs chez le turbot, telles que des altérations du sac vitellin, et des anomalies du squelette. D'après EC_{50} (96 h), la toxicité des métaux est la suivante, classée en ordre croissant : 47,4 (46,8–59,2) μg L^{-1} Cu ; 51,5 (20,9–91,7) μg L^{-1} Cd, et 83,2 (62,3–125) μg L^{-1} Hg. Pour les hydrocarbures aromatiques, leur toxicité respective est 5,2 (4,3–6,0) μg L^{-1} pour le pyrène, 12,3 (6,4–12,7) μg L^{-1} fluoranthène, 52,2 (30,4–82,7) μg L^{-1} phénanthrène, et 142 (55–228) μg L^{-1} pour le naphthalène ; le pyrène est le plus toxique. De plus, des expositions à la lumière sont effectuées, ainsi la photo-toxicité résultante est observée uniquement pour le fluoranthène et le pyrène. Les embryons de turbot semblent plus tolérants aux métaux et hydrocarbures que les stades larvaires. Ces résultats suggèrent que sur une durée réduite, les tests effectués sur les premiers stades de développement du turbot sont très sensibles et sont adéquats pour des tests standards chez les poissons marins.

1 Introduction

Sea-urchin and bivalve early life stages (ELS) are the most common models in marine ecotoxicology (Kobayashi 1995; His et al. 1999). However, for regulatory reasons, fish tests

⋆ Supplementary tables are only available in electronic form at www.alr-journal.org

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must be developed as an indispensable component of integrated toxicity testing strategies for the aquatic environment (e.g. European Council 2000). Acute toxicity tests on vertebrates play an important role in environmental risk assessment and hazard classification because they allow first estimates to be made of the relative toxicity of chemicals in ecologically and economically important species (Wedekind et al. 2007). The egg, embryo, yolk-sac larva, and feeding larva stages are more sensitive to environmental quality variations than are juvenile fish (Rose et al. 1993).

Early ontogenetic stages of fish are generally regarded as the most sensitive life-history stages to toxic agents (Power 1997; Hutchington et al. 1998). This sensitivity may be due to a large body surface: volume ratio, undifferentiated epithelia, and the vulnerability of the developmental processes (Oberemm 2000). During early ontogenesis critical development of tissues and organs takes place, a process which can easily be disrupted by unfavourable environmental conditions, including exposure to toxic compounds (Foekema et al. 2008). For this reason, toxicity tests with ELS of fish are often used to assess the toxic potential of substances and environmental samples. Besides their sensitivity, these tests have practical advantages over tests with older fish in that they require less test volume and space. This enables the use of higher numbers of test organisms and replicates and thus improves the statistical power of the test results (Wedekind et al. 2007).

The available test guidelines for fish ELS testing place their emphasis on fresh water species from temperate or tropical regions (OECD 1992, 1998 & 2006). However, the utilisation of marine species is scarce, especially with respect to species relevant for western European regions. We therefore investigated the potential of the marine flatfish turbot, *Psetta maxima* (Pleuronectiformes, Scophthalmidae) for use as a test species in ELS development tests. Turbot is a native European species of both ecological and economic importance. Adult mature stocks are available throughout the year as the species is reared under controlled conditions for aquaculture purposes. Bioassays with marine organisms for the assessment of marine pollution must meet certain fundamental requirements: (i) ecological relevance; (ii) feasibility, as the bioassays must be easy to standardize based on precisely defined protocols and use simple, rapid and cost-effective methods; and (iii) sensitivity, offered by the early life stages, which are less tolerant to toxicants than adults (e.g. Ringwood 1991; His et al. 1999), but can be used to record sublethal responses. This paper describes a standard ELS test with fertilised eggs of turbot, using hatching success, larval survival and malformations as endpoints. It reports the effects of two different kinds of toxicants, metals and polycyclic aromatic hydrocarbons (PAHs), on the early development of the turbot (*Psetta maxima*).

2 Materials and methods

2.1 Biological material

Turbot (*Psetta maxima*) eggs from a single stock of adults were obtained in kind from a fish hatchery (PESCANOVA Insuiña, Mougás, Galicia, Spain). Eggs were transported to the laboratory in a portable ice-box, in plastic bags containing seawater, and maintained in aquaria with running natural seawater (salinity 34). Eyed eggs were acclimated to laboratory conditions for 24 h at 14 ± 1 °C (hatchery rearing temperature) before the experimental exposures to toxicants were started.

2.2 Experimental solutions and exposures

Metal stock solutions were made up from copper ($CuSO_4 \cdot 5H_2O$), cadmium ($CdCl_2 \cdot 2^1/_2\ H_2O$) and mercury ($HgCl_2$), obtained from Sigma–Aldrich, (Steinheim), dissolved in Milli-Q water (Direct-Q^{TM} ultrapure water systems Millipore, Canada). Copper, cadmium and mercury were obtained by dissolving $CuCl_2 \cdot 2H_2O$, $CdCl_2 \cdot H_2O$ and $HgCl_2$ in chemically defined artificial seawater (ASW), made up from analytical grade reagents and Milli-Q water following the formulation of Lorenzo et al. (2005). Preliminary trials showed no significant differences in hatching success when using 0.22 μm filtered natural seawater (FSW) compared with ASW. For each toxicant, six concentrations in a 2× geometric scale were tested, plus one control with no metal added, using four replicates for each condition. Fresh solutions were prepared daily. Nominal concentrations of 5, 10, 20, 40, 80 and 160 $\mu g\ L^{-1}$ were used for Cu and Cd, and 3, 9, 27, 81, 243 and 729 $\mu g\ L^{-1}$ for Hg.

Naphthalene, phenanthrene, fluoranthene and pyrene (Sigma–Aldrich, Steinheim) stock solutions were made up in dimethyl sulfoxide (DMSO, Sigma–Aldrich, Steinheim) and stored in amber glass vials. All treatments, including controls, contained <0.01% (v/v) DMSO, so this was constant in all treatments for a given experiment. A preliminary test was performed to evaluate DMSO toxicity in the turbot ELS assay. A stock solution of 10% DMSO in FSW was serially diluted using FSW to make up 0.05%, 0.1%, 0.15% and 0.3% (v/v) DMSO solutions for these experimental exposures.

For PAHs, glass vials containing at least 200 ml of each exposure solution and 50 eggs per condition were used. Naphthalene exposures were conducted at the nominal concentrations 15.75, 31.5, 62.5, 125, 250 and 500 $\mu g\ L^{-1}$, fluoranthene and phenanthrene exposures at 6.25, 12.5, 25, 50, 100 and 200 $\mu g\ L^{-1}$, and pyrene at 1.25, 2.5, 5, 10, 20 and 40 $\mu g\ L^{-1}$. The experimental concentrations were obtained by diluting the DMSO-stock solutions in ASW, as prepared in Lorenzo et al. (2005). The stock solutions were prepared daily and stored at room temperature in the dark.

Experimental concentrations were chosen on the basis of range-finding trials and data from the literature. Tested concentrations for each compound were below their water-saturation levels. Incubations were made in 1000 ml plastic beakers (for metals) or 250 ml glass bottles with airtight Teflon-lined screw caps (for PAHs), to avoid losses of the tested compounds from the solutions. All glassware was acid-washed (HNO_3 10% vol.) and rinsed with acetone and distilled water before the experiments.

The experimental design followed the recommendations of OECD guidelines (OECD 1998) and the EU Commission Directive 92/69/EEC, with the modifications indicated below.

All of the experiments were performed using a semi-static test with water renewal every 48 h.

2.3 Fish embryo exposure and toxicity assay

Immediately after their arrival at the laboratory, within 72 hours post-fertilization, the floating fertilised eggs were collected and the non-fertilised eggs at the bottom discarded. The eggs were examined under a dissecting microscope, and those embryos exhibiting normal development that had reached the blastula stage were selected for subsequent experiments. Briefly, 50 normal fertilized eggs were randomly selected and carefully distributed into exposure Pyrex-glass bottles or plastic beakers containing 200 and 500 ml FSW or ASW and spiked with the test solutions. Treatments were incubated per quadruplicate in an isothermal room (18 ± 1 °C) in the dark, except for the PAHs which were tested both in the absence and presence of artificial visible light. (14:10 h light:dark) in order to study their potential photoinduced toxicity. Cool daylight lamps (Osram L15W/765) were used in order to simulate natural irradiation (emission spectrum range: 380–780 nm; photosynthetically active radiation). Control beakers were set up similarly. Neither food nor aeration was provided during the bioassays. Eggs were transferred into each beaker from the lowest to the highest concentration to minimize the risk of cross-contamination.

The effects of the toxicants on turbot embryos and larvae were observed daily throughout the 6-day exposure period. The number of dead eggs/embryos was recorded 48 h after incubation. Hatching was defined as the rupture of the egg membrane. Partially- as well as fully-hatched larvae were counted as hatched.

Survival and malformation of larvae were observed and recorded every day after hatching. Embryos and larvae were considered dead when no heartbeat could be observed. Mortality was identified by coagulation of the embryos, missing heartbeat, failure to develop somites and a non-detached tail. Sublethal endpoints recorded included embryo malformation, hatching success and mortality.

The observations were made using a thick slide with a concave chamber, which was filled with clean seawater. Each larva was carefully placed in the chamber and observed under a binocular dissection microscope (magnification 1.5 × 1.6) using MultiScan (Nikon SMZ1500) computer image analysis.

2.4 Statistical analyses

Differences between treatments were tested for significance by means of one-way analysis of variance (ANOVA). When differences among groups were significant, the Dunnett's test was employed to compare the control group with each of the experimental groups for calculation of the lowest observed effect concentration (LOEC). Statistical significance was set at $p < 0.05$. The EC_{10} and EC_{50} (median effective concentration) and their 95% confidence intervals (95CI) were calculated according to the probit method, after normalizing data to the mean control response using Abbot's formula (Emmens 1948). For analysis, data were first arcsine-transformed to achieve normality (Hayes 1991). The results of the experiments conducted in the dark and under artificial light were analyzed using two-way ANOVA. Also, significant differences between parameters for pairs of curves from the dark and light exposure experiments were tested using an extra sum-of-squares F-tests were used to determine whether curves were statistically indistinguishable (Motulsky and Christopoulos 2004). Statistical analyses were conducted using SPSS statistical software version 16.0.

3 Results

3.1 Hatching 48-hour exposure success

Metal and PAH exposures caused a significant reduction in hatching success and larval survival according to monotonic dose-response patterns, which allowed the calculation of toxicity parameters, as well as the identification of a number of body malformations (Figs. 1–2, Tables 1–2, and S1 to S5).

Concerning the metals (Tables S1 and S2), at 5 μg Cd L^{-1} exposure a 98% hatching success was recorded. The first developmental abnormalities and mortalities of the embryos were observed above 40 μg Cd L^{-1}. At 20 μg Cu L^{-1} embryo development had 99.5% success, whilst at 160 μg L^{-1} only 60% of hatching was successful. For both, Cd and Cu exposures, alterations in yolk sac and non-rupture of egg membrane were observed. At Hg concentrations of 243 and 729 μg L^{-1} only 18.5 and 1.3% hatching success, respectively, was observed.

The median effective concentration (EC_{50}) was obtained for Cd at 112 μg L^{-1} (92.5–132), for Cu: 110 μg L^{-1} (64.5–116) and Hg: 79 μg L^{-1} (66.1–98.7) (Table 1). The three elements ranked consistently in the following order from highest to lowest toxicity: Cd> Cu> Hg.

The development of the eggs/larvae in <0.1% (v/v) solvent controls (DMSO) was not significantly different from that in control seawater. At the end of this experiment, 90% of the eggs from both controls (seawater and DMSO) had developed into normal turbot larvae (data not shown). This control experiment ensures that the toxicity shown in the PAH exposures is not attributable to the solvent. In contrast, abnormalities in the pericardial edema, somites failure, and alterations in the yolk sac were found for 0.3% (v/v) DMSO, a concentration higher than those used in the PAH exposures.

In the PAH exposures, we observed high levels of hatching success (i.e. 98, 98.5, 97.5 and 95.5% for fluoranthene, phenanthrene, pyrene and naphthalene, respectively) for the lowest PAH concentrations tested (Table S3), and a sharp increase in malformations (alterations of yolk sac, pericardial edema and coagulation of eggs) as PAH concentrations increased. The most common abnormalities were alterations in the yolk sac, followed by embryo deformities (Tables S4, S5).

3.2 Larval 96-hour exposure response

An average 93% survival in the controls shows good experimental conditions, which is in accordance to the Organisation for Economic Co-operation and Development (OECD) guideline for ELS tests (OECD 1998). The number of dead larvae and malformations in the metal and PAH exposure

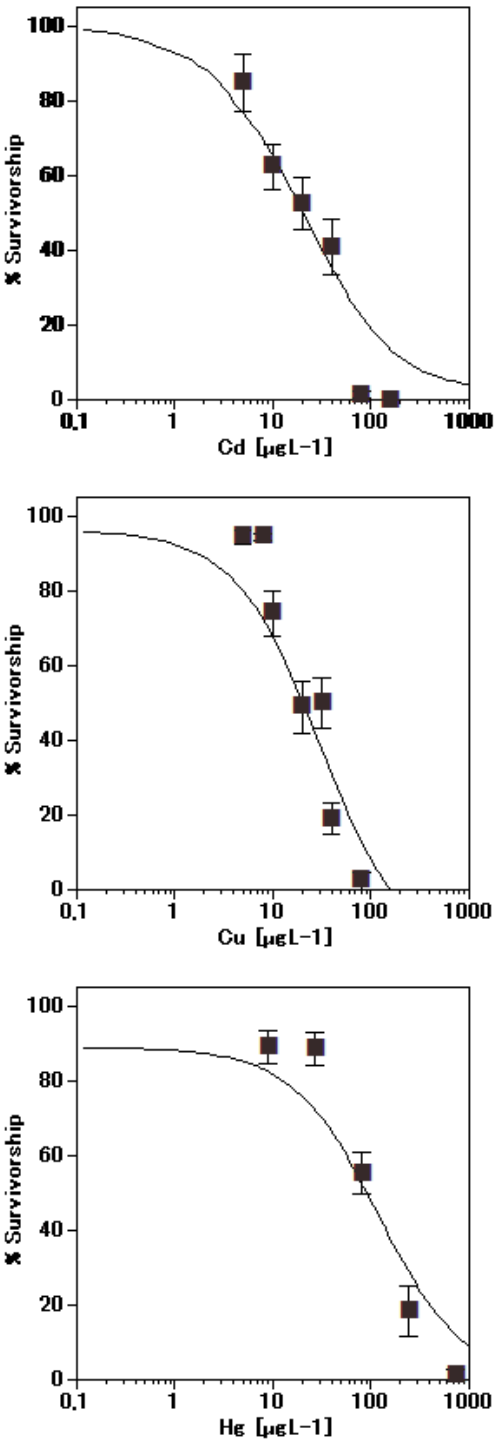

Fig. 1. Survival of turbot embryos and larvae exposed to Cd, Cu and Hg.

treatments increased significantly with increasing concentrations at 24 and 96 hours post-hatching (hph) exposure ($p < 0.05$) (Table S3).

In both Cu and Cd exposed groups, the same types of body malformations were observed (Table S2). Alterations in the yolk sac were the most frequent, and were noted in 16 and 12 occasions for Cu and Cd, respectively; pericardial edema was observed in 13 and 12 cases, respectively. In contrast, no skeletal deformities were observed in Cd exposure groups. EC_{10}, EC_{50}, NOEC and LOEC values at 96 hph were calculated for all test compounds (Table 2).

In the case of PAH toxicity bioassays, both in darkness and artificial light, the dominant effects were mortality, edema and skeletal abnormalities (Tables S4 and S5). The major abnormalities found were the skeletal deformities: 9 and 15 cases for pyrene, 7 and 11 cases for fluoranthene and 8 and 7 cases for phenanthrene, in dark and light conditions, respectively. The second most important abnormality recorded was pericardial edema: with pyrene these occurred in 6 and 10 cases and with fluoranthene in 7 and 9 cases, in dark and light, respectively). Lastly, alterations in the yolk sac occurred in 13 and 12 cases for fluoranthene and 6 and 5 cases for naphthalene exposure, in dark and light, respectively.

Fluoranthene caused malformations and mortality in turbot larvae according to a sigmoidal toxicity curve (Fig. 2). The EC_{10} and EC_{50} values were 4.7 (3.8–10.3) and 31.0 (19–63) $\mu g\ L^{-1}$ in dark conditions and 2.6 (0.4–2.8) and 12.1 (6.4–12.7) $\mu g\ L^{-1}$ under artificial light, respectively (Table 2). Pyrene showed toxicity to turbot larvae both in dark condition (EC_{10} = 18.7 (8.5–25.3), EC_{50} = 33.6 (11.4–40.7) $\mu g\ L^{-1}$) and under light (EC_{10} = 3.4 (1.5–3.4), EC_{50} = 5.2 (4.3–6) $\mu g\ L^{-1}$). Similarly, phenanthrene showed EC_{10} and EC_{50} values of 6.7 (3.4–8.3) and 56.4 (30.4–82.7) $\mu g\ L^{-1}$ for dark and 4.5 (2.3–6.6) and 41.0 (17.1–46.3) $\mu g\ L^{-1}$ under light condition. Naphthalene was less toxic to turbot larvae and showed less dark-light difference (Fig. 2). Both, pyrene and fluoranthene were significantly more toxic under light than in the dark ($p < 0.05$).

4 Discussion

Exposure to metals and PAHs in ecologically relevant concentrations, either in darkness or under artificial light, caused significant lethal and sublethal effects in turbot such as alterations in yolk sac, pericardial edema and skeletal abnormalities. Pyrene and fluoranthene appeared to be more toxic under light conditions. Turbot embryos were found to be more tolerant to metals and PAHs than were hatched larvae.

Our findings are similar to those of other studies that reported skeletal deformities as being the most frequent teratogenic effects in fish ELS exposed to metals (Weis and Weis 1991). Metal exposure caused significant reduction of hatching success and numerous body malformations. It also caused an increase in the number of deformed larvae among the newly hatched fishes. The results showed that cadmium at >20 $\mu g\ L^{-1}$ reduced the success of hatching and caused a number of deformities such as alterations in yolk sac and pericardial edema, in agreement with Cheng et al. (2000), Von Westernhagen (1988), and Nguyen and Janssen (2001). In contrast, some authors found only low effects of Cd on hatching success (Williams and Holdway 2000; Nguyen and Janssen 2001, 2002). However, no skeletal deformation was observed for Cd in our study, which is similar to findings by Nguyen and Janssen (2001). The EC_{50} is higher than the promulgated guidelines or standards for the protection of marine life (3 $\mu g\ Cd\ L^{-1}$) (GRNC, 2002). Generally, the acute toxicity of cadmium to fishes is highly variable, with EC_{50} ranging

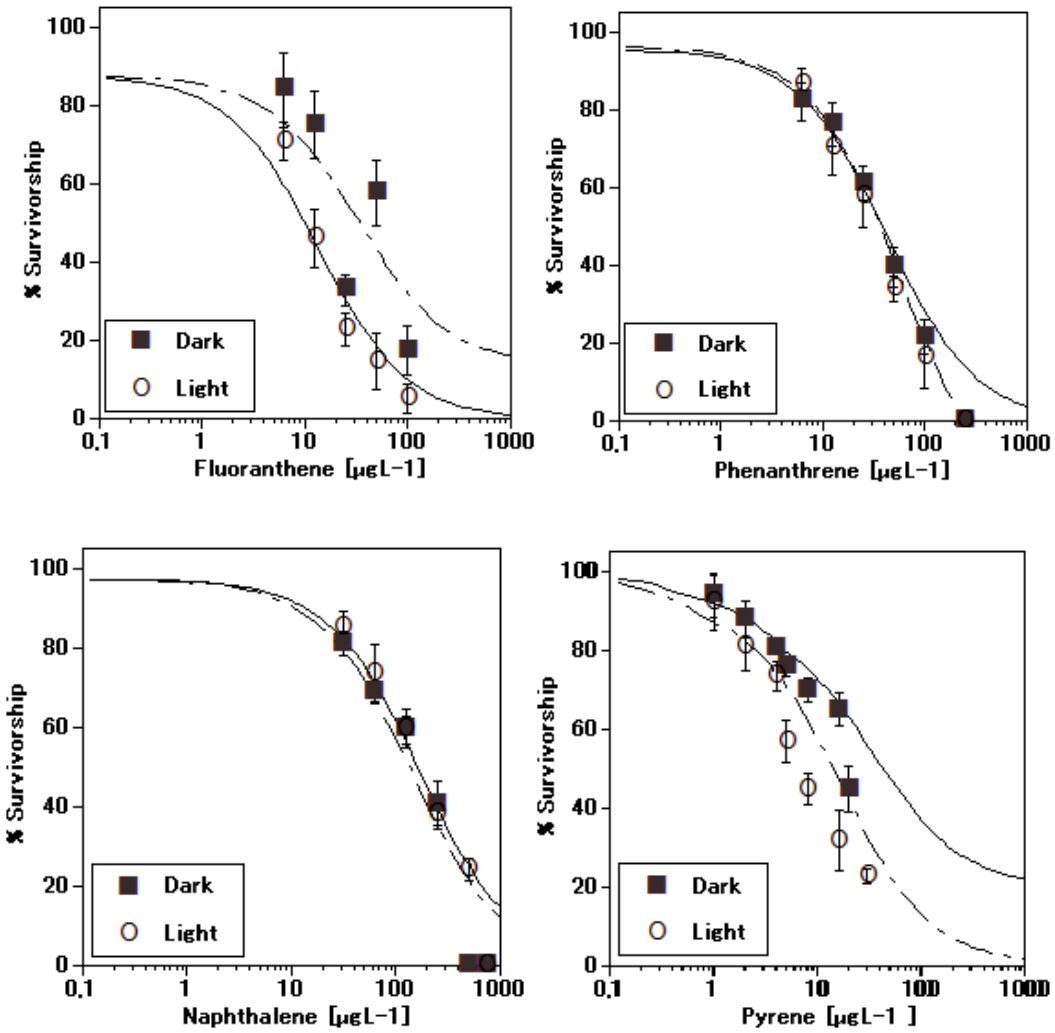

Fig. 2. Survival of turbot embryos and larvae exposed to fluoranthene, phenanthrene, naphthalene and pyrene.

from as low as 1–30 μg L^{-1} for salmonids to 600 μg L^{-1} in flounder (*Pseudopleuronectes americanus)* (Mance 1987). The low EC_{50} calculated in our study highlights the sensitivity of turbot to Cd. Witeska et al. (1995) reported that 47% of mirror carp larvae were deformed after exposure to 200 μg L^{-1} of Cd. The proportion of deformities seems to increase with increasing Cd concentration (Calta 2001). The toxicity of Cd for ELS of turbot appears to be more elevated after hatching and may be in relation to a considerable increase of Ca^{2+} uptake (Chen et al. 2003).

Copper toxicity can vary with water chemistry, temperature and fish species, and can induce damage leading to the death of organisms (Grosell et al. 2002). The NOEC for Cu in our study was 4 μg L^{-1}, a level in line with results reported by Grosell et al. (2002). This study proved that Cu above 20 μg L^{-1} significantly affected the hatching success and increased the mortality and frequency of gross deformities of turbot embryo-larvae after 48-h exposure. Nonetheless, decreased hatching success was observed in embryos exposed to concentrations > 50 μg Cu L^{-1}, in agreement with Johnson et al. (2007). Similarly, studies conducted on other species have shown that fish larvae are very sensitive to low levels of Cu (Wu et al. 2003; Stasiûnaitë 2005).

Acute exposures to Hg had adverse effects on turbot embryo-larval development. The calculated acute EC_{50} and EC_{10} for exposure to various Hg concentrations in turbot larvae resulted in values of 83.22 (62.3–125) and 43.27 (36.2–58.8) μg L^{-1}, respectively. This is similar to the LC_{50} and LC_{10} values of 86.8 and 73 μg L^{-1} found by Gharaei and Esmaili-Sari (2008) for *Rutilus frisii*. Comparing the EC_{50} values from our study with data reported by the World Health Organization (WHO 1989) we found a similar toxicity in turbot to that obtained for striped bass (*Roccus saxatilis*) at 90 μg L^{-1}.

Recent studies indicated that chronic PAH exposures in fish embryos can cause early life-stage mortality and a suite of sublethal effects similar to the blue-sac disease, which are attributed to more persistent alkylated aromatic compounds (Heintz et al. 1999). Here we assessed the effects of the four most abundant PAHs in Ría de Vigo (Galicia, NW Iberian peninsula). The results are ecologically relevant considering the background levels of PAHs found in the water in the Galician Rias (0.05 to 2.86 μg L^{-1}; González et al. 2006).

Table 1. Effective concentrations EC10, EC50 (μg L^1) and their confidence intervals (C.I.) for hatching success of turbot eggs exposed to Cu, Cd, naphthalene, fluoranthene, phenanthrene and pyrene for 48 h.

Exposure	Light				Dark			
	EC_{10}		EC_{50}		EC_{10}		EC_{50}	
	values	CI 95%	values	CI 95%	values	(CI 95%)	values	(CI 95%)
Cu	35.1	28–38	109.5	65–116	n.r.	n.r.	n.r.	n.r.
Cd	55.4	31–73	112.3	93–132	n.r.	n.r.	n.r.	n.r.
Hg	26.4	17–39	79	66–99	n.r.	n.r.	n.r.	n.r.
Pyrene	16.6	10–20	36.3	23–47	32.5	21–56	78.9	72–103
Fluoranthene	94.5	71–124	135.5	100–163	81.2	60–92	130.7	53–142
Phenanthrene	102.5	77–114	74.5	67–94	42.9	32–92	63.5	345–82
Naphthalene	152.4	112–243	354.2	77–413	121	62–123	283.5	134–352

n.r.: not recorded

Table 2. No observed effect concentrations (NOEC), the lowest observed effect concentrations (LOEC), EC10, EC50 (μg L^1) and their confidence intervals (C.I.) for survival of turbot larvae exposed to Cu, Cd, naphthalene, fluoranthene, phenanthrene and pyrene for 96 h.

Exposure type	NOEC	LOEC	EC_{10}		EC_{50}	
			values	CI 95%	values	CI 95%
Cu	4	5	7.3	6.1–9.4	47.39	46.8–59.2
Cd	5	10	11.1	5.2–13.8	51.54	20.9–91.7
Hg	9	27	43.3	36.2–58.8	83.22	62.3–125
Pyrene						
Light	1.3	2.5	3.4	1.5–3.4	5.17	4.3–6.0
Dark	2.5	5	18.5	8.5–25.3	33.6	11.4–40.7
Fluoranthene						
Light	n.r.	6.3	2.6	0.4–2.8	12.3	6.4–12.7
Dark	n.r	6.3	4.7	3.8–10.3	31.1	19–63
Phenanthrene						
Light	6.3	12.5	4.5	2.3–6.6	41.0	17.1–46.3
Dark	6.3	12.5	6.66	3.4–8.3	52.2	30.4–82.7
Naphthalene						
Light	15.8	31.5	50.0	36.2–72.4	141.9	55.2–228
Dark	15.8	31.5	52.6	17.1–93.1	160.2	74.6–196

Because DMSO was used as the PAH solvent, its toxicity was independently tested in a preliminary experiment. The findings showed a NOEC value of 0.1% DMSO to be non toxic in the preliminary test. In contrast, higher concentrations produced several effects, including mortality and many malformations. Foekema et al. (2008) determined NOEC values of 0.1% for Sole (*Solea solea*), similar to the present study. Other authors have pointed out that DMSO had no effect on zebrafish embryo development (Hallare et al. 2004).

Under both dark and light conditions, all PAHs tested were toxic to turbot embryos and larvae at the experimental concentrations tested; however, only pyrene and fluoranthene were significantly more toxicity to turbot when exposures were conducted under light.

We observed a significant increase in the effects on hatching success, malformations and death of larvae at increasing PAH concentrations. The differences in the number of dead larvae between 24, 48, 72 and 96 h were found significant for each concentration of each of the PAHs ($p < 0.05$). In a similar way to our results, Carls et al. (1999) found that embryonic herring consistently responded to a suite of aqueous PAHs in the low parts-per-billion range. The observed skeletal, pericardial edema, and yolk sac alterations appear to be the most important effect of increasing PAH concentrations, which finally lead to a decreased survival. Other fish species subjected to PAHs suffered similar types of abnormalities (e.g. Carls et al. 1999; Pollino and Holdway 2002; Boudreau et al. 2009; Gonzalez-Doncel et al. 2008). Among all studied PAHs, pyrene was the most toxic, in line with results reported by Pollino and Holdway (2002). The four compounds ranked consistently in the following order from highest to lowest toxicity: pyrene > fluoranthene > phenanthene > naphthalene.

Photoinduced toxicity of PAHs has been demonstrated since early 1980s in many studies with freshwater organisms (reviewed by Arfsten et al. 1996), and the effect is especially remarkable when using UV irradiation (Pelletier et al. 1997). In this work, phenanthrene and naphthalene did not show enhanced toxicity to turbot larvae under artificial visible light. There were no significant differences in the effect of concentrations between presence and absence of light for those two PAHs, in agreement with Bellas et al. (2008). However, significant light-darkness differences were found for pyrene and fluoranthene ($p < 0.05$). These observations are in agreement with previous studies where phototoxicity has been shown in a variety of fishes, including bluegill sunfish (*Lepomis macrochirus*) and fathead minnow (*Pimephales*

promelas) (Ankley et al. 1994). In contrast, Kagan et al. (1985) did not find toxicity of fluoranthene and pyrene to embryos and larvae of *Pimephales promelas.*

The irradiance value used in our experiments may be easily reached or even exceeded in the water column (Barron et al. 2000), indicating that photo-enhanced toxicity of certain PAHs may occur in the marine environment. This factor should thus be taken into account in risk evaluation procedures for these substances. On the basis of the present data, and ignoring the photoactivation of PAHs, worst-case-scenario environmental concentrations of phenanthrene, fluoranthene and pyrene are considered to pose a risk to turbot larvae. The risk values increase when we consider light exposure, especially for pyrene and fluoranthene. Although the fish acute toxicity test is a mandatory requirement under environmental protection agency policies, long-term sublethal effects of these contaminants would increase the overall risk to aquatic biota.

5 Conclusion

This study shows that the flatfish turbot is a suitable marine test organism to perform early life stage tests. As a test species turbot is relevant for the European ecosystem (Atlantic Sea area) and turbot eggs are readily available since the species is reared on fish farms all year round.

Both 48-h hatching success and 96-h post hatching larval survival are suitable endpoints for the standard test, but the latter increases approximately doubles the sensitivity.

The high sensitivity of turbot ELS to metals and PAHs supports their use for ecological risk estimations in coastal areas under the influence of harbour activities or other inputs of hydrocarbons into the sea.

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Supplementary tables

Table S1. 48 h-hatching success and 96 h larval survival of turbot exposed to dissolved copper, cadmium and mercury (n = 200).

Table S2. Morphological abnormalities of turbot embryos and larvae exposed to metals: (A) yolk sac alterations, (B) no rupture of the eggs membrane, (C) pericardial edema, (D) skeletal deformities.

Table S3. 48 h-hatching success and 96 h-larval survival of turbot exposed to PAHs in dark and under artificial light (n = 200).

Table S4. Morphological abnormalities of turbot embryos larvae exposed to PAHs in dark: (A) yolk sac alterations, (B) no rupture of the eggs membrane, (C) pericardial edema, (D) skeletal deformities.

Table S5. Morphological abnormalities of turbot embryos and larvae exposed to test toxicants under artificial light: (A) yolk sac alterations, (B) no rupture of the eggs membrane, (C) pericardial edema, (D) skeletal deformities.

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