

Full Length Research Paper

Toxicity of three selected pesticides (Alachlor, Atrazine and Diuron) to the marine fish (turbot *Psetta maxima*)

Mhadhbi Lazhar^{1*}, Toumi Hela¹, Boumaiza Moncef¹ and Aloui Néji²

¹Laboratoire de Biosurveillance de l'Environnement Unité d'Hydrobiologie, Faculté des Sciences de Bizerte, Université de Carthage, Zarzouna 7021, Tunisie.

²Institut National des sciences et technologies de la Mer de Salammbôo Tunisie.

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The present study aimed to evaluate acute toxicity tests for three selected herbicides: Alachlor, Atrazine and Diuron using turbot flatfish. Larvae were more sensitive than turbot embryos to all pesticides. Median lethal concentrations of the selected pesticides during a 48 h and 96 h exposure for turbot embryos and larvae were, respectively: alachlor, 2177 and 2233; diuron, 10076 and 7826 and atrazine, 11873 and 9957 µg/L. On the basis of the obtained acute toxicity data, all compounds were included among substances highly toxic to fish, in the following order: alachlor > atrazine > diuron. At higher concentrations, pesticides caused a significant increase in embryo mortality. Surviving organisms suffered a significant decrease in hatching success, malformations (embryos), pericardial oedema and skeletal deformation (larvae). All herbicides appear to be teratogenic to the turbot early life stage. Furthermore, the three selected pesticides differed in their toxicity to the fish.

Key words: Turbot, early life stage, acute toxicity, sublethal effects, herbicides.

INTRODUCTION

Fishes are good suitable bio-indicators of environmental pollution monitoring and can play significant roles in assessing potential risk associated with contamination in aquatic environment since they are directly exposed to chemicals resulting from agricultural production or indirectly through food chain of ecosystem (Lakra and Nagpure, 2009). The turbot (*Psetta maxima*) is currently the most commonly cultivated fish species in Galicia, Spain. It is cultivated in various regions, with an annual production of 13, 000 to 14,000 tonnes. Turbot is a good biological model for toxicological studies (Mhadhbi et al., 2010) due to diverse characteristics, namely their high growth rates, efficiency in adapting to diverse diets, great resistance to diseases and handling practices, easy reproduction in captivity at prolific rate and finally, good tolerance to a wide range of environmental conditions. Turbot is constantly at risk of being exposed to contaminants or parasites. In addition, the application of

pesticides in agriculture and in areas located close to wetlands has resulted in toxicity to many non-target species such as fish and aquatic invertebrates (Giari et al., 2008).

Pesticides are widely used all over the world to control the harmful effects of pests on agriculture productions and fish farms. However, despite the good results of using pesticides in agriculture, their use in the environment is usually accompanied by deleterious environmental and public health effects. These pesticides are not always useful. The pesticides after being used ultimately find their way into different aquatic ecosystems and have been found to be highly toxic to non-target organisms, especially aquatic life forms and their environment (Nwani et al., 2010). Pesticide/herbicide pollution severely affects aquatic organisms at higher trophic levels including human beings. The effects of pesticides on fishes are of great concern (Bagheri and Nezami, 2000; Nwani et al., 2010). The effects of insecticides on fish are well documented; however, the effects of herbicides on fish as well as other non-target aquatic organisms are not well documented (Phyu et al.,

*Corresponding author. E-mail: lazhar@uvigo.es.

2006).

Among all forms of chemical pesticides, alachlor, atrazine and diuron are considered to be the most hazardous with respect to environmental pollution, since they are very persistent, non-biodegradable and capable of bio-magnification as they move up in the food chain (Jaffery et al., 1990). Exposure to these compounds can result in mortality as well as sub-lethal impacts. In recent years, some insecticides/herbicides are highly and widely used in Galician area and are on the list of priority substances (According to Annex II of the Directive 2008/105/EC). Alachlor (2-chloro-N-[2,6-diethylphenyl]-N-[methoxymethyl] acetamide) is used for the control of annual grasses and many broadleaf weeds. Its extensive use and its persistence in ground and surface water have posed potential risks for human exposure, not only to professional applicators and farmers, but also to general populations. There have been numerous studies on alachlor, but much less investigation of its effects on fish and aquatic species. Alachlor has been listed as carcinogenic (Potter and Carpenter, 1995; Charizopoulos and Papadopoulou-Mourkidou, 1999) and toxic to humans despite low acute toxicity (Wilson et al., 1996; Orme and Kegley, 2005).

Also, Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is one of the most commonly used herbicides found in the rural environments. It is extensively used on corn, sorghum, sugarcane, and to some extent on landscape vegetation. Rated as moderately toxic to aquatic species, atrazine are one of the most significant water pollutants in rain, fresh, marine and ground waters (Battaglin et al., 2003, 2008). In addition, diuron is a systematic substituted phenyl urea herbicide which is a broad spectrum herbicide used for weed, grass and bush control and it stops photosynthesis which in turn causes plants to stop growing. It is known for its environmental persistence and global concern and has detrimental effects on the environment as well as humans (Jones and Kerswell, 2003). The majority of the diuron entering the aquatic environment is a result of agricultural and residential runoff (Moncada, 2004; Green and Young, 2006).

The pesticides may exert their toxic effects in various forms ranging from alteration within a single cell, whole organism or even changes in whole population (Giari et al., 2008). Thus, contamination by pesticides is a serious water pollution problem, which may cause an environmental imbalance and increase the incidence of poisoning of fish and other aquatic species (Aguar et al., 2004; Barbieri, 2009). In recent years, incidences of fish mortality due to pesticides, industrial effluents and sewage pollution have been reported (Bagheri and Nezami, 2000). Previous studies has been evaluated the acute toxicity of these pesticides on fish. However, the early life stages (embryo, larval, and early juvenile stages) of fish are generally regarded as the most sensitive life-history stages to toxic agents (Power, 1997;

Hutchington et al., 1998), they are ideal for determining responses to environmental contaminants.

However, there have been very few studies of the consequences of embryonic and larval fish exposure to pesticides and its lethal and sublethal effects is not fully understood. 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; Kammann et al., 2009). In tests with early life stage of fish, the toxicant effects can be examined through diverse endpoints, such as hatching success, embryo-larval morphology malformations and larval survival (Mhadhbi and Boumaiza, 2012). Therefore, our objective was to test the toxicity of the most abundant insecticides/herbicide used in Galicia on the early life stages (ELS) of turbot.

MATERIALS AND METHODS

Turbot (*Psetta maxima*) eggs from a single stock of adults were obtained in kind from a fish hatchery (Insuiña S.L., Mougás, Galicia, Spain). The eggs were transported to the laboratory in plastic bags inside portable ice-boxes, and maintained in aquaria with running natural seawater (salinity 34‰). Eyed eggs were acclimated to laboratory conditions for 24 h at $14 \pm 1^\circ\text{C}$ (hatchery-rearing temperature) before the experimental exposures to the toxicants.

Experimental solutions and exposures

Technical-grade atrazine (97.5% purity), alachlor (99.2% purity) and diuron (98% purity) were obtained from Sigma Chemical (Co., St. Louis, MO) (West Chester, PA). The stock solutions of each pesticide were made in 100% Dimethyl sulfoxide (DMSO, Sigma-Aldrich, Steinheim). Pesticide grade DMSO was used as a carrier (0.1%, as this was found to be non-toxic in the preliminary test) in all tests. DMSO was added to the control groups equal to the amount of carrier solvent used for the toxicity tests. Six concentrations in a 2x geometric scale, plus one solvent control and one control with no toxicants added were tested, using four replicates for each condition. All experiments were repeated twice consecutively. Experimental concentrations were chosen on the basis of range-finding trials and data from the literature.

The concentrations tested for each compound were below their water-saturation levels. Incubations were made in 1000 ml glass beakers, 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. Physico-chemical conditions of the experiments were 34.20 ± 0.15 ppt salinity, 7.32 ± 0.70 mg/L O_2 and 8.29 ± 0.11 pH (mean \pm STD, $n = 15$). The experimental design followed the recommendations from OECD guidelines (OECD, 1998) and the EU Commission Directive 92/69/EEC, with the modifications indicated below. The deviations from the guidelines were the higher number of eggs per experiment and the number of reported non-lethal endpoints. All of the experiments were performed using a semi-static test with water renewal every 48 h.

Fish embryo exposure and toxicity assay

Immediately after their arrival at the laboratory, within 72 h post-

fertilization, the floating fertilized eggs were collected and the non-fertilized 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 glass beakers containing 500 ml filtered seawater (FSW) and spiked with the test solutions. The treatments were incubated per quadruplicate in an isothermal room ($18 \pm 1^\circ\text{C}$), in dark. The control beakers were similarly set up. Neither food nor aeration was provided during the bioassays. The eggs were transferred into each beaker from the lowest to the highest concentration to minimize the risk of cross-contamination.

The tests were run for 6 days. The effects of the toxicants on the turbot embryos and larvae were observed daily throughout the 6 days exposure period (from 0 to 2 days embryonic exposure and from 2 to 6 days larval exposure). The number of dead eggs/embryos was recorded 48 h after incubation. Hatching was defined as the rupture of the egg membrane, and partially as well as fully hatched larvae were counted as hatched. Survival and malformation of larvae were observed and recorded every day after hatching.

Mortality was identified by coagulation of the embryos, missing heartbeat, failure to develop somites and a non-detached tail. Recorded sublethal endpoints included embryo malformations -yolk sac alterations, no rupture of the egg membrane, pericardial oedema and skeletal deformities- and hatching success. 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 (magnification 1.5 x 1.6) using MultiScan (Nikon SMZ1500) computer image analysis.

Statistical analysis

The dose-response relationships were described using the modified Weibull model. Fitting procedures and parametric estimations were performed by minimization of the sum of quadratic differences between experimental and model-predicted values, using the non-linear least-squares (quasi-Newton) method provided by the 'Solver' macro of the Microsoft Excel spreadsheet. Parametric estimates were confirmed in the non-linear section of the Statistica 8.0 pack, which was also used to calculate the parametric confidence intervals and model consistency (Student's *t*- and Fisher's *F*- tests, respectively, in both cases with $\alpha = 0.05$). The maximum no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) were established through ANOVA and Dunnett's post-hoc test, using the SPSS application, version 19.0. Non-parametric tests, Kruskal-Wallis and the Mann-Whitney *U*, were used when data did not meet the requirements of normality and homogeneity. Differences were considered as significant when $P < 0.05$.

RESULTS

Exposure to herbicides led to non-lethal and lethal malformations in the early life stages of turbot according to a sigmoidal dose-response pattern shown in Figure 1. Pesticides caused a significant increase in embryo mortality just after 48 h. Surviving organisms suffered a significant decrease in hatching success, malformations (embryos), pericardial oedema and skeletal deformations (larvae), at 96 h post-hatching (hph). The controls

showed normal embryo-larval development, with control mortality always below 10%, as required for test validity. The results indicate no significant difference in mortality between the standard controls and the solvent controls for both the embryos and the 96 hph larvae. The control group showed normal embryo development as described by Kimmel et al. (1995).

For concentrations up to 1250 $\mu\text{g/L}$ alachlor, 2500 $\mu\text{g/L}$ atrazine and 5000 $\mu\text{g/L}$ diuron no significant differences in hatching success were observed for any exposure group of eggs. At higher concentrations a significant difference in hatching success was observed (Figure 1). In the controls and in the groups with lower pesticide concentrations (that is, concentrations aforementioned), the fish showed a well-developed head, body and tail. For those concentrations, occasional malformations were noticed although they were not statistically significant. Fish mortality increased with the concentration of pesticides and the exposure period. Mortality rates of 100% were observed after 96 h exposure to the highest concentrations of the selected pesticides and differences between concentrations were observed (Kruskal-Wallis $H = 30.7$, $P < 0.001$). Pesticide-induced typical morphological abnormalities in embryo-larval development, including no rupture of the egg membrane, yolk sac alterations, pericardial oedema and skeletal deformities, are illustrated in Figure 2.

No observed effect concentration (NOEC), lowest observed effect concentration (LOEC), LC_{10} , LC_{50} and their 95% confidence intervals (CI) of all the pesticides were calculated for both embryogenesis and larval stage of the turbot (Table 1). NOEC/LOEC values were higher for corion-protected embryos (625 and 1250 $\mu\text{g/L}$ for alachlor and diuron, 1250 and 2500 $\mu\text{g/L}$ for atrazine) than for naked larvae (625 and 1250 $\mu\text{g/L}$ for atrazine, alachlor and diuron) (Figure 1). Upon exposure to high concentrations of pesticides, several common malformations have been induced in the embryos: they stopped their development in the epiboly stage and after 48 h, the exposed eggs coagulated (Figure 2). Pesticides induced a spinal malformation which was restricted to the embryo's tail. We observed a special alteration in the shape of the larval tail bud comparable to the embryonic malformation (Figure 2).

DISCUSSION

Acute toxicity of pesticides to early life stage (ELS) fish

Fish embryo-larvae lethal (mortality) and teratogenic (deformity) assays are useful because they can rapidly provide information on developmental toxicants. While so far most research into fish has examined acute toxicity to adults or juveniles as well as endocrine disruption, especially acetyl cholinesterase (AChE) inhibition, there

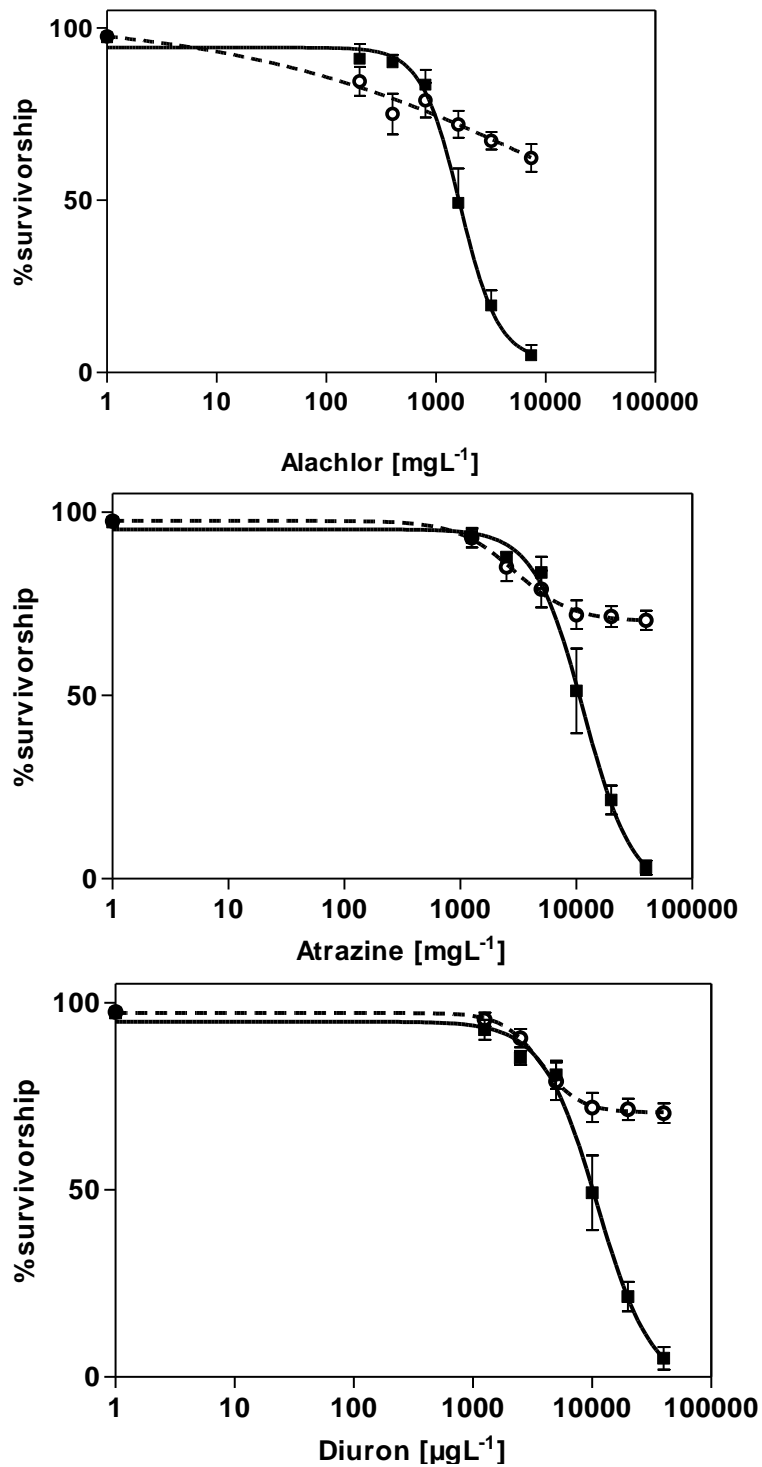


Figure 1. Dose–effect curves of lethal malformations of turbot embryos (48 h) and larval (96 h) caused by selected pesticides exposure. Filled-in squares represent experiments carried out with embryos. Open circles represent experiments carried out with larvae. Error bars represent standard deviations.

is only limited information on the acute toxicity and teratogenic effects of some insecticides and herbicides,

such as diuron, atrazine and alachlor, on the fish early life stage. In fact, we are forced to compare our results with

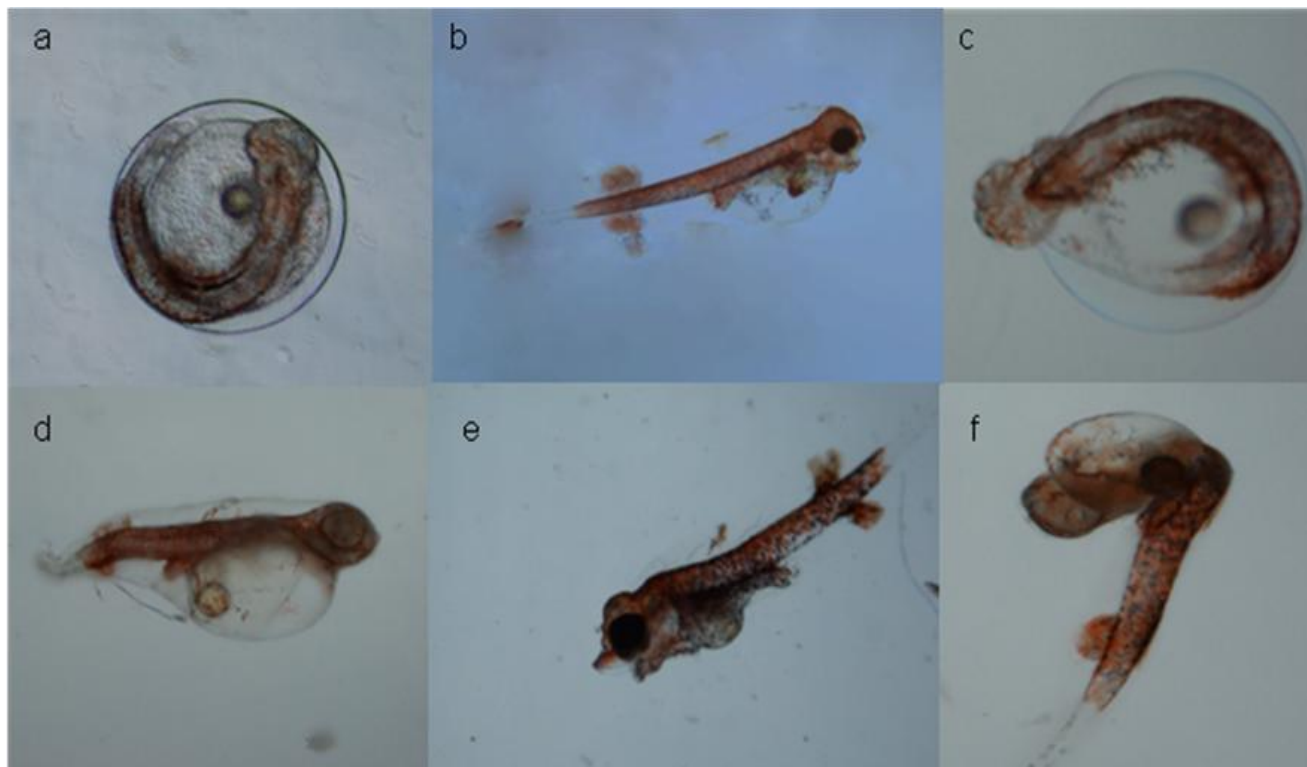


Figure 2. Morphological abnormalities of turbot embryos and larvae exposed to selected pesticides compounds: (A) Normal embryo, (B) Normal larvae, (C) no-rupture of the egg membrane, (D) yolk sac alterations, (E) pericardial edema, (F) skeletal deformities.

Table 1. Toxicity thresholds (NOEC, LOEC and LC₁₀) and semi-maximum response concentration (LC₅₀) for pesticides tested.

Stages	Chemical	NOEC	LOEC	LC ₁₀	LC ₅₀
Embryo	Alachlor	625	1250	1412 (978 - 1918)	2177 (1631 - 2945)
	Atrazine	1250	2500	2987 (2704 - 3193)	11873 (10862 - 12309)
	Diuron	625	1250	1396 (931 - 1833)	1076 (8887 - 11277)
Larval	Alachlor	625	1250	1352 (237 - 672)	1838 (856 - 2076)
	Atrazine	625	1250	1753 (1208 - 2130)	9957 (9152 - 1125)
	Diuron	625	1250	1617 (1395 - 1987)	7826 (6802 - 8730)

Values are in µg/L, with 95% confidence intervals in parentheses.

juvenile and adult fish. During the toxicity assays course of our study, no significant alterations were observed in the control solvent groups, in which a 0.1% DMSO concentration was used. Our results are in agreement with the data obtained in the tests of embryos of zebrafish *Danio rerio* (Hallare et al., 2006), where not even higher DMSO concentrations (0.3 to 1%) did alter the survival, development and growth of the organisms.

The three pesticides differed in their toxicity to the turbot early life stage. LC₅₀ values for alachlor in turbot embryos and larvae were 2,177 and 1,838 µg/L, respectively. LC₅₀ values for alachlor varying widely from

380 to 17,000 µg/L have been reported depending on the species of fish and test conditions. Moderately high 96 h LC₅₀ values were found for bluegill (PED, 2000), Indian catfish (Chaturvedi and Agrawal, 1991) and fathead minnow (Broderius et al., 1995). The 96 h LC₅₀ value for Nile tilapia was comparable to that for rainbow trout as reported by PED (2000). The turbot is generally more sensitive to alachlor than other species of fish except juvenile Nile tilapia.

Acute toxicity data demonstrate that diuron and atrazine were lowly toxic or non-toxic to turbot based on classification scheme of Zucker (1985). The results show

that the toxicity of atrazine and diuron to *P. maxima* is both time and concentration dependent, as described in previous studies. It was found that the 96h LC₅₀ values for both atrazine and diuron from the present study are consistent with the values from the above literature. LC₅₀ values for atrazine and diuron in fish range widely in the literature.

As for atrazine, the LC₅₀ value obtained for turbot in this study is lower than that reported by Neškovic et al. (1993) and Nwani et al. (2010) for other fish species, but similar to that for juvenile rainbow fish (Phyu et al., 2006), adult Nile tilapia and *Chrysichthys auratus* (Hussein et al., 1996). Besides, the acute toxicity of diuron to fish has been shown to be in a similar range (1.2 to 27.16 mg/L), and the toxicity data for diuron in adult lake trout (APVMA, 2005), sheepshead minnow (USEPA, 2004), rainbow trout (Giacomazzi and Cochet, 2004) and bluegill (USEPA, 2004) were lower than those in turbot embryos and larvae. However, striped bass larvae and fathead minnow's embryo-larvae and juvenile (Hughes, 1973; Nebeker and Schuytema, 1998) prove less sensitive to diuron than the turbot embryo-larvae from the present work. Besides, the present results are similar to those obtained by Call et al. (1987) for adult fathead minnows.

Teratogenic effects

According to the present findings, the effects of the selected pesticides, such as mortality, hatching success and morphological abnormalities (yolk sac alteration, pericardial oedema, skeletal deformities) showed a significant increase in toxic effects, which varied depending on the pesticides, concentrations, exposure time, and stage of development of fish as compared to the controls. This might be due to the sensitivity of each fish species and the chemical characteristics of the compounds. Under our experimental conditions, mortality and deformities caused by pesticides are confirmed by the decreased number and motility of the fish. In fact, both herbicides were capable of affecting survival and development of embryos and larvae, although mainly at high concentrations.

The results also show that pesticides above the threshold concentrations 1250 µg/L for alachlor, 2500 µg/L for atrazine and diuron, respectively reduced hatching success and caused many deformities in turbot. One-way ANOVA using ranks showed that the treatment had a statistically significant effect ($P < 0.05$) on the morphological abnormalities. The most conspicuous effect of alachlor was abnormal skeletal formation, whereas for atrazine and diuron it was pericardial oedema (Table 2). In addition, very low mortality and malformations were observed in the solvent control and control groups.

No studies were found in the literature to support teratogenic responses to various pesticides during early

life stage development in fish. In our study, the most pronounced effect in larvae exposure was abnormal skeletal formation. It was observed that by increasing all pesticide concentrations by 96 hph, significant effects on hatching success and mortality as well as deformities and malformations in larvae were produced. This is in line with the work of Gagnon and Rawson, (2009), who reported that exposure to diuron (50 µg/L) triggered a significant increase in the rate of spinal deformities in hatched pink snapper (*Pagrus auratus*) and a decrease in the proportion of eggs that hatched and had normal development in the early life stages of the pink snapper.

The turbot embryo-larvae in our study were even less sensitive to high atrazine concentrations than catfish embryo-larvae (at concentration > 65 µg/L) (Birge et al., 1983), Zebrafish (at concentration > 1300 µg/L) (Görge and Nagel, 1990) and Bluegill (at concentration > 46 µg/L at 90 days) (Macek et al., 2003). Birge et al. (1983) suggested that exposure of fish embryos to atrazine may induce abnormalities. Specific types of abnormalities associated with atrazine exposure were not reported, although the report notes that defects of the head and the vertebral column, dwarfed bodies and absent or reduced eyes and fins were reportedly the most common symptoms across studies and species. The relatively high embryo mortality and reduced survival rate reported here and in other studies (for example, Birge et al., 1983; Brown et al., 1998) could be due to the fact that the high lipophilicity of pesticides allows them to partially overcome the chorion barrier that protects the egg.

Serious effects of pesticides on the early life stages of turbot were observed in this study. Embryos were less sensitive to pesticides than larvae, possibly because the chorion protected the embryos. For some toxicants the egg chorion acts as a barrier that protects the embryo (Hallare et al., 2006). These findings are similar to those of previous studies (Barry et al., 1995; Humphrey and Klumpp, 2003), who exposed post fertilized eggs and larval of *M. fluviatilis* to esfenvalerate and rainbowfish to chlorpyrifos, and found that newly hatched larvae were more sensitive than eggs. This is consistent with the morphological abnormalities reported for catfish exposure to atrazine (Birge et al., 1983) and pink snapper (*Pagrus auratus*) exposure to diuron (Gagnon and Rawson, 2009) during embryogenesis. The reduced larval survival observed in our study also agrees with the findings of Humphrey and Klumpp (2003) and Kienle et al. (2009), who observed reduced juvenile survival in killifish and rainbow fish exposed to pesticides. For adult fish, the literature indicates lack of effects (Humphrey and Klumpp, 2003; Belden and Lydy, 2006; Eder et al., 2009).

Our results support the conclusion that the turbot seems to be more sensitive than the classical toxicological model zebrafish. The results from our study suggest that the fish in their early life stages are more sensitive than juveniles or adults. Therefore, the use of juveniles and adults, instead of larvae, may

Table 2. Morphological abnormalities of turbot embryos larvae exposed to tested pesticides.

Toxicant	Concentration (µg/L)	Embrionary stage (48 h)			Larval stage (96 h)			
		A	B	C	A	B	C	D
Alachlor	312.5	-	-	-	-	-	+	-
	625	-	+	-	-	-	-	+
	1250	-	-	+	-	-	-	+
	2500	+	+	-	+	+	+	++
	5000	++	+	+	+	+	++	+++
	10000	+++	++	+++	++	+	++	+++
Atrazine	1250	-	-	+	-	-	+	-
	2500	+	-	+	-	-	-	+
	5000	++	++	-	+	++	+	+
	10000	++	++	+	++	+++	+	++
	20000	+++	++	+	++	++++	+	+++
	40000	+++	-	-	-	-	-	+++
Diuron	1250	-	++	-	-	+	-	-
	2500	-	-	+	-	-	-	-
	5000	+	+++	+	+	+	+	+
	10000	+++	+++	++	++	+	++	-
	20000	++	-	++	-	++	+	+++
	40000	+++	++	+	+	+	++	+++
Control	-	+	-	+	-	-	+	+

(A) Yolk sac alterations, (B) no rupture of the egg membrane, (C) pericardial edema, (D) skeletal deformities, + indicate number of individuals affected. n=200.

underestimate the toxicity to the species in question. In addition, there is some evidence that saltwater fish are more susceptible to pesticide exposure than freshwater fish. The results of this investigation may help in guarding against the toxicity hazard to sea fish and the environment through judicious and careful use of these pesticides in agricultural and non-agricultural areas.

Conclusion

The findings in this study have revealed that alachlor is moderately hazardous and atrazine and diuron were less hazardous to *P. maxima*. The larvae of *P. maxima* were more sensitive to pesticides than the embryos. Pesticides proved to be teratogenic to the embryo-larval stages at concentrations above, 1250 µg/L for alachlor and 2500 µg/L for atrazine and diuron, leading to malformations in embryo development, failure to hatch and consequent egg coagulation at 48 h. At higher concentrations, pesticides caused a significant increase in embryo mortality. Surviving organisms suffered a significant decrease in hatching success, malformations (embryos), pericardial oedema and skeletal deformation (larvae). Combining all data, including hatching success and

morphological abnormalities, with the fact that pesticides are frequently used directly in water, it is suggested that insecticides are a potentially harmful compound for fish, including turbot.

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