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Toxicity of a commercial herbicide containing terbutryn and triasulfuron to seabream (*Sparus aurata* L.) larvae: a comparison with the Microtox test

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Abstract

The present study aimed to compare the sensitivity of larvae of the gilthead seabream (*Sparus aurata*), a valuable fish species of the Spanish South Atlantic littoral, with the extensively used Microtox test on a commercial herbicide formulation containing terbutryn (59.4%) and triasulfuron (0.6%). To this purpose, mortality displayed by endogenous feeding *S. aurata* larvae exposed during 72 h post-hatching to nominal concentrations of the commercial formulation and bioluminescence of the marine bacterium *Vibrio fischeri* were compared. Histomorphological changes were also studied. Clearly, the *S. aurata* assay was the more sensitive indicator of toxicity for this herbicide. The 72-h concentration lethal to 50% of the individuals (LC_{50}) found for yolk sac larvae was 1.41 mg/L. This value was more than one order of magnitude below the 15-min EC_{50} found for *V. fischeri* (15.94 mg/L). Growth of the larvae was not significantly affected by a terbutryn–triasulfuron mixture at concentrations up to 1.56 mg/L, the maximum at which there was some proportion of survival.

Keywords: Gilthead seabream; *Sparus aurata*; Early life stage tests; Yolk sac larvae; Terbutryn; Triasulfuron

1. Introduction

Symmetrical triazines (s-triazines) constitute a group of similar herbicides used extensively in agriculture and nonagricultural use sites, primarily to control broadleaf and some grassy weeds, that have become ubiquitous contaminants of the environment.

Atrazine is the most heavily used and most frequently detected representative of this group (Solomon et al., 1996). However, because this compound was initially considered possibly carcinogenic to humans (IARC, 1991), it was banned in some European countries and replaced by novel s-triazine herbicides such as simazine and terbutryn.

These compounds may be disseminated to adjacent untreated areas as a result of evaporation, drift, leaching, and runoff, but they may further contaminate large areas (Chevreuil et al., 1996). This means that they may be significant pollutants not only in rain (Richards et al., 1987; Doerfler and Scheunert, 1997), surface and ground water (Davies et al., 1994; Guilliom et al., 1999; Carabias Martínez et al., 2000), but also in marine ecosystems (Bester and Huehnerfuss, 1993; Bester et al., 1995; Readman et al., 1993; Kucklick and Bidleman, 1994), where they may potentially affect many nontarget organisms. The short half-lives given for these chemicals (days to months) are clearly shifted to higher values (300–500 days) at pH values >7, as found in seawater (Weigel, 2000). Eight s-triazines (atrazine, cyanazine, prometryn, propazine, sebuthylzine, simazine, terbuthylazine, and terbutryn) have been identified as relevant (European Commission, 1999), taking into account a compilation of monitoring data from freshwaters in the member states of the European Community. Terbutryn was also encountered in some Mediterranean coastal waters (Tolosa et al., 1996).

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In contrast, sulfonylurea herbicides are a relatively new class of herbicides characterized by broad-spectrum weed control at very low use rates (2–75 g/ha), reducing the amount of chemicals applied to the field by a factor of 100–1000 compared with conventional herbicides such as atrazine (Beyer et al., 1988), from which the sulfonylureas are derived. These herbicides are readily soluble in water, and some have rather high persistence in alkaline soils (Moyer, 1995). These properties indicate a high potential for leaching and transportation in runoff of some sulfonylurea herbicides such as chlorsulfuron and triasulfuron (Ferris, 1993; Stork, 1995), and they are reported to occur in various aquatic systems (Nilsen, 1989; Battaglin et al., 2000; Carabias Martínez et al., 2000).

In contrast to the vast quantity of information available on the toxicity of herbicides to freshwater organisms, there are relatively few data on the effects of such substances on marine and estuarine organisms.

The marine bioluminescent bacterium, *Vibrio fischeri*, bioassay or Microtox test is one of the assays frequently used for assessment of the acute toxicity of such environmental samples as water and sediments (Dutka et al., 1991), as well as pure compounds (Kaiser and Palabrica, 1991). The Microtox test has many advantages. It is relatively inexpensive, provides well-reproducible results, and offers a fast testing procedure. For many compounds and samples, the toxicity data obtained with the Microtox test correspond well with acute toxicities obtained with standard toxicity tests (Kaiser and Palabrica, 1991; Toussaint et al., 1995; Weideborg et al., 1997); therefore, toxicity in *V. fisheri* can applied to predict toxicity to other aquatic organisms (Blum and Speece, 1990; Chen and Que Hee, 1995; Ribo and Kaiser, 1983; Zhao et al., 1995).

Toxicity tests with early life stages of aquatic organisms have been proposed as a faster and more cost-efficient way of testing chemicals and environmental samples. Moreover, experience shows that these developmental stages of fishes are often the most sensitive to toxic effects, although the various embryonic and larval stages differ in their susceptibility as a result of physiological and biochemical differences (McKim, 1995). Newly hatched larvae constitute a particularly critical and sensitive life stage, because at hatching the embryos lose their protective membrane and are fully exposed to potential toxicants.

Gilthead seabream (*Sparus aurata*) is an important and abundant commercial fish in the Atlantic littoral of the southern Iberian Peninsula (Suau and López, 1976; Arias and Drake, 1990) that can easily be produced in the hatcheries. In fact, its aquaculture grew very fast in the Mediterranean countries (Josupeit, 1995). The Bay of Cádiz (southern Spain) is a very important production zone of alevins of this species, which are intensively reared in local estuaries or exported. As a result of its wide distribution, importance from the commercial point of view, disponibility, and the vast knowledge about it, this species was chosen in this study to assess the ecotoxicity of a commercially formulated s-triazine herbicide.

Accordingly, the objectives of this study were (1) to measure the toxicity of the commercial herbicide Logran Extra with terbutryn (59.4%) and triasulfuron (0.6%) as active ingredients, on seabream yolk sac larvae, (2) to investigate the susceptibility of two endpoints (survival and growth) to this agent, (3) to describe histopathological alterations induced by the exposure to the herbicide, and (4) to compare the sensitivity of this bioassay to Logran Extra with that of the Microtox acute test.

2. Materials and methods

2.1. Exposure of gilthead seabream (*S. aurata*) larvae

Newly hatched seabream larvae were obtained from captive broodstocks at the Laboratory of Marine Culture at the Marine and Environmental Sciences Faculty of the University of Cádiz. The stage of postembryonic development tested was the pre-larva from hatching (day 0) to mouth opening (day 3 post-hatching), when endogenous yolk sac reserves are depleted and exogenous feeding begins. During the experiment, test larvae were kept at a temperature of $19 \pm 1^{\circ}C$, salinity of $37 \pm 1‰$, pH 8 ± 0.1, photoperiod 12 h light/12 h darkness, and dissolved oxygen 90% saturation.

The commercial formulation Logran Extra (Industrias Químicas de Navarra, S.A.) comprising 59.4% terbutryn and 0.6% triasulfuron, an herbicide of the sulfonylurea class with an s-triazine moiety, was used in this study. Hereafter, this formulation will be identified as Commercial Formulation LE. A stock solution of Commercial Formulation LE (25.0 mg/L) was prepared by dissolving the commercial herbicide in natural, filtered (0.45 μm pore size) seawater collected in the Bay of Cádiz, without any carrier solvent, and subsequently diluted to give the final concentrations of the testing media. The highest concentration used in these tests approximated three-fifths the solubility of terbutryn, the less soluble compound in Commercial Formulation LE.

The test was designed to include three replicates per treatment, with 25 newly hatched larvae per replicate randomly placed into glass vessels of 1-L capacity. Each test comprises one control and seven concentrations using standard semistatic acute toxicity procedures, solutions being replaced every 24 h. The nominal concentrations were as follow: 0, 0.31, 0.63, 1.56, 3.13, 6.25, 12.5, and 25 mg/L for the Commercial Formulation

LE. Replicates of 0.31, 0.63, 1.25, and 2.5 mg/L of the herbicide were used to evaluate larval histopathological alterations.

Dead larvae were recorded daily throughout the 3-day exposure period, and dead larvae were removed from test containers. Larvae were considered dead when immobility and no heartbeat were observed. The growth of the fish larvae was estimated at the end of the tests by measuring the dry weight of surviving larvae. Survivors from each replicate were pooled, dried at 60°C for 24 h, and the body dry weight determined.

2.2. Histomorphology and histopathology

For the histological study, the surviving specimens in the corresponding replicates were sampled at 72 h and fixed in 10% formaldehyde in 0.1 M phosphate buffer (pH 7.2), dehydrated in alcohol series, and embedded in paraffin wax. Sagittal and cross-sections (6–8 μm thick) were stained with H&E for observation by the light microscope. Previously, larvae were observed for gross morphological alterations using a light microscope by placing them on a cavity slide with a drop of water.

2.3. Statistical analysis

The toxicity endpoint data were LC_{50} and NOEC (no observed effect concentration) for mortality and growth. The lethal concentration values and 95% confidence limits were estimated using the Probit routine in the statistical package TOXSTAT Version 3.4 (Western Ecosystems Technology Inc.; Gulley, 1994), with correction for control mortalities. Statistical differences in survival and growth between herbicide treatments and the controls for seabream larvae were analyzed by ANOVA and Dunnett's test. Data on mortality were normalized by using arcsin square roots transformation.

2.4. V. fischeri toxicity test (Microtox)

A Microtox Model 500 Analyzer (Azur Environmental, Carlsbad, CA) was used for the toxicity tests on the marine bioluminescent bacteria *V. fisheri*. Freeze-dried bacteria and reconstitution solution were supplied by Azur Environmental. The bioassays were carried out according to the manual (Azur Environmental, 1998) using the duplicate basic test procedure. Each test consisted of one control and four serial dilutions of the agent (11.25, 5.63, 2.81, 1.40 mg/L). The 5-, 15-, and 30-min EC_{50} and EC_{10} (concentrations of toxicant required to decrease by 50% and 10%, respectively, the bacterial bioluminescence, with 95% confidence intervals, CIs) were calculated for each test using MicrotoxOmni Software Version 1.18 available from the manufacturer (Azur Environmental, 1999).

3. Results

3.1. Mortality and growth data

Mortality of the seabream *S. aurata* yolk sac larvae in different concentrations of the herbicide and exposure times, from which the LC_{50} was calculated, is shown in Fig. 1. Larval mortality significantly increased after 24 h ($F_{7,16} = 74.31$, $P < 0.05$), 48 h ($F_{7,16} = 52.59$, $P < 0.05$), and 72 h ($F_{7,16} = 70.94$, $P < 0.05$) with Commercial Formulation LE exposure. Survival of larvae was unaffected by exposure to herbicide concentrations up to 1.56 (24-h NOEC), 0.63 (48-h NOEC), and <0.31 mg/L (72-h NOEC) (Table 1). An average survival of 93% in the controls indicates good experimental conditions, according to the OECD guideline for ELS tests (OECD, 1998).

LC_{50} values and their 95% CIs for the formulated terbutryn–triasulfuron mixture are summarized in Table 1.The 24-h, 48-h, and 72-h LC_{50} values were 3.66 mg/L (3.35–3.70 mg/L), 2.18 mg/L (1.97–2.39 mg/L), and 1.41 mg/L (1.26–1.57 mg/L), respectively.

Dry weight of the larvae that survived till the end of the experiments (72 h) with Commercial Formulation LE is shown in Table 2. Weight of the larvae was not influenced by terbutryn–triasulfuron mixture up to 1.56 mg/L (NOEC), the maximum concentration at which there was some proportion of survival ($F_{3,8} = 1.34$, $P > 0.05$).

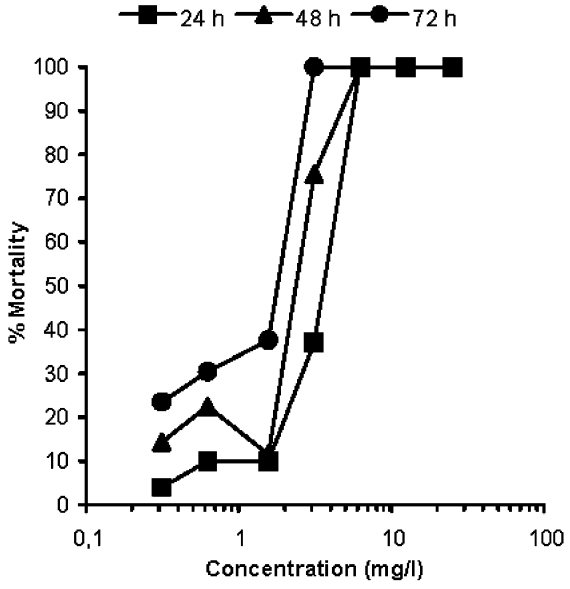


Fig. 1. Seabream yolk sac larvae mortality after exposure to different concentrations of formulated terbutryn–triasulfuron mixture for (a) 24 h (■), (b) 48 h (▲), or (c) 72 h (●).

Table 1
Toxicity values of formulated terbutryn–triasulfuron for *S. aurata* yolk sac larvae and *V. fischeri*

Species	Endpoint	Parameter	Toxicity value (mg/L)[a]
S. aurata	Larval survival	24-h LC_{50}	3.66 (3.35–3.70)
	Larval survival	48-h LC_{50}	2.18 (1.97–2.39)
	Larval survival	72-h LC_{50}	1.41 (1.26–1.57)
	Larval survival	24-h NOEC	1.56
	Larval survival	48-h NOEC	0.63
	Larval survival	72-h NOEC	$\leqslant$0.31
	Larval growth (weight)	72-h NOEC	1.56
V. fischeri	Bioluminescence inhibition	5-min EC_{10}	1.87 (1.67–2.09)
	Bioluminescence inhibition	15-min EC_{10}	2.27 (2.01–2.58)
	Bioluminescence inhibition	30-min EC_{10}	2.39 (2.07–2.77)
	Bioluminescence inhibition	5-min EC_{50}	14.79 (12.65–17.29)
	Bioluminescence inhibition	15-min EC_{50}	15.94 (12.93–19.66)
	Bioluminescence inhibition	30-min EC_{50}	15.90 (12.36–20.46)

[a] 95% confidence limits are shown in parentheses.

Table 2
Dry weight of *S. aurata* yolk sac larvae exposed to different treatment levels of formulated terbutryn–triasulfuron mixture during 72 h

Concentration (mg/L)	Weight (mg/individual)
Control	0.047 ± 0.007
0.31	0.048 ± 0.002
0.63	0.056 ± 0.02
1.56	0.052 ± 0.01
3.13	—[a]
6.25	—[a]
12.5	—[a]
25	—[a]

Note: Data are expressed as mean ± SD ($n = 3$).
*Significantly different from the control ($P < 0.05$).
[a] Larval mortality was 100%.

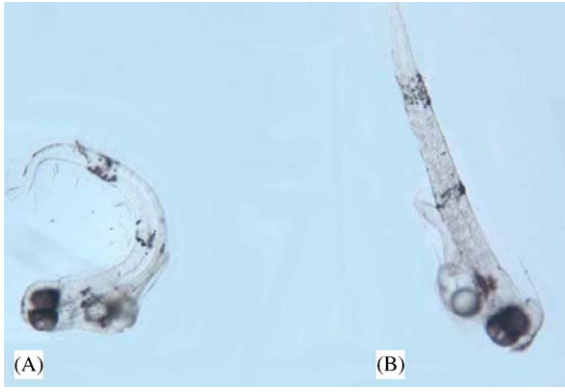


Fig. 2. Morphological aspects of seabream yolk sac larvae. (a) Abnormal seabream larva exposed to Logran, showing an inflexible, extremely wavy structure of the vertebral column (× 25). (b) Control larva at hatch (× 25).

3.2. Histomorphology and histopathology

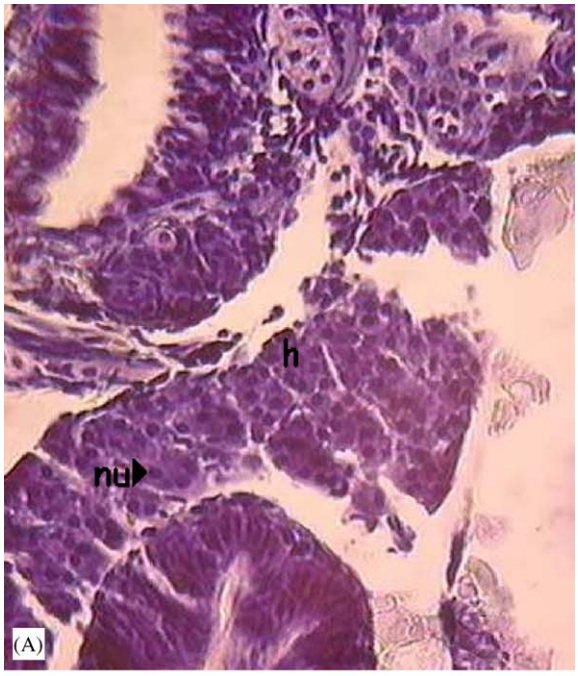
Although this aspect was not studied quantitatively, curvatures of the vertebral column were identified in some exposed individuals (Fig. 2). No abnormal larvae were observed in control groups. Histological examination of larvae focused on the liver. In control larvae, the liver showed the hepatocytes forming slackly arranged cords. In larvae exposed to the Commercial Formulation LE at concentrations of 2.5 mg/L, histopathological alterations were detected in the liver, showing cellular alterations related to loss of cellular shape of hepatocytes. This appearance of hepatic cells could be affected by the amount of lipid inclusions, which were less in controls and organisms exposed to low concentrations than in those exposed to high concentrations (Fig. 3). Likewise, an intense nuclear pyknosis was detected in the hepatocytes from larvae exposed to terbutryn–triasulfuron mixture at 2.5 mg/L.

3.3. Microtox assay

The effects of Commercial Formulation LE on the bioluminescence of *V. fischeri* are summarized in Table 1 and Fig. 4. This herbicide inhibited the bacterial bioluminescence in a concentration-dependent manner. Microtox 5-, 15-, and 30-min EC_{50} values were 14.79 mg/L with a 95% CI of 12.65–17.29 mg/L, 15.94 mg/L (CI, 12.93–19.66 mg/L), and 15.90 mg/L (12.36–20.46 mg/L), respectively. It is clear that the EC_{50} value was quite insensitive to the length of incubation time.

4. Discussion

Published toxicity data for early life stages of *S. aurata* are scant. Previous short-term embryo–larval assays with *S. aurata* have been developed to study the



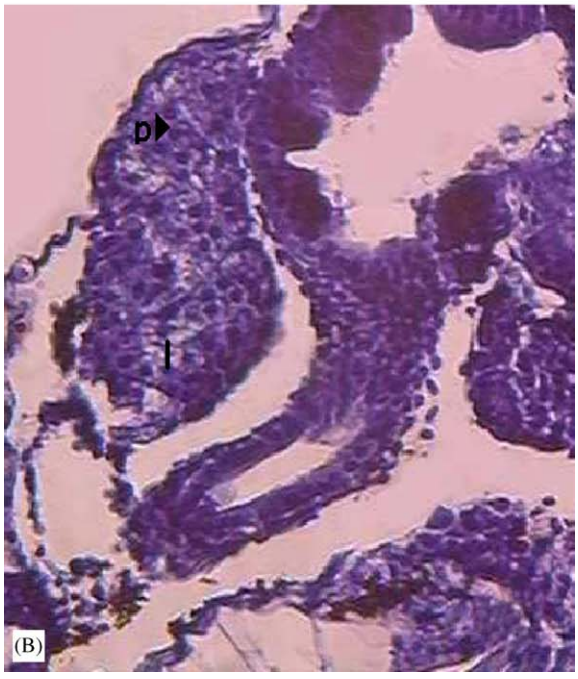


Fig. 3. Histological sections of liver from seabream yolk sac larvae. (a) Control treatment. (b) Larva exposed to 2.5 mg/L Commercial Formulation LE (59.4% terbutryn and 0.6% triasulfuron) during 72 h. l, lipid droplets; nu, nucleus; p, nuclear pyknosis.

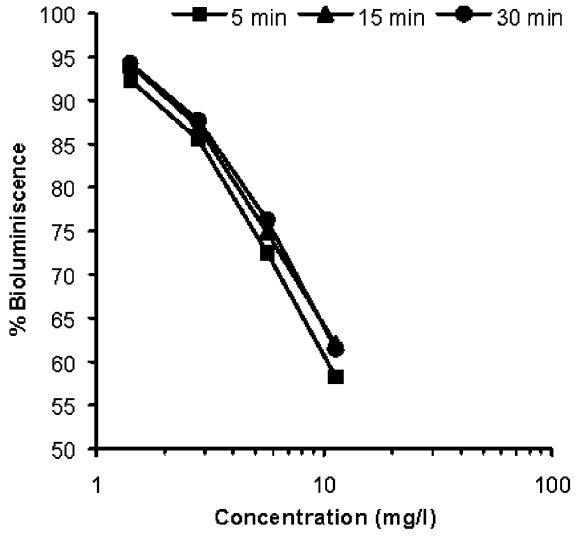


Fig. 4. Bioluminescence inhibition of *V. fischeri* bacteria after exposure to different concentrations of formulated terbutryn–triasulfuron mixture for (a) 5 min (■), (b) 15 min (▲), or (c) 30 min (●). Data expressed relative to mean value in respective unexposed controls.

toxicity of total ammonia and nitrite (Parra and Yufera, 1999), linear alkylbenzene sulfonate, and one long-chain degradation intermediate, sulfophenyl carboxylic acid (Hampel and Blasco, 2002), as well as organotin compounds (Dimitriou et al., 2003). However, there are no published data on the toxicity of the herbicide tested in this study to allow a direct comparison with other work.

Our findings indicate that total mortality of yolk sac seabream larvae was a more sensitive parameter than weight upon exposure to Commercial Formulation LE, resulting in 72-h NOECs of <0.31 and 1.56 mg/L, respectively. This is in agreement with the study of Görge and Nagel (1990), which showed that survival in early life stages of zebrafish was the most sensitive parameter upon exposure to the s-triazine herbicide atrazine. Accordingly, the usefulness of measuring the growth response in routine applications of early life stage toxicity tests have been questioned (Woltering, 1984).

Moreover, the seabream larvae 72-h LC_{50} (1.41 mg/L) of Commercial Formulation LE is roughly three times lower than the 24-h LC_{50} (3.66 mg/L) (Table 1). The NOEC values based on larval survival decreased with time from 1.56 mg/L at 24 and 48 h to <0.31 mg/L at 72 h. This means that there was an increase of toxicity of the herbicide as the exposure time increased.

Comparison of our results to published information indicates that our data for the formulated mixture terbutryn–triasulfuron, the 72-h LC_{50} value for seabream yolk sac larvae derived from this study, are in general agreement with values published in the literature for formulated terbutryn for other saltwater species; most notably, the estimated 72-h LC_{50} (1.41 mg/L) is similar to terbutryn 96-h LC_{50} (1.0 mg/L) and 48-h LC_{50} (1.0 mg/L) found by Lowe et al. (1970) for the American oyster (*Cassostrea virginica*) and the longnose killifish (*Fundulus similis*), respectively. Terbutryn 96-h LC_{50}

values for the freshwater fishes *Lepomis macrochirus* and *Oncorhynchus mykiss* are 4 and 3 mg/L, respectively (Bathe et al., 1973), and the 48-h EC_{50} for *Daphnia magna* is 7.1 mg/L (Marchini et al., 1988). However, the LC_{50} and EC_{50} values for triasulfuron are well above these values, reported to be > 100 mg/L for the species *Cyprinodon variegatus*, *Lepomis macrochirus*, *O. mykiss*, and *D. magna* (Office of Pesticide Programs, 2000), indicating a significantly lower acute toxicity.

According to the results of other work conducted in our laboratory, this herbicide formulation was about three times more toxic than another s-triazine formulation containing simazine for seabream larvae (unpublished data).

In contrast, the livers of *S. aurata* larvae exposed to Commercial Formulation LE showed such important cellular alterations as loss of cellular shape in hepatocytes, lipid inclusions, and abundant nuclear pyknosis in the hepatocytes. Rudolph et al. (2001) observed similar alterations in the liver of *O. mykiss* exposed to the water-accommodated fraction of petroleum hydrocarbons (WAF), related to loss of the cellular shape in hepatocytes. This hepatic alteration was shown by changes in the cytoplasm, where the quantity of lipids present increases with concentration of contaminant WAF. Lipid vacuoles are limited in number in healthy hepatocytes but increase notably under reproductive and nutritive conditions (Vethaak and Wester, 1996; Arellano, 1999), as well as in pathological conditions (Geneser, 1993). Alterations in metabolism of hepatic lipids were observed in various fish species contaminated by heavy metals such as copper (Arellano et al., 1999), including ambiental stress and inadequate artificial feeding (Deplano et al., 1989; Braunbeck and Segner, 1992).

In addition to the histological alterations, exposed larvae showed spinal curvatures. A similar outcome was reported in medaka embryos exposed to fenitrothion (Hiraoka, 1989), diazinon (Hamm and Hinton, 2000), and thiobencarb (Villalobos et al., 2000), as well as in African catfish larvae following malathion exposure (Lien et al., 1997). Likewise, medaka fry were shown to develop a lump in the notochord after parathion exposure (Tomita and Matsuda, 1961).

In terms of mortality, the results show differences in sensitivity to the Commercial Formulation LE between the two marine species tested, *S. aurata* larvae and the prokaryote *V. fischeri*. The herbicide was more than an order of magnitude less toxic to *V. fischeri* (15-min EC_{50}, 15.94 mg/L) than to yolk-sac *S. aurata* larvae (72-h LC_{50}, 1.41 mg/L). This was biochemically the most complex test system used and was found to be the most sensitive. The 15-min EC_{50} (15.94 mg/L) of the terbutryn–triasulfuron mixture using the Microtox test was within the range of that reported for pure terbutryn (13 mg/L) by other authors (Gaggi et al., 1995). Tchounwou et al. (2000) reported 15-min EC_{50} values of 39.87, 273.2, 226.80, and 11.80 mg/L for the s-triazine herbicides atrazine, propazine, prometryn, and ametryn, respectively.

To conclude, our results have shown that larval toxicity assays with the seabream *S. aurata* larvae provided various types of information on the lethal and sublethal effects of the commercial formulation comprising terbutryn and triasulfuron. Larval growth appeared to be less sensitive in comparison to larval lethality in characterizing exposures to this herbicide. In addition, the Microtox test revealed comparatively lower sensitivity to the formulation. In any case, the concentrations that we found that adversely affected survival and induced histological alterations in seabream yolk sac larvae after 72 h are well above the expected environmental concentrations, because, for example, total triazine concentrations in the North Sea and the Mediterranean vary from several tenths of a nanogram per litre in the open sea to tens of nanograms in coastal areas, and hundreds of nanograms in estuarine waters close to river mouths (Bester and Huehnerfuss, 1993; Readman et al., 1993; Zhou et al., 1996).

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