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Comparative toxic effects of formulated simazine on *Vibrio fischeri* and gilthead seabream (*Sparus aurata* L.) larvae

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Abstract

The use of Early Life Stage (ELS) tests is a useful tool in risk assessment. The purpose of this study was to compare the sensitivity of the seabream (*Sparus aurata*) larvae with the extensively used Microtox® test on a commercial formulation containing simazine, an s-triazine herbicide. To this end, survival, growth and histopathological changes displayed by seabream yolk sac larvae exposed during 72 h post-hatching to nominal concentrations of the commercial preparation up to its saturating concentration in water, and bioluminescence of the marine bacteria *Vibrio fischeri* (Microtox®) were studied. Survival of larvae after three days of exposure was significantly reduced in the highest (4.5 mg/l) concentration, but no effects on growth were found in any of the simazine treatments. The 72 h LC50 value for yolk sac larvae was estimated as 4.19 mg/l. Commercial grade simazine did not exert any significant toxicity to the marine bacterium *V. fischeri* at the concentrations tested.

Keywords: Gilthead seabream; *Sparus aurata*; Early Life Stage Tests; Yolk sac larvae; Simazine

1. Introduction

Simazine is a symmetrical triazine (s-triazine) herbicide whose mechanism of action is blockage of electron flow in photosystem II of photosynthesis (Rochaix and Erickson, 1988; Hansson and Wydrzynski, 1990). This compound has a relatively low solubility in water (0.025 mM) (Erickson and Lee, 1989), as well as a low sorption coefficient (Nicholls et al., 1984). s-Triazines constitute a group of herbicides used extensively in modern agriculture that, in some extent, are considered to be persistent in water and mobile in soil, and have become ubiquitous contaminants in both, fresh (Davies et al., 1994; Guilliom et al., 1999; Carabias Martínez et al., 2000) and marine ecosystems (Bester and Huehnerfuss, 1993; Readman et al., 1993; Kucklick and Bidleman, 1994; Bester et al., 1995).

In a review by Strandberg and Scott-Fordsmand (2002) about toxicological effects of simazine, it was

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pointed out that in both soil and water, degradation studies have in most cases shown DT50 times (denoting the 50% disappearance of the compound) that vary between a few days and 150 days. Hence, total or near total disappearance time may be at least three times longer; moreover, low temperatures and drought may prolong the dissipation time by a factor of two or more. On the other hand, these authors pointed out that, in fresh water, concentrations approximately 4 μg/l simazine have been recorded.

Simazine and other seven s-triazines have been identified as relevant in a study on the prioritisation of substances dangerous to the aquatic environment in the member states of the European Community (European Commission, 1999). Simazine, atrazine, and terbutylazine have been frequently found in the German Wadden Sea (Bester and Hühnerfuss, 1996), the North Sea (Hühnerfuss et al., 1997), and the Southern Baltic off Poland (Pempkowiak et al., 2000). Total triazine concentrations in the North Sea and the Mediterranean Sea 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).

The seabream *Sparus aurata* is a marine species with high commercial value occurring in the North Eastern Atlantic Ocean and the Mediterranean Sea (Bauchot and Hureau, 1986; Arias and Drake, 1990). In fact, this autochthonous species to the Andalusian littoral (Suau and López, 1976) it is commonly exploited in extensive aquacultural production in the Cádiz bay (southern Spain). Due to 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 simazine.

On the other hand, Fish Early Life Stage (ELS) tests have been considered as faster and cost-efficient bioassays for toxicity testing of chemicals and environmental samples. Experience shows that these developmental stages are often the most sensitive to toxic effects (McKim, 1995). Particularly, newly hatched larvae are a critical and sensitive life stage because at hatching the embryos lose their protective membrane and are fully exposed to potential toxicants.

The luminescence inhibition bioassay with marine *Vibrio fischeri* photobacteria (Microtox® test) is a useful tool that has been used to estimate the acute toxicity of pure chemicals (Kaiser and Palabrica, 1991), contaminated water (Bulich and Isenberg, 1981; Dutka et al., 1991; Fernández et al., 1995) and sediments (Guzzella, 1998; Johnson and Long, 1998). The method is sensitive, relatively rapid and inexpensive, the results are comparable to other acute toxicity tests (Kaiser and Palabrica, 1991; Toussaint et al., 1995; Weideborg et al., 1997) and can be applied to predict toxicity to other aquatic organisms (Ribo and Kaiser, 1983; Blum and Speece, 1990; Chen and Que Hee, 1995; Zhao et al., 1995). Likewise, Microtox® test has been used to assess the environmental quality of marine coastal areas (Carr et al., 1996; Cook and Wells, 1996; Cotou et al., 2002).

In contrast to the vast quantity of information available on the toxicity of herbicides to freshwater organisms, there are very few data on the effects of such substances to marine and estuarine organisms. Accordingly, to obtain valuable information to be used in ecotoxicological assessment of simazine in marine waters, the objectives of this study were: (1) to measure the toxicity of simazine on seabream yolk sac larvae, (2) to investigate the susceptibility of two endpoints (survival and growth) to this agent (3) to describe histopathologic alterations induced by the short-term exposure to the herbicide and (4) to compare the sensitivity of this bioassay to simazine with that of the Microtox® acute test.

2. Material and methods

2.1. Seabream (*Sparus aurata*) larvae toxicity test

Newly hatched seabream larvae were obtained from eggs obtained by natural spawning from captive broodstocks at the Laboratory of Marine Culture at the Marine and Environmental Sciences Faculty (University of Cádiz). At 3 h to 5 h post-hatching, the yolk sac larvae were collected and exposed to the herbicide for three days. Main test conditions were: temperature of 19 ± 1 °C, salinity of 37 ± 1‰, pH 8 ± 0.1, photoperiod of 12 h light/12 h darkness, and 90% dissolved oxygen.

The working stock solution of formulated simazine was prepared by dissolving the commercial herbicide Gesatop® GW (CIBA-GEIGY, S.A.) containing 90% simazine, in natural and filtered (0.45-μm) sea water, collected in the Bay of Cádiz, without any carrier solvent, for a final concentration of 5 mg/l of the formulation, and subsequently diluted to give the final concentrations of the testing media. Test concentrations were corrected for purity and are expressed as milligrams simazine per liter. The test comprised one control and five toxicant concentrations, with three replicates per treatment. The static-renewal method was used for the exposures, solutions being replaced every 24 h with freshly-prepared solutions. Newly hatched larvae were randomly placed into glass beakers ($n = 25$ per replicate) containing 1-l of test solution of commercial simazine at the nominal concentrations of 0, 0.28, 0.56, 1.13, 2.25 and 4.5 mg/l. A fourth replicate of 0.28 and 4.5 mg/l was utilized to evaluate larval histopathological alterations. The highest concentration utilized in the test approximated the solubility of simazine.

Larval mortality (defined as immobility and absence of heart beat) was recorded in each vessel at 24 h intervals and dead individuals were removed. Tests were considered valid if the survival rate was at least 90% in the controls. The test was terminated after three days and the growth of the fish larvae was estimated 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.

For the histological observations, the surviving specimens in the corresponding replicates were sampled at 72 h and processed by a routine histological method. They were fixed in 10% formaldehyde in phosphate buffer 0.1 M (pH 7.2), dehydrated in ethanol series and embedded in paraffin wax. Sections of 6–8 μm thickness were stained with haematoxylin-eosin for observation by the light microscope.

The toxicity endpoint data were LC10, LC50 and LC90 (concentrations lethal to 10%, 50% and 90% of population), NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) for mortality and growth. Lethal concentration values with 95% confidence limits ($p \leqslant 0.05$) were estimated by probit analysis, using XLSTAT-Pro 7.1. Average dry weight of the surviving fish larvae and survival by replicate data were checked for normality and homogeneity of variance using Bartlett's test and the Shapiro–Wilk's test, respectively using the statistical package TOXSTAT Version 3.4 (WEST and Gulley, 1994). Survival data were arcsin-square root-transformed prior to analysis (Zar, 1984) and proportions of 0/*n* were replaced with 1/4*n* to improve the arcsine transformation (Bartlett, 1937). One-way analysis of variance (ANOVA) was performed with Dunnett's multiple range test for significance compared to controls ($p \leqslant 0.05$) (Zar, 1984) and the NOEC and LOEC estimates for larval survival and growth (measured as dry weight) were determined.

2.2. *Vibrio fischeri* toxicity test

The Microtox® system used was supplied by AZUR Environmental (Carsbad, CA), and consisted of lyophilized bacterial reagent *V. fischeri*, reconstitution reagents and the Model 500 Toxicity Analyzer (AZUR Environmental). The short-term luminescent bacteria assay was done according to the supplier's protocol for the duplicate basic test (Azur Environmental, 1998). In brief, four 1:1 serial dilutions (45%, 22.5%, 11.25% and 5.6%) of the stock solution containing 5 mg/l of the commercial composition were made in 2% sterile NaCl solution (Microtox® diluent) and toxic effects were monitored as a percent decrease of the light emission of *V. fischeri* after 5, 15 and 30 min of incubation at 15 °C using the Microtox® calculation software (version 1.18).

3. Results and discussion

Statistically significant differences in the lethality occurred among treatments after 72 h ($F_{5,12} = 75.11$, $p < 0.05$) of exposure with simazine. Survival of yolk sac seabream *S. aurata* larvae was unaffected by exposure to the herbicide at concentrations up to 2.25 mg/l compared to the controls, however larvae exposed to 4.5 mg/l experienced a significant increase in lethality to 58.3% after 72 h of exposure. According with these results, the no observed effect concentration (NOEC) for mortality was 4.5 mg/l and the lowest observed effect concentration (LOEC) was above this value (Table 1), the maximum test concentration. Despite weak trends toward greater mortality in simazine exposed larvae after the 24-h (8.6%) and 48-h (10.6%) exposure periods, the ANOVA test revealed no significant effect of simazine on mortality compared with controls. An average survival of 100%, 95% and 94% in the controls at 24,

Table 1
Toxicity values of simazine for *S. aurata* yolk sac larvae and *V. fischeri*

Species	Endpoint	Parameter	Toxicity value (mg/l)[a]
S. aurata	Larval survival	72-h-LC50	4.19 (3.68–4.97)[b]
	Larval survival	72-h-LC10	2.36 (1.65–2.81)
	Larval survival	72-h-LC90	7.42 (5.92–12.15)
	Larval survival	72-h-NOEC	2.25
	Larval survival	72-h-LOEC	4.50
	Larval growth (weight)	72-h-NOEC	4.50
	Larval growth (weight)	72-h-LOEC	>4.50
V. fischeri	Bioluminescence inhibition	5-min-EC50	>2.03
	Bioluminescence inhibition	15-min-EC50	>2.03
	Bioluminescence inhibition	30-min-EC50	>2.03

[a] In brackets: 95% confidence limits.
[b] Probit regression line: Slope ± Standard error: 5.154 ± 1.067; Intercept ± Standard error: −3.204 ± 0.632.

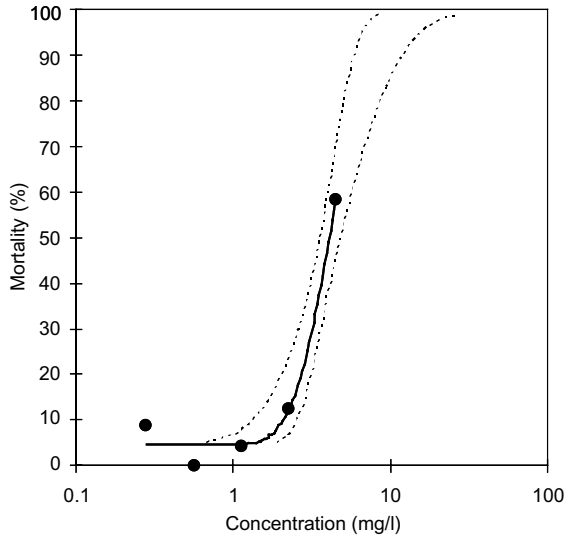

Fig. 1. Dose–response curve of simazine for seabream yolk sac larvae mortality after exposure to different concentrations of formulated simazine for 72 h.

48 and 72 h of exposure, respectively, indicates good experimental conditions, according to the OECD guideline for ELS tests (OECD, 1998).

Mortality of larvae after exposure to different concentrations of formulated simazine for 72 h, from which the LC50 was calculated, is shown in Fig. 1. Simazine was moderately toxic to seabream larvae, with a 72-h LC50 value of 4.19 mg/l (95% confidence limits, 3.68–4.97 mg/l). Other LCs estimates and their 95% confidence intervals are summarized in Table 1. The 24-h and 48-h LC values were not calculable because sufficient mortality did not occur in these exposure periods.

Unfortunately, no literature data for simazine toxicity to *S. aurata* could be found to allow a direct comparison with a later stage of development. Comparison of our results to published information, indicates that our data for the formulated simazine are below the ranges reported in the literature for other species of fish. For example, Munn and Gilliom (2001), in a review of toxicity data collected from different databases, reported simazine 96-h LC50 toxicity values for fathead minnow (*Pimephales promelas*) ranged from 5 to 510 (median, 6.4 mg/l), for bluegill (*Lepomis macrochirus*) ranged from 16 to 118 mg/l (median, 95 mg/l), and for rainbow trout (*Oncorhynchus mykiss*) ranged from 40.5 to 70.5 mg/l (median, 56 mg/l), well above the value for *S. aurata* larvae (72-h LC50, 4.76 mg/l). On the contrary, the 48-h EC50 for water flea (*Daphnia magna*) was 1.1 mg/l (Munn and Gilliom, 2001).

Very few studies have been conducted on the toxicity of simazine on marine organisms according to the literature reviewed and the data found in the EPA's AQUIRE database (Table 2). In acute tests with invertebrates using different exposure techniques (static or static-renewal), the 96-h LC50 value for *Penaeus duorarum* was 113 mg/l and for *Neopanope texana* was above 1000 mg/l (Office of Pesticide Programs, 2000); meanwhile, the 48-h LC50s for adult specimens of *Crangon crangon* and *Cerastoderma edule* (Portmann and Wilson, 1971) and larval stages (Portmann, 1972) were above 100 mg/l (Portmann, 1972), and above 10 mg/l for *Carcinus maenas* (Portmann and Wilson, 1971), the maximum test concentrations used in each case. Finally, the 96-h LC50 for the saltwater fish, sheepshead minnow (*Cyprinodon variegatus*) using flow-through test conditions has been reported as >4.3 mg/l (Office of Pesticide Programs, 2000). All these values are well above the 72-h LC50 value (4.19 mg/l) found for *S. aurata* larvae in this study, using a static-renewal test. Instead, the approximate estimate of 96-h EC50 (immobilization) for *Crassostrea virginica* spats was above 3.7 mg/l. In any case, according to the results of other work conducted in our laboratory, simazine was about three times less toxic than other s-triazine formulation comprising 59.4% terbutryn and 0.6% triasulfuron for seabream yolk sac larvae (Arufe et al., 2004).

Table 2
Ecotoxicity data found on AQUIRE database for simazine on marine organisms

Test species	Life stage/size	Exposure period and endpoint	Exposure type	Toxicity value EC/LC50 (mg/l)	Reference
C. maenas	Adult	48 h lethality	Static-renewal	>10	Portmann and Wilson (1971)
C. edule	Larval	48 h lethality	Static	>100	Portmann (1972)
C. edule	Adult	48 h lethality	Static-renewal	>100	Portmann and Wilson (1971)
C. crangon	Larval	48 h lethality	Static	>100	Portmann (1972)
C. crangon	Adult	48 h lethality	Static-renewal	>100	Portmann and Wilson (1971)
C. virginica	Spat	96 h inmobilization	Flow-through	>3.7	Office of Pesticide Programs (2000)
C. variegatus	0.36 g	96 h lethality	Flow-through	>4.3	Office of Pesticide Programs (2000)
N. texana	15 mm	96 h lethality	Static	>1000	Office of Pesticide Programs (2000)
P. duorarum	55 mm	96 h lethality	Static	113	Office of Pesticide Programs (2000)
S. aurata	Larval	72 h lethality	Static-renewal	4.19	This study

Table 3
Dry weight of yolk sac larvae of seabream exposed to different treatment levels of formulated simazine during 72 h

Concentration (mg/l)	Weight (mg/individual)
Control	0.039 ± 0.003
0.28	0.037 ± 0.013
0.56	0.047 ± 0.015
1.13	0.058 ± 0.010
2.25	0.048 ± 0.008
4.50	0.052 ± 0.012

Data are expressed as mean ± SD; $n = 3$.
No treatments were significantly different from the control ($p \leqslant 0.05$).

Evidently, the higher toxicity of simazine to primary producers (in particular phytoplankton) is attributable to its specific mechanism of action, i.e. inhibition of the photosynthetic electron transport at photosystem II (Rochaix and Erickson, 1988; Hansson and Wydrzynski, 1990). Thus, Fairchild et al. (1997) reported an effective concentration resulting in 50% growth inhibition (96h-EC50) for the algae *Selenastrum capricornutum* and the floating vascular plant *Lemna minor* of 1.24 mg/l and 0.166 mg/l, respectively.

On the other hand, dry weight of the larvae that survived till the end of the experiments (72 h) with formulated simazine is shown in Table 3. Weight of the larvae was not influenced by this herbicide up to 4.5 mg/l, the maximum test concentration ($F_{5,12} = 1.55$, $p > 0.05$). According to these results, the no observed effect concentration (NOEC) for growth was >4.5 mg/l (Table 1). Our findings indicate that total mortality of yolk sac seabream larvae was a more sensitive parameter that larval growth weight. 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.

Histological examination of larvae focused on liver because, based on available data, the liver appears to be the most common target organ for toxicity of many contaminants. In this respect, certain hepatic lesions in fish, especially in comparison to lesions in other organ systems (Hinton et al., 1992), are currently regarded as having utility as histological indicators or biomarkers of contaminant exposure and effects (Myers et al., 1991; Hinton et al., 1992; Varanasi et al., 1992; Myers et al., 1994) and have become useful indicators of environmental degradation in marine ecosystems. Liver toxicity results in altered fat metabolism, and digestion, thereby resulting in generalized stress of the fish (Klaassen, 1996).

In control larvae, the liver showed the hepatocytes forming slackly arranged cords. In larvae exposed to formulated simazine at concentrations of 4.5 mg/l, histopathological changes were detected in the liver, showing

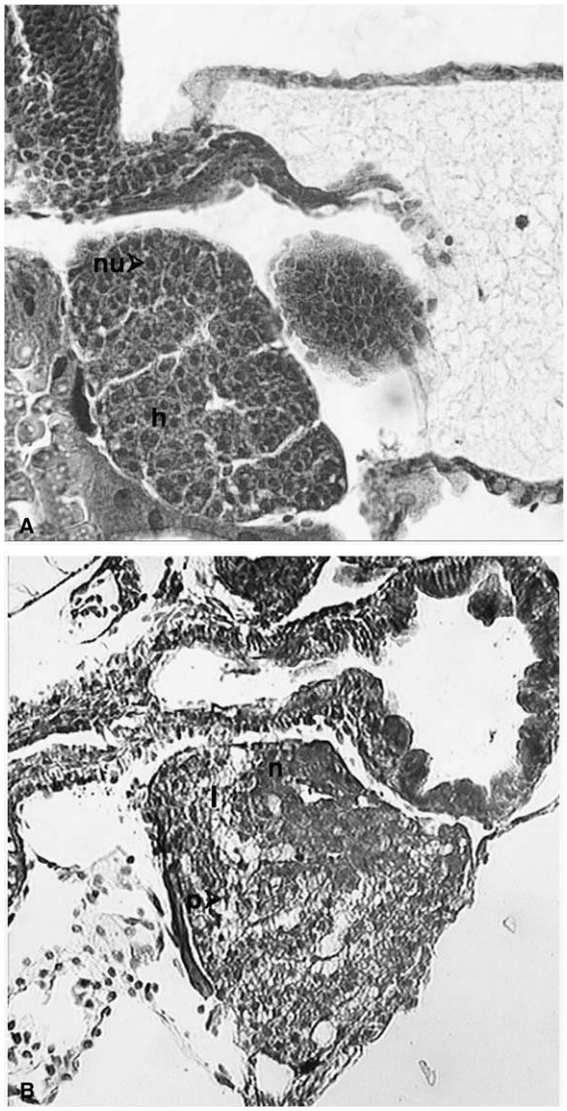

Fig. 2. Histological sections of liver from seabream yolk sac larvae: (A) control treatment, (B) exposed to 4.5 mg/l simazine during 72 h (l: lipid droplets, n: necrosis, nu: nucleus, p: nuclear pyknosis, h: hepatocyte).

cellular alterations related to loss of cellular shape in hepatocytes, lipid inclusions, focal necrosis (cell death) and abundant nuclear pyknosis (first stage of necrosis where the nucleus in shrunken and very dark) in the hepatocytes. This appearance of hepatic cells could be affected by the amount of lipid inclusions which was less in controls and organisms exposed to low concentrations than in those exposed to the highest concentration (Fig. 2).

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) and in pathologic conditions (Geneser, 1993). Alterations in metabolism of hepatic lipids were observed in different fish species contaminated by heavy metals, i.e. copper (Arellano et al., 1999), so as environmental stress, inadequate artificial feeding. etc. (Deplano et al., 1989; Braunbeck and Segner, 1992).

Conversely, commercial grade simazine did not exert any significant toxicity to the marine bacterium *V. fischeri* at the concentrations and exposure periods tested as evidenced by any significant reduction in bioluminescence (highest percentage effect of about 8%). Because the highest concentration in the incubation vial was 2.03 mg/l, it may be concluded that the 5-min, 15-min and 30-min EC50 values for this herbicide exceeded 2.03 mg/l. In terms of acute toxicity, the results show differences in sensitivity to simazine between the two marine species tested, *S. aurata* larvae and the prokaryote *V. fischeri*. The first was biochemically the most complex test system used and was found to be the most sensitive. Thus, the low level of solubility of simazine meant that an EC value for *V. fischeri* was not reached. Similarly, effective concentrations values were not possible to obtain for four different lux-marked (luminescence-based) bacterial biosensors (*Escherichia coli*, *Pseudomonas fluorescens*, *Pseudomonas putida*) exposed to simazine (Strachan et al., 2001). However, other s-triazines are reported to be more toxic. For example, the IC50-15 min of terbutryn using the Microtox® test was 13 mg/l (Gaggi et al., 1995). On the other hand, Tchounwou et al. (2000) reported 15 min-EC50 values of 39.87, 273.2, 226.80 and 11.80 mg for atrazine, propazine, prometryne and ametryne, respectively.

The interspecies relationships between *V. fischeri* and numerous other aquatic species, which can be exploited to make confident predictions of one end point from another, works particularly well for compounds of relatively simple chemical structure with one reactive or functional group. However, less reliable predictions can be made for chemicals of more complex structure, such as those with several functional groups interacting, that are frequently associated with highly specific effects on a particular organism or biochemical function (Kaiser, 1998).

In conclusion, our results have shown that the concentrations of simazine that adversely affected survival and induced histological alterations in seabream yolk sac larvae after 72 h are significantly greater than the levels of this pollutant that are commonly detected in the marine environment. In addition, larval growth (measured as dry weight) appeared to be less sensitive in comparison to larval lethality in characterizing exposures to this herbicide. Finally, it is particularly noteworthy that *S. aurata* larvae not only were more sensitive to the reference toxicant than the marine bacteria *V. fischeri*, but they also demonstrated more sensitivity than adult and larval stages of other marine invertebrate and fish species reported in literature.

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