Histol Histopathol (2018) 33: 157-169

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Toxicity of malathion at early life stages of the Senegalese sole, *Solea senegalensis* (Kaup, 1858): notochord and somatic disruptions

Juan B. Ortiz-Delgado[1], Emanuele Scala[1], Juana M. Arellano[2], María Úbeda-Manzanaro[1] and Carmen Sarasquete[1]

[1]Instituto de Ciencias Marinas de Andalucía (ICMAN-CSIC) and [2]CEIMAR, Universidad de Cádiz, Campus Universitario Río San Pedro, Puerto Real, Cádiz, Spain

Summary. The toxicity of malathion to *Solea senegalensis* was studied in a static renewal bioassay for 24, 48 and 72 h, with toxicant concentrations ranging from 1.56 until 100 μgL^{-1}. The LC_{50} values of malathion for 48 and 72-h was 63.5 (95% C.I: 50.83-79.34) and 22.94 (95% C.I: 17.16-30.68) μgL^{-1} respectively. The survival of larvae was non-affected by exposure to malathion at concentrations up to 25 μgL^{-1} (24 h NOEC), 6.25 μgL^{-1} (48 h NOEC) and <1.6 μgL^{-1} (72 h NOEC). At the end of the experiment, surviving larvae from concentrations smaller than the 72h-LC_{50} were chosen to study morphological changes during malathion exposure. Results revealed a strong disruption in the notochord and trunk musculature integrity as a result of toxicant exposure. Noticeable changes in the composition and reduction of collagen fibers from the perinotochordal connective sheath and perimysium were clearly detected. The trunk musculature was also altered, showing a general disorganization of fibers. Moreover, malathion exposure provoked pericardial and yolk-sac oedemas and histopathological alterations in some other organ- systems and tissues (i.e. liver, pancreas, intestine).

Key words: Malathion, Notochord, Histopathology, Senegalese sole, LC_{50}, Larvae

Offprint requests to: Dr. Juan B. Ortiz-Delgado, Instituto de Ciencias Marinas de Andalucía (ICMAN-CSIC), Campus Universitario Río San Pedro, 11510 Puerto Real, Cádiz, Spain.

DOI: 10.14670/HH-11-899

Introduction

In coastal ecosystems burdened with high levels of pollutants, deformities, pathologies and diseases have been reported as high as 90% (Bengtsson, 1975; Boglione et al., 2006; Weis, 2014). A variety of natural environmental factors (i.e. temperature, salinity, and others) have been identified as responsible for inducing deformities in early life stages (ELSs) of wild and reared fish species (Boglione et al., 2006; Weiss, 2014). The natural background of malformations in flatfish (i.e. Senegalese sole) is generally expected to be less than 20% (Gavaia and Cancela, 2002; Gavia et al., 2009). However, this frequency could increase significantly in parallel with nutritional and environmental stressors (Gisbert et al., 2008; Weiss, 2014).

Organophosphorus pesticides (OPs) have been widely used in agriculture and public health, accounting for approximately 50 percent of global insecticide use. Despite its ban by the European Commission since June 2007 (Regulation EC 1376/07, 07/389), the non-systemic and wide-spectrum organophosphate insecticide malathion [1,2-Di(ethoxycarbonyl)ethyl O,O-dimethyl phosphorodithioate], is still widely used throughout the world causing harmful environmental problems in coastal areas because of its runoff from agriculture and urban sources (Köck-Schulmeyer et al., 2012). Under the present legislation, the use of the OP pesticides is prohibited and thereby these pollutants might not be present in the environment. However, data of OPs pollution have been registered recently in Mediterranean coastal areas (Gómez-Gutiérrez et al., 2006; Köch et al., 2010). Pesticides such as dichlorvos,

malathion, quinalphos and other contaminants have been measured in coastal aquaculture systems (Patnaik, 2010). Moreover, the RASFF (Rapid Alert System for Food and Feed; from European Commission) reported 13 notifications during 2006 to 2014 of pesticide residues in fish fillets from Indian areas (Chatterjee et al., 2015).

Malathion is a phosphorothioate compound especially toxic to most insects and possesses from moderate to high toxicity in vertebrate species, where concentrations as low as 4 parts per billion (ppb) have been shown to adversely affect several freshwater fish species (U.S. EPA, 2006). It is established that many OP pesticides can produce toxic and adverse effects on many biological processes and in different organ systems and tissues of non-target organisms, such as fish species (Klaassen et al., 1986; Khan, 2006). In fish, malathion is metabolized to the bioactive form malaoxon via oxidative desulfuration by the cytochrome P450 (CYPs). Malaoxon can react with the hydroxyl group of serine in the active site of acetylcholinesterase (AChE) and thereby may affect its activity (Aker et al., 2008). The inhibition of AChE by OP pesticides (i.e. malathion) is the most critical toxic effect because it results in the accumulation of the neurotransmitter acetylcholine in the synapses, interrupting neural transmission and thus provoking uncontrolled muscle contractions. Moreover, these pesticides can also exert non-neurotoxic effects, such as developmental alterations, skeletal deformities and defective behaviours, among other critical disorders (Scott and Sloman, 2004; Weis, 2014). Malathion, parathion and their metabolites-oxons provoke many other serious disorders that are commonly consistent with collagen defects. Thus, among other alterations, there is evidence of circulatory disturbances, bent, curved and wavy notochords and shortening of embryos axis (Snawder and Chambers, 1990). Furthermore, growth retardation, haematological and biochemical alterations (glycogen, cholesterol, proteins), swimming disturbances and morpho-structural deformities, are also effects induced by malathion in several fish species (Lien et al., 1997; Huculeci et al., 2009; Lal et al., 2013).

Unlike the vast quantity of information available on the toxicity of pesticides on freshwater organisms, there are relatively few data on the effects of these insecticides on marine and estuarine organisms. Some studies analyzing the toxicity of pesticides have been performed in different commercial and reared marine teleostean species (Arufe et al., 2007; Solé et al., 2012), enclosing commercial flatfish species However, investigations related with pesticide toxicity in bentic species like *Solea senegalensis*, are scarce (Varó et al., 2003; Costa et al., 2011; Fonseca et al., 2011; Sanchez-Nogué et al., 2013).

The pleuronectiform Senegalese sole (*Solea senegalensis*) was used as a target species in our study because of its wide distribution in littoral and estuarine environments in the Easter Atlantic from the gulf of Biscay to the coasts of Senegal (Desoutter, 1990; Cabral and Costa, 1999). Additionally, this flatfish has a great economic potential and it is cultured at a commercial scale (Dinis et al., 1999; Imsland et al., 2003). The ontogenetic pattern during larval development and metamorphosis as well as different behaviour, habits and ecological life styles of this species have been previously studied (Sarasquete et al., 1996, 1998, 2001; Ribeiro et al., 1999; Ortiz-Delgado et al., 2006; Padrós et al., 2011, among others). Nevertheless, high mortalities and several diseases or pathologies (skeletal deformities, pigmentation disorders, eye migration failures among other pathological problems) are often detected in this species under intensive rearing conditions (Dinis et al., 1999; Power et al., 2008; Gavaia et al., 2009; Boglino et al., 2012). As causative agents of the presence of such pathologies, genetic, nutritional and environmental perturbations or a mixture of them have been pointed out. In this context, the use of different biochemical and cell biomarkers are adequate approaches for assessing the effects of human-induced environmental stressors, and/or for detecting alterations induced by zootechnical and nutritional non-optimal conditions (Cajaraville et al., 2000; Sarasquete and Segner, 2000; Ortiz-Delgado and Sarasquete, 2004; Gisbert et al., 2008).

Several test guidelines are useful tools for testing of chemical effects on biotic systems. In fact, fish acute toxicity tests are widely used in order to identify the concentration and time of exposure to xenobiotics associated with 50% mortality (LC_{50}). Lethal and sublethal effects are assessed and compared with control values to either determine the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC). Among other parameters or induced toxicant responses, fish weight and length, abnormal appearance and behaviour, hatching and survival rates, among other responses can be analyzed (OECD, 2013).

Early life stages (ELSs) of *Solea senegalensis* were used in our study in order to assess the safe environmental level of malathion in this estuarine marine species. ELS toxicity tests are advantageous due to their time- and cost-efectiveness and the reduced toxic waste generation. Moreover, previous experiences show that ELSs are often the most sensitive stages of life in fish species and are adequate to analyze several toxic and harmful effects for different environmentally relevant pollutants (Macek and Sleight, 1977; McKim, 1977) and, in most cases, long-term toxicity could be predicted by studies with ELSs (McKim, 1977, 1985; Woltering, 1984; Weiss, 2014). For toxicity tests and in order to diminish the potential natural mortality that usually occurs in *Solea senegalensis* from hatching until the first exogenous feeding period (from 0 to 3-4 dph (days post hatching)), 4 dph larvae were used. Since LC_{50} was studied at 24, 48 and 72 h, malathion exposure period was extended for 3 days, until the premetamorphic phase (larvae at 7 dph).

Several field studies have reported the presence of malathion in water samples at concentrations ranging between 18.12 (Karyab et al., 2013), 29.84

(Sankararamakrishnan et al., 2005) and 105.2 μgL^{-1} (Chowdhury et al., 2013). Based on these considerations, seven nominal concentrations of malathion (1.65, 3.12, 6.25, 12.5, 25, 50 and 100 μgL^{-1}) were selected in the present study. Acute toxicity of malathion in *Solea senegalensis* larvae (from first feeding phase (4 dph) until 7 dph was determined by means of LC_{50} (lethal dose at 24, 48 and 72h)) as well as NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) values. In addition, histopathological assessment on notochordal structures, trunk musculature and alterations in organ systems and somatic tissues (yolk-sac, heart-pericardium, liver, exocrine pancreas and intestine) were carried out in experimental groups below LC_{50} at 72 h in an attempt to elucidate the impact of sub-lethal and environmentally relevant malathion doses of exposure.

Materials and methdos

Biological material and toxicity test

Newly hatched Senegalese sole larvae were supplied by IFAPA-El Toruño (Cadiz, Spain) and transported to the Institute of Marine Sciences of Andalusia facilities (ICMAN-CSIC, Cadiz, Spain). Larvae and experimental procedures were treated in agreement with the European Convention for the Protection of Animals used for Experimental and Scientific purposes. The facilities of ICMAN-CSIC were approved for experimentation by the Ministry of Agriculture and Fisheries (REGA-ES110280000311) in accordance with the current EU and Spanish legislation.

Static-renewal toxicity tests were conducted for *S. senegalensis* larvae during 24, 48 and 72 h. The ELSs analyzed were from 4 (endo-exotrophic larvae) until 7 dph (before stating the metamorphosis process by 9-10 dph). Toxicity tests consisted of a control, a solvent control and seven malathion concentrations (Pestanal > 98% purity, Fluka, Sigma-Aldrich Química, Madrid, Spain) with three replicates per treatment. Nominal concentrations of malathion tested were: 1.65, 3.12, 6.25, 12.5, 25, 50 and 100 μgL^{-1}. All experimental treatments and controls received the same carrier solvent (0.05% acetone).

In each beaker, 50 larvae and 1 L of daily-prepared malathion test solutions were added (9 conditions x triplicate x 50 larvae per condition: 1350 total larvae used). Senegalese sole larvae were fed during the experimental period (rotifers, 15 individuals per ml; *Nannochloropsis sp.*: 300.000 and *Isocrhrysis sp.*: 50.000 cells per ml). Surviving larvae were gently transferred by pipette into beakers with fresh solution every 24 hours. After each water renewal, food was freshly added. The toxicity assays were performed at 19±1°C, pH 8±0.1, salinity 37±1‰, dissolved oxygen higher than 85% saturation and photoperiod 12L:12D. The larval mortality rates were evaluated as immobility, absence of heartbeat and opaque coloration of larval body.

Statistical analysis

The LC_{50} values for malathion toxicity were estimated by using the trimmed Spearman-Karber method (Hamilton et al., 1977). Statistical differences of surviving larvae from controls and malathion treatments were analyzed by ANOVA and Dunnett's test. Shaphiro-Wilk's and Barlett's test were used to test the normality of the data distribution and the homogeneity of the variances, respectively. The Computer Program TOXSTAT 3.5 (Gulley and Western Inc., 1996) was used to perform these analyses. For statistical evaluation, control and solvent control were pooled, as they did not differ significantly with regard to survival.

Histomorphology and histopathology

At the end of the experimental designs (72 h), malathion concentrations below LC_{50} (1.65, 3.12, 6.25 and 12.5 μgL^{-1}) were selected for histopathological records. Surviving larvae for the corresponding replicates (3 fish per treatment x triplicate= 9 fish per each treatment condition) were sampled, anesthetized with clove oil and selected for macroscopical examination under a stereomicroscope or processed for histopathological purposes. Briefly, surviving larvae were fixed in formaldehyde (4% v/v, phosphate buffered, pH 7.2), dehydrated in alcohol series, and embedded in paraffin wax. Serial histological sections were stained with Haematoxylin-eosin/H&E for morphological study. Afterwards, sections were observed under a light microscope and histopathological findings in terms of occurrence (percentage of affected fish) or severity (intensity of alteration) were recorded in a table (Table 2).

Additionally, individuals from the highest sublethal malathion concentration tested (72h-LOEC/ 3.12 μgL^{-1}) were used for performing a detailed description of the most characteristic histopathological findings. Picrosirius Red (PSR) and Reticulin (R) stainings (Kit DC, Panreac, following manufacturer's recommendations) were applied for collagen fibers. For PSR technique (Ortiz-Delgado et al., 2014), the histological sections were incubated in 0.1 % (w/v) fast green in saturated picric acid solution for 10 min. Afterwards, samples were rinsed 3 times in 1 % acetic acid and stained for 20 min in a solution of 0.2 % Sirius red F3B with 0.2 % picric acid. Finally, sections were washed in 1 % acetic acid, air dried at room temperature and mounted. A BX41 Olympus light microscope equipped with two filters (polarizer and analyzer) was used to provide linearly polarized illumination. Digital images were obtained with Olympus digital camera (C3030).

Results

Toxicity assays

According to the test guidelines for toxicity in ELSs of fish species (OECD, 2013), a larval survival of 93% in controls is indicative of good experimental designs. In

the present study, the 24-h LC_{50} could not be calculated since for all malathion concentrations assayed, rates of larval mortality remained below 48%. As expected, the toxicity of malathion in early life stages (ELSs) of Senegalese sole (4-7 dph) increased with both the time of exposure and concentrations of malathion.

The mortality rates in Senegalese sole are shown in Figure 1. The larval survival rate was not affected by exposure to malathion at concentrations up to 25 μgL^{-1} (24-h NOEC), 6.25 μgL^{-1} (48-h NOEC) and ≤1.6 μgL^{-1} (72-h NOEC). The LC_{50} and their 95% confidence intervals (C.I.) as well as NOEC and LOEC values for malathion toxicity are summarized in Table 1. After 72 h of exposure, malathion induced a statistically significant reduction of larval survival, for almost all concentrations tested (Fig. 1). The estimated LC_{50} values (48 h and 72 h) for Senegalese sole larvae were 63.5 μgL^{-1} (95% C.I.: 50.83-79.34) and 22.94 μgL^{-1} (95% C.I.: 17.16-30.68), respectively.

Notochord and trunk alterations

The most significant effects induced by malathion on *S. senegalensis* larvae are summarized in Table 2 and Figs. 2-5.

Macroscopically, environmentally relevant sub-lethal exposures to malathion (concentrations below LC_{50} : 1.65, 3.12, 6.25 and 12.5 μgL^{-1}) induced at 72 h noticeable morphological abnormalities, i.e. curved larval body and enlargement of the front portion of the yolk sac, that finally consisted of a severe body trunk bending (inward dorsal curving) and pericardial and yolk-sac oedemas (Table 2, Fig. 2A-D).

Microscopically, in control larvae at 7 dph, the notochord is constituted by well-defined and homogeneously sized polygonal chordoblasts, with smooth boundaries and gradually decreasing diameters towards the caudal region (Fig. 3A-D). The perino-tochordal connective sheet was also smooth and regular and was composed of collagen type I and III fibers as revealed by reticulin (Fig. 3B) and PSR (Fig. 3C,D) techniques. The branches of the perimysium penetrate perpendicularly to the perinotochordal connective sheet at intervals supporting muscle masses (PSR positive fibers). Well-developed and arranged muscle bundles were also evidenced (Fig. 3C,D). The collagen fibers arranged around the notochord, in longitudinal and circular bundles, exhibited a homogeneous green coloration (small collagen fibers) when they were stained with PSR technique (Fig. 3D).

In Senegalese sole exposed larvae, a microscopical description of histological alterations was performed at the highest sublethal malathion concentration tested (72 h LOEC: 3.12 μgL^{-1}) at the end of the experimental assay (larvae at 7 dph). Abnormal notochords (56% of affected fish) with irregular diameters having widening and narrowing, especially at the curved region, were detected in exposed larvae at 3.12 μgL^{-1}. The perinotochordal connective sheet was quite irregular and discontinuous and showed many infoldings as revealed by means of PSR technique. The perinotochordal sheet was not uniform, showing an irregular profile with many undulations and a severe disorganization of the arrangement and spatial distribution of collagen fibers (Fig. 3E-H). These fibers exhibited few orange surfaces (big collagen fibers) embedded in a general dark green colour affinity when stained with PSR (Fig. 3F,H). Moreover, many canaliculi-like structures could be identified. The chordoblasts lost their polygonal shape and appeared shrunk and rounded. In the bended body areas, the notochord showed severe disorganization with the chordoblasts replaced by a heterogeneous and fibrous substance, appearing with an intense orange dye-

Table 1. Toxicity data for malathion in *Solea senegalensis* larvae.

Final endpoint	Species	Parameter	Value (μgL^{-1})
Survival	*Solea s.*	LC_{50}-24h	-
		NOEC-24h	25
		LOEC-24h	50
		LC_{50}-48h	63,5 (50,83-79,34)*
		NOEC-48h	6,25
		LOEC-48h	12,5
		LC_{50}-72h	22,94 (17,16-30,68)*
		NOEC-72h	1,6
		LOEC-72h	3,12

*Estimated value by using trimmed Spearman-Karber method. Between brakets: 95% confidence limit.

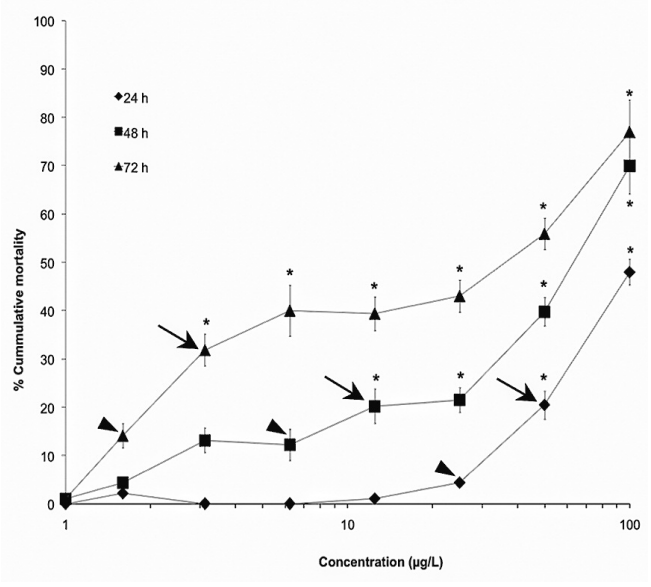

Fig. 1. Concentration-response curve for Senegalese sole larvae after exposure to different concentrations of malathion for 24, 48 or 72 hours. LOEC values are represented as arrows and NOEC as arrowheads. Asterisks denote statistically significant differences from control ($P<0.05$).

affinity (PSR staining), which filled the inter-cellular spaces (Fig. 3E,F).

Trunk musculature was also affected by malathion (78% of affected fish), showing a general disorganization of the muscle fibers and severe shrinkage of the myomers with increased inter-myotomal spaces in comparison with control larvae (Fig. 4A,B). Furthermore, the intramuscular connective branches (myoseptal branches) were reduced in number and showed a severe disorganization as revealed by reticulin staining technique (Fig. 4C-E).

Other somatic alterations

In general, exposure to malathion (72 h LOEC: 3.12 μgL^{-1}, 7 dph larvae) provoked in Senegalese sole larvae

Table 2. Histopathological findings in different organ/tissues of *S. senegalensis* larvae (72 h) exposed to malathion.

Type of alteration	Occurrence[a]/severity[b]				
	Malathion (μgL^{-1})				
	Control	1.65	3.12	6.25	12.5
Pericardial and yolk sac oedemas	(11)/(0-1)	(33)/(1)	(44)/(1-2)	(56)/(2-3)	(78)/(3)
Trunk bending	(0)/(0)	(11)/(0-1)	(56)/(1-2)	(56)/(2-3)	(67)/(3)
Abnormal notochords	(0)/(0)	(22)/(1)	(56)/(1-2)	(67)/(2-3)	(78)/(3)
Disorganization of trunk musculature	(11)/(0-1)	(33)/(1)	(78)/(2)	(100)/(3)	(100)/(3)
Delayed organogenesis	(0)/(0)	(56)/(2)	(89)/(3)	(100)/(3)	(100)/(3)
Atrophy of hepatocytes and nuclear pycnosis	(0)/(0)	(44)/(1-2)	(89)/(3)	(100)/(3)	(100)/(3)
Disorganization and atrophy of pancreocytes	(0)/(0)	(33)/(1)	(89)/(3)	(89)/(3)	(89)/(3)
Atrophy of mucose layer from intestine	(11)/(0-1)	(44)/(1)	(89)/(3)	(100)/(3)	(100)/(3)

[a]: Occurrence is the percentage of affected fish (n=9). [b]: Severity is the intensity of alteration (0= no alteration; 1= mild alteration: only a small portion of the organ is affected; 2= moderate alteration half of the organ presented the alteration; 3= strong alteration: pathology is presented in almost the entire organ).

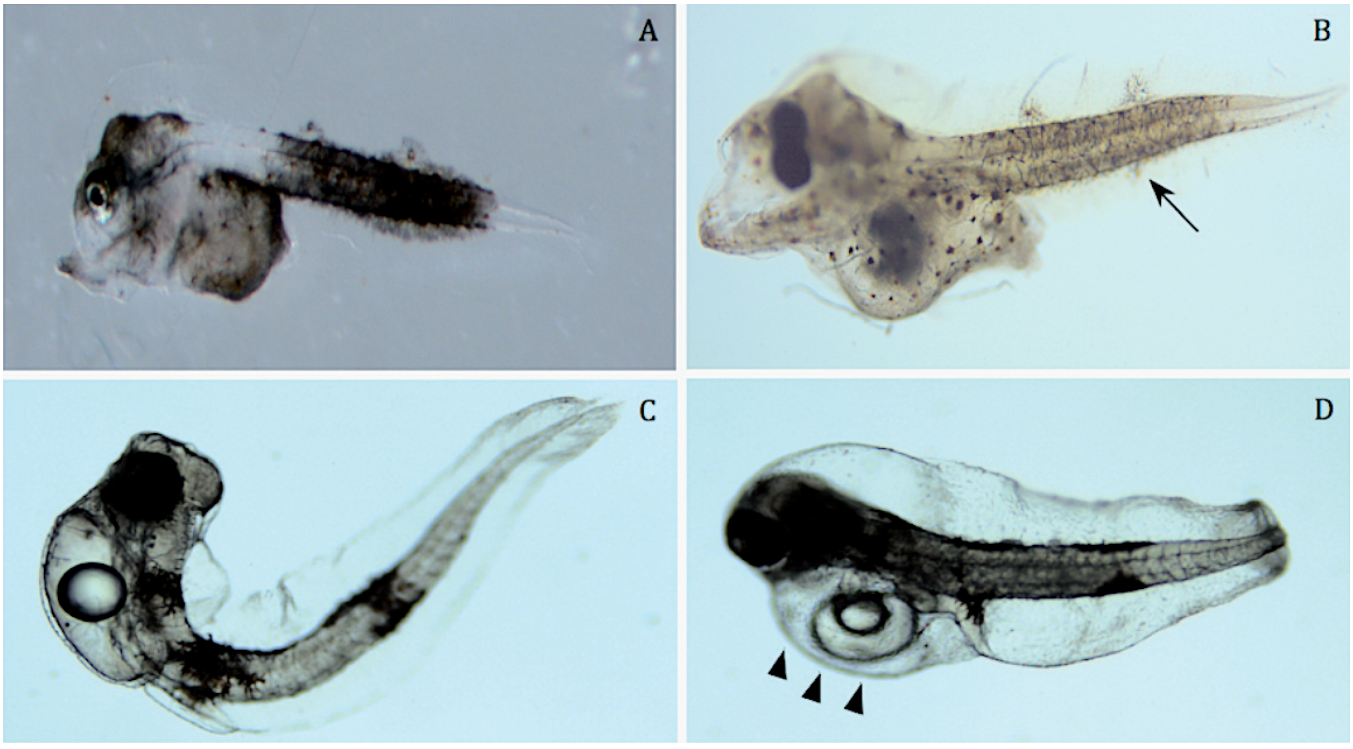

Fig. 2. Macroscopical observations in Senegalese sole larvae at 24, 48 and 72 h of exposure. General view of a control **(A)** and malathion **(B to D)** exposed specimens. **A.** Macroscopical view of a control larvae (72 h) showing the mouth opened and complete resorption of the yolk sac. **B.** Donwards bending (arrow) of the lumbar region in affected larvae (72 h, 1.65 μgL-1). **C.** Abnormal larvae showing an inwards curving of the dorsal body region (24 h, 3.12 μgL-1). **D.** Pericardial and yolk sac oedemas (arrowheads) in exposed larvae (48 h, 1.65 μgL-1).

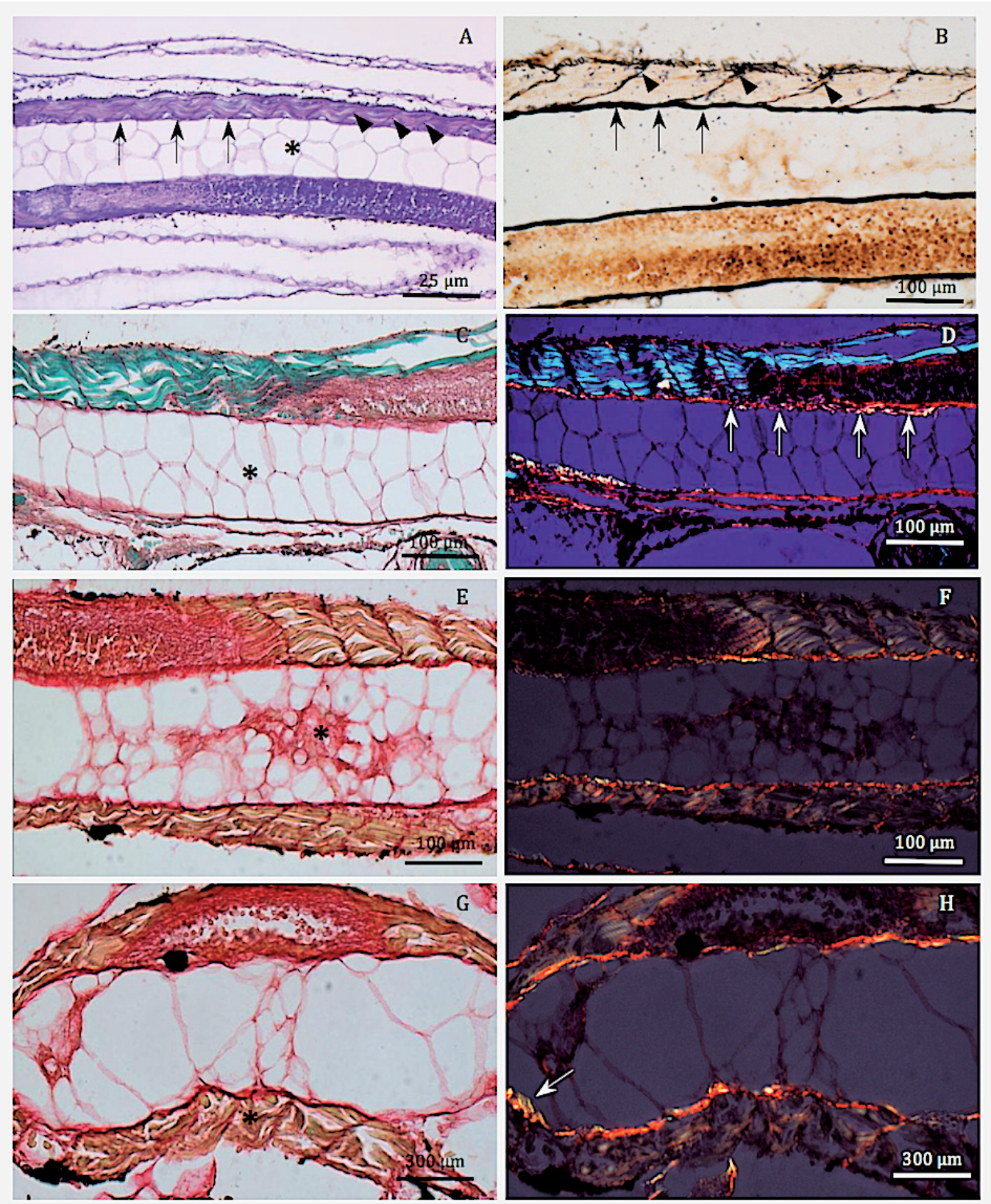

Fig. 3. Sagittal sections of normal and deformed *Senegalese sole* larvae. **A-D.** control larvae, note the regular arrangement of muscle bundles surrounding the notochord of control larvae that displays polygonal cells (chordoblasts) (star) **(A and C)**. Perinotochordal (arrows) and intermuscular conective sheets (arrowheads) are clearly discernible and composed of collagen type I and III as evidenced by reticulin **(A and B)** and PSR under polarized light **(C and D)** stainings, respectively. **E-H.** 7 dph, 3.12 μgL-1, deformed larvae presenting chordoblasts replaced by heterogeneous and fibrous substances (asterisk, **E and F**); note the disorganized muscle bundles arranged in different planes (asterisk, **G**) and the presence of an irregular and discontinuous perinotochordal sheet exhibiting a severe disorganization of collagen fibers (arrow, **H**).

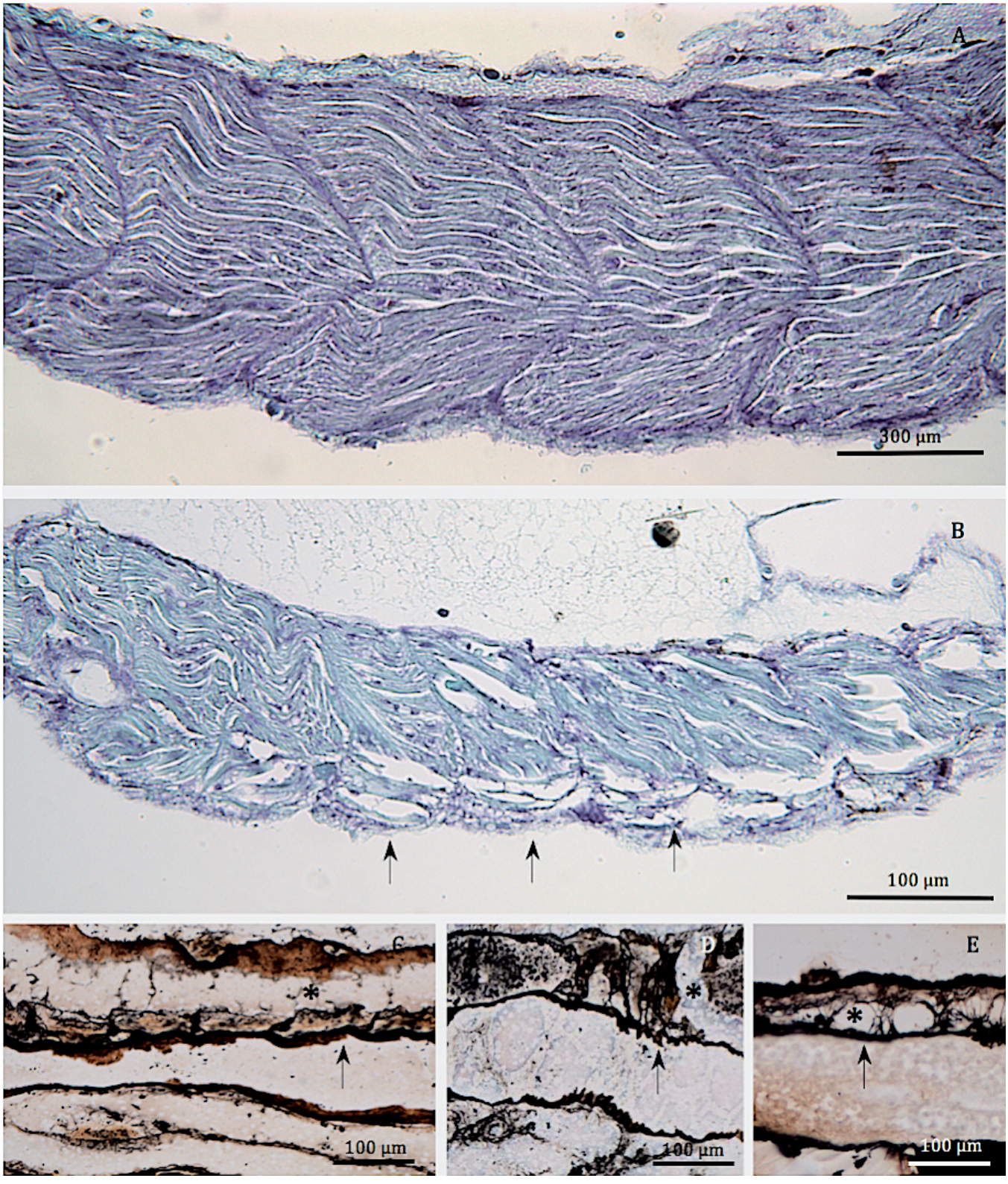

Fig. 4. Detail of trunk musculature of normal and deformed *Senegalese sole* larvae. **A.** normal pattern of controls showing the regular arrangement of muscle bundles. **B.** Deformed larvae showing severe shrinkage of the myomers (arrows) and increases of inter-myotomal spaces of deformed larvae. **C, E.** myoseptal branches are reduced in number and show a severe disorganization (asterisk). Perinotochordal sheet presents an irregular profile (arrows).

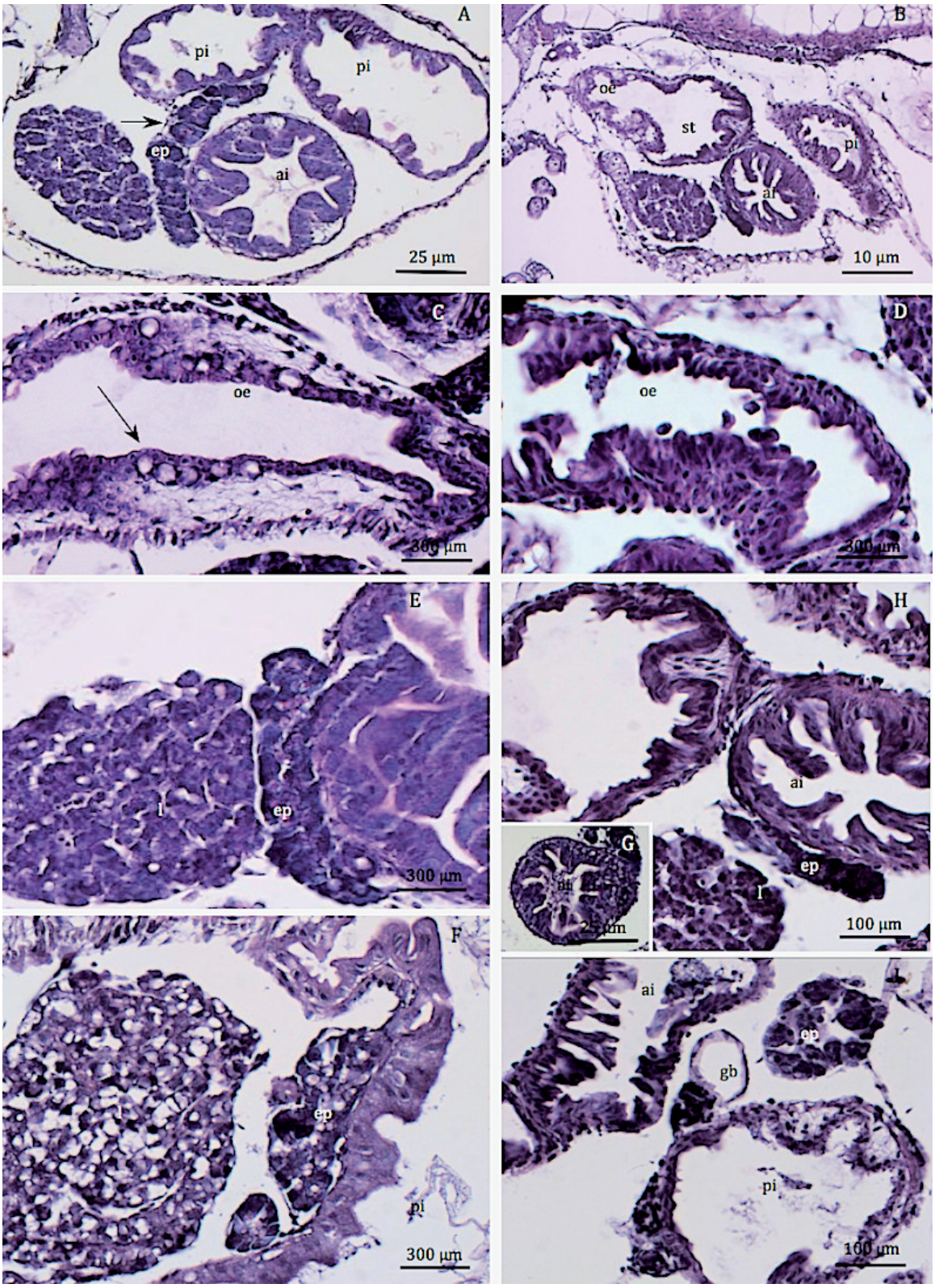

Fig. 5. Histological sections of the visceral cavity in Senegalese sole larvae. Controls: **A, C, E, F and G**. 7 dph, 3.12 µgL-1**: B, D, H, and I.** General overview of the digestive tract and accessory organs of controls **(A)** and malathion exposed larvae **(B)** showing the digestive tract, liver and exocrine pancreas. Note a general delay of the digestive tract development and maturation, translated into a lack of oesophageal mucous cells (arrows) in exposed larvae in comparison with controls **(C and D).** Comparing to controls **(E, F and G**) note the absence of lipid inclusions into anterior intestine and liver and lack of pancreatic exocrine zymogen granules in exposed larvae **(H and I)**. Discernible absence of supranuclear vesicles into enterocytes of the posterior intestinal region in exposed larvae when compared with controls **(F and I)**. ai: anterior intestine; ep: exocrine pancreas; gb: gall bladder; l: liver; oe: oesophagus; pi: posterior intestine; st: stomach.

reduced growth and a delayed organogenesis (89% of affected fish) in comparison with controls (Table 2, Fig. 5A,B). The oesophagus from control larvae (Fig. 5C) showed a typical pseudo-stratified epithelium with numerous mucous cells, whereas in malathion exposed larvae, no mucous cells were evidenced (Fig. 5D). Additionally, the liver from controls presented polygonal hepatocytes with vacuolated cytoplasms arranged in cords of two cells surrounding the sinusoids and the typical hepatic parenchymal structure (Fig. 5E,F). However in exposed larvae, homogeneous and non-vacuolated cytoplasms of hepatocytes were evidenced (Fig. 5H). Moreover, the hepatic cells lost the polygonal appearance and acquired rounded shapes. Nuclear pycnosis was also observed in the hepatocytes (Fig. 5H). On the other hand, while pancreocytes from control larvae were organized in acini showing acidophilic exocrine zymogen granules (Fig. 5E,F), in malathion exposed larvae, exocrine pancreatic cells showed a noticeable disorganization and signs of cellular atrophy, and zymogens were not detected at this larval age-7 dph-(Fig. 5H,I). Finally, the gut from control larvae contained lipid droplets (Fig. 5G) and supranuclear protein inclusions (Fig. 5F) in the anterior and posterior intestinal regions, respectively, which were absent in malathion exposed larvae (Fig. 5H,I). Furthermore, the intestinal mucosa layer also showed signs of cellular atrophy and a slight shrinkage in the *lamina propria*/submucosa (Fig. 5H,I).

Discussion

Toxicity test

This study examines the effect of lethal and sublethal concentrations of malathion on larval Senegalese sole perfomance using a concentration- and time- response desingn. Survival and development were affected by malathion even at concentrations below those expected in the environment (Sankararamakrishnan et al., 2005; Chowdhury et al., 2013; Karyab et al., 2013; Agarwal et al., 2015).

As shown in this paper, the early-life stages of fish (ELSs) are adequate *in vivo* animal models for evaluating the chemical toxicity of different contaminants (OECD-2013). Particularly, in Senegalese sole larvae (at 7 dph) after 72 h of malathion exposure, before starting the metamorphosis and the eye migration processes by 9-10 dph onwards, under optimal rearing conditions (Dinis et al., 1999; Ortiz-Delgado et al., 2006), the current results indicate that the mortality rates increased from 14.1% at 1.65 μgL^{-1} to 77% at 100 μgL^{-1}, suggesting that the larval survival decreased as malathion concentrations increased.

The LC_{50} values were estimated at 63.5 μgL^{-1} (LC_{50}) and 22.94 μgL^{-1} (72h-LC_{50}), respectively, which could indicate that Senegalese sole larvae showed an increased sensitivity to malathion exposure as a function of stages of larval development from 4 to 7 dph. Studies in different fish species (Durkin, 2008; Faria et al., 2010, Ahmad, 2012; Naserabad et al., 2015) pointed out that malathion displays a wide range of toxicity extending from very highly toxic (LC_{50} less than 1 mgL^{-1}) in the walleye (*Stizostedion vitreum*, 72h-LC_{50} of 0.08 mgL^{-1}) to highly toxic (LC_{50} of 0.1-1 mgL^{-1}) in brown trout, rainbow trout and carp (72h-LC_{50} 0.13, 0.12 and 0.14 mgL^{-1}, respectively), moderately toxic (LC_{50} from 1 to 10 mgL^{-1}) in killifish, gold fish and Nile tilapia (72h-LC_{50} 1.06, 5.38 and 4.6 mgL^{-1}, respectively) and slightly toxic (LC_{50} from 10 to 100 mgL^{-1}) in fathead minnow, black bullhead and Eastern mosquitofish (72h-LC_{50} 15.49, 14.98 and 12.68 mgL^{-1}, respectively) among others. Considering LC_{50} values obtained for ELSs of Senegalese sole larvae, ranging around very high toxicant values (0.063.5 mgL^{-1} at 48h-LC_{50} and 0.02294 mgL^{-1} at 72h-LC_{50}) and the sublethal histopathological effects observed, a greater sensitivity to acute malathion exposure in this flatfish species in comparison with other fish species, might be assumed.

It is known that the sensitivity of animals and toxicant effects of different xenobiotics, enclosing contamination by pesticides such as malathion, present inter and intra-specific variations, depending on species (freshwater, marine), ecology (benthonic, planktonic) and stage of development, since there are different mechanisms implicated in the metabolism, detoxification and elimination of xenobiotics (Cajaraville et al., 2000; Weiss, 2014). In seabream, *Sparus aurata* larvae exposed to dioxins and benzopyrene, Ortiz-Delgado and Sarasquete (2004) evidenced a higher sensitivity to both contaminants in larvae at first feeding in comparison with in yolk-sac larvae. This fact may be due to the lack of development and/or maturation of some of the most important organ systems (digestive, skin, gills, etc.) in these ELSs when compared with more advanced developmental stages (i.e. metamorphosed larvae, juveniles).

Notochord and trunk alterations

Malathion is a known and potent acetylcholine-sterase-AChE- inhibitor. Nevertheless, the mechanisms by which this OP pesticide causes axis deformations are not well understood, but it could be related to the integrity of the extracellular matrix (Chemotti et al., 2006). In teleostean fish species, it is assumed that skeletal deformities are linked with dysfunctions in collagen synthesis and/or metabolism (Green et al., 1968; Santamaría et al., 1994). Moreover, it has been suggested that pesticides such as malathion may target the collagen extracellular transport and cross-linking (Snawder and Chambers, 1993; Chemotti et al., 2006). In this sense, Santamaría et al. (1994) observed that in both normal and lordotic seabream larvae, the presence of type II collagen, non-sulphated glycosaminoglycans, and elastic fibers in perinotochordal connective sheet were similarly detected. However, while in normal seabream larvae a part of the glycosaminoglycans from

the notochord were transformed into proteoglycans, the formation of proteoglycans in lordotic seabream larvae was impaired and low levels of collagen-proteoglycan interactions occurred. These authors also evidenced during the normal vertebral development of the seabream, a time-shifting associated to collagen maturation as well as sequential changes from green to an orange colour-affinity during the maturation of collagen-fibers by using the PSR technique (Fernández et al., 2012; Ortiz-Delgado et al., 2014).

In our study, whereas the notochord of control Senegalese sole larvae presented small collagen fibers (green coloration) in both perinotochordal connective sheet and perimysial supporting tissue, in malathion exposed Senegalese sole larvae notochordal structure changed its collagen composition showing few orange surfaces suggesting the presence of big collagen fibers, as well as a severe disorganization of the arrangement and spatial distribution of collagen fibers. In this sense, Snawder and Chambers, (1993) pointed out that collagen makes up the notochord sheath, and changes in collagen composition induce abnormalities consistent with collagen defects, bent notochords, shortened body lengths and circulatory disorders. Thus, disruptions of the notochord sheath may contribute to abnormal bending and shortening of early life stages (Chemotti et al., 2006). The pesticide malathion can perturb the collagen synthesis and/or processing, and as a consequence, the affected tissues could be constrained to develop and to form improper connective sheath, with adverse effects in notochord and vertebral development (Snawder and Chambers, 1993).

Furthermore, notochord alterations induced by this OP pesticide could be attributed to undersulphation of chondroitin (4 and 6) sulphates; which are the more relevant glycosaminoglycan constituents of the sheath (Garrison and Wyttenbach, 1985). In *Xenopus* embryos, Chemotti et al. (2006) showed that malathion reduced the number of extracellular collagen fibers, due to decreases in ascorbic acid and hydroxyproline levels and a consequent inhibition of collagen prolyn-hydroxylase activity during collagen biosynthesis. In *S. senegalensis* larvae, malathion induced reduction of fibers and changes in collagen composition, which are typical fibers of both perinotochordal connective sheet and perimysial supporting tissues.

Moreover, notochord disturbances induced by malathion in Senegalese sole larvae, could also be due to uncontrolled contractions or irregular spasms of the trunk musculature, which act directly onto notochordal supporting tissues, resulting in a bending and even a looping of the notochord. These body contractions may occur due to disturbances of the impulse transport from the nerves (AChE inhibition) to the trunk musculature (Scott and Sloman, 2004; Weis, 2014). In this sense, Lien et al. (1997) suggested that axial skeletal alterations and deformities detected in catfish, *Clarias gariepinus* exposed to malathion could be caused by irregular spasms of the body musculature.

On the other hand, as the development of bony vertebral centra occurs around notochord sheath, the normal development of vertebral structures could be affected by malathion exposure (Chemotti et al., 2006). Furthermore, spatial notochord alterations also can deform the neural tube covering the dorsal face of the notochord and thus, it will affect the correct ontogeny and the formation of the subsequent elements, i.e. neural arches. In fact, in mammals, the initiation of chondrogenesis by the notochord has been demonstrated to depend on the developmental state of the latter (Cooper, 1965). As was suggested by Lien et al. (1997), this fact might explain the vertebral deformities observed in fish (juvenile and adults) which have been exposed to malathion, from the notochordal-stage onwards. According to these last authors, the abnormal vertebrae are originated from deformed notochords (erratic muscular contractions), instead of deformation of the vertebral elements themselves.

Additionally, it was indicated that OP pesticides (i.e. malathion) significantly induce decreases in the activity of enzymes related with bone metabolism, such as carbonic anhydrase (Thomas and Murthy, 1976) which can provoke a disruptive effect on the calcification process of the bone tissues. Furthermore, Videira et al. (1994) pointed out that malathion affected the Ca^{2+} pump activity of sarcoplasmic reticulum by interacting with the boundary lipids of the pump. Nevertheless, in Senegalese sole larvae (4-7 dph), as is reported in other larval fish species, i.e. *Clarias gariepinus* (Lien et al., 1997), notochordal disturbances induced by sublethal concentrations of malathion could not be attributed to alterations during the process of vertebral calcification, since no bony vertebral structures are developed at these early stages of larval life (4-7 dph), as was previously pointed out in this flatfish species (Gavaia et al., 2006).

Other somatic alterations

The exposure to malathion induced biological, morphological and histopathological responses in Senegalese sole larvae, which consisted of growth retardation and delayed organogenesis of different somatic organ systems and tissues. Yolk sac and pericardial oedemas detected in this species under malathion treatment, are abnormalities commonly reported in several fish species exposed to many other different contaminants (Lien et al., 1997; Ortiz-Delgado and Sarasquete, 2004; Scott and Sloman, 2004; Weis, 2014). It is hypothesized that yolk sac oedemas can be caused by over hydration of adjacent areas due to osmoregulatory failures associated with accumulation of contaminants (Cook et al., 2005).

Senegalese sole larvae exposed to malathion (72 h LOEC: 3.12 μgL^{-1}, 7 dph larvae) showed cellular atrophy of pancreatic acinar cells and in the digestive mucosa layer as well as necrosis of hepatic cords in the liver. The presence of neutral lipids (vacuoles) within hepatocytes and into intestinal enterocytes, as well as the

presence of protein inclusions (micropynocitosis, intracellular digestion) in the posterior intestinal region are typical features of digestive development, partial maturation and nutritional functionality, respectively, on fish ELSs (Gisbert et al., 2008). This normal pattern and the presence of acidophilic protein zymogens within pancreocytes were evidenced in Senegalese sole control larvae, as was previously described in this flatfish and in many other fish species (Zambonino et al., 2008). Nevertheless, in malathion exposed Senegalese sole larvae these digestive characteristics (lipid globules, protein inclusions, zymogens) were not detected suggesting a delayed or disrupted ontogenesis induced by malathion and a minor digestive functionality at these early and critical larval stages. These metabolic disturbances can eventually lead to decreases in the absorption, synthesis and mobilization of different nutrients. In Nile tilapia (*Oreochromis nitolicus*) exposed to sublethal concentrations of malathion a significant decrease in body weight, as well as minor glycogen and protein contents in skeletal muscle were evidenced (Al-Ghanim, 2012). Similarly, Lal et al. (2013) in a freshwater fish species (*Clarias batrachus*) exposed to malathion, evidenced a reduction in hepatic protein content as well as lipolysis in both muscle and hepatic tissues. Chen et al. (2006) in malathion treated carp cell lines detected cytotoxicity and increased apoptotic processes. As was suggested by Bradbury et al. (1987) most of the above mentioned alterations and/or disturbances could lead to an impairment of protein synthesis machinery and consequently to a delayed larval ontogeny, development and growth.

Finally, other authors (Cook et al., 2005; Lal et al., 2013), in several different fish species exposed to malathion evidenced a noticeable reduction in body-length and minor eye-diameters, as well as several hormonal disturbances (growth hormone, steroids) corroborating the known effect of this OP pesticide, as a typical endocrine disruptor (Weiss, 2014).

In conclusion, our results showed that toxicity assays provide adequate information on the lethal and sublethal effects of malathion on *Solea senegalensis* larval specimens. Particularly, the sensitivity of the Senegalese sole larvae to malathion was found to be higher than those registered for many other fish species, as is largely referenced in the present study. In addition, the concentrations that we found that adversely affect larval survival and induced histopathological alterations in Senegalese sole larvae were below those expected environmental concentrations pointed out in many other different ecosystems and fish species (Sankararamakrishnan et al., 2005; Chowdhury et al., 2013; Karyab et al., 2013; Agarwal et al., 2015).

Acknowledgements. This work was funded by Ministry of Science and Innovation (MICIIN) of the Spanish government (project AGL2010-15951). The authors are grateful to IFAPA for providing biological material and to Mr. Juan A. Canales for technical assistance.

References

Agarwal A., Prajapati R., Singh O.P., Raza S.K. and Thakur L.K. (2015). Pesticide residue in water--a challenging task in India. Environ. Monit. Assess. 187, 54.

Ahmad Z. (2012). Toxicity bioassay and effects of sub-lethal exposure of malathion on biochemical composition and haematological parameters of *Clarias gariepinus*. African J. Biotech. 11, 8578-8585.

Aker W., Hu X., Wang P. and Hwang H.M. (2008). Comparing the relative toxicity of malathion and malaoxon in blue catfish *Ictalurus furcatus*. Environ. Toxicol. 23, 548-554.

Al-Ghanim K.A. (2012). Acute toxicity and effects of sublethal malathion exposure on biochemical and haematological parameters of *Oreochromis nitolicus*. Sci. Res. Assays 7, 1674-1680.

Arufe M.I, Arellano J.M., García L., Albendín G. and Sarasquete C. (2007). Cholinesterase activity in gilthead seabream (*Sparus aurata*) larvae: characterization and sensitivity to the organo phosphate azinphosmethyl. Aquac. Toxicol. 84, 328-336.

Bradbury S.P., Symonic D.M., Coats J.R. and Atchison G.J. (1987). Toxicology of fenvalerete and its constituent isomers to the fathead minnow (*Piephales promeos*) and blue gill (*Lepomis macrochirus*). Bull. Environ. Cont. Toxicol. 38, 727-735.

Bengtsson B.E. (1975). Vertebral damage in fish induced by pollutans. In: Sublethal effects of toxic chemic als on aquatic animals. Koeman J.H. and Strik J.J.T.W.A. (eds). Elsevier, Amsterdam. pp 23-30.

Boglino A., Darias M.J., Ortiz-Delgado J.B., Özcan F., Estévez A., Andree K.B., Hontoria F., Sarasquete C. and Gisbert E. (2012). Commercial products for Artemia enrichment affect growth performance, digestive system maturation, ossification and incidence of skeletal deformities in Senegalese sole (*Solea senegalensis*) larvae. Aquaculture 324-325, 290-302

Boglione C., Costa C., Giganti M., Cecchetti M., Di Dato P., Scardi M., and Cataudella S. (2006). Biological monitoring of wild thicklip grey mullet (Chelonlabrosus), golden grey mullet (Lizaaurata), thinlip mullet (Lizaramada) and fathead mullet (Mugilcephalus) (Pisces: Mugilidae) from different Adriatic sites: meristic counts and skeletal anomalies. Ecol. Indic. 6, 712-732.

Cabral H.N. and Costa M.J. (1999). Differential use of nursery areas within the Tagus estuary by sympatric soles, Solea solea and *Solea senegalensis*. Environ. Biol. Fish. 56, 389-397

Cajaraville M.P., Bebianno M.J., Blasco J., Porte C., Sarasquete C. and Viarengo A. (2000). The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Total Environ 247, 295-311

Chatterjee N.S., Banerjee K., Utture S., Kamble N., Rao M., Panda S.K. and Mathew S. (2015). Assessment of polyaromatic hydrocarbons and pesticide residues in domestic and imported pangasius (*Pangasianodon hypophthalmus*) fish in India. J. Sci. Food and Ag. 96, 2373-2377.

Chemotti D.C., Davis S.N., Cook L.W., Willoughby I.R., Paradise C.J. and Lom B. (2006). The pesticide malathion disrupts xenopus and zebrafish embryogenesis: an investigative laboratory exercise in developmental toxicology. Bioscene 32, 4-18.

Chen X.Y., Sao J.Z., Xiang L.X. and Liu L.X. (2006). Involvement of apoptosis in malathion-induced cytotoxicity in agrass carp (Ctenopharyngodon idellus) cell line. Com. Biochem. Physiol. C. 142, 36-45.

Chowdhury M.A., Banik S., Uddin B., Moniruzzaman M., Karim N. and Gan S.H. (2013). Organophosphorus and carbamate pesticide

residues detected in water samples collected from paddy and vegetable fields of the Savar and Dhamrai Upazilas in Bangladesh. Int. J. Environ. Res. Public Health 9, 3318-3329.

Cook L.W., Paradise C.J. and Lom B. (2005). The pesticide malathion reduces growth and survival in the decvelopment of zebrafish, Danio rerio. Environ. Toxicol. Chem. 24, 1745-1750.

Cooper G.W. (1965). Induction of the somite chondrogenesis by cartilage and notochord: a correlation betweeninductive activity and specific stages of cytodifferentiation. Dev. Biol. 12, 185-212.

Costa P.M., Caeiro S., Lobo J., Martins M., Ferreira A.M., Caetano M., Vale C., DelValls T.A. and Costa M.H. (2011). Estuarine ecological risk based on hepatic histopathological indices from laboratory and in situ tested fish. Mar. Pollut. Bull. 62, 55-65.

Desoutter M. (1990). Soleidae. In: Check list of the fishes of the eastern tropical atlantic (CLOFETA) Vol. II. Quero J.C. (ed). Unesco, Portugal. pp 1037-1049.

Dinis M.T., Ribeiro L., Soares F. and Sarasquete C. (1999). A review on the cultivation potencial of *Solea senegalensis* in Spain and in Portugal. Aquaculture 176, 27-38.

Durkin P.R. (2008). Malathion, Human health and ecological risk assesment. PCR, USDA/forest service, Surthern region, Atlanta Georgia. Sera TR-05202-02c, 325.

European Commision (2007). Study on the prioritisation of substances dangerous to the aquatic environment. Office for official publications of the european comunities, Luxembourg.

Faria I.R., Palumbo A.J., Fojut T.L. and Tjeerdema R.S. (2010). Water quality criteria report for malathion. Phase III: Application of the pesticide water quality criteria methodology. UCDAVIS 7, 64.

Fernández I., Ortiz-Delgado J.B., Sarasquete C. and Gisbert E. (2012). Vitamin A effects on vertebral bone tissue homeostasis in gilthead sea bream (*Sparus aurata*) juveniles. J. Appl. Ichthyol. 28,419-426.

Fonseca V.F., Franca S., Serafim A., Company R., Lopez B., Bebianno M.J. and Cabral H.N. (2011). Multi-biomarker responses to estuarine habitat contamination in three fish species: Dicentratus labrax, *Solea senegalensis* and Pomatoschistus microps. Aquat. Toxicol. 102, 216-227.

Garrison J.C. and Wyttenbach C.R. (1985). Teratogenic effects of the organophosphate insecticide dicrotophos (bidrin). histological characterization of defects. Anat. Rec. 213, 464-472.

Gavaia, P.J., Simes, D.C., Ortiz-Delgado, J.B., Viegas, C.S., Pinto, J.P., Kelsh, R.N., Sarasquete, M.C. and Cancela, M.L. (2006). Osteocalcin and matrix Gla protein in zebrafish (Danio rerio) and Senegal sole (*Solea senegalensis*): Comparative gene and protein expression during larval development through adulthood. Gene Expr. Patterns. 6, 637-652.

Gavaia P.J.and Cancela M.L. (2002). Osteological development and abnormalitiesof the vertebral column and caudal skeleton in larval and juvenile stages of hatchery reared senegal sole (*Solea senegalensis*). Aquaculture 211, 305-323.

Gavaia P.J., Domingues S., Engrola S., Drake P., Sarasquete C., Dinis M.T. and Cancela M.L. (2009). Comparing skeletal development of wild and hatchery-reared Senegalese sole (*Solea senegalensis*, Kaup 1985): evaluation in larval and postlarval stages. Aquacult. Res. 40, 585-1593.

Gisbert E., Ortiz-Delgado J.B. and Sarasquete C. (2008). Nutritional cellular biomarkers in early life stages of fish. Histol. Histopathol. 23, 1525-1539.

Gómez-Gutiérrez A.I., Jover E., Bodineau L., Albaigés J.M. and Bayona J.M. (2006). Organic contamination loads into the western mediterranean sea: estimate of Ebro river imputs. Chemosphere 65, 224-236.

Green H., Goldberg B., Schwart M. and Brown D. (1968). The synthesis of collagen during the development of *Xenopus laevis*. Dev. Biol. 18, 391-400.

Gulley D. and West Inc. (1996). TOXSTAT 3.5. University of Wyoming, Western Ecosystem Technology, Inc. Cheyenne, Wyoming, USA.

Hamilton M.A., Russo R.C. and Thruston R.V. (1977). Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11, 714-719.

Huculeci R., Dinu D., Staicu A.C., Munteau M.C. and Dinischiotu A. (2009). Malathion-induced alteration of the antioxidant defence system in kidney, gill and intestine of Carassius auratus. Environ. Toxicol. 24, 523-530.

Imsland A.K., Foss A., Conceiçao L.E.C., Dinis M.T., Delbare D., Schram E., Kamstra A., Rema P. and White P. (2003). A review of the culture potential of Solea solea and *Solea senegalensis*. Rew. in Fish Biol. Fisheries 13, 379-407.

Karyab H., Mahvi A.H., Nazmara S. and Bahojb A. (2013). Determination of water sources contamination to diazinon and malathion and spatial pollution patterns in Qazvin, Iran Bull. Environ. Contam. Toxicol. 90, 126-131.

Khan M.S. (2006). Protective effect of black tea extract on the levels of lipid peroxidation and antioxidant enxymes in liver of mice with pesticide induced liver injury. Cell. Biol. Funct. 24, 332-372.

Klaassen C.D., Amdur M.O. and Doull J. (1986). Casarett and Doull's Toxicology: The basic science of poisons. N.Y., Macmillan Publishing Co.

Köch M., Farre M., Martínez E., Gajda-Schrantz K., Ginebreda A., Navarro A., López de Alda M. and Barceló D. (2010). Integrated ecotoxicological and chemical approach for the assesment of pesticide pollution in the Ebro River Delta (Spain). J. Hydrol. 383, 73-82.

Köck-Schulmeyer M., Ginebreda A., González S., Cortina J.L., de Alda M.L. and Barceló D. (2012). Analysis of the occurrence and risk assessment of polar pesticides in the Llobregat River Basin (NE Spain). Chemosphere. 86, 8-16.

Lal B., Sarang M.K. and Kumar P. (2013). Malathion exposure induces the endocrine disruption and growth retardation in *Clarias batrachus*. Gen. Comp. Endocrinol. 181, 139-145.

Lien N.T.H., Adrien D. and Janssen C.R. (1997). Morphological abnormalities in African catfish (*Clarias gariepinus*) larvae exposed to malathion. Chemosphere 35, 1475-1486.

Macek K.J. and Sleight B.H. III. (1977). Utility of toxicity tests with embryos and fry of fish in evaluating hazards associated with the chronic toxicity of chemicals to fishes. In: Aquatic Toxicology and Hazard Evaluation. STP 634. Mayer F.L. and Hamlink J.L. (eds). American Society for Testing and Materials, Philadelphia, PA, pp 137-146.

McKim J.M. (1977). Evaluation of tests with early life stages of fish for predicting long-term toxicity. Can. J. Fish Aquat. Sci. 34, 1148-1154.

McKim J.M. (1985). Early Life Stage Toxicity Test. In: Fundamentals of Aquatic Toxicology-Methods and Applications. Rand G.M. and Petrocelli S.R. (eds). Hemisphere Publ. Corp. Washington DC. pp 58-95.

Naserabad S.S, Mirvaghefi A., Gerami M.H. and Farsani H.G. (2015). Acute toxicity and behavioral changes of the goldfish (*Carassius auratus*) exposed to malathion and Hinosan. Iranian J. Toxicol. 8,

1203-1208.

OECD (2013). Fish embryo acute toxicity test. OECD guidelines for testing of chemicals, OECD, Paris, France, pp 22.

Ortiz-Delgado J.B. and Sarasquete C. (2004). Toxicity, histopathological alterations and immunohistochemical CYP1A induction in the early life stages of the seabream, *Sparus aurata*, following waterborne exposure to B(a)P and TCDD. J. Mol. Histol. 35, 29-45.

Ortiz-Delgado J.B., Ruane N.M., Pousao-Ferreira P., Dinis M.T. and Sarasquete C. (2006). Tyroid gland development in Senegal sole (*Solea senegalensis* Kaup 1858) during early life stages: a histochemical and immunohistochemical approach. Aquaculture 260, 346-356.

Ortiz-Delgado J.B., Fernández I., Sarasquete C. and Gisbert E. (2014). Normal and histopathological organization of the opercular bone and vertebrae in gilthead sea bream *Sparus aurata*. Aquat. Biol. 21, 67-84.

Padrós F., Villalta M., Gisbert E. and Estévez A. (2011). Morphological and histological study of larval development of the Senegal sole *Solea senegalensis*: an integrative study. J. Fish Biol. 79, 3-32.

Patnaik L. (2010). Biochemical alterations induced by sevin in *Clarias batrachus*. Asian J. Exp. Biol. Sci. 1, 124-127.

Power D.M., Einarsdóttir I.E., Pittman K., Sweeney G.E., Hildahl J., Campinho M., Silva N., Saele O., Galay-Burgos M., Sráradottir H. and Bjorsnsson B.T. (2008). The molecular and endocrine basis of flatfish metamorphosis. Rev. Fish Sci. 16, 95-111.

Ribeiro L., Zambonino-Infante J.L., Cahu C. and Dinis M.T. (1999). Development of the digestive enzymes in larvae of *Solea senegalensis*, Kaup 1858. Aquaculture 179, 465-473.

Sanchez-Nogué B., Varó I. and Solé M. (2013). Comparative analysis of selected biomarkers and pesticide sensitivity in juveniles of Solea solea and *Solea senegalensis*. Environ. Sci. Pollut. Res. 20, 3480-3488.

Sankararamakrishnan N., Kumar Sharma A. and Sanghi R. (2005). Organochlorine and organophosphorous pesticide residues in ground water and surface waters of Kanpur, Uttar Pradesh, India Environ. Int. 31, 113-120.

Santamaría J.A., Andrades J.A., Herráez P., Fernández-Llebrez P. and Becerra J. (1994). Perinotochordal connective sheet of gilthead seabream larvae (*Sparus aurata*, L.) affected by axial malformations: an histochemical and immunocytochemical study. Anat. Rec. 240, 248-254.

Sarasquete C. and Segner H. (2000). Cytochrome P4501A (CYP1A) in teleost fishes. A review of immunohistochemical studies. Sci. Total Environ. 247, 313-332.

Sarasquete C., González de Canales M.L., Arellano J., Muñoz-Cueto J.A., Ribeiro L. and Dinis M.T. (1996). Histochemical aspects of the yolk sac and digestive tract of larvae of the Senegal sole, *Solea senegalensis* (Kaup, 1858). Histol. Histopathol. 11, 881-888.

Sarasquete C., González de Canales M.L., Arellano J., Muñoz-Cueto J.A., Ribeiro L. and Dinis M.T. (1998). Histochemical study of skin and gills of Senegal sole, *Solea senegalensis* lavae and adults. Histol. Histopathol. 13, 727-735.

Sarasquete C., Gisbert E., Ribeiro L., Vieira L. and Dinis M.T. (2001). Glycoconjugates in epidermal branchial and digestive mucous cells and gastric glands of gilthead seabream, *Sparus aurata*, Senegal sole, *Solea senegalensis* and Siberian sturgeon, Acipenser baeri development. Eur. J. Histochem. 45, 267-278.

Scott G.R. and Sloman K.A. (2004). The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquat, Toxicol. 68, 369-392.

Snawder J.E. and Chambers J.E. (1990). Crictical time period and the effect of tryptophan in malathion-induced developmentals deffects in Xenopus embryos. Life Sci., 46, 1635-1642.

Snawder J.E. and Chambers J. (1993). Osteolathyrogenic effects of malathion in Xenopus embryos. Toxicol. Appl. Pharmacol. 121, 210-216.

Solé M., Manzanera M., Bartolomé A., Tort L. and Caixach J. (2012). Persistent organic pollutants (POPs) in sediments from fishing grounds in the NW Mediterranean: ecotoxicological implications for the benthic fish Solea sp. Mar. Pollut. Bull. 15, 158-165.

Thomas P.C. and Murthy T.L. (1976). Acid phosphatase activity in a fresh-water air breathing fish Heteropneustes fossilis& the effect of certain organic pesticides on it.Indian J. Biochem. Biophys.13, 347-349.

U.S. EPA (2006). Technical overview of ecological risk assessment analysis phase: ecological effects characterization, U.S. Environmental Protection Agency, Washington, D.C.

Varó I., Navarro J.C., Amat F. and Guillermino L. (2003). Effect of dichlorvos on cholinesterase activity of the european seabass (*Dicentrarchus labrax*). Pestic. Biochem. Phys. 75, 61-72.

Videira R.A., Peça L.P.S., Antunes-Madeira M.C. and Madeira V.M.C. (1994). Effects of malathion on membrane fluidity and its implications for the mechanisms of toxicity Med. Sci. Res. 22, 551-553.

Weis J.S. (2014). Delayed behavioral effects of early life toxicant exposures in aquatic biota. Toxics 2, 165-187.

Woltering D. (1984). The growth response in fish chronic and early lifestage toxicity tests: a critical review. Aquat. Toxicol. 5, 1-21.

Zambonino J., Gisbert E., Sarasquete C., Navarro I., Gutiérrez J. and Cahu C.L. (2008). Ontogeny and physiology of the digestive system of marine fish larvae. In: Feeding and digestive functions of fish. Science Publishers, Inc, Enfield (NH), Jersey, Plymouth. pp 281-348.

Accepted April 28, 2017