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ACUTE TOXICITY OF A COMMERCIAL GLYPHOSATE FORMULATION ON EUROPEAN SEA BASS JUVENILES (*Dicentrarchus labrax L.*): GENE EXPRESSIONS OF HEME OXYGENASE-1 (*ho-1*), ACETYLCHOLINESTERASE (*AChE*) AND AROMATASES (*cyp19a and cyp19b*)

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Abstract

Acute toxicity of Roundup, a commercial glyphosate-based herbicide, was evaluated in a teleost marine fish, the European sea bass, after 96h of exposure. The LC_{50} 96-h value of Roundup was 529 mg/L. Juveniles (*Dicentrarchus labrax* L.) were exposed to a sublethal concentration (35% of the LC_{50}, i.e. 193 mg/L) of Roundup for 96-h. The study of *heme oxygenase-1* (*ho-1*) gene expression was performed in four tissues (liver, gills, brain and gonads) and highlighted the disruption of antioxidant defence system. Results showed that *ho-1* mRNA levels in liver and gills significantly decreased ($p<0.001$ and $p<0.01$ respectively) in fish exposed to 193 mg/L of Roundup, whereas in brain and gonads, *ho-1* mRNA level was not altered. The analysis of *acetylcholinesterase* expression was used to evaluate the overall neurotoxicity of the herbicide and *aromatase* genes to assess the alteration of the endocrine system. Results showed that *AChE* and *cyp19b* gene transcriptions significantly increased ($p<0.01$) in brain of sea bass, whereas aromatase gene expression (*cyp19a*) in gonads was not significantly altered. Our results showed complex tissue-specific transcriptional responses after 96h of exposure to a sublethal concentration. All these disruptions confirmed the deleterious effects of this glyphosate-based herbicide in a marine species.

Key words: Roundup toxicity, European sea bass, LC_{50} 96-h, gene expressions, heme oxygenase-1, aromatases, acetylcholinesterase.

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INTRODUCTION

Roundup, a broad-spectrum, non-selective herbicide is the most extensively used herbicide worldwide. Roundup formulations are composed of their active ingredient, glyphosate, and various adjuvants such as polyoxyethyleneamine (POEA) which enables glyphosate to penetrate the plant cuticle (11). Glyphosate inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme which acts on in the biosynthesis of aromatic amino acids and of shikimic acid in plants. Glyphosate is stable to photodegradation at pH 5 to 9 and its hydrolysis half-life is greater than 35 days, then it is degraded by microflora into its major metabolite, aminomethylphosphonic acid (AMPA) which has an equal or lower toxicity than glyphosate. Even if glyphosate is moderatly toxic to aquatic animals, Roundup is considered more toxic due to the addition of its surfactant (33, 84, 85). After run-off events, typical values of total pesticide concentrations found in European coastal water ranged between 1 to 3.2 µg/L (79, 98). Noteworthy, all components of Roundup or its metabolite (AMPA) were found in surface waters in France (27, 50). This environmental occurrence (low but pertinent doses) is due to its frequent use to manage crops, forest and railways tracks over extended periods. For all these reasons, this herbicide may present in the aquatic environment and thus organisms are in contact for a longer time with it. Recently, Hanana et al. (2012) (42) showed the bioaccumulation of Roundup formulation in clam (*Ruditapes decussatus*) owing to an effective method, 31P HRMAS NMP. Glyphosate also is biomagnified in the trophic chain (34). Glyphosate-based herbicides were reported to be responsible for histological alterations (64), oxidative stress in fish tissues (73), metabolic disruption such as acetylcholinesterase (AChE) inhibition (105) or decrease in testosterone levels on rat testicular cells (20). Whatever the studied organism, mammals or fish, this herbicide acts as endocrine disruptor on many target-endocrine molecules such as vitellogenin (68, 126), StAR (104, 123), steroid receptors of cells (32) and aromatases (103).

In response to oxidative stress, animals up-regulate their antioxidant system based on stimulation of low molecular weight thiol (as tripeptide glutathione) synthesis and/or reactive oxygen species- inactivating enzymes (e.g. superoxide dismutase (SOD), catalase, glutathione peroxidase) and phase 2 detoxifying enzymes (e.g. NAD(P)H quinone oxidoreductase, Glutathione S-transferase (GST), Heme oxygenase-1) (43). Heme oxygenase-1 (HO-1) is a microsomal enzyme that catalyzes intracellular heme to biliverdin, free iron and carbon monoxide. To date, three isozymes of HO have been identified: HO-1 (75), HO-2 (77) and HO-3 (81). These isoforms are the products of different genes. Whereas HO-2 and HO-3 are constitutive isozymes, HO-1 is a 32 kDa stress protein induced by numerous stimuli associated with the production of reactive oxygen species (ROS). *ho-1* gene expression is regulated by its own substrate, heme that is a pro-oxidant product, but also by chemical stress such as heavy metals (5, 12), PCB (65), pesticides (49, 97), lipopolysaccharide (125), abiotic stresses such as temperature (2, 40), hypoxia (38), hyperoxia (66), UV-light (60), oxidative stresses (55), and by physiological stress such as inflammation (93). Recent studies have shown that the role of this enzyme in the cellular defence mechanism is crucial. Moreover, HO-1 is ubiquitously distributed in vertebrate tissues. For all these reasons, *ho-1* gene expression is commonly used as a physiopathological biomarker in order to predict several human disease states (51): for instance, neurodegenerative

injury such as Alzheimer disease (107), traumatic brain injury (7), kidney ischemia-reperfusion (100), immune dysfunction (122), inflammatory diseases (93), organ transplantation (23, 45). Since *ho-1* gene expression is considered as a sensitive biomarker in many human pathological conditions, we speculated that HO-1 mRNA level may be used as a broad spectrum biomarker in other species such as marine teleosts.

In the brain, acetylcholinesterase is often a target enzyme of pesticides. This enzyme modulates acetylcholine (ACh) levels, and plays a crucial role in the regulation of the cholinergic neurotransmission. AChE activity is a specific biomarker in fish exposed to pesticides to assess their neurotoxicity in aquatic species. Organophosphate (parathion, chlorpyrifos) and carbamate pesticides are known to inhibit AChE activity (95, 127) but also to alter its gene expression (112). Recent studies highlighted the neurotoxic effect of glyphosate-based herbicide in freshwater fish through the inhibition of AChE activity (15, 105).

Pesticides can also disrupt the endocrine system. The hypothalamic-pituitary-gonadal (HHG) axis is regulated by different complex signalling pathways controlled by hormones and enzymes. Among the latter, the cytochrome P450 aromatase (P450arom), a key steroidogenic enzyme, catalyzes the irreversible and rate-limiting conversion of androgens into estrogens. In fish, two distinct *aromatase* genes (*cyp19*) encode two structurally and functionally different isoforms, commonly termed CYP19A1 (or CYP19A) and CYP19A2 (or CYP19B) (18). These enzymes have similar affinities to their natural ligand such as testosterone or androstenedione. These two forms have been reported in several freshwater fish and in the European sea bass, a marine teleost fish (8, 22). Aromatase expression in fish has been identified preferentially in gonads and brain, but also at lower levels in liver and other peripheral tissues (kidney, adipose tissue, intestine) (96). In teleost fish, CYP19A is predominantly expressed in ovaries and is involved in sex differenciation, in oocyte growth and in the reproductive cycle. However, this isoform is also found in testis and brain and to a lesser level in other tissues (37). Generally, *cyp19a* gene expression is higher in females than in male fish (testis). In ovaries, aromatase expression progressively increases (in zebrafish, its expression is higher during stage III oocytes) under the action of estrogens (mainly estradiol) but also androgens which enhance FSH-stimulated aromatase expression in rat granulosa cells (114). Conversely, CYP19B is mainly expressed in neural tissues. In teleost fish, aromatase is involved in the development and the activity of estrogen target tissues such as brain (24, 25) but also in reproductive tactics (108). In European sea bass, the level of *cyp19b* mRNA begins to increase at early development (50 dph) and continues during growth, because aromatase is involved in neurogenesis (8). Whatever fish species, high levels of aromatase activity are reported in the forebrain regions and the hypothalamic-pituitary complex (10, 83) and its level is 100- to 1000- fold higher than in adult mammal brain (13). In teleosts, *cyp19b* gene is upregulated by estrogen but also by high levels of circulating androgens (87). *Cyp19a* and *cyp19b* genes are characterized by distinct and tissue-specific promoters. In teleosts, promoters are composed by several cis-regulatory elements of which some are highly conserved. They include an estrogen-responsive element (ERE), half-EREs (120), a potential androgen-responsive element (ARE) (14), aryl hydrocarbon responsive elements (AhRE), dioxine-responsive elements (DRE) or cAMP responsive elements (CRE) (19). All these features led to presume a possible interference of endocrine disruptors on *cyp19* genes expression.

Since glyphosate is expected to chronically contaminate coastal waters, and considering its physiological effects on organisms, we assumed that Roundup could impact the widely distributed European sea bass (*Dicentrarchus labrax* L.) in the Mediterranean Sea. At the juvenile stage, this species is found in coastal areas, hence it is exposed to chronic pesticide contaminations since pollutants are transported to marine environment by surface run-off. The main objectives of this work were to determine, for the first time, the acute toxicity (LC_{50} 96-h) of Roundup on marine fish and to evaluate the expression of four stress-responsive genes in fish. Acute non-environmental concentration was deliberately chosen to observe and quantify putative effects of the glyphosate-based herbicide on the expression of interest genes. The analysis of *ho-1* mRNA levels in four tissues (liver, gills, gonads and brain) was used to show the effect of the potential oxidative stress induced by a short-term exposure to herbicide whereas *AChE* expression was used to evaluate the overall neurotoxicity of the herbicide, and *aromatase* genes to assess the alteration of the endocrine system.

MATERIALS AND METHODS

Fish and rearing conditions

One hundred seventy 290 dph European sea bass (*Dicentrarchus labrax L.*) juveniles (23 ±1.13 g BW and 12.8 ±0.2 cm), purchased from a local fish farm "Tamaris Sud Fish" (Seyne-sur-Mer, France), were maintained in aerated 75 liters glass aquariums (fish-loading density: 1.5 g/L; dissolved oxygen: 7 ±0.1 mg/L) at 15°C, containing 31.7 ±0.2 g/L salinity sea water with a pH of 6.9 ±0.1 and nitrite concentration < 0.3 mg/L. Fish have been acclimated for 3 weeks during which they were fed once a day with commercial diet pellets (Le Gouessant) containing 50% crude protein.

Chemical analysis of seawater

Commercially available Roundup (360 g glyphosate/L, Mosanto) was purchased from a local store. For each experiment, stock solutions were prepared in seawater and added in aquarium at day 0 to give the initial theoretical concentration. At zero and 96 hours, 250 ml of seawater samples were collected from each aquarium (with the presence of fish) and stored at 4°C in specific bottles until chemical analysis, in order to correlate theoretical and actual concentrations. Two components of the Roundup formulation were analyzed by IDHESA laboratory (Brest, France): glyphosate and its degradation product, the aminomethyl phosphonic acid (AMPA). Glyphosate and AMPA were detected using liquid chromatography with fluorescence detection after liquid–liquid extraction and derivatization with 9-fluorenylmethylchloroformate (FMOC-Cl). The initial glyphosate and AMPA concentrations were < 0.05µg/L in seawater.

Acute toxicity test

After acclimation period, groups of 6 juveniles were subjected to static acute toxicity tests with different pre-

nominal Roundup concentrations (3, 30, 60, 300 and 800 mg/L) during 96-h. All concentrations are related to mg of glyphosate present in glyphosate formulation (Roundup). The Roundup concentrations used for the definitive LC_{50} 96-h-test were: 120, 350, 400, 500, 600, 680 and 760 mg/L. Fish in the control group were treated in the same conditions, but Roundup was omitted. Seawater was not changed during the course of the experiment and fish did not receive food during the experimental period. Mortality was recorded at 1, 2, 6, 24, 48, 72 and 96-h after the start of the experiment. Dead individuals were counted and removed immediately. After 96-h exposure all fish were anaesthetized with 2-phenoxyethanol (3% - v/v) and quickly killed by cervical section. Four tissues (liver, gills, brain and gonads) were immediately removed, submerged in RNA*later*® (Ambion, Applied Biosystems, France) and stored at -80°C until further analysis.

Sublethal Roundup exposure

A short-term (96-h) static toxicity test was performed to evaluate the (1) oxidative stress, (2) neurotoxicity and (3) potential endocrine disrupting effects of 193 mg of glyphosate /L of Roundup on 10 juveniles. This concentration corresponds to 35% of the previously calculated LC_{50} 96-h to *D. labrax*. After 96-h exposure, all control and exposed fish were sampled. Livers, gills, brains and gonads were immediately removed, submerged in RNA*later*® and stored at -80°C until further analysis.

RNA isolation

Samples of liver, gills, brain and gonads were collected from juveniles sedated by 2 phenoxyethanol (3% -v/v) and euthanized by cervical dislocation. Tissues were immediately submerged in RNA*later*® (Ambion, Applied Biosystems, France) and stored at -80°C until use. Tissues (50-100 mg) were crushed in 1 ml of EXTRACT-ALL (Eurobio, France) in a lysing matrix D® (MP Biomedicals), and vortexed for 40s (x2) with FastPrep-24®. After incubation at RT for 3 minutes, samples were centrifuged at 12000 g for 15 min. at 4°C. Following centrifugation, aqueous phase was transferred to a new tube and 500 µl of isopropanol was added and incubated for 10 min. at room temperature. After centrifugation (12000 g, 10 min., 4°C), supernatant was discarded and the RNA pellet was washed in 70% ethanol. Following centrifugation (7500 g, 5 min., 4°C), supernatant was removed and RNA pellet was air dried for 10 min. The RNA pellet was redissolved in 50 µl of RNase-free water. Concentration and quality of RNA samples were assessed by spectrophotometry and by 2% (w/v) agarose gel electrophoresis. A digestion step with 2U/µl DNase I, RNase-free (Ambion) at 37°C for 30 min., was performed to prevent any potential DNA contaminations.

RT -PCR

The cDNA were synthesized at 37°C for 60 min. then 14°C for 15 min. by incubating 1 µg of total RNA in a 20 µl reaction volume: 1X RT buffer (Qiagen), 1µl of 4 U/µl Omniscript RT (Qiagen), 5 mM of dNTPs (Qiagen), 1µl of 10 U/µl RNase Inhibitor (Eurobio) and 100 µM of Random primers. All PCRs were performed using 1 µl of the first-strand mixture in a 50 µl total volume combining 10X Hot Master TaQ buffer (including 25 mM of $MgCl_2$), 5 mM of dNTPs, 0.2 µl of 5 U/µl of Hot Master TaQ polymerase (5 Prime®) and specific primers (10 µM) (see Table 1). The amplification procedure for *ho-1* was performed as previously described (99). The amplified fragment is 201 bp in length. The amplification procedures for *cyp19a* and *cyp19b* PCRs were similar: 2 min. at 94°C followed by 35 cycles at 94°C for 45 s, 58°C for 45 s, 72°C for 2 min. and the extension phase of the last cycle was prolonged by 5 min. The sizes of *cyp19a* and *cyp19b* PCR products are 493 bp and 230 bp respectively. *AChE* cDNA amplification (400 bp) was obtained as follows: 2 min. at 94°C followed by 35 cycles at 94°C for 45 s, 53°C for 45 s, 72°C for 2 min. and a 5 min. extension phase.

Tissue-specific expression of heme oxygenase-1, aromatase and acetylcholinesterase genes

Semi-quantitative PCR were used to measure *HO-1* mRNA expression in the four studied tissues (liver, gills, brain and gonads), *cyp19a* and *cyp19b* mRNA expressions in gonads and brain respectively, and *AChE* mRNA expression in brain. Primers (Table 1) used for European sea bass HO-1 were designed in a previous study (99): sense primer HO1Dir201 and antisense primer HO1Rev201 corresponding to the amplification between positions +494 and +694 of the HO-1 mRNA sequence (GeneBank accession no **EF139130**). Primers used for sea bass brain aromatase were as in Blásquez and Piferrer (2004)(8): sense primer, Dir-P450Brain and antisense primer, Rev-P450Brain. Sea bass gonad aromatase primers were as in Dalla Valle et al. (2002)(22): sense primer, Dir-P450Gonad and antisense primer, Rev-P450Gonad. Finally to design brain *AChE* primers we used the consensus sequence obtained from alignment of three fish mRNA GenBank sequences: *Danio rerio* (GeneBank accession no **NM131846**), *Cyprinus carpio* (GeneBank accession no **AB361595**) and *Torpedo californica* (GeneBank accession no **X03439**): Dir-AChE, sense primer and Rev-AChE, antisense primer.

Relative gene expression was evaluated using either the housekeeping gene *RPL17*, that displayed the most stable expression in most tissues and condition or the *beta-actin* gene. The use of this gene was recommended as a reference gene by Zheng et al. (2011)(129). Sea bass *RPL17* primers were designed from the cDNA sequence available in the GenBank accession no **AF139590** with Primer3Plus (GNU General Public License software). Semi-quantitative PCRs were performed with both specific primers and housekeeping primers on the same cDNA sample. After a migration on 2% agarose/TAE gel containing ethidium bromide, the two PCR product lengths were different and they were analyzed with ImageJ software (Public Domain License). *ho-1*, *cyp19a* and *cyp19b* mRNA levels were standardized against the *RPL17* mRNA level, whereas *beta-actin* was used as housekeeping gene to quantify *AChE* mRNA expression. Beta-actin was deliberatly chosen in order to obtain two amplicon sizes significantly different. Duplicates were averaged and results were shown as mean value ± S.E.M (Standard Error of the Mean).

Statistical analysis

Before statistical analysis (using the Mann-Withney test), all data were tested for normality via a Shapiro-Wilk test with GNU-R (GNU General Public License software). Difference (between tested fish and control group) was considered significant when $p<0.05$.

Table 1. Primer sequences used to tissue-specific expressions of *heme oxygenase-1*, *aromatases* and *acetylcholinesterase* genes in *Dicentrarchus labrax* L.

Primer name	Primer sequence (5' → 3')	Amplicon size (bp)
HO1Dir201	CCC TGA ATT TCT AGT TGC CCA TGC	201
HO1Rev201	TCC GTC AGC TCC ACG CTG TTC ATC	
Dir-P450Brain	GTC GTT TCT TCC AGC CCT TC	230
Rev-P450Brain	TGA AGG TTG TGC TGT TCG AG	
Dir-P450Gonad	GGC AGA CTG TGC TGA TCA AA	493
Rev-P450Gonad	TTC GGT ACC CTG TAG CCA TC	
Dir-RPL17	GGT GGT TCA TCT GGA GCC AGG TTC C	489
Rev-RPL17	GCG TTA GAG GCT ATC CGG GGC C	
Dir-AChE	TGG CCT GAG TGG ATG GG	400
Rev-AChE	TCT TCC AGT GCA TCA TGT AGG	
Dir-bactin	CAG AGC AAG AGA GAG GTA TCC TGACC	895
Rev-bactin	ATC CAC ATC TGC TGG AAG GTC GAC	

RESULTS

Chemical analysis of seawater

Chemical analysis of the LC_{50} 96-h concentration is 529 mg/L for Roundup in seawater. AMPA concentrations was <125 mg/L. Similarly, the nominal sublethal concentration has been measured at 193 mg/L of glyphosate and inferior to 125 mg/L of AMPA.

Acute toxicity of Roundup: behavioral effects and LC_{50} value

During the first hour after exposure, fish showed various symptoms: rapid and erratic swimming, respiratory difficulties displayed by an increased operculum ventilation and a relocalization of the fish to the surface to "gulp air" (Fig. 1).

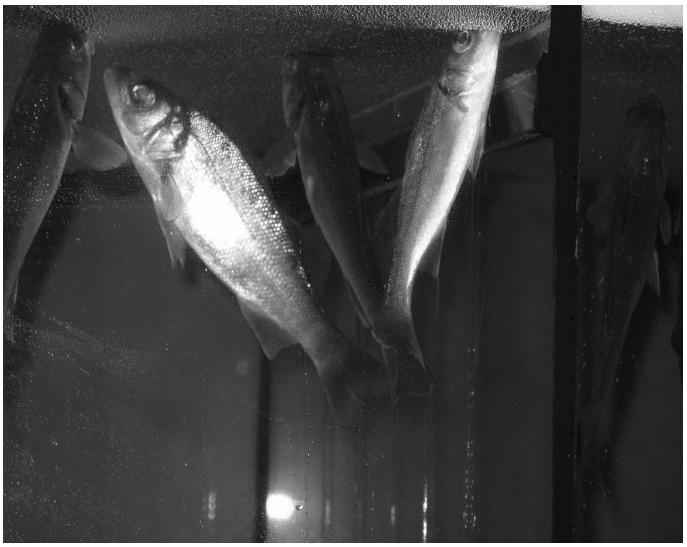

Figure 1. European sea bass juveniles exposed to Roundup (193 mg/L). Erratic swimming and respiratory distress displayed by a moving (vertical position) to the surface to gulp air.

Roundup effects on the European sea bass (n = 170) behavior are similar whatever the concentration (3 mg/L to 760 mg/L), but their intensity was dose-dependent. The erratic movements were associated with a lethargy period where fish stay at the bottom of the aquarium. One hour after exposure and only from the 278 mg/L Roundup concentration, some fish collapsed and died. Fish showed also a darkly pigmentation in lateral parts of their body.

The behavior of the 193 mg/L Roundup-exposed fish, during the sublethal toxicity experiment, was similar to signs described above (erratic swimming and respiratory distress) but a stable and normal behavior reappeared after one hour and none of the fish died.

The median lethal concentration LC_{50} 96-h of European sea bass juveniles to Roundup was estimated through the Trimmed Spearman-Karber method, based on the probit analysis (41). The LC_{50} 96-h was 529 mg/L and 95% lower and upper confidence limits were 424 and 595 mg/L respectively for Roundup (figure of probit analysis not shown).

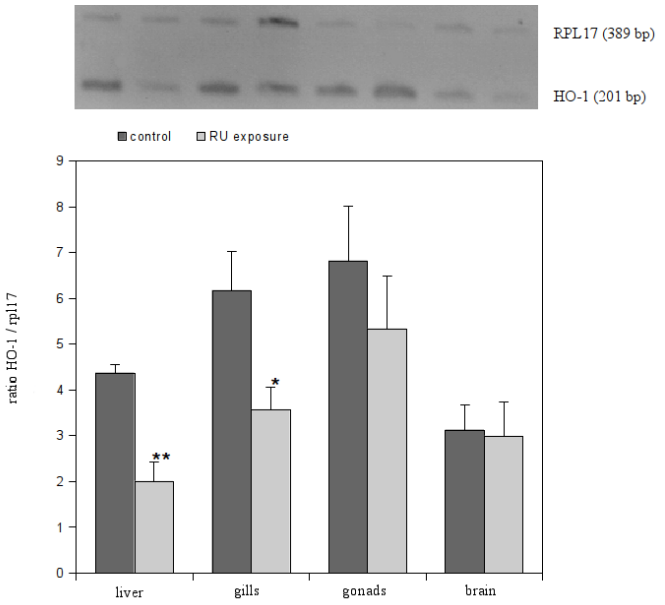

Figure 2. *HO-1* mRNA levels in liver, gills, gonads and brain of juveniles (*D. labrax*) after 96 h-exposure to RU (193 mg/L). Significant difference, against appropriate control, is labelled with asterisks (* $p<0.01$, ** $p<0.001$).

Roundup effects on mRNA levels of ho-1, cyp19a, cyp19b and AChE

Effects of Roundup (193 mg of glyphosate/L of Roundup) on *ho-1* mRNA expression in four tissues (liver, gills, gonads and brain) of European sea bass (n =10) were measured at 96 h (Fig. 2). Compared to the control group (n=12), a significant 1.7-fold decrease in HO-1 mRNA levels was observed in liver ($p<0.001$) and gills ($p<0.01$) whereas no significant effect on *ho-1* gene expression was detected in the brain and gonads.

Several classes of endocrine disrupting chemicals are known to interfere with *cyp19* gene expression in most teleosts. Through this study, we expected to observe the

endocrine disruption effect of Roundup at a sublethal concentration (193 mg/L) by measuring its impact on the gene expression of the aromatase genes, *cyp19a* and *cyp19b*. The short-term exposure (96 h) to Roundup did not significantly (p=0.104) alter *cyp19a* gene expression in gonads of the juveniles but an increase (p=0.01) was noted for the *cyp19b* gene expression in brain (Fig. 3).

After a 96 h-exposure to Roundup, we determined in brain of juvenile fish a significant 1.9-fold increase (p<0.01) in *AChE* mRNA levels compared with the control group (Fig. 4).

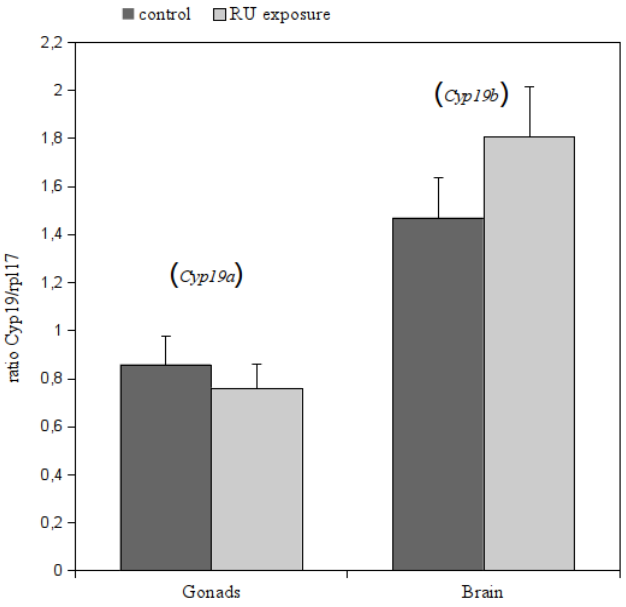

Figure 3. *Cyp19a* and *cyp19b* mRNA levels in gonads and brain of juveniles (*D. labrax*) after 96 h-exposure to RU (193 mg/L).

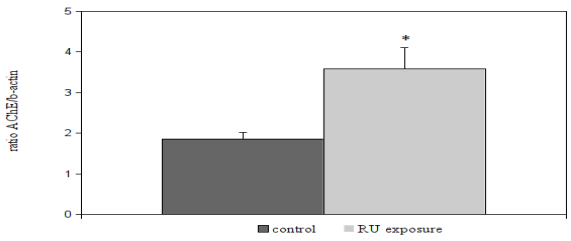

Figure 4. *AChE* expression in brain of juveniles (*D. labrax*) after 96h-exposure to RU (193 mg/L). Significant difference, against appropriate control, is labelled with asterisk: * p<0.01.

DISCUSSION

LC_{50} 96-h of Roundup

In the present study, the LC_{50} 96-h of Roundup in sea bass juveniles was estimated to 529 mg/L. The LC_{50} 96-h of the formulated product Roundup varies from 7.3 to 94.5 mg/L (Table 2) depending on the fish species, the life stage but also on abiotic parameters such as salt, temperature and pH (21). On the basis of the aquatic toxicology classification (34), if the value of the LC_{50} 96-h concentration is lower than 1 mg/L, the chemical compound is considered be very toxic, whereas for concentrations between 1-10 mg/L, the substance is moderately toxic. To date LC_{50} values of Roundup in seawater fish are very scarce. Our LC_{50} 96-h value is higher than the value range obtained in other freshwater fish species and can be classified as slightly toxic in juvenile European sea bass**.**

Table 2. LC_{50} 96-h values of the formulated product Roundup in fish.

Species	LC_{50} values (mg RU/L)	References
Piaractus brachypomus	94.47	(101)
Salmo gairdneri	54.8	(109)
Oncorhynchus kisutch	42	(109)
Goodea Atripinnis	38.95[(b)]	(92)
Jenynsia multidentata	19.02	(46)
Gambusia yucatana	17.8	(102)
Oreochromis niloticus	16.8-36.8[(a)]	(53)
Prochilodus lineatus	13.69[(a)]	(64)
Crassostrea gigas	10	(118)
Rhamdia quelen	7.3[(a)]	(62)

(a) Roundup: 360 g of glyphosate/L

(b) Yerbimat, commercial herbicide similar to RU 360 g/L

Behavioral effects

Effects of Roundup on the European sea bass behavior are similar to those reported in other Roundup-exposed fish such as silver catfish (*Rhamdia quelen*) and Nile tilapia (*Oreochromis niloticus*) (26, 62). The symptoms observed in fish in the present study, such as erratic movements and respiratory distress, are typical from exposures to carbamates and organophosphates. The main biological effect of these last insecticides, stems from their ability to inhibit the AChE enzyme reversibly (carbamates) or irreversibly (organophosphates), with resultant excessive accumulation of acetylcholine in synaptic clefts. This accumulation leads to a disturbance of the nervous system associated to the uncoordinated movements of fish (48). *AChE* activity was reduced by 25 to 27% in brain of silver catfish, depending on the Roundup concentration (ranging from 0.2 to 0.4 mg/L) (35). The same effect has also been observed in piava (*Leporinus obtusidens*) and neotropical fish (*Prochilodus lineatus*) (85, 105). Modesto and Martinez (2010) reported for the first time an equivalent inhibition of *AChE* in muscle and concluded that Roundup acts as an acetylcholinesterase inhibitor in both brain and muscles (85). Consequently, this property could affect, in a long term, the survival of fish population due to a disturbance of the reproductive behavior (95). In our experiment, juveniles exposed to 193 mg/L Roundup recovered a normal behavior within 2 hours, with no immediate mortality. Hence Roundup might act as a potential short/medium-term reversible AChE inhibitor.

Effects of sublethal concentration of Roundup on mRNA levels of ho-1, cyp19a, cyp19b and AChE

In this work, the Roundup concentration tested corresponds to 35% of the LC_{50} 96-h, i.e. 193 mg/L. This concentration was chosen because, at this value, no mortality (in relation to control) of fish was observed. This acute non-environmental concentration and the short period exposure (96-h) were deliberately chosen to observe and evaluate the effects of the formulated product Roundup on the expression of four genes in four different tissues because no data on marine fish had been published.

Tissue-specific expression of HO-1 after acute Roundup-exposure

This study evidences the inhibitory effect of the glypho-

sate-based formulation of Roundup on transcription in the liver, gills, gonads and brain of juvenile sea bass.

The liver is the major detoxification organ and is very sensitive to anthropic pollutants. Sometimes the biotransformation of xenobiotics produces reactive oxygen species and secondary metabolites potentially deleterious for the organism. In order to neutralise oxidative stress, the expression of genes encoding many antioxidant and phase II enzymes is stimulated. Roundup exposure induces mild oxidative stress (73), DNA adducts and/or other oxidative DNA damages (39, 94). Noteworthy, Monroy et al. (2005) (86) showed the genotoxic activity of glyphosate amplified by AMPA and POEA (16). In our present study, we observed a significant decrease of *HO-1* mRNA ($p < 0.001$) in the liver. This down-regulation could be explained by a transcriptional disruption and a histological alteration of the liver. Some studies on the effects of oxidative stress reported that the expression of *heme oxygenase-1* is stimulated by the redox-sensitive transcription factor Nrf2 (Nuclear factor erythroid 2-related factor-2) (56) and its specific repressor, Kelch-like ECH-associated protein-1 (Keap1) (74). This cytosolic complex plays a key role in the response of oxidative stress. In teleost fish, under oxidative stress, the thiol dimer, Keap1a and Keap1b is oxidized (70). Free Nrf2 can migrate into the nucleus and binds to cis-element of *HO-1* promoter after heterodimerizing with other nuclear transcription factors such as CREB, cJun, ATF4, fos or Maf (4). This heterodimer complex initiates Nrf2-target gene transcription through antioxidant/electrophile response elements (ARE/EpRE) (52, 128). Generally, promoters of antioxidant and phase II enzyme genes contain a multiple functional AREs. Alam et al. (1999) (3) have shown that one of the partners of Nrf2 which is involved in the initiation of the *HO-1* gene transcription is Maf. In zebrafish the Nrf2-Keap1-Maf pathway leads to the up-regulation of *HO-1* gene and other antioxidant and phase II enzymes (76, 106). Under basal conditions, Nrf2 binds to homodimers of Keap1 that leads to ubiquitination of Nrf2 and its proteasomal degradation.

Stimuli and their regulation pathways that inhibit the *HO-1* gene expression are less known. Nevertheless, this down-regulation can be induced by the hetero-oligomer Bach1/Maf, in which the transcription factor Bach1 is known to be a repressor of the *maf* recognition element (90), by hypoxia and thermal stress (88, 91) or under treatment with interferon-γ in the nervous system (116).

The repression of *ho-1* gene expression can also be due to a feedback regulation mechanism mediated by a lower intracellular heme level (111). Indeed, in presence of high heme levels, the transcription factor Bach-1 is inactivated upon binding to heme, hence allowing the Nrf2-Maf pathway to be initiated (90). In contrast, under low levels of heme, the heterodimer Bach1-Maf translocates into nucleus and inhibits transcription through the *cis*-element MARE of HO-1 promoter (110). Another explanation for this significant down-regulation of HO-1 could be due to physiological and histological liver alterations in fish exposed to Roundup for 96-h. Dissected livers of contaminated fish presented a "liquefied" appearance compared to control (hydropic degeneration), so that the normal functioning of the organ could be impaired.

As a consequence, its antioxidant defense function may be disturbed and lead to a significant decrease of *HO-1* mRNA level. These histological changes have been already observed after Roundup exposures, leading to an alteration of the structural pattern of hepatocytes in carps (*Cyprinus carpio*) (89, 115) or in *Oreochromis niloticus* (53). These alterations result in various lesions such as myelin-like structures, mitochondria swelling and disappearance of mitochondria internal membrane, infiltration of leukocytes, focal necrosis, hydropic degeneration in liver.

Hued et al. (2012) (46) also highlighted that Roundup altered in a concentration-dependent manner the liver and gills histology of the neotropical fish *Jenynsia multidentata.* In our study, gills showed macroscopic alterations such as loss of usual red color and branchial structures (erosion of filaments). The fish gills, which have respiratory, excretory and osmoregulory fuctions, are considered to be the first target of waterborne contaminants. In accordance with recent studies in fish, the histological alterations in gills are less critical than those observed in liver such as epithelial hyperplasia, subepithelial edema, leukocyte infiltration or hypertrophied cells (92). Besides the histological damages induced by Roundup, the *HO-1* mRNA levels are also significantly reduced by 58 % in gills ($p<0.01$) as shown in liver. It is likely that this decrease is mainly due to histopathological alterations, partially induced by oxidative stress triggered by Roundup.

Whereas morphological changes were observed in liver and gills after a short-term Roundup exposure, brain and gonads seem not be affected by the herbicide. In normal brain of teleosts, among the two HO isoforms (124), HO-2 accounts for the main HO form whereas HO-1 is weakly expressed in neurons (1, 99). In the same manner, HO-1 is poorly induced in gonads. Few studies have shown the neuroprotective role of HO-1 in the brain (6, 7) and its up-regulation in response to oxidative stress (47). In our study, the *HO-1* mRNA expression level ($p>0.05$) was not affected in brain and gonads. It would seem that oxidative stress induced by Roundup in these two tissues was too weak to trigger the antioxidant defence system. Indeed, our results are similar to those observed in brain of goldfish (*Carassius auratus*) exposed to Roundup where the concentration of tripeptide glutathione is reduced by 29% in opposite to protein thiol levels (high molecular antioxidant enzymes) that are not affected (73). The authors concluded that oxidative stress induced by Roundup was negligible in this organ. Nevertheless Roundup exposure can lead to a partial inhibition of the activity of antioxidant enzymes in brain (SOD, GST, GR and G6PDH) (82). Thus, our results confirm the low impact of Roundup in brain and gonads, however we cannot exclude a HO-1 expression inhibition such as described above.

Roundup effects on cyp19a and cyp19b mRNA levels

Studies showed that the glyphosate alone or the Roundup formulation act as endocrine disruptors through three major mechanisms: (1) inhibition of hormone production such as progesterone (69), (2) interaction with nuclear receptors (NRs)(72) and (3) disruption of steroidogenic gene expression such as *StAR* gene (78, 123). In our present study, we focused on the *aromatase* which converts androgens into estrogens because it is expressed both in juveniles, males and females, and is sensitive to different hormones and endocrine disrupting compounds.

In contrast to mammals, most of teleost fish possess two distinct genes which encode two isoforms, one ovarian form (cyp19a) and one brain form (cyp19b). The structure

of these two *cyp19* genes have been characterized in zebrafish (61), channel catfish (59), goldfish (119), medaka (117), tilapia (17), rainbow trout (22) and European sea bass (8, 31). The promoters of *aromatase* genes display identical regulatory elements such as TATA box, half-ERE (Estrogen-Responsive Element), putative ARE (Androgen-Responsive Element), CREs (cAMP-Responsive Elements), a CCAAT DNA sequence (C-enhancer binding protein), DRE (Dioxin-Responsive Element), GRE (Glucocorticoid-Responsive Element), a recognition site (AhR)/(AhR) (arylhydrocarbon receptor), various nuclear receptors (PPARα, RXRα): PPRE, RXRE and putative DNA sequences SOX/SRY. Specifically, the 5'-flanking region of *cyp19a* gene includes a CAAT box and SF-1/ad4BP responsive element (Steroidogenic Factor 1/adrenal 4 Binding Protein-Responsive Element), whereas the *cyp19b* gene promoter includes at least an ERE, a PRE (Progesterone-Responsive Element), COUP (Chicken Ovalbumin Upstream Promoter) in zebrafish and various transcription factor responsive elements which are specific to neurogenesis in sea bass (96). Thus, the regulation of the *cyp19* genes expression can be mediated by different signalling pathways and likely affected by endocrine disrupting chemicals. In teleost fish the expression of the neural form, Cyp19b, is mainly responsive to sexual steroids via their nuclear receptors. *Cyp19b* gene is up-regulated by 17 beta-estradiol (E_2) but is also triggered by xenoestrogens (nonylphenol and ethinylestradiol) in tilapia (121), black porgy (67), goldfish (14) and zebrafish (58), and by androgens such as testosterone (T) or 5 alpha dihydrotestosterone (DHT) in zebrafish (24, 87). In this latter case, the androgen regulation was mediated by ERE and not by ARE (87). Moreover, 11-ketotestosterone (11-KT), the principal male androgen in teleosts which is not converted into estrogen, cannot induce *cyp19b* gene expression. All these results highlighted the key role of ERE in the regulation of reproductive axis (HHG axis).

In the present work, juveniles sea bass showed a *cyp19b* mRNA level two fold higher than *cyp19a* which is in accordance with the literature. As shown in figure 3, *cyp19b mRNA levels* in brain of all sea bass exposed to Roundup were higher compared to control fish. As the expression of cyp19b isoform is predominantly under the control of factors that interact with the ERE promotor sequence, two hypotheses regarding the up-regulation of *cyp19b* gene can be made. Firstly, it was shown that adjuvants in glyphosate-based herbicide enhanced the glyphosate biodisponibility at cellular level (80, 103).

Thus in presence of the surfactant, POEA, the permeability of cell membrane is increased which facilitates the penetration of glyphosate. It can inhibit the aromatase activity by direct interaction with its active site (20). This partial inhibition of aromatase was observed in Roundup-exposed Jundiá (*Rhamdia quelen*) with a decrease of serum E2 concentration (113). Since aromatase can not convert androgen into estrogen, E2 cannot initiate *cyp19b* gene transcription. However *cyp19b* gene expression requires low estrogenic compound concentration (19, 58, 83). In the present work, Roundup could behave like an E2 agonist and initiate *cyp19b* gene transcription because this herbicide affects estrogen-regulated genes (44). Secondly, our hypothesis is based on the low anti-androgenic activity of Roundup (32). Indeed androgens stimulate the up-regulation of *cyp19b* gene expression via ARE. In channel catfish, testosterone significantly increased both mRNAs of *cyp19b* and *LHβ* (59). Two molecular signalling pathways modulated *cyp19b* gene expression: one is mediated by the conversion of T into E_2, the second one is under the control of gonadotropin-releasing hormones (GnRH). This co-expression (*cyp19b* and/or *FSHβ*, *LHβ* and *glycoprotein hormone-α*) was also observed in *Ictalurus punctatus* (63).

Recent studies showed that Roundup leads to a decrease in serum concentration of T (20, 104). In our work, Roundup, like androgens, would interact with ER to stimulate *cyp19b* gene expression. In counterpart, it was shown that androgens such as alpha-methyltestosterone inhibit the *cyp19a* transcripts in gonads of zebrafish (29, 120). Similar effects were observed in sea bass exposed to Roundup (Fig. 3): gonadal *cyp19a* mRNA levels tend to decrease ($p=0.104$) compared to control fish. This down-regulation could be due to the disruption of the cAMP/PKA/CREB pathway which modulates the *cyp19a* gene expression in mammals and teleost fish. Several studies highlighted that FSH stimulates the production of a second intracellular messenger, cAMP, which activates PKA and CRE. Both interacted with the *cis*-element cAMP-CREB to initiate *cyp19a* gene transcription (114). In a Roundup exposure, three signalling pathways could explain the decrease of *cyp19a* transcripts in sea bass gonads. **(i)** The circulating E2 concentration is decreased in the presence of Roundup. Estrogens cannot up-regulate FSH secretion. This pituitary gonadotropin could not activate the cAMP/PKA/CREB pathway and *in fine* the *cyp19a* gene expression will be disturbed (71). **(ii)** In normal conditions, androgens stimulate mRNA alpha subunit FSH transcription and this up-regulation is mediated by AR-ARE interaction (17). Roundup had a low anti-androgenic activity and alters the production of serum testosterone concentration (32). So FSH could not stimulate the cAMP/PKA/CREB pathway. **(iii)** Finally, Gasnier et al. (2009) (32) have suggested that glyphosate could bind to the aromatic cycle of steroidogenic membrane receptors such as FSH. Overall, organophosphate pesticides showed anti-gonadic action which directly altered testosterone production and led to a severe depletion of gonadal development and functioning (28). It becomes obvious that Roundup exposure leads to reduced *cyp19a* mRNA level via nuclear receptor interactions, but it does not exclude a direct toxicity of Roundup towards gametogenesis in this species.

Roundup effect on brain AChE gene expression

Numerous studies about glyphosate effects on catalytic activity of AChE were reported, but results on *AChE* mRNA expression are scarce in marine species exposed to Roundup. Strikingly glyphosate, the active compound of Roundup displays a general structure quasi-similar to organophosphate insecticides (OPs), which are known to inhibit AChE activity.

AChE consists of a gorge with an acylation site (A-site) composed of a catalytic triad (Ser, His, Glu) where substrate (acetylcholine or allosteric ligands) hydrolysis occurs and a peripheral site (P-site) with binding sites (His and Trp) (9). One of key steps of the AChE catalytic mechanism is the acylation of the serine hydroxyl moiety. Then deacetylation performs rapidly after hydrolysis and enzyme returns to its free form.

The inhibition of AChE by organophosphates is due to the serine phosphorylation of the catalytic site (Fig. 5.A).

The phosphorylated enzyme is more stable than acetylated AChE, and this configuration, depending on groups (R or R'; glyphosate groups are hydroxyl) bound to phosphorus atom, can lead to an irreversible inhibition (30). AChE inhibition can also be due to ligand binding to the P-site leading to a "*steric blockade"* (54). In both cases, AChE is inactivated and ACh is not degraded and accumulates in synapses. Inactivation of AChE enzyme and ACh accumulation can be correlated to an induction of *AChE* transcription in order to offset this imbalance. In our case, we did not attempted determine the inhibitory mechanism of AChE by Roundup (Fig. 5.B). Nevertheless we observed similar molecular responses to organophosphates (127) or Roundup in *Cyprinus carpio* (15), *Leporinus obtusidens* and *Rhamdia quelen* (35, 36) where Roundup formulation was shown as an anti-AChE.

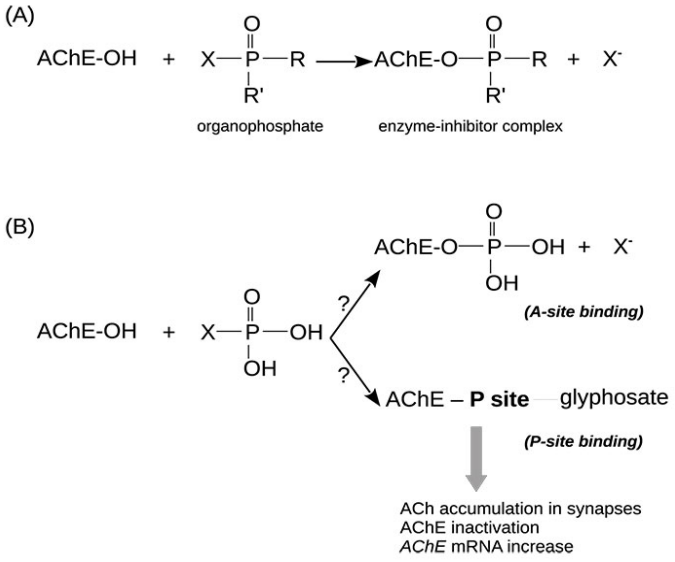

Figure 5. Inhibition of *AChE* by organophosphate insecticide (A) and putative inhibition by glyphosate, (N-phosphomethyl)glycine, the active ingredient of Roundup (B). *AChE, acetylcholinesterase; R and R' groups are linked to phosphorus atom and their nature can lead to an irreversible inhibition of AChE. X is the leaving group*.

Our present results showed that 96-h Roundup exposure resulted in a significant up-regulation of brain *AChE* mRNA (Fig. 4) in European sea bass juveniles. This induction has also been observed in hybrid catfish (*Clarias gariepinus* x *Clarias macrocephalus*) after a 24-h exposure to chlorpyrifos (OP) (112). This up-regulation of *AChE* mRNA level has been ascribed to an autologous feedback response which is initiated by the enzyme-inhibitor complex and ACh accumulation. Anticholinesterase compounds (such as organophosphate and carbamate insecticides) act on intracellular signalling pathways (e.g. intracellular Ca^{2+} increase; activation of *c-fos,* a transcriptional factor of *AChE* gene) which increase *AChE* mRNA levels (57).

The results of the present study revealed that, in European sea bass, a short-term exposure (96-h) to sublethal-non-environmental concentration of Roundup (193 mg/L) led to a significant down-regulation of *heme oxygenase-1* mRNA levels in liver and gills showing an impact on the antioxidant defence system. Roundup exposure also led to a significant increase of *AChE* gene expression in brain: this result highlighted the neurotoxicity of glyphosate-based herbicide in *Dicentrarchus labrax*. Finally a sublethal concentration of Roundup impaired the regulation of the hypothalamic-pituitary-gonadal axis with an alteration of aromatase (*cyp19a* and *cyp19b*) gene expression in gonads and brain respectively. Further research is needed to (1) know and understand metabolic pathways disturbed by Roundup, in particular by study on pathways leading to the disruption of *aromatase* genes' expression; (2) evaluate the impact of a long-term exposure at relevant environmental concentrations of Roundup (< 3 µg/L) (or < 0.05 µg/L in the harbor of Toulon) on the development and the reproduction of this marine species.

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