Marine Pollution Bulletin xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Effects of subchronic exposure to glyphosate in juvenile oysters (*Crassostrea gigas*): From molecular to individual levels

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Crassostrea gigas
Glyphosate
Condition index
Histopathology
Enzyme activities
Gene expression

ABSTRACT

Glyphosate-based herbicides are extensively used and can be measured in aquatic ecosystems, including coastal waters. The effect of glyphosate on non-target organisms is an issue of worldwide concern. The aim of this study was to investigate the effects of subchronic exposure to glyphosate in juvenile oysters, *Crassostrea gigas*. Yearling oysters were exposed to three concentrations of glyphosate (0.1, 1 and 100 μg L^{-1}) for 56 days. Various endpoints were studied, from the individual level (e.g., gametogenesis and tissue alterations) to the molecular level (mRNA quantification), including biochemical endpoints such as glutathione-S-transferase (GST) and catalase activities and malondialdehyde content. No mortality and growth occurred during the experiment, and individual biomarkers revealed only slight effects. The levels of gene expression significantly increased in oysters exposed to the highest glyphosate concentration (GST and metallothioneins) or to all concentrations (multi-xenobiotic resistance). These results suggested an activation of defence mechanisms at the molecular level.

1. Introduction

In Europe, littoral ecosystems, including shellfish farming areas in the English Channel, can be impacted by various contaminants originating primarily from terrestrial inputs (Colas, 2011; European Environmental Agency, 2011). Among them, pesticides from agricultural and non-agricultural activities could exert an impact on littoral ecosystems (Renault, 2011). Besides these terrestrial inputs, some herbicides containing glyphosate as an active matter have been punctually used to control invasive weeds such as *Spartina alterniflora* (Simenstad et al., 1996; Kilbride and Paveglio, 2001). However, the quantification of pollutants including pesticides in the littoral ecosystems remains quite rare compared to the surveys of terrestrial and freshwater environments. In European surface waters, measurements of pesticides during application periods have shown that the concentration patterns are highly dynamic and significantly influenced by point sources of pollution (Holvoet et al., 2007). However, it has been reported that total pesticide concentrations can reach up to a few tens of μg L^{-1} (e.g., Herrero-Hernández et al., 2013), and glyphosate concentration could be as high as 137 μg L^{-1} 3 days after a glyphosate application to control riparian vegetation in a Spanish river (Puértolas et al., 2010).

In marine waters, the level of contamination by pesticides mainly includes organochlorine insecticides, such as hexachlorocyclohexanes (HCHs) and dichlorodiphenyltrichloroethanes (DDTs), which have been reported to be highly toxic and persistent. For example, in the coastal environment of Mumbai, HCHs and DDTs could reach values of 15.92 ng L^{-1} and 33.21 ng L^{-1}, respectively, and approximately 33–34 ng g^{-1} in marine biota from the same area (Pandit et al., 2006). Analyses of HCHs and DDTs performed from 1994 to 2001 in various countries from the Asia–Pacific region revealed very high levels of these insecticides in mussels (up to 0.43 μg g^{-1} of lipid and 61.00 μg g^{-1} of lipid, respectively) (Monirith et al., 2003). In Europe and North America, these insecticides have been banned or strictly regulated. Nevertheless, in the framework of chemical monitoring (ROCCH: Réseau d'Observation de la Contamination CHimique), the only pesticides routinely quantified in French coastal waters are DDTs (banned from 1972) and lindane (γ-HCH; banned from 1998): median national values

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http://dx.doi.org/10.1016/j.marpolbul.2014.10.026
0025-326X/

in oysters remained as high as 2.83 ng g^{-1} and 0.74 ng g^{-1}, respectively, during the period from 2003 to 2007 (ROCCH, 2014). In regard to other pesticides including herbicides, the level of contamination of marine waters is poorly documented. Mai et al. (2013) reported that 20 representatives of six chemical classes of currently used pesticides (e.g., triazine, thiadiazine, dinitroaniline and chloroacetanilide herbicides) were quantified in the central North Sea in 2009 and 2010. However, the concentrations were generally as low as a few pg L^{-1} with a maximum of 1.27 ng L^{-1} and 1.13 ng L^{-1} for atrazine and its main metabolite, respectively. In French coastal environments, few studies have aimed to quantify the level of contamination of shellfish farming areas by pesticides. These studies were mainly justified by the economic importance of shellfish farming in France, which is the leading producer of oysters (*C. gigas*) in Europe and the fourth leading producer in the world (Association des CESER de l'Atlantique, 2012). Oysters are reared notably in the English Channel (Normandy and North Brittany which contribute to approximately 44% of the French production). In this context, Auby and collaborators (2007) reported the contamination of Arcachon Bay by seven insecticides, six herbicides and their metabolites, including bifentrine and permetrine (insecticides), which showed the highest concentration in seawater (0.27 μg L^{-1}) and oyster flesh (3 ng g^{-1}), respectively. In Normandy, no herbicides were detected in oyster meat but six herbicides were quantified in seawater for a total of 15 investigated herbicides and a maximum value of 0.132 μg L^{-1} was recorded for diuron (Buisson et al., 2008). Using Polar Organic Chemical Integrative Samplers (POCIS), Munaron and collaborators (2012) highlighted the contamination of Mediterranean lagoons and coastal waters by 15 herbicides and a biocide, out of the 27 total pesticides analysed. The studies mentioned above did not investigate glyphosate because this herbicide requires a separate analysis procedure and is thus not routinely sought in marine waters. However, Burgeot et al. (2008) reported concentrations of total pesticides exceeding 0.5 μg L^{-1}, with peaks of 0.4 μg L^{-1} of isoproturon and 0.1 μg L^{-1} of glyphosate, in the Seudre estuary. Data regarding the concentrations of glyphosate in marine coastal waters remain very scarce, but the potential effect of glyphosate on coastal organisms cannot be excluded with regard to the important use of this herbicide. Indeed, glyphosate-based herbicides are widely used and are among the most used pesticides in the world (Howe et al., 2004). Glyphosate shows a non-selective mode of action with a broad application spectrum and is widely applied in agricultural, silvicultural and urban environments (Hanke et al., 2010).

Glyphosate is considered moderately toxic to birds, most aquatic organisms, earthworms and honeybees (PPDB, 2014). Williams and collaborators (2000) concluded "under present and expected conditions of use, glyphosate does not pose a health risk to humans". For aquatic environments, ecotoxicological data of pesticides including glyphosate mainly concern aquatic plants and algae, various fish species (e.g., *Cyprinus carpio* and *Oncorhynchus mykiss*) and invertebrate models commonly used in ecotoxicology, such as crustaceans (e.g., *Daphnia magna*) or insects (e.g., *Chironomus plumosus*) (Maycock et al., 2012; Agritox, 2014; PPDB, 2014). Compared to other organisms including algae and arthropod species, data are scarce regarding the impact of glyphosate on freshwater mollusc species. However, Bringolf and collaborators' (2007) work on the mussel *Lampsilis siliquoidea* can be included; the authors studied the survival of larvae and 1- to 2-month-old juveniles for 48–96 h and juvenile growth for 21 days. In the freshwater species mentioned above, the aquatic toxicity can correspond to short-term (acute) or long-term (chronic) effects, depending on the investigated endpoints (e.g., immobility versus growth or reproduction) and the life span of the studied stage or species. Moreover, a subchronic exposure can be defined as the repeated exposure of a toxicant for a comparatively shorter period of time than chronic exposure, i.e., 1–3 months (Pani, 2010). Similar to marine environments, both short-term and long-term toxicities of glyphosate can be distinguished, but the number of available model species is lower compared with freshwater environments (Tsui and Chu, 2003). In this context, Tsui and Chu (2003) studied the acute toxicity of glyphosate and glyphosate-based formulations on two species of microalgae, protozoa and crustaceans (freshwater and marine). Regarding invertebrates, most of the studies that addressed toxicity could be considered as acute (short-term). Bivalve molluscs such as clams, mussels and oysters have been postulated as ideal indicator organisms because of their wide geographical distribution, sedentary lifestyle and sensitivity to environmental pollutants (Renault, 2011). Elandallousi et al. (2008) assessed survival of the clam *Ruditapes decussatus* (25 mm) exposed to glyphosate for 96 h, and a report edited by the World Health Organization (1994) indicated the survival of the oyster *Crassostrea virginica* exposed to glyphosate for 48 h. In both cases, LC_{50} exceeded 10 mg L^{-1}. These results, and the high survival rates recorded in 21-day-old pediveliger larvae of *C. gigas* exposed to glyphosate (up to 100 mg L^{-1}) for 24 h (Mottier et al., 2013), suggested the relatively strong resistance of marine bivalves to glyphosate in terms of survival. Therefore, it is important to consider the sub-lethal effects of glyphosate in these bivalves during a rather long exposure time. These studies become more relevant because the oyster farming represents an important commercial activity in coastal areas.

Several authors have investigated various endpoints to study the effects of pesticides in molluscs including *C. gigas* exposed naturally or experimentally to pesticides. In situ investigations reveal the advantage of chronic exposures but the disadvantage to be difficult to interpret because of the multitude of factors able to influence bivalve physiology (mixture of pollutants, trophic resources, temperature, etc.). Some of these abiotic parameters may act as confounding factors. In contrast, experimental subchronic exposures are less environmentally realistic but easier to interpret because the only varying factor is the contaminant concentration. Greco et al. (2011) used an array of biomarkers to study the physiological status of soft-shell clams, *Mya arenaria*, exposed for 28 days to a mixture of herbicides (2,4-D, mecoprop and dicamba): survival, condition and gonadosomatic indexes, immune parameters and activities of various enzymes notably implied in the regulation of oxidative stress (e.g., catalases). The activities of enzymes involved in stress regulation were also measured in the brown mussel *Perna perna* and in the mangrove oyster *Crassostrea rhizophorae* exposed to furadan (carbamate biocide) but the exposure time (96 h) was too short to be considered as subchronic (Alves et al., 2002). In *C. gigas*, immune parameters (e.g., haemocyte mortality and phagocytosis) were used to assess the toxicity of a mixture of eight pesticides including glyphosate for 7 days (Gagnaire et al., 2007) and the toxicity of diuron (herbicide) for 11 weeks (Bouilly et al., 2007). Moreover, Bouilly and collaborators (2007) studied the potential effect of diuron on oyster aneuploidy and Gagnaire et al. (2007) quantified the expression of 19 genes involved in haemocyte functions. Quantification of gene expression in oyster and mussel species was also used to evaluate the toxicity of subchronic exposures to different pesticides: glyphosate or an herbicide mixture (atrazine, diuron, isoproturon) for 30 days (Tanguy et al., 2005a,b); a cocktail of three pesticides (lindane, metolachlor and carbofuran) for 30 days (Collin et al., 2010); and a mixture of two neonicotinoid insecticides for 4 days (Dondero et al., 2010). Finally, it is noticeable that the histopathology approach was mainly used in bivalves exposed to pollutants in situ (and not experimentally).

Considering both the important use of glyphosate and the lack of data on the toxicity of this herbicide in non-target marine

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organisms, we aimed to assess the toxicity of glyphosate on different life-cycle stages in *C. gigas* including juvenile oysters. In the framework of the European Interreg IV project "Chronexpo" dealing with organisms from the English Channel, the aim of the present study was to assess the effects of a subchronic exposure to glyphosate in yearling oysters by studying different organisational levels of physiology, from individuals (e.g., reproduction) to molecular levels (e.g., expression of target genes).

2. Materials and methods

2.1. Organisms and chemical compounds

Juveniles of the Pacific oyster *C. gigas* (Thunberg, 1793) were obtained from the "Société ATlantique de MARiculture" (SATMAR®; Barfleur, France) hatchery. They were produced by the fertilisation of wild origin spawners. At the beginning of the experiments, oysters were 12 months old and averaged 1.09 g (±0.38) in weight and 20.58 mm (±2.73) in length. It is important to note that since 2008 in France, significant mortalities have been observed in juvenile oyster livestock in relation with OsHV-1 µVar. We assessed the risk of abnormally high death rates due to viral disease during the experiment, and viral loads were measured at the "Centre de Référence sur l'Huître" (CRH) using real-time TaqMan® PCR. Ten pools of five oysters were analysed, and only one pool showed a low viral load.

Glyphosate [N-(phosphonomethyl)glycine, $C_3H_8NO_5P$] is the active ingredient in commercial Roundup® herbicides. In the present study, glyphosate acid (97% purity; CAS number: 1071-83-6) was obtained from Dr. Ehrenstorfer GmbH® (Augsburg, Germany). This compound is considered as a non-persistent molecule in soils (half-life, DT50 = 12 days) but is highly water soluble (Battaglin et al., 2005) and relatively persistent in this matrix considering its hydrolysis (>30 days for pH ranging from 5 to 9) and photolysis times (69 and 77 days, respectively, for pH of 7 and 9) (Agritox, 2014; PPDB, 2014). Three concentrations were tested: 0.1 µg L^{-1} (considered as a realistic environmental concentration), 1 µg L^{-1} (frequently measured in freshwater) and 100 µg L^{-1} (corresponding to concentration peaks in rivers). The solutions of herbicides used were prepared with natural sterilised open seawater (0.22 µm, Steritop® Millipore). During experiments, pollutant concentrations in tanks could not be analysed. However, in a preliminary study, glyphosate analyses were conducted in similar conditions. Glyphosate concentrations were checked in 10 liters of seawater right after contamination and after 24 h of contamination. The analysis method was previously described in Mottier et al. (2013). Measured concentrations at T0 were close to those expected (e.g. 0.06 µg L^{-1} measured for 0.1 µg L^{-1} expected and 0.87 µg L^{-1} measured for 1 µg L^{-1} expected), and no statistical differences were recorded after 24 h (e.g. 0.08 µg L^{-1} measured for 0.1 µg L^{-1} expected and 0.99 µg L^{-1} measured for 1 µg L^{-1} expected). A slow diffusion and a non-complete dissolution at T0 could explain the slight increase of glyphosate concentrations between T0 and T24 h.

2.2. Experimental structure and design

A total of 592 juvenile oysters were randomly distributed in eight tanks (74 individuals per tank corresponding to 70 oysters sampled at 5 dates and 4 extra individuals to constitute the sample at T0 and to compensate potential mortalities; each concentration and control in duplicate). At the beginning of the experiment, the oysters of these batches were homogeneous in size and weight (ANOVAs, $p > 0.05$). Polypropylene tanks were filled with 10L of sterilised natural open seawater and equipped with a sieve and air-driven filter. Total water removal occurred twice per week, and water was partially (1/10) changed daily. The 1L of removed water was replaced by 1L of *Isochrysis galbana* (T-Iso clone) culture to feed the animals at an average concentration of 4.47×10^7 cells per oyster (by referring to a practical manual for bivalve hatcheries; Helm et al., 2006). After each water change, the glyphosate concentrations were readjusted according to nominal concentrations in order to compensate for the removal of water (e.g. for partial changes, 10% of glyphosate nominal concentration was added). Oysters were maintained at the temperature of 17 °C (±0.5) and a photoperiod of LD 10:14 h.

The experiment was conducted from 11 April to 6 June 2012, and oysters were sampled at six dates: T0 and T24 h and T7, T14, T28 and T56 days. At each sampling date, 14 animals per tank were removed, in total, for the different endpoints.

2.3. Studied endpoints

2.3.1. Individual and histological biomarkers

Mortality was checked daily to discard dead oysters and calculate daily mortality rates. At each sampling date, except for T24 h, all oysters from the different tanks were measured in length (to the nearest 0.01 mm) and weight (to the nearest 0.01 g) to determine growth during the exposure. Furthermore, at these same dates, oysters sampled for the various endpoints were also used to determine the AFNOR condition index calculated as follows: CI_{AFNOR} = (flesh weight/total weight) × 100.

At each study date except T24 h, cross sections of oysters (~4 mm thick) were cut behind the labial palps and fixed in Davidson's solution: 1 vol. glycerol, 2 vol. formaldehyde 37%, 3 vol. ethanol 95% and 3 vol. filtered seawater. Tissue samples were then routinely processed for histology, and 3-µm paraffin-embedded sections were stained according to the trichrome protocol of Prenant Gabe (Gabe, 1968). In each specimen, sex was determined and individuals were classified as female, male and undetermined sex when the gametogenesis was not far enough advanced. Gametogenesis stage was determined according to Lubet's modified classification of microscopic observations; the four stages and substages observed in the present study are described in Table 1. The histological slides were also examined to determine tissue alterations such as destructurations of the connective tissue (corresponding to reserve tissue poorly developed), hemocytic infiltrations (essentially in connective tissue) and atrophies of the wall of the digestive tubules (with lumen enlargement) (Fig. 1). The tissue disturbances were semi-quantified according to a scale with four levels (indices) from slight to severe (Buisson et al., 2008) (Table 2). At each date, an average index (±SEM) was calculated for each condition and tissue alteration.

2.3.2. Enzymatic activities and markers of lipid peroxidation

During sampling, the digestive glands of six oysters per condition were individually homogenised with a Potter–Elvehjem homogeniser in phosphate buffered saline (PBS) (NaCl 500 mM, Na_2HPO_4 10 mM, KH_2PO_4 3.1 mM, pH 7.4) containing 1% Halt Protease Inhibitor Cocktail, EDTA-Free (100X) Thermo Fisher Scientific® (Waltham, USA) in a ½ w/v (weight/volume) ratio. Homogenates were then centrifuged at 12,500*g* for 15 min at 4 °C (S9 fraction), and supernatants were aliquoted and stored at −80 °C. The aliquots were used for determining the amount of proteins, the malondialdehyde content and enzymatic activities.

2.3.2.1. Protein content. Protein contents were determined using the Bio-Rad Protein Assay kit (Bio-Rad®) according to Bradford (1976). Samples were diluted at 1/200 in PBS. Ten microlitres of the samples or bovine serum albumin (BSA) was deposited in the microplate wells, and 200 µL of Bio-Rad reagent was added

Table 1
Description of distinct phases of bivalve gonadal maturity observed in the present study and based on microscopic analysis. Stages and sub-stages were determined according to Lubet's modified classification (Lubet, 1959).

Stages	Gonad	Gonadal maturity	Follicle maturityin male	Follicle maturity in female
0	Reduced; follicles are collapsed and very unobtrusive	Inactive; connective tissue between tubules is very abundant	Very regressed tubules possessing a single gonial cell layer	Very regressed tubules possessing a single gonial cell layer
I	Early developing	Early active; connective tissue remains very abundant	Phase of gonial mitoses; tubules contain spermatogonia	Phase of gonial mitoses; tubules contain oogonia
Beginning II	Developing	Active; connective tissue in regression but tubules remain well separated from each other	Tubules contain spermatogonia and spermatocytes	Beginning of vitellogenesis; tubules contain oogonia and early oocytes
II	Developing	Fully active; connective tissue in regression and certain tubules are well separated from each other, whereas other ones are close	Tubules contain all categories of cells, from spermatogonia to spermatozoa	Vitellogenesis process is well underway; tubules contain oogonia and both early and mature oocytes

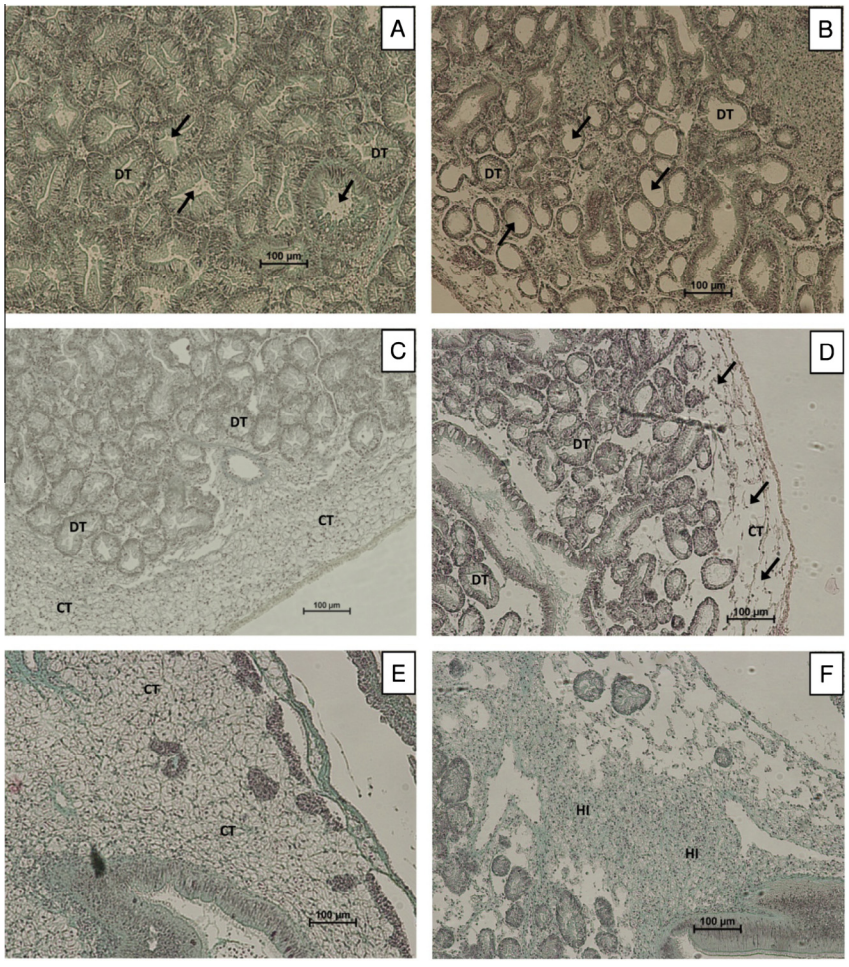

Fig. 1. Histological sections showing normal condition (A; C; E) and three types tissue alterations (high level: 4; see Table 2): atrophies of the wall of the digestive tubules (in an individual exposed to 0.1 µg L^{-1} of glyphosate); arrows indicate the lumen of the digestive tubule (B), destructuration of the connective tissue (in a control individual); arrows indicate tissue gaps due to destructuration of the connective tissue (D) and hemocytic infiltrations (F) (in an individual exposed to 100 µg L^{-1} of glyphosate). Bar: 100 µm. DT = digestive tubule; CT = connective tissue; HI = area of hemocytic infiltrations.

Table 2
Semi-quantitative scales chosen to qualify the degree of tissue alteration (% in relation to total surface occupied by a given tissue). The thresholds correspond to those determined by Buisson et al. (2008).

	Level 1 (slight)	Level 2 (moderate)	Level 3 (severe)	Level 4 (very severe)
Atrophies of the digestive tubules	5–25%	25–50%	50–75%	>75%
Connective tissue destructuration	5–10%	10–33%	33–50%	>50%
Hemocytic infiltrations	5–10%	10–33%	33–50%	>50%

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(diluted at 1/5 in ultrapure water). Absorbance was determined at 595 nm by a microplate reader (Flexstation 3, Molecular Device®). The results were expressed as mg total protein per mL (mg proteins/mL). For enzymatic activities and lipid peroxidation, the results were normalised to total protein content in supernatants.

2.3.2.2. Glutathione-S-transferase (GST). Glutathione-S-transferase (GST) activities were measured according to Habig et al. (1974) (adapted for microplate reading) by monitoring the increase in absorbance due to GS-DNB (S-(2,4-dinitrobenzyl)glutathione) formation (extinction coefficient: 0.0096 $\mu M^{-1}\,cm^{-1}$ at 340 nm). Ten microlitres of sample was added to 170 µL of PBS. Next, 50 µL of the previous solution was deposited in microplate wells with 200 µL of the solution reagent (GSH 1 mM, Hepes 10 mM, NaCl 125 mM, CDNB 1 mM, pH 6.5). The kinetics was measured at 340 nm for 20 min at 25 °C in the microplate reader (Flexstation 3, Molecular Device®). The results were expressed as µmol of GS-DNB per min and mg of proteins (µmol $min^{-1}mg^{-1}$ proteins).

2.3.2.3. Catalase. Catalase activities were measured according to Babo and Vasseur (1992) and modified for microplate by reading the decrease in absorbance due to hydrogen peroxide consumption. Samples were diluted by 500 in PBS. Next, 100 µL of the samples or catalase standard was deposited in UV microplate wells (UV star, Greiner Bio-One Gmbh®) and H_2O_2 (28 mM) was added. Kinetic measurements were made at 240 nm for 15 min at 25 °C in the microplate reader (Flexstation 3, Molecular Device®). The results were expressed in µmol of H_2O_2 min^{-1} mg $prot^{-1}$ corresponding to a catalase unity per mg of proteins (U CAT $prot^{-1}$).

2.3.2.4. Malondialdehyde content. Malondialdehyde (MDA) accumulation was used as a lipid peroxidation marker. MDA content was measured with an MDA-586 assay kit (Oxis-Research®) according to the manufacturer. Ten microliters of the sample was added to 50 µL of PBS to obtain a protein concentration from 1 to 8 mg proteins/mL. Next, 30 µL of samples or MDA standard was mixed on ice with 1.5 µL of probucol, 96 µL of diluted R1 reagent (acetonitrile diluted by ¼ in methanol), and with 22.5 µL of R2 reagent. After 60 min of incubation at 45 °C in a dry bath incubator, samples were centrifuged at 10,000*g* for 10 min. Finally, 80 µL of the supernatants was then deposited in the microplate wells. Endpoint measurements were made at 586 nm (Gérard-Monnier et al., 1998) in the microplate reader (Flexstation 3, Molecular Device®). The results were expressed as quantity in µmol of MDA per mg of proteins (µmol MDA mg^{-1} proteins).

2.3.3. mRNA quantification

At each sampling date, eight juveniles were dissected and the isolated digestive glands and gills were immediately rinsed and mixed with TRI REAGENT (Sigma Aldrich®) in a proportion of 10 mL per 1 mg of tissue. Samples were stored at −80 °C before analysis. The detailed protocol for RNA extraction, RNA quantification, DNAse treatment and retrotranscription has been previously described in Farcy et al. (2007). A MyiQ Cycler (Bio-Rad®) was used to perform real-time PCR, and the primers corresponding to the different genes are given in Table 3. The expression of the following mRNAs was analysed: heat shock genes (Hsc 72, Hsp 70, Hsp 90), metallothineins (MT1&2), cell cycle regulation gene (p53), genes involved in detoxification mechanisms: cytochrome p450 (CYP450), glutathione-S-transferase (GST), multixenobiotic resistance gene (MXR) and gene involved in regulation of oxidative stress: superoxide dismutase (SOD). These stress markers were selected on the basis of literature concerning cellular functions expected to be involved in response to xenobiotics including pesticides (Eufemia and Epel, 1998; Hanioka et al., 1998; Snyder et al., 2001; Oropeza-Hernandez et al., 2003; Boutet et al., 2004; Saint-Jean et al., 2005; Eder et al., 2007; Gagnaire et al., 2007; Farcy et al., 2008; Erdoğan et al., 2011; Ceyhun et al., 2012).

Protocols and primers were chosen in accordance with previous studies on *C. gigas* (Farcy et al., 2007; Devos et al., 2012). Briefly, PCR analysis was performed in 96-well plates in a final volume of 15 µL: cDNA obtained from reverse transcription of 5 ng of total RNA, 500 nM of each primer and 7.5 µL of 2X iQ SYBR Green Supermix (Bio-Rad®). The following thermocycling program was applied: 40 cycles of 15 s at 95 °C and 45 s at 60 °C. A melting curve analysis was performed from 55 °C to 95 °C with an increase of 0.5 °C, and the specificity of the reaction was confirmed by observing a single peak at the expected melting temperature (Tm). All of the amplicon sequences obtained were previously analysed by Farcy et al. (2007).

All of the determinations were performed in duplicate, and Q-PCR runs were analysed using MyIQ optical system software (Bio-Rad®) that provided Cycle threshold (Ct) values for each gene. To normalise the mRNA level between samples, three different housekeeping genes were used: actine, Glyceraldhyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA. The expression level of a specific gene was determined using the following formula:

$$\text{Normalised mRNA level} = 2^{|\text{Ct reference gene} - \text{Ct gene of interest}|}.$$

2.4. Statistical analyses

Differences recorded between sampling times (six dates) and conditions (control and three concentrations) were statistically tested. Data that did not show homoscedasticity and/or that did not meet the assumption of normality were analysed with a Kruskal–Wallis (K–W) non-parametric test. Comparisons between concentrations or dates were then performed with *post hoc* Dunn tests. Data that met the conditions above were tested using one-way ANOVAs, and differences among concentrations were then analysed with *post hoc* Student Newman Keuls (SNK) tests. The proportions of the different gametogenesis stages of the control and exposed oysters were tested with Fisher tests. The statistical analyses were performed using STATISTICA 8.0 software (Statsoft®, Tulsa, OK, USA).

3. Results

3.1. Individual endpoints: survival, growth and reproduction

No mortality occurred during the exposures despite the sampling date or glyphosate concentration. During the 8 weeks of the experiment, exposed or control oysters showed no growth, both in length and in weight, and no significant differences were recorded between the oysters exposed to the different glyphosate concentrations or control (ANOVAs: $p > 0.05$). The condition index (CI) slightly decreased during the experiment for the oysters placed in all conditions; indeed, the CI values at T56d were from 92.78% (100 µg L^{-1}) to 88.29% (0.1 µg L^{-1}) of the value calculated at the beginning of the experiment (11.08 ± 0.94). Only a significant difference was observed at the lowest concentration (0.1 µg L^{-1}) between T14d (CI: 11.66 ± 0.45) and T56d (CI: 9.78 ± 0.50) (ANOVA and SNK tests: $p < 0.05$). Nevertheless, no significant differences were found between the three conditions and control at T56d: 9.93 ± 0.60 for control oysters and 9.78 ± 0.50, 9.97 ± 0.54, 10.28 ± 0.82 for oysters exposed to 0.1, 1 and 100 µg L^{-1}, respectively.

At the beginning of the experiment, it was impossible to reliably determine oyster sex. After 56d of exposure, only 21% of the individuals could be precisely sexed: 14% were female and 7% male.

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Table 3
Primer sequences used for mRNA quantification in *Crassostrea gigas* and accession numbers.

Gene	GenBank accession No.	Forward primer	Reverse primer
GST	AJ557140	5′AACGCCACCATTCACGAC 3′	5′AAGACCCCACCCAATGCT 3′
CYP450	AF075692	5′CCCTGGGAGTTCAAACCTG 3′	5′CGAGCGCAAATCCAATAAA 3′
MXR	AJ422120	5′CCGAGAACATCCGCTACG 3′	5′GCCCTGTGGGAGTTCCTT 3′
Hsc72	AJ305315	5′GAGGATCGCAGCCAAGAA 3′	5′TATCGCCCTCGCTGATCT 3′
Hsp70	AF144646	5′AGCAAGCCAGCACAGCA 3′	5′GCGATGATTTCCACCTTC 3′
Hsp90	AJ431681	5′GGAGAGCAAAACCCTCACC 3′	5′TGGCAATGGTTCCAAGGT 3′
MT1&2	AJ243263 AJ242657	5′TGTCTGATCCATGTAACTGCACT 3′	5′TCAGGCTGCAAAGTCAAGTG 3′
SOD	AJ496219	5′AACCCCTTCAACAAAGAGCA 3′	5′TTTGGCGACACCGTCTTC 3′
p53	AM236465	5′ACCCAGCTCCGACTCATTT 3′	5′TCATGGGGGATGATGACAC 3′
Actine ♦	AF026063	5′GCCCTGGACTTCGAACAA 3′	5′CGTTGCCAATGGTGATGA 3′
18S ♦	AB064942	5′CGGGGAGGTAGTGAC GAA 3′	5′ACCAGACTTGCCCTCCAA 3′
GAPDH ♦	AJ544886	5′TTGTCTTGCCCCTCTTGC 3′	5′CGCCAATCCTTGTTGCTT 3′

Regarding sexual maturity, no significant differences were observed between oysters under the four conditions (Fisher tests: $p > 0.05$) whatever the sampling date and only results at T0 and T56d are thus illustrated in Fig. 2. On the 11th of April (corresponding to T0), 65.52% of oysters were in stage 0 corresponding to sexual resting, and the remaining individuals were in stage I (gonial mitoses). After 56d of exposure, the oysters showed three stages and sub-stages: stage I, beginning stage II and stage II. The latest stage corresponded to the oysters that were the most advanced in the gametogenesis and represented 9.09%, 10.53% and 4.55% of the individuals exposed to 0.1, 1 and 100 µg L^{-1}, respectively. Control oysters could be classified as stage I and beginning stage II and appeared globally less advanced in gametogenesis.

3.2. Histopathology

Tissue alterations were studied at five dates (T0, T7, T14, T28 and T56d). For clarity, the results in Fig. 3 were restricted to T0 and T56d but the mean indices are given for each date in Table 4. Atrophies of the wall of the digestive tubules were less pronounced at the beginning of the study (average index of 0.67 ± 0.11) and reached 1.00 ± 0.23, 1.33 ± 0.25 and 1.56 ± 0.25 at T56d for the individuals exposed to 100, 0.1 and 1 µg L^{-1}, respectively (Figs. 1B and 3A); however, no significant differences were revealed between conditions (K–W: $p = 0.065$). The mean values of the connective tissue destructurations ranged from 2.21 (±0.17) in individuals at T0 to 2.85 (±0.27) in control oysters at T56d, but no significant differences were recorded at T56d (K–W: $p > 0.05$) (Figs. 1D and 3B). Hemocytic infiltrations increased during the course of experiment regardless of the condition and significant differences were observed between individuals at T0 (2.36 ± 0.16) and exposed oysters after 56d; nevertheless, no significant differences were observed between the oysters placed in the four conditions at T56d (K–W: $p > 0.05$) (Figs. 1F and 3C).

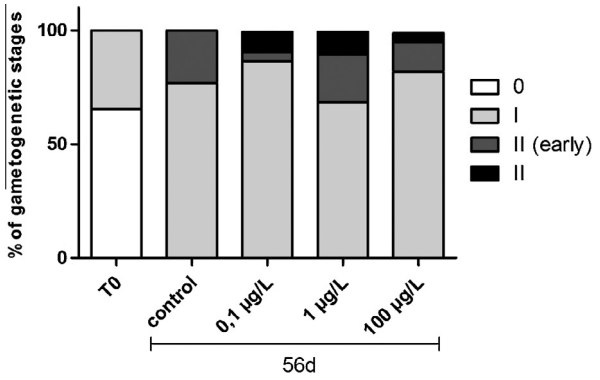

Fig. 2. Proportion of the four oyster gametogenesis stages and sub-stages (0, I, beginning II and II) observed at the beginning of the experiment (T0) and after the 56-d exposure to three concentrations of glyphosate: 0.1 µg L^{-1}, 1 µg L^{-1} and 100 µg L^{-1}. For the description of gametogenesis stages, see Table 1.

3.3. Enzyme activities and lipid peroxidation

GST activities ranged from 0.60 ± 0.13 (control at T56d) to 1.88 ± 0.30 µmol min^{-1}mg $prot^{-1}$ (control at T24 h) during the entire experiment (Fig. 4A). These activities decreased at T56d compared to the four other dates but the differences were significant only between T56d and T0, T24 h, T14d for control oysters and T0 and T56d for oysters exposed to 0.1 µg L^{-1} (ANOVAs and SNK tests: $p < 0.05$). At each sampling date, no significant differences were observed between the four conditions (ANOVAs: $0.53 < p < 0.86$), suggesting no effect of glyphosate on GST activities.

When examining temporal variations, measurements of catalase activities revealed only significant differences for oysters exposed to 0.1 µg L^{-1} and 1 µg L^{-1} (ANOVAs and SNK tests: $p < 0.05$) with CAT activities comparatively high at T7d (Fig. 4B). At each sampling date, no significant differences were detected between the four conditions (ANOVAs: $0.35 < p < 0.75$), except at T24 h when control oysters showed a significantly higher catalase activities reaching 527.01 ± 179.11 µmol min^{-1}mg $prot^{-1}$ (ANOVA and SNK tests: $p < 0.05$).

The MDA content reached a maximum value for oysters after 56d of exposure at 100 µg L^{-1} (0.031 µmol mg^{-1} proteins ±0.004), and the minimum was observed for oysters exposed to the intermediate concentration (1 µg L^{-1}) at T7d (0.016 µmol mg^{-1} proteins ±0.002) (Fig. 4C). Only two significant differences were computed for the lowest concentration between T7d and T0 and between T7d and T14d (SNK tests: $p < 0.05$). At the four dates, MDA content did not differ significantly between the four conditions (K–W: $0.07 < p < 0.85$).

3.4. Levels of mRNA expression

The data were provided only if statistical differences were observed regardless of the gene used for normalisation" (i.e., actin, GAPDH or 18S). Furthermore, for the sake of brevity, the results that are presented correspond to GAPDH normalisation. During the experiment, no significant differences were observed for mRNA levels of the control group regardless of experiment duration. After glyphosate exposure, only three genes were differentially expressed (i.e., GST, MT1&2 and MXR), and no significant differences were observed for CYP450, Hsc72, Hsp70, Hsp90, SOD and p53.

At 24-h of exposure, the GST mRNA level was significantly higher in oysters exposed to the highest dose of glyphosate (100 µg L^{-1}) compared to the control (5.5-fold) or the 0.1 and 1 µg L^{-1} group (ANOVA and SNKs: $p < 0.05$) (Fig. 5A). Moreover,

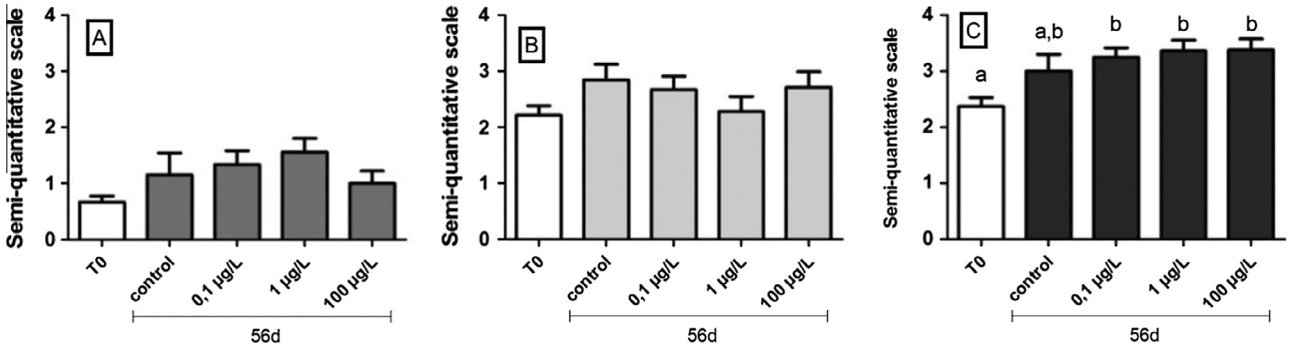

Fig. 3. Mean (±SEM) of tissue alteration indices calculated at the beginning of the experiment (T0) and after the 56-d exposure to three concentrations of glyphosate: 0.1 µg L^{-1}, 1 µg L^{-1} and 100 µg L^{-1}. The oyster tissue alterations correspond to atrophies of the wall of the digestive tubules (A), destructuration of the connective tissue (B) and hemocytic infiltrations (C). For the index thresholds of the three tissue alterations, see Table 2. The concentrations (at T0 and T56d) that do not share a letter are significantly different.

Table 4
Average indices (±SEM) calculated by considering the scales (indices) chosen to qualify the degree of the three types of tissue alteration (see Table 2) at each date.

	Conc.	T0	T7d	T14d	T28d	T56d
Atrophies of the digestive tubules	ctrl	0.67 ± 0.11	1.00 ± 0.30	0.71 ± 0.29	1.00 ± 0.52	1.15 ± 0.39
	0.1		1.00 ± 0.37	1.33 ± 0.42	1.43 ± 0.43	1.33 ± 0.25
	1		1.29 ± 0.42	1.00 ± 0.38	0.86 ± 0.34	1.56 ± 0.25
	100		0.57 ± 0.30	1.00 ± 0.44	0.50 ± 0.27	1.00 ± 0.23
Connective tissue destructuration	Ctrl	2.21 ± 0.17	2.71 ± 0.36	2.57 ± 0.43	2.5 ± 0.50	2.85 ± 0.27
	0.1		2.17 ± 1.00	1.83 ± 1.00	2.14 ± 0.46	2.67 ± 0.24
	1		2.00 ± 0.38	1.71 ± 0.42	2.29 ± 0.36	2.28 ± 0.27
	100		2.00 ± 0.44	2.29 ± 0.36	2.25 ± 0.16	2.71 ± 0.28
Hemocytic infiltrations	Ctrl	2.36 ± 0.16	2.14 ± 0.34	3.00 ± 0.44	3.67 ± 0.21	3.00 ± 0.30
	0.1		2.50 ± 0.56	3.00 ± 0.45	3.71 ± 0.18	3.25 ± 0.16
	1		2.00 ± 0.44	2.43 ± 0.20	3.00 ± 0.31	3.36 ± 0.19
	100		2.43 ± 0.43	3.43 ± 0.30	3.25 ± 0.31	3.38 ± 0.20

at 14d, the GST mRNA levels were significantly higher for exposed oysters, although the increases were less evident than at T24 h. With respect to MT1&2 mRNA, levels were significantly higher for juveniles exposed to 100 µg L^{-1} of glyphosate compared to the control group (3.50-, 5.81- and 3.91-fold at 24 h, 7d and 14d of exposure, respectively) (ANOVA and SNKs: $p < 0.05$), whereas no differences were recorded at 28d (Fig. 5B). MXR mRNA levels were significantly higher at 28-d of experiment for all of the exposed oysters (ANOVA and SNKs: $p < 0.01$) and reached 3.08-, 2.64- and 4.72-fold the level of the control group for 0.1, 1 and 100 µg L^{-1}, respectively (Fig. 5C).

4. Discussion

4.1. Individual and histological biomarkers

Pesticide databases provide values of LC_{50} corresponding to the lethal concentration for 50% of the individuals of a given population after an exposure of 48, 72 or 96 h (PAN, 2014; Agritox, 2014). For glyphosate and aquatic animal species, LC_{50} values are available for the crayfish, *Orconectes nais* ($LC_{50\ (96h)}$ = 7 mg L^{-1}), zooplankton ($LC_{50\ (96h)}$ values from 20 mg L^{-1} in *D. magna* to 79 mg L^{-1} in *Americamysis bahia*), amphibians ($LC_{50\ (96h)}$ = 39 mg L^{-1} in *Rana clamitans* and $LC_{50\ (72h)}$ = 72 mg L^{-1} in *Crinia insignifera*) and finally fish ($LC_{50\ (96h)}$ = 39 mg L^{-1} in *O. mykiss* and 115 in *C. carpio*). In the marine bivalves, *R. decussatus* and *C. virginica* exposed to glyphosate for 48 h and 96 h, respectively, the LC_{50} values exceeded 10 mg L^{-1} (Elandallousi et al., 2008; WHO, 1994). All of these values are far higher than the highest concentration tested in the present study (100 µg L^{-1}), and it is thus not surprising that no mortalities occurred in control and exposed oysters during the 56-d exposure. This result suggests that glyphosate did not induce mortalities even at the highest concentration but also that the experimental conditions were sufficient for oyster survival. However, the experimental conditions did not allow significant oyster growth even in the control batch. Considering the relatively high growth rates reported by various authors in *C. gigas* similar in size and/or age (e.g. Héral and Deslous-Paoli, 1991; Grabowski et al., 2004), a growth, even moderated, could be expected (~3 mm). To explain this lack of growth, it could be hypothesised that the amount and/or the quality of the provided microalgae, *Isochrysis galbana T-Iso*, was not sufficient from a quantitative and/or qualitative standpoint. Although some pesticides can affect the bivalve growth (e.g. Bringolf et al., 2007), this point could not be analysed in this work because the conditions of maintenance could not be suitable to induce growth. Sokolova et al. (2012) have defined stress in terms of increased expenditure on maintenance, defence and repair in response to stress, resulting in a reduction of net energy gain to invest in production (somatic growth and reproduction). Nicholson and Lam (2005) highlighted that in mussels exposed to stressful conditions, energy reserves are channelled into energy-consuming detoxification processes, and consequently, mussels inhabiting polluted waters often have retarded growth and poor tissue condition. Changes in glycogen and lipid contents greatly reflected the pollution level of study sites where zebra mussels had been transplanted (Smolders et al., 2004). In oysters, energy reserves are mainly formed by glycogen and in a future study, it would be very interesting to measure the glycogen content in control and exposed oysters. However, since the connective tissue is composed of glycogen storage cells (Berthelin et al., 2000); histopathology regarding connective tissue destructuration can be considered as an alternative semi-quantitative approach of the energy reserves.

In the present study, the index of connective tissue destructuration was relatively high (2.21 ± 0.17) at the beginning of the

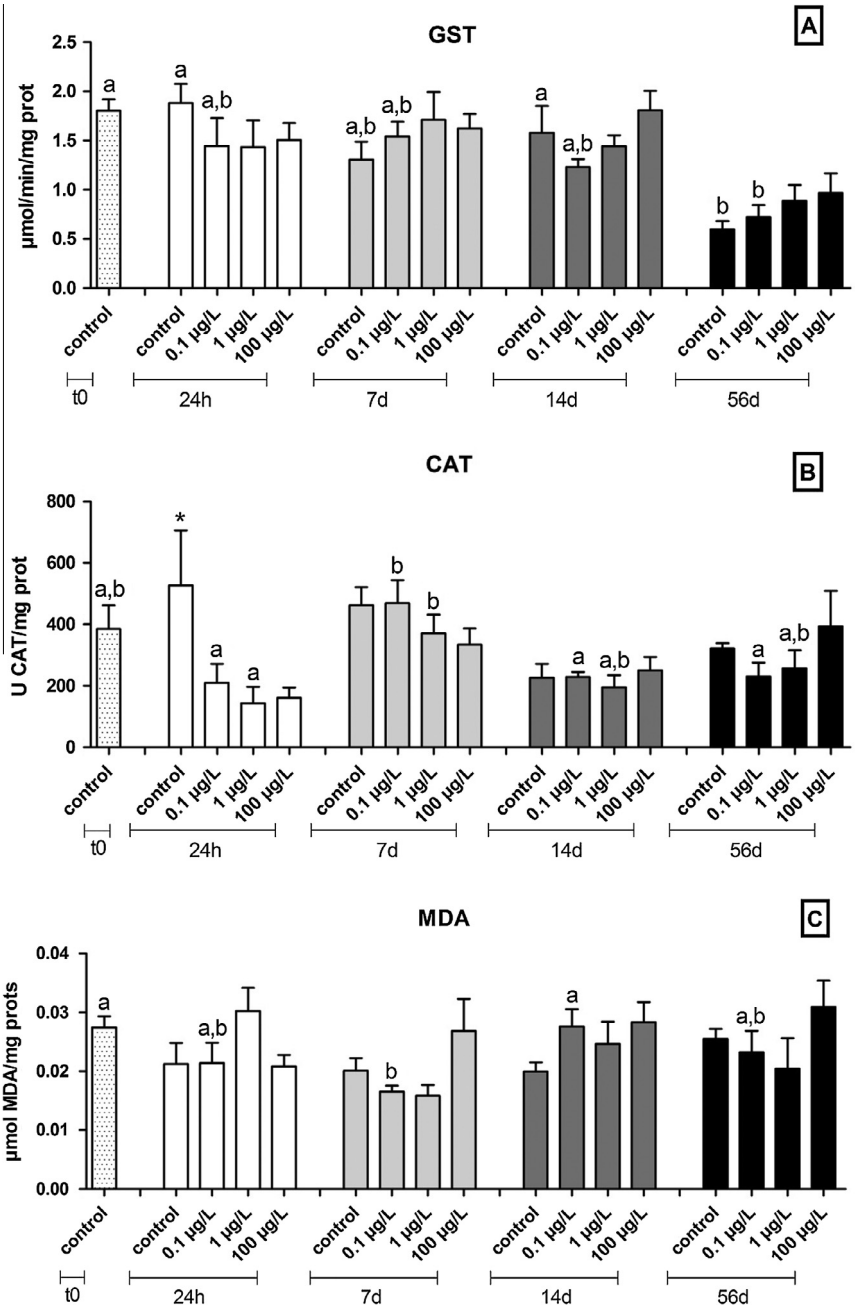

Fig. 4. Mean (±SEM) of biochemical endpoints studied in juvenile oysters at T0 and after 24 h, 7d, 14d and 56d of exposure to three concentrations of glyphosate: 0.1 µg L^{-1}, 1 µg L^{-1} and 100 µg L^{-1}. The studied endpoints are glutathione-S-transferase (GST) activities (A), catalase (CAT) activities (B) and malondialdehyde (MDA) content (C). Because of technical problems, data at T28d are lacking. If significant differences were recorded for temporal variation at a given concentration, letters are indicated and sampling dates that do not share a letter are significantly different. The only significant difference between concentration at a given date was recorded for CAT activities at T24 h and this difference is illustrated by *.

experiment in April, and for oysters from Normandy (France), it was not surprising because at this date, oysters had not yet benefited from the spring algal bloom. However, the connective tissue destructurations remained at this high level or even slightly increased after 56d of exposure. This result confirmed that the oysters failed to build up their reserve tissue again despite the condition (control or three glyphosate concentrations). However, we observed that oysters fed and produced faeces, confirmed by the index relative to the atrophies of the wall of the digestive tubules. Indeed, the values of this index were always low (with a maximum of 1.56 for the oysters exposed to 1 µg L^{-1}), whereas atrophy of digestive tubule walls is pronounced in starving or fasting individuals (Morton, 1977; Winstead, 1995). The condition index (corresponding to the ratio of the meat weight to total weight) slightly decreased during the experiment but only the oysters exposed to the lowest concentration (0.1 µg L^{-1}) showed a significant decrease. This quite surprising result deserves to be verified in a further study. Because the shell size (length) did not significantly increase, this condition index decrease could illustrate a meat weight loss, which would be prejudicial to oysters. Low condition indices have been reported in various marine bivalve species inhabiting or transplanted in polluted sites such as *M. arenaria*, *Mytilus edulis* (McDowell et al., 1999) and *C. virginica* (Scott et al., 2002). An experimental study by Greco et al. (2011) indicated an

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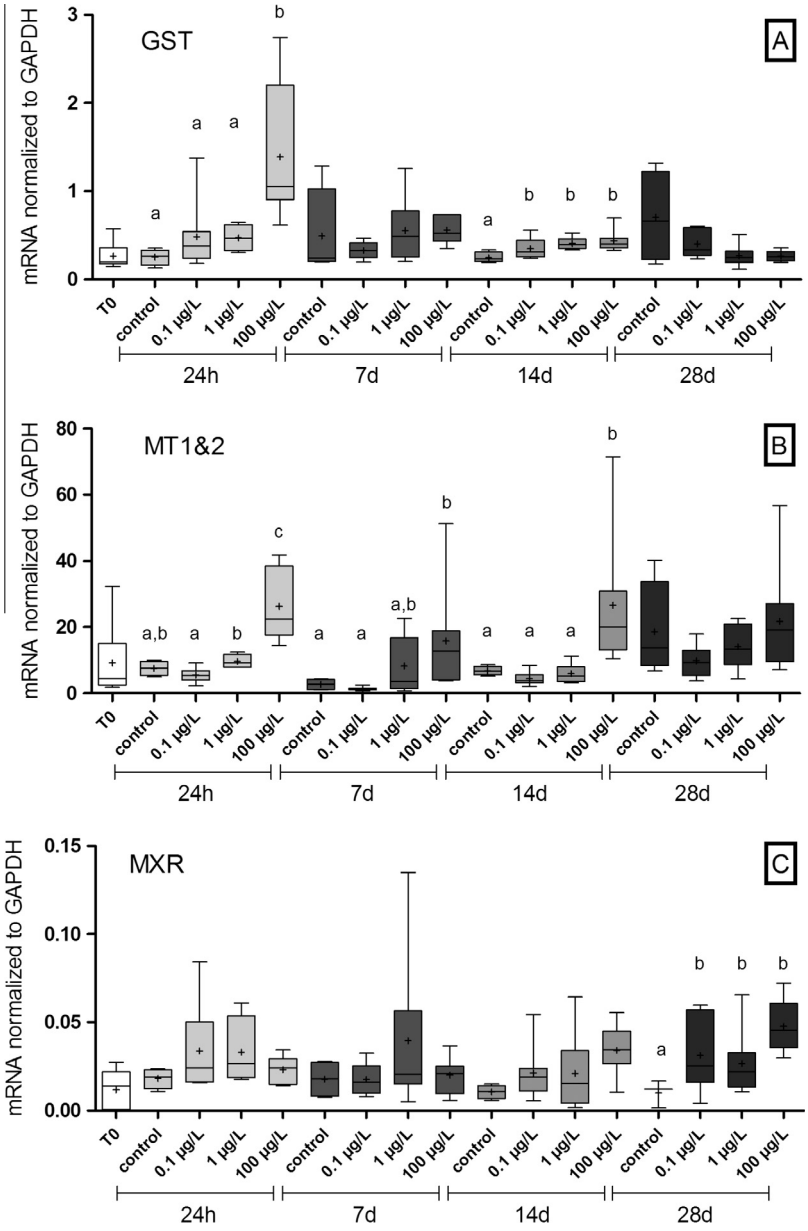

Fig. 5. Transcriptional expression levels of genes encoding for glutathione-S-transferase (GST) (A), metallothionein (MT1 and MT2) (B) and multi-xenobiotic resistance (MXR) (C) in *C. gigas* juveniles sampled after 0, 24 h, 7d, 14d and 28d of exposure to glyphosate at three concentrations:0.1 µg L^{-1}, 1 µg L^{-1} and 100 µg L^{-1}. Data are normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Statistics are given for each sampling time separately. The concentrations that do not share a letter are significantly different. Data are illustrated with a box and whisker diagram: the bottom and top of the box correspond to the 25th and 75th percentile, the band near the middle of the box represents the median, and the black cross is used to represent the mean. Finally, the ends of the whiskers represent the minimum and the maximum of all of the data for a given concentration. Because of technical problems, data at T0 and T56d are lacking.

enhancement of several endpoints including the condition index in *M. arenaria* acclimated at 7 °C and exposed to a mixture of herbicides (2,4-D; mecoprop and dicamba); however, this condition index increase was not significant for oysters exposed to the same mixture but placed at 18 °C.

Atrophy of the digestive tubule walls and destructuration of the connective tissue can be clearly related to the oyster physiological status, whereas hemocytic infiltrations are more in line with histopathology (except during the post-spawning period when haemocytes are concentrated in gonadal tubules and residual gametes are resorbed). At the beginning of the study, we observed that the mean level of hemocytic infiltration was rather high and increased during the course of experiment in all oyster groups. At T56d, hemocytic infiltrations in exposed but not control oysters were significantly more severe compared to the level recorded at T0. However, at T56d, no significant differences were calculated between the four conditions (control and three glyphosate concentrations). The histopathology approach can be considered as a long-term biomarker, and it cannot be excluded that differences would become significant if the exposure was longer than 56 d. Without a link to an infection by pathogens, hemocytic infiltrations into connective tissue could be related to chemical contamination in *M. edulis* (Auffret, 1988) and the hard clam *Mercenaria mercenaria* (Nasci et al., 1999). In other cases (e.g., De Los Ríos et al., 2012), hemocytic infiltrations were not more frequent and/or marked in marine bivalves from polluted sites, and Au (2004) reported that the cause and effect relationships between histopathological alterations and pollutant exposure were not fully understood.

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At the beginning of the experiment on the 11th of April, the majority of the studied oysters were in the sexual resting stage (stage 0) and the other oysters were in stage I, corresponding to gonial mitoses. The experimental conditions allowed the oysters to progress in the course of gametogenesis, and after the 56-d exposure, the oysters were in stages I or (beginning or more advanced) II. We did not observe histological abnormalities such as gamete degenerations or gonad necrosis. No significant differences were observed between the proportions of stage I versus stage II in the oysters maintained in the four conditions, but it is noticeable that the exposed oysters appeared slightly more advanced in gametogenesis by comparison with control oysters. In older individuals of *C. gigas* exposed to diuron (substituted urea herbicide) for 7 days, Buisson and collaborators (2008) reported an acceleration of the maturation processes and a stimulation of spawning. Similarly, *M. arenaria* placed at 7 °C showed an increased gametogenesis after an exposure to an herbicide mixture for 14 days (Greco et al., 2011). These authors hypothesised that the observed increase in the rate of metabolism could be related to an increase in energy demand because of pesticide exposure. This hypothesis was supported by Gagné and collaborators' (2007) study that reported that pollution could enhance the metabolism rate in clams for the maintenance of homeostasis and integrity.

4.2. Activities of enzymes involved in detoxification and oxidative stress regulation

Catalases correspond to a family of antioxidant enzymes involved in the dismutation of hydrogen peroxide. In the present study, catalase activities were found to be significantly lower in exposed oysters compared to the control group at T24 h. Inhibition of catalases has already been mentioned in fish exposed to metals such as copper and cadmium (Roméo et al., 2000; Radhakrishnan, 2009) and in earthworms exposed to a mixture of herbicides (Schreck et al., 2008). In molluscs, Greco and collaborators (2011) observed an inhibition of catalase activities after a 28-d exposure of *M. arenaria* to a 10 $\mu g\,L^{-1}$ mixture of 2-4 D, mecoprop and dicamba. Toxic effects of pollutants often depend on their capacity to generate ROS (Viarengo et al., 2007). Kono and Fridovich (1982) demonstrated that catalases could be converted into inactive compounds and then inhibited by the presence of excess superoxide anion (O_2^-). Besides this inhibition at T24 h, no additional significant effects were observed at a given date and from T7d, catalases recovered an activity similar to that of control oysters. Similarly, no changes were observed in the mussel *Limnoperna fortunei* exposed for 26 days to glyphosate at concentration ranging from 1000 to 6000 $\mu g\,L^{-1}$ (Iummato et al., 2013). For a given concentration throughout the experiment, the only significant differences were observed at 0.1 and 1 $\mu g\,L^{-1}$ with higher CAT activities at T7d. However, they were similar to CAT activities recorded in the control group at this sampling date.

Malondialdehyde (MDA) is the final product of lipid peroxidation resulting from free radical reactions in biological membranes, which are rich in polyunsaturated fatty acids. Despite the catalase inhibition observed at T24 h, the quantity of MDA was not statistically increased at this date, suggesting that catalases are not the only enzymes implicated in oxidative stress regulation. At a given date, no significant differences were recorded between control and exposed oysters. By contrast, Iummato collaborators (2013) reported a high level of lipidic peroxidation in mussels exposed to the glyphosate concentration of 3000 $\mu g\,L^{-1}$ (30-fold higher than our highest concentration) after 26d exposure. Regards to temporal variation, the only significant differences were observed between the oysters exposed to the lowest concentration (0.1 $\mu g\,L^{-1}$) at T7d and 1) this oyster group at T14d and 2) the control group at T0. Nevertheless, at these two dates, the amounts of MDA did not differ significantly between control individuals and the oysters exposed to 0.1 $\mu g\,L^{-1}$ of glyphosate suggesting that the lower quantity of MDA at T7d could not be easily related to glyphosate exposure.

Glutathione S-transferases (GST) are crucial enzymes of the cellular detoxification system (phase II enzymes). They are involved in the catalysis of glutathione with xenobiotics or compounds previously modified by phase I enzymes (to make them more hydrosoluble and then easily removed by organisms) (Chatterjee and Bhattacharya, 1984). Our results showed that glyphosate had no significant effects on the GST activities for the first 14 days. Similarly, Alves and collaborators (2002) reported no changes of GST (and CAT) activities in the mussel *P. perna* exposed to furadan, a carbamate insecticide for 96 h. However, in the present study, all of the GST activities decreased at T56d, but the temporal differences were significant only for the two lowest concentrations of glyphosate. The results recorded at T56d could not reflect an inhibition of the detoxification machinery by glyphosate because control oysters also showed the same trend. Data from the literature often demonstrated an increase in GST activities after exposures to different types of contaminants, for example, in *Elliptio complanata* exposed to municipal effluents (Farcy et al., 2011) or in *C. gigas* juveniles exposed to carbofuran (Damiens et al., 2004). GST activities in *L. fortunei* were higher in individuals exposed to 1 $mg\,L^{-1}$ of glyphosate for 26 days (Iummato et al., 2013).

Glyphosate had very moderate effects on the studied enzymatic activities and lipid peroxidation in our experimental conditions. However, the enzymatic biomarkers were found to be sensitive endpoints after exposure of bivalves to herbicides (Greco et al., 2011; Iummato et al., 2013). In future experiments, it would be interesting to study other enzymatic biomarkers of oxidative stress regulation such as superoxide dismutase (SOD). This enzyme is involved in the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide and therefore acts upstream from CAT activities in antioxidant enzymes. Despite the link between these two enzymes, they are not always correlated as described by Iummato et al. (2013). In fact, in this study on *L. fortunei* exposed to glyphosate, it was shown that a decrease occurred in SOD activity without changes in CAT activities.

4.3. Levels of mRNA expression

The RT-PCR used to study the expression of target genes is commonly recognised to be a sensitive (small amount of RNA needed), precise and reproducible method. However, even if a first normalisation step occurs with the use of 5 ng of cDNA for each sample, a normalisation step using "housekeeping" genes is still needed and could lead to potential bias. The synthesis of these genes is considered to occur in all nucleated cell types because they are necessary for cell survival (Thellin et al., 1999). However, the expression of these genes may vary according to various parameters including seasonality (Farcy et al., 2007) or pesticide exposure (Tanguy et al., 2005a). In this study, no significant differences between the different groups were detected in the expression of reference genes within the same time of exposure. Furthermore, the use of three different genes for normalisation (i.e., actin, GAPDH and 18S RNA) allowed us to obtain robust results.

The increased expression of GSTs after the 24-h exposure to the highest concentration of glyphosate (100 $\mu g\,L^{-1}$) suggests that early defence mechanisms were put in place after herbicide exposure. The increase in GST expression in oysters exposed to glyphosate has previously been reported by Boutet and collaborators (2004). These authors showed that GST sigma, which is the GST class targeted in this study, was over-expressed in the digestive gland of oysters exposed to 2 $\mu g\,L^{-1}$ of glyphosate for 30 days.

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The kinetics of the response of GST expression after exposure to glyphosate was not similar in our study, but it appeared that the GST sigma mRNA level could be a good marker of pesticide exposure. Nevertheless, the increase in the GST mRNA level was not observed at the enzymatic level. Furthermore, the over-expression of GST recorded after the 14-d exposure for all of the glyphosate concentrations was weak, and we could hypothesise that other defence mechanisms were put in place from 24 h of exposure to face the glyphosate contamination. For example, metallothionein mRNA levels were higher in the digestive gland of oysters exposed to 100 $\mu g\,L^{-1}$ glyphosate compared to the control group and remained significantly higher after the 7-d and 14-d exposure. The metallothioneins are involved in many mechanisms and are essential in metal sequestration, detoxification and oxidative stress regulation (Roesijadi et al., 1997; Anderson et al., 1999; Leung and Furness, 2001). Transcriptional induction of metallothioneins has been previously described in bivalves from different studies: Fang et al. (2013) after exposure of *Mactra veneriformis* to cadmium and mercury for 7 days; Devos et al. (2012) after chronic exposure of oyster (*C. gigas*) larvae to zinc; and Ivanković et al. (2010) in zebra mussels (*Dreissena polymorpha*) exposed to cadmium for a period of 1 week. The induction of metallothioneins has also been demonstrated after exposure to various categories of pesticides. Ceyhun et al. (2012) and Erdoğan et al. (2011) showed transcriptional induction of metallothioneins MT-A and MT-B after exposure of trout (*O. mykiss*) to deltamethrin (insecticide) and dichlorvos (acaricide) for 21 and 30 days, respectively.

At 28-d of exposure, statistical tests indicate that only MXR (multi-xenobiotic resistance) was differentially expressed in the studied samples. The MXR equivalent in mammals is the MDR system (multi-drug resistance), which encodes for a glycosylated membrane protein (P-glycoprotein) and can carry a wide variety of compounds (Endicott and Ling, 1989). The MXR system is considered a defence mechanism as part of the general response to organic contaminants (Eufemia and Epel, 1998). Eufemia and Epel (1998) have reported that MXR could be induced at the protein level in *M. edulis* by different compounds such as chlorthal-dimethyl (herbicide) or DDE, a manufacturing by-product of the DTT insecticide.

RT-qPCR allowed us to precisely determine the variation in the expression of target genes. However, no differences in the level of mRNA expression were observed between exposed and control oysters for SOD, CYP450, Hsc72, Hsp70, Hsp90 and p53. These stress markers were selected on the basis of literature concerning cellular functions expected to be involved in response to xenobiotics: SOD (Gagnaire et al., 2007), chaperone proteins (Eder et al., 2007; Snyder et al., 2001), CYP450 (Oropeza-Hernandez et al., 2003; Hanioka et al., 1998) and P53 (Saint-Jean et al., 2005; Farcy et al., 2008). Glyphosate does not appear to induce oxidative stress since no differences were observed for SOD mRNA level. The lack of differences in the mRNA level of chaperone genes may show that glyphosate would not induce protein unfolding, misfolding or aggregation. The absence of alteration in CYP450 (i.e. CYP2E1) seems to indicate that glyphosate is not a substrate neither an inducer nor an inhibitor of this CYP450 isoform. Finally, the lack of differences in P53 level suggested that no perturbations of the cell cycle occurred during the exposure to glyphosate in our experimental conditions.

Glyphosate subchronic exposure did not lead to any mortality, and no or few adverse effects were recorded at the individual level. The experimental conditions allowed the oysters to progress in the course of gametogenesis but not to grow in weight and length. The failure to build up energy reserves was illustrated by relatively high index of connective tissue destructuration in addition to an increased index of hemocytic infiltration, especially in exposed oysters. Regarding the studied biochemical endpoints, the main result was a transient inhibition of catalase activities after 24 h exposure in exposed oysters and, then no significant differences were recorded between conditions at a given sampling date. Nevertheless, defence mechanisms appeared to be set up at the molecular level in individuals exposed to the highest concentrations of herbicide. It can be hypothesised that the low toxicity of this molecule comes from its chemical properties (high water solubility, low bioaccumulation potential) that do not allow it to bioaccumulate in cells. Some results have to be confirmed in a further study and finally it will be necessary to study not only the active matter but also commercial formulations to yield stronger conclusions regarding the toxicity of glyphosate on marine bivalves. Indeed, in these formulations, adjuvants are used to promote the penetration of the active ingredient (glyphosate) into the plant cuticle, and several studies have shown that these formulations are much more toxic due to surfactants.

Acknowledgments

We gratefully acknowledge the staff of the laboratory UMR BOREA in the University of Caen and especially Béatrice Adeline. We are also grateful to our colleagues of the "Chronexpo scientific community" for fruitful discussions. We thank Dr. Aude Jouaux, who quantified viral loads in oysters at the "Centre de Référence sur l'Huître" (CRH). We also thank the SATMAR team, who kindly provided us juvenile oysters and the "Centre de Recherche en Environnement Côtier" (CREC) in Luc-sur-Mer that provided technical facilities. This study was supported by the Region Basse-Normandie (France) (Grant 09P00702) and the European Program Interreg IVA Chronexpo. Finally, we would like to thank the anonymous reviewers for their constructive comments for improving the manuscript.

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