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Sub-lethal effects of a glyphosate-based commercial formulation and adjuvants on juvenile oysters (*Crassostrea gigas*) exposed for 35 days

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ABSTRACT

Glyphosate-based herbicides include active matter and adjuvants (e.g. polyethoxylated tallow amines, POEAs). In addition to a previous investigation on the effect of glyphosate on oysters, the aim of the present study was to investigate the effects of sub-chronic exposures (35 days) to three concentrations (0.1, 1 and 100 μg L^{-1}) of Roundup Express® (R_{EX}) and POEAs on oysters belonging to the same age group. Low mortality rates were calculated, and only few significant differences (i.e. shell length) between exposure conditions were observed at a given date. However, when comparing the biomarker's temporal variations, some different patterns (e.g. condition index, reproduction, parameters of oxidative stress) were observed depending on the molecules and concentrations. These results suggest that a longer exposure to an environmental concentration (0.1 μg L^{-1}) of R_{EX} and POEAs could induce harmful effects on oysters.

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1. Introduction

Bivalves such as oysters are considered ideal bio-indicators because of their wide geographical distribution, sedentary lifestyle and sensitivity to environmental pollutants (sub-lethal effects) (Renault, 2011). Among them, the Pacific oyster, *Crassostrea gigas*, is the second main species cultivated throughout the world after the clam *Ruditapes philippinarum* (FAO, 2015). In Europe, oysters are mainly reared by using off-bottom cultures in the intertidal zone (Buestel et al., 2009). This rearing location offers many advantages including easy access to cultures and the trophic richness allowing a rapid oyster growth. However, this location also has the disadvantage of being submitted to various pollutants from terrestrial inputs and marine activities. Indeed, estuaries and coastal habitats are continuously subjected to important xenobiotic inputs resulting from agriculture, industrialisation, high population density and heavy shipping traffic. Therefore, there are growing concerns regarding the adverse effects of various pollutants including pesticides on non-target aquatic organisms, particularly littoral species inhabiting the ultimate receptacle for waste. Nevertheless, the level of contamination of marine waters by pesticides including herbicides is poorly documented in Europe, but some data from French fishing and shellfish farming areas are available because of the great economic importance of these activities including oyster farming in this country (Robert et al., 2013). In the central North Sea, 20 representatives of six chemical classes of currently used pesticides (e.g. triazine, thiadiazine, dinitroaniline and chloroacetanilide herbicides) were reported by Mai et al. (2013) in 2009 and 2010, but the concentrations were generally very low (few pg L^{-1} with a maximum of 1.27 ng L^{-1} for atrazine). In a shellfish area located in Normandy (north-west France), no herbicides were detected in oyster flesh, but six of the 15 investigated herbicides were quantified in seawater, and a maximum value of 132 ng L^{-1} was recorded for diuron (Buisson et al., 2008). Pesticides in Arcachon Bay (south-west France) showed higher concentrations, with the values of insecticides reaching 270 ng L^{-1} of bifentrine and 3 ng g^{-1} of permetrine in the flesh of oysters (Auby et al., 2007). Using passive samplers (POCIS), Munaron et al. (2012) highlighted the contamination of Mediterranean lagoons and coastal waters by 15 herbicides and a biocide out of the 27 pesticides analysed. The three studies mentioned above did not investigate glyphosate because this herbicide requires a particular analysis procedure and is thus not routinely sought in marine waters. As a consequence, data about the concentrations of glyphosate in coastal waters remain very scarce (Mercurio et al., 2014). Burgeot et al. (2008) reported the following data: in the Seudre estuary (France),

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the concentrations of total pesticides exceeding 500 ng L^{-1} with peaks of 100 ng L^{-1} of glyphosate were detected in 2003, whereas the highest glyphosate concentration was as high as 1.2 μg L^{-1} in 2004. Mercurio et al. (2014) showed that glyphosate is moderately persistent in marine waters under low light conditions (47 days at 25 °C); they added that the persistence of this herbicide may be affected by the toxicity of formulation surfactants on microbial communities, but data on co-occurrence with glyphosate in the field is lacking.

Glyphosate-based herbicides are used in agricultural, silvicultural and urban environments (Hanke et al., 2010), and they are currently the most commonly used herbicides in the world (Howe et al., 2004; European Commission, 2007; Benbrook, 2016). These pesticides are sold as non-selective, broad-spectrum and post-emergent herbicides that inhibit the plant growth by interfering with the biosynthetic pathway of the essential aromatic amino acids (Williams et al., 2000; Monheit, 2003). This biosynthetic pathway is not shared by animals, and glyphosate-based herbicides were thus reported to be relatively non-toxic to terrestrial and aquatic fauna. Indeed, the effects of glyphosate on aquatic organisms are generally considered to be low to moderate (Giesy et al., 2000; Williams et al., 2000; Monheit, 2003; PPDB, 2016). In molluscs, values of LC_{50} (lethal concentration for 50% of the individuals of a given population) exceeding 10 mg L^{-1} were calculated in the clam *R. decussatus* exposed to glyphosate for 96 h (Elandalloussi et al., 2008) and in the oyster *Crassostrea virginica* after 48 h of exposure (WHO, 1994).

In commercial formulations such as Roundup Express® (R_{EX}), adjuvant compounds are added to the active ingredient to improve the efficiency of the commercial product, for example, by promoting the penetration of the active ingredient into plant cuticle. These adjuvants are generally considered biologically inert by manufacturers and protection agencies, with no or few consequences in terms of ecotoxicology. In R_{EX}, the most common adjuvants (i.e. surfactants) in glyphosate-based formulations are polyethoxylated tallow amines (POEAs) in the proportion of 10–20% (Howe et al., 2004; Pérez et al., 2011). POEAs are characterised by the ratio of oxide:tallow amine ranging from 5:1 to 25:1 (Brausch and Smith, 2007). A great variety of glyphosate-based herbicides are sold worldwide, and some of these commercial formulations are ready to use. Among them, R_{EX} (Monsanto Company, St. Louis, MO, USA) was chosen because it is widely marketed in the French garden centres. Legislation on pesticides is mainly based on active molecules; however, various studies have shown that commercial formulations are more toxic than active ingredients (e.g. Folmar et al., 1979; Tsui and Chu, 2003; Howe et al., 2004; Bringolf et al., 2007; Moore et al., 2012). The higher toxicity of commercial formulations has been reported to be due to the addition of surfactants. In addition to acute ecotoxicity tests (e.g. Brausch et al., 2007; Tsui and Chu, 2003), some studies more rarely investigated the chronic or sub-chronic (repeated exposures or exposures for several weeks or months) effects of pesticides and/or surfactants on aquatic organisms. Bringolf et al. (2007) conducted acute toxicity tests through 'chronic tests' that consisted of studying the survival and growth of freshwater mussels, *Lampsilis siliquoidea*, for 28 days. The effect of glyphosate and cocktails of pesticides was also investigated in *C. gigas* by using biomolecular approaches (Tanguy et al., 2005; Collin et al., 2010).

Considering both the important use of glyphosate and the lack of data on the toxicity of this herbicide in non-target marine organisms, we previously assessed the toxicity of glyphosate in juvenile oysters (*C. gigas*) and showed low sub-lethal effects (Mottier et al., 2015). The present experiment conducted for 56 days was completed by exposing the oysters to R_{EX} and POEAs for a shorter period (35 days) but by maintaining improved feeding conditions. In the framework of the European Interreg IV project 'Chronexpo', the present study aimed to assess the effects of a sub-chronic exposure to R_{EX} and POEAs in yearling oysters by using individual, histological and biochemical biomarkers.

2. Materials and methods

2.1. Biological materials and chemical compounds

Juveniles of the Pacific oyster *C. gigas* (Thunberg, 1793) were obtained from the 'Société ATlantique de MARiculture' (SATMAR®; Barfleur, France) hatchery. At the beginning of the experiments, the studied oysters, produced from the fertilisation of wild-origin spawners, were 18 months old and averaged 2.12 g (±0.58) in weight and 28.28 mm (±3.78) in length. Since 2008 in France, significant mortalities have been observed in juvenile oyster livestocks in relation to OsHV-1 μVar. Therefore, 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) by using real-time TaqMan® PCR. Ten pools of five oysters were analysed, and all individual pools were revealed to be infected. However, the viral loads were low with a mean value of 4265 UGs (±4265 UGs). These viral loads were below the threshold (440,000 UGs) that is susceptible to trigger mortalities according to Oden et al. (2011).

R_{EX} contains adjuvants and 7.2 g L^{-1} of glyphosate acid (corresponding to 9.6 g L^{-1} of glyphosate isopropylamine salt, glyphosate IPA) as the active ingredient. In pesticides, the exact composition of the mixtures of adjuvants is confidential information, but according to Giesy et al. (2000) and Pérez et al. (2011), a mixture of POEAs synthesised from animal-derived fatty acids is the predominant surfactant used in glyphosate-based products; the adjuvant (corresponding to a non-ionic surfactant) is typically 15% or less of the formulation. In the present study, we tested a mixture of ethoxylated alkylamines (POE-15) provided by Dr. Ehrenstorfer GmbH® (Augsburg, Germany) (CAS number: 61791-26-2).

Glyphosate 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 for pH of 7 and 9, respectively) (Agritox, 2015; PPDB, 2016). Data on POEAs are scarce, but a range of aquatic half-life values are estimated to be from 21 to 42 days by Giesy et al. (2000). For R_{EX}, three exposure concentrations were chosen by considering the concentrations of glyphosate measured in aquatic ecosystems: 0.1 μg L^{-1} (considered as a realistic environmental concentration in estuarine and coastal environments), 1 μg L^{-1} (frequently measured in freshwater) and 100 μg L^{-1} (corresponding to concentration peaks in rivers). To our knowledge, no values of POEA concentration were available for aquatic ecosystems at the beginning of the study, and it was thus decided to test both molecules at the same concentrations to compare the potential toxicity of both molecules. The solutions of herbicides and adjuvants were prepared with natural sterilised open seawater (0.22 μm, Steritop® Millipore). We previously showed that nominal and measured concentrations of glyphosate were close and no differences appeared after 24 h in the exposure tanks (Mottier et al., 2015). Because of the chemical complexity of the POEA molecules, it was not possible to measure the concentrations of the adjuvants in the water of the experimental tanks.

2.2. Experimental design

Before the beginning of the experiment, 840 juvenile oysters were randomly distributed into 14 tanks (each condition in duplicate), and the bivalves were maintained during 1-week acclimatisation. In these tanks, the oyster batches were homogeneous in size and weight (ANOVAs, $p > 0.05$). Polypropylene tanks, which have previously been aged in seawater, were filled with 10 L of sterilised natural open seawater and were equipped with a sieve and air-driven filter. Total water removal occurred twice per week, and the water was partially (1/10) changed daily. The 1 L of removed water was replaced by 1 L of *Tetraselmis suecica* culture (maintained at a mean concentration of 2.09×10^6 cells mL^{-1}). This diet was supplemented by *Chaetoceros calcitrans* provided in the form of a frozen pulp (1.00×10^6 cells mL^{-1})

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defrosted just before using. The studied oysters were fed through a drip system, and finally, the daily ration was assessed to 8.86×10^8 algal cells per individual. After each water change, the R_{EX} and POEA concentrations were readjusted. Oysters were maintained at a temperature of 15.42 °C (±1.00) and a photoperiod of L:D 10:14 h. The experiment was conducted from 29 April to 3 June 2013, and oysters from all tanks were sampled at five dates: T0; T24h; and T7, T14 and T35d. At each sampling date and for each experimental condition, oysters were randomly and equally removed from the two duplicate tanks.

2.3. Studied biomarkers

2.3.1. Individual biomarkers

At the end of the exposures, cumulative mortality rates were calculated from the total number of individuals that died during the experiment. However, the oysters' population decreased because of samplings; therefore, daily mortality rates were also calculated. Mortality was thus checked daily to discard dead oysters, and daily mortality rates were calculated as follows: $(N_d / N_t) \times 100 / (T_2 - T_1)$, where N_d represents the number of dead oysters at T_2, N_t the number of live oysters at the beginning of the interval and $(T_2 - T_1)$ the interval between two successive countings (5 countings during the experiment).

At each sampling date, except at T24h, 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 sacrificed for other endpoints were also used to determine the AFNOR condition index (CI) calculated as follows: CI_{AFNOR} = (flesh weight / total weight) × 100.

2.3.2. Histological biomarkers

As histological biomarkers do not correspond to early biomarkers, they were studied only at the beginning and the end (T35d) of the experiment. As already described (Di Poi et al., 2016; Mottier et al., 2015), cross sections of oysters (~4 mm thick) were cut behind the labial palps and fixed in Davidson's solution. 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 advanced enough. Gametogenesis stage was determined according to Lubet's modified classification of microscopic observations; the three stages and sub-stages 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 poorly developed reserve tissue), haemocytic infiltrations in the connective tissue and atrophies of the wall of the digestive tubules (with lumen enlargement); the tissue disturbances were quantified according to an ordinal scale with four levels from slight to severe (Table 2) (for illustrations, see Di Poi et al., 2016; Mottier et al., 2015).

2.3.3. Enzymatic activities and markers of lipid peroxidation

2.3.3.1. Sample preparation. Biochemical biomarkers were studied 24 h after the beginning of the experiment, and then after 7 and 14 days of exposure. Immediately after collecting individuals in the experimental tanks, the digestive glands of six oysters per condition were individually homogenised using 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 (100×), Thermo Fisher Scientific® (Waltham, USA) in a 1:2 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 finally stored at −80 °C. The aliquots were used for determining the amount of proteins, the malondialdehyde (MDA) content and enzymatic activities.

2.3.3.2. Protein content. For enzymatic activities and lipid peroxidation, the results were normalised to the total protein content in supernatants. Protein contents were determined using the Bio-Rad Protein Assay kit (Bio-Rad®) according to the method by Bradford (1976). Samples were diluted 1:200 in PBS. Ten microlitres of the samples or bovine serum albumin was deposited in the microplate wells, and 200 μL of Bio-Rad reagent was added (diluted at 1:5 in ultrapure water). Absorbance was determined at 595 nm using a microplate reader (Flexstation 3, Molecular Device®). The results are expressed as mg total protein per mL (mg proteins mL^{-1}).

2.3.3.3. Enzymatic activities: glutathione-S-transferases and catalases. Catalases (CATs) and glutathione-S-transferases (GSTs) are enzymes involved in reactive oxygen species regulation and redox regulation at the cellular level (Regoli and Giuliani, 2014). Indeed, GSTs are crucial enzymes of the cellular detoxification system (phase II enzymes), and CATs are involved in the dismutation of hydrogen peroxide. The CAT activities were measured using the protocol described by Babo and Vasseur (1992) with some modifications to allow microplate reading by considering the decrease in absorbance due to decrease in hydrogen peroxide. Samples were diluted by 2400 in PBS. Next, 100 μL of the samples were deposited in UV microplate wells (UV star, Greiner Bio-One Gmbh®) and H_2O_2 was added. Kinetic measurements were made at 240 nm for 15 min at 25 °C in the microplate reader (Flexstation 3). The results are expressed in μmol of H_2O_2 min^{-1} mg $prot^{-1}$ corresponding to a CAT unity per mg of proteins (μmol min^{-1} mg^{-1} prot).

GST activities were measured according to Habig et al. (1974) (adapted for microplate reading) by increasing the absorbance by GS-DNB formation. Two microlitres of the sample was added to 178 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 were measured at 340 nm for 20 min at 25 °C in the microplate reader (Flexstation 3). The results are expressed as μmol of GS-DNB per min and mg of proteins (μmol min^{-1} mg^{-1} proteins).

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 maturity in male	Follicle maturity in female
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

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Table 2
Ordinal scales allowing 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%
Haemocytic infiltrations	5–10%	10–33%	33–50%	>50%

2.3.3.4. Lipid peroxidation: malondialdehyde content. The content of MDA, which corresponds to the final product of lipid peroxidation, is a marker of oxidative stress damage. Various kinds of environmental stress including contaminants may induce oxidative stress in organisms. The MDA contents were measured with an MDA-586 assay kit (Oxis-Research®) according to the manufacturer's instructions and the protocol from Gérard-Monnier et al. (1998). First, samples were diluted by a factor of 15, and 30 μL of the samples or MDA standard was then mixed on ice with 1.5 μL of probucol (used to minimise interference from other lipid peroxidation products such as 4-hydroxyalkenal), 96 μL of diluted R1 reagent (*N*-methyl-2-phenylindole in acetonitrile diluted by 1:4 in methanol), and 22.5 μL of R2 reagent (concentrated hydrochloric acid). After 60 min of incubation at 45 °C in a dry bath incubator, the samples were centrifuged at 10,000 × *g* for 10 min. Finally, 80 μL of the supernatants was then deposited in microplate wells. Endpoint measurements were made at 586 nm in the microplate reader (Flexstation 3). The results are expressed as quantity in μmol of MDA per mg of proteins (μmol MDA mg^{-1} proteins).

2.4. Statistical analyses

Differences recorded between sampling times (four dates) and conditions (control and three concentrations of R_{EX} or POEAs) were statistically tested. Data that did not show homoscedasticity and/or did not meet the assumption of normality were analysed with Kruskal-Wallis (K-W) non-parametric tests. 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. Mortality data (2 × 2 tables) were tested by Chi^2 tests by applying Yates' correction. The proportions of the different gametogenesis stages of the control and exposed oysters were tested with Fisher's exact tests. The statistical analyses were performed using STATISTICA 8.0 software (Statsoft®, Tulsa, OK, USA).

3. Results

3.1. Individual biomarkers: survival and growth

During the 35 days experiment, 19 individuals in total died: 2 control oysters, 4 individuals exposed to R_{EX} and 13 exposed to POEAs (mean daily mortality rates ranging from 0% to 0.20%; Table 3). It is notable that nine of these 19 individuals died during the first week. The mean cumulative mortality rates ranged from 0% (100 μg L^{-1} R_{EX}) to 4.17% (1 μg L^{-1} POEAs), and no significant differences (even for these extreme values: Chi^2 with Yates' correction; $p = 0.0768$) were calculated.

Oyster growth was followed throughout the experiment by considering shell length and whole weight. The shell of control oysters tended to increase between the beginning (28.66 mm ± 0.36) and the end (29.61 mm ± 0.57) of the experiment, whereas this tendency was not observed in the oysters exposed to R_{EX} or POEA regardless of the concentration (Fig. 1A). Therefore, after 35 days of exposure, the shell of the individuals exposed to the lowest and highest REX concentrations and those exposed to any of the three POEA concentrations were significantly smaller than that of the control oysters (ANOVA and SNK tests; $p < 0.05$). With regards to the whole weight, control oysters showed the highest increase during the experiment (from 2.11 ± 0.06 g to 2.26 ± 0.11 g: +7.11%), but this difference is not enough to be considered as a significant increase (Fig. 1B). The whole weight of control or exposed oysters did not significantly differ except that of oysters exposed to 0.1 μg L^{-1} R_{EX} compared to the weight of control individuals at T14d (1.92 g ± 0.08 versus 2.19 g ± 0.08, respectively).

CI illustrating the filling of the shell by flesh did not significantly differ between exposure conditions regardless of the sampling date (ANOVAs: $p > 0.05$) (Fig.1C). Nevertheless, this index was significantly higher after 35 days in controls (12.04 ± 0.32 versus 10.55 ± 0.35 at the beginning of the experiment) and oysters exposed to the three concentrations of R_{EX} (mean CI of 12.18 ± 0.23) (ANOVA and SNK: $p < 0.05$) but not in the individuals exposed to the three concentrations of POEAs: at T35d, $11.32 < CI < 11.62$ (ANOVAs: $0.0574 \leq p \leq 0.1341$).

3.2. Histological biomarkers: reproduction and histopathology

At the beginning of the experiment, all oysters had begun the gametogenesis cycle, and most of them (~77%) were in stage I (gonial mitosis) during which sex is impossible to be reliably determined (Fig. 2). After 35 days of exposure, all tested oysters progressed during gametogenesis, and the total percentages of female and male oysters were 29% and 27%, respectively (44% remaining sexually indeterminable). For example, the percentage of control individuals remaining in stage I was 27% and the percentage of oysters in the beginning of stage II (IIe) or in stage II was 36.5%. Nevertheless, the oysters exposed to 0.1 and 1 μg L^{-1} of R_{EX} and POEAs (but not to the highest concentration of both compounds) did not significantly differ from individuals at T0, suggesting a delay in gametogenesis because the individuals in sub-stages IIe and II were under-represented. Finally, when the proportions of oysters in stage I or in sub-stages II (IIe + II) at T35d were compared, no exposure conditions significantly differed from that of the controls (Fisher tests, $p > 0.05$), and the only significant difference was found between the oysters exposed to 1 and 100 μg L^{-1} R_{EX} (Fisher test, $p = 0.03$).

Three types of tissue alterations could be examined at T0 and T35d. Regarding atrophies of the wall of the digestive tubules, the severity level was moderated at T0 (mean level of 1.50 ± 0.16) (Fig. 3A). At

Table 3
Daily mortality rate (number of dead individuals per day ± SEM) and cumulative mortality rate (%) after 35 days of exposure in control individuals and oysters exposed to three concentrations of Roundup Express® (R_{EX}) or polyethoxylated long-chain alkylamines (POEAs).

	Control	R_{EX}			POEA		
Concentration (μg L^{-1})	0	0.1	1	100	0.1	1	100
Daily mortality rates (±SEM)	0.06 ± 0.04	0.15 ± 0.07	0.04 ± 0.04	0.00 ± 0.00	0.16 ± 0.09	0.14 ± 0.10	0.20 ± 0.14
Cumulative mortality (%)	1.67	2.50	0.83	0.00	3.33	4.17	3.33

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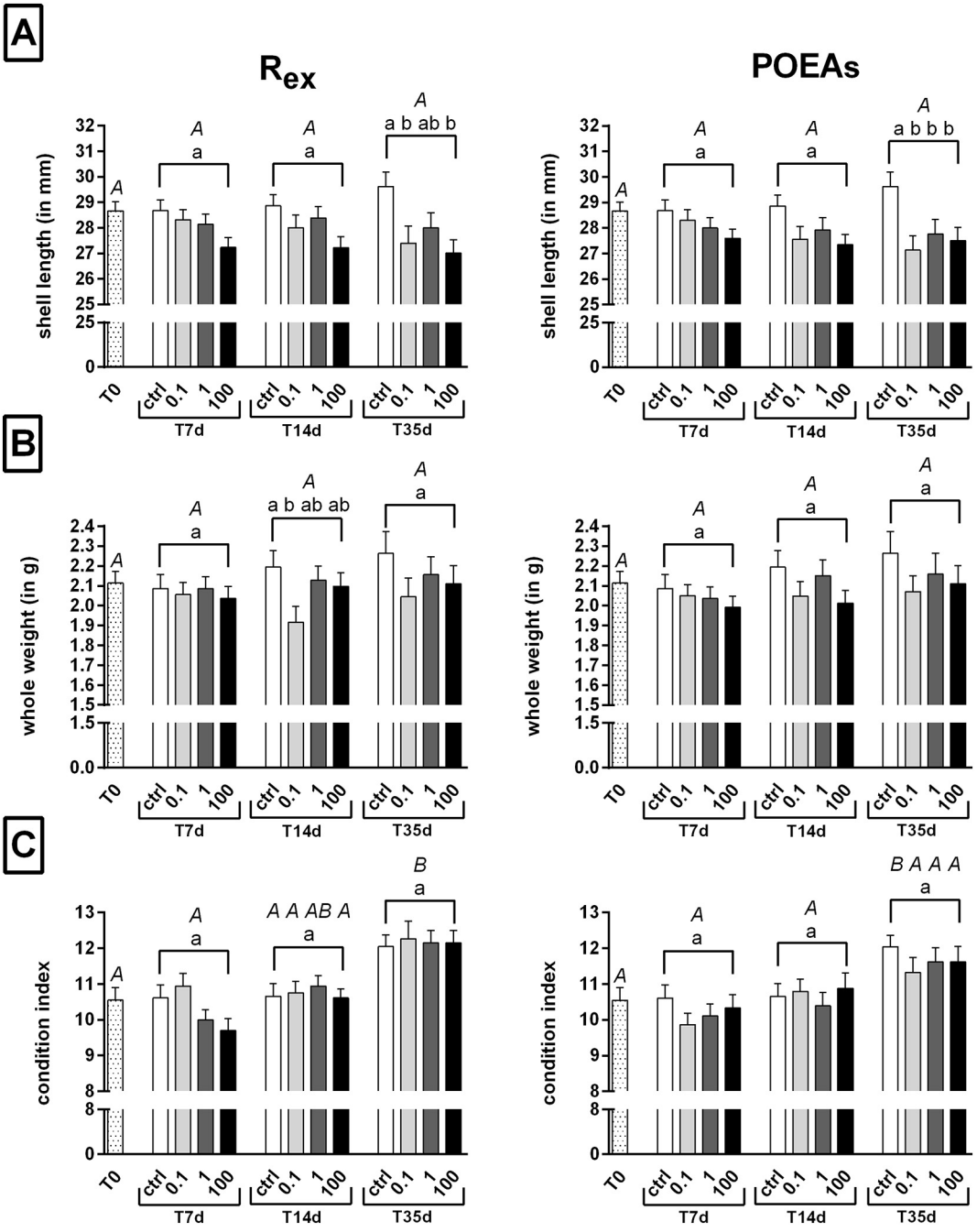

Fig. 1. Biometric parameters recorded in control individuals and oysters exposed to three concentrations of Roundup Express® (R_{EX}) or polyethoxylated long-chain alkylamines (POEAs) at T0 and three sampling dates: A: mean shell length (±SEM); B: mean whole weight (±SEM) and C: mean AFNOR condition index (±SEM). For shell length and whole weight (A and B), the mean oyster numbers per experimental condition were: n = 120 at T0, 86 at T7d, 62 at T14d and 38 at T35d; for AFNOR condition index (C), n = 55 at T0, 24 at T7d and T14d, and 32 < n < 44 at T35d. Groups that do not share a common letter are significantly different. Letters in lowercase illustrate significant differences ($p < 0.05$) between experimental conditions at a given sampling date; letters in uppercase and italics illustrate significant differences ($p < 0.05$) between sampling dates for a given experimental condition.

the end of the exposures (T35d), these atrophies were less pronounced, especially in control individuals and oysters exposed to 0.1 and 100 µg L^{-1} of R_{EX} and 1 µg L^{-1} of POEAs, significant differences being revealed by K-W test followed by Dunn tests ($p < 0.001$). The mean values of the connective tissue destructurations ranged from 1.73 (±0.17) in control individuals at T0 to 2.81 (±0.29) and 3.00 (±0.42) in oysters exposed for 35 days to intermediate concentrations of R_{EX} and POEAs, respectively (Fig. 3B). At T35d, a significant difference was recorded between conditions (K-W: $p = 0.021$), but Dunn tests did not reveal different homogeneous groups. Minimal (1.09 ± 0.31) and maximum (2.20 ± 0.18) levels of haemocytic infiltrations were observed in control oysters at T35d and T0, respectively (Fig. 3C). All the calculated values indicated a slight or moderate level of haemocytic infiltration, which did not significantly differ according to control or exposure conditions (K-W test: $p = 0.53$).

3.3. Enzyme activities and lipid peroxidation

Protein and MDA contents and enzymatic activities were measured from T0 to T14d. Mean protein content ranged from 16.20 ± 2.58 g L^{-1} (control at T0) to 25.83 ± 3.91 g L^{-1} (1 µg L^{-1} POEAs at T7d), and no significant differences were detected regardless of the sampling dates and exposure conditions (*data not shown*) (ANOVAs: $p > 0.05$). The values of CAT activities ranged from 29.31 ±

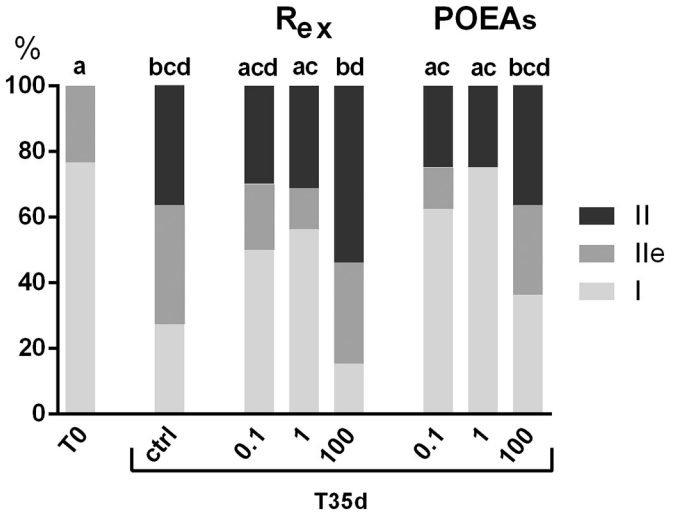

Fig. 2. Percentage of the three oyster gametogenesis stages and sub-stages [I, IIe (e = early) and II (*for stage description*, see Table 1)] observed at T0 and after 35 days of exposure to three concentrations of Roundup Express® (R_{EX}) or polyethoxylated long-chain alkylamines (POEAs). To test the inter-group differences, the gametogenesis sub-stages (II) were pooled, and Fisher's tests were applied. The number of studied oysters was n = 30 at T0 and 11 (on average) at T35d. Groups that do not share a common letter are significantly different.

2.41 μmol min^{-1} mg^{-1} prot (0.1 μg L^{-1} POEAs at T7d) to 55.78 ± 10.36 μmol min^{-1} mg^{-1} prot (1 μg L^{-1} R_{EX} at T24h) (Fig. 4A). Similar to the protein contents, CAT activities did not show significant differences with sampling dates or exposure conditions (ANOVAs: $0.08 \leq p \leq 0.87$).

Compared to CATs, GST activities were more variable with the minimum and maximum average values of 11.2 ± 2.4 nmol min^{-1} mg $prot^{-1}$ (0.1 μg L^{-1} R_{EX} at T7d) and 35.1 ± 6.0 nmol min^{-1} mg $prot^{-1}$ (0.1 μg L^{-1} POEAs at T7d), respectively (Fig. 4B). When examining temporal variations, significant differences in GST activities were detected only in oysters exposed to the lowest concentration of POEAs (ANOVA, $p = 0.01$); indeed, individuals sampled at T7d showed significantly higher GST activities than those sampled at the three other dates ($p < 0.05$) (SNK tests). However, POEAs did not induce significant differences in the GST activities regardless of the sampling date (ANOVAs or K-W tests: $0.23 \leq p \leq 0.94$). In contrast, the oysters exposed to the three concentrations of R_{EX} did not show significant temporal variations in GST activities, but at T7d, the GST activities of the individuals exposed to 0.1 μg L^{-1} of R_{EX} were significantly lower than those of the individuals exposed to 100 μg L^{-1} of R_{EX} (SNK test: $p = 0.04$).

MDA content showed the highest levels at the beginning of the experiment, with values reaching 13.77 ± 2.56 mmol mg $prot^{-1}$ at T0 and 14.34 ± 2.54 mmol mg $prot^{-1}$ in oysters exposed to 0.1 μg L^{-1} R_{EX} after 24 h (Fig. 4C). Nevertheless, the control group did not show significant temporal variations in MDA content (ANOVA: $p = 0.11$). In contrast, this index of lipid peroxidation significantly decreased from T7d regardless of the concentration of R_{EX}. (ANOVAs or K-W tests followed by SNK or Dunn tests: $p < 0.05$). During POEA exposures, MDA content also significantly decreased but only in animals exposed to the concentrations of 0.1 and 1 μg L^{-1} from T7d and T14d, respectively. However, at each sampling date, no significant differences were calculated between the four conditions (ANOVAs or K-W tests: $0.08 < p < 0.84$), suggesting no effects of R_{EX} and POEAs on MDA content.

4. Discussion

4.1. Effects of glyphosate-based formulations and POEAs on lipid peroxidation and enzymes involved in detoxification and oxidative stress regulation

MDA content and CAT and GST activities are commonly included in a battery of biomarkers in toxicology, ecotoxicology and biomonitoring surveys (e.g. Viarengo et al., 2007; Iummato et al., 2013; Nahrgang et al., 2013). Because of an extensive literature, the discussion is focused on the effects of commercial formulations of adjuvants and pesticide on various organisms in comparison with the effect of the active substances in the pesticide when data are also available. Studies relative to aquatic organisms including bivalves are especially considered.

In the present study, CAT activities in the digestive gland of oysters did not significantly vary between exposure conditions, and temporal fluctuations were non-significant. It was only shown that the CAT activities of the oysters exposed to 0.1 μg L^{-1} of POEAs tended to be lower at T7d than that at T24h and T14d ($p = 0.0799$). In a previous study, we showed that similar concentrations of glyphosate also had low effect on CAT activities in oysters belonging to a similar age class (Mottier et al., 2015). Indeed, significant temporal variations (over a period of 56d) were only observed at the two intermediate concentrations of glyphosate (0.1 and 1 μg L^{-1}), and at a given date, only one significant difference was recorded at T24h when control oysters showed higher CAT activities than those of exposed individuals. Such results suggest that glyphosate-based herbicides had no effects or non-dose-effect responses at concentrations and durations tested in our two studies. According to Viarengo et al. (2007), the antioxidant enzyme response to toxic chemicals generally shows a bell-shaped trend, with an initial increase due to the activation of enzyme synthesis followed by a decrease in enzymatic activity (due to the enhanced catabolic rate and/or a direct inhibitory action of toxic chemicals on the enzymes); hence, these authors added that enzyme assays should be used in association with other biomarkers. Similarly, for CAT activities, we did not find significant differences in MDA content regardless of the exposure condition and analysis date. Moreover, the two highest levels of MDA were

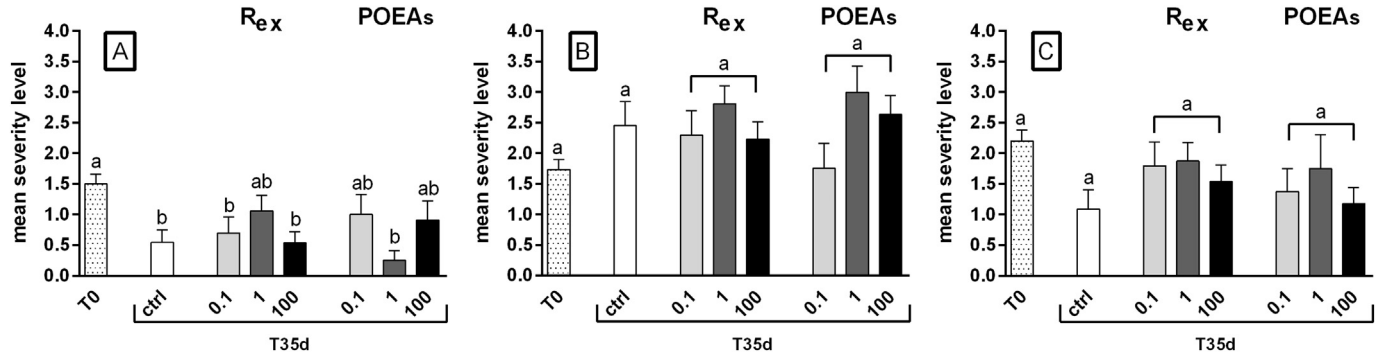

Fig. 3. Mean (±SEM) level of tissue alterations observed in control oysters at T0 and T35d and in individuals exposed for 35 days to three concentrations of Roundup Express® (R_{EX}) or polyethoxylated long-chain alkylamines (POEAs): A: atrophies of the wall of the digestive tubules; B: destructuration of the connective tissue (indicating a poorly developed reserve tissue) and C: haemocytic infiltrations of the connective tissue. The number of studied oysters was n = 30 at T0 and 11 (on average) at T35d. Groups that do not share a common letter are significantly different.

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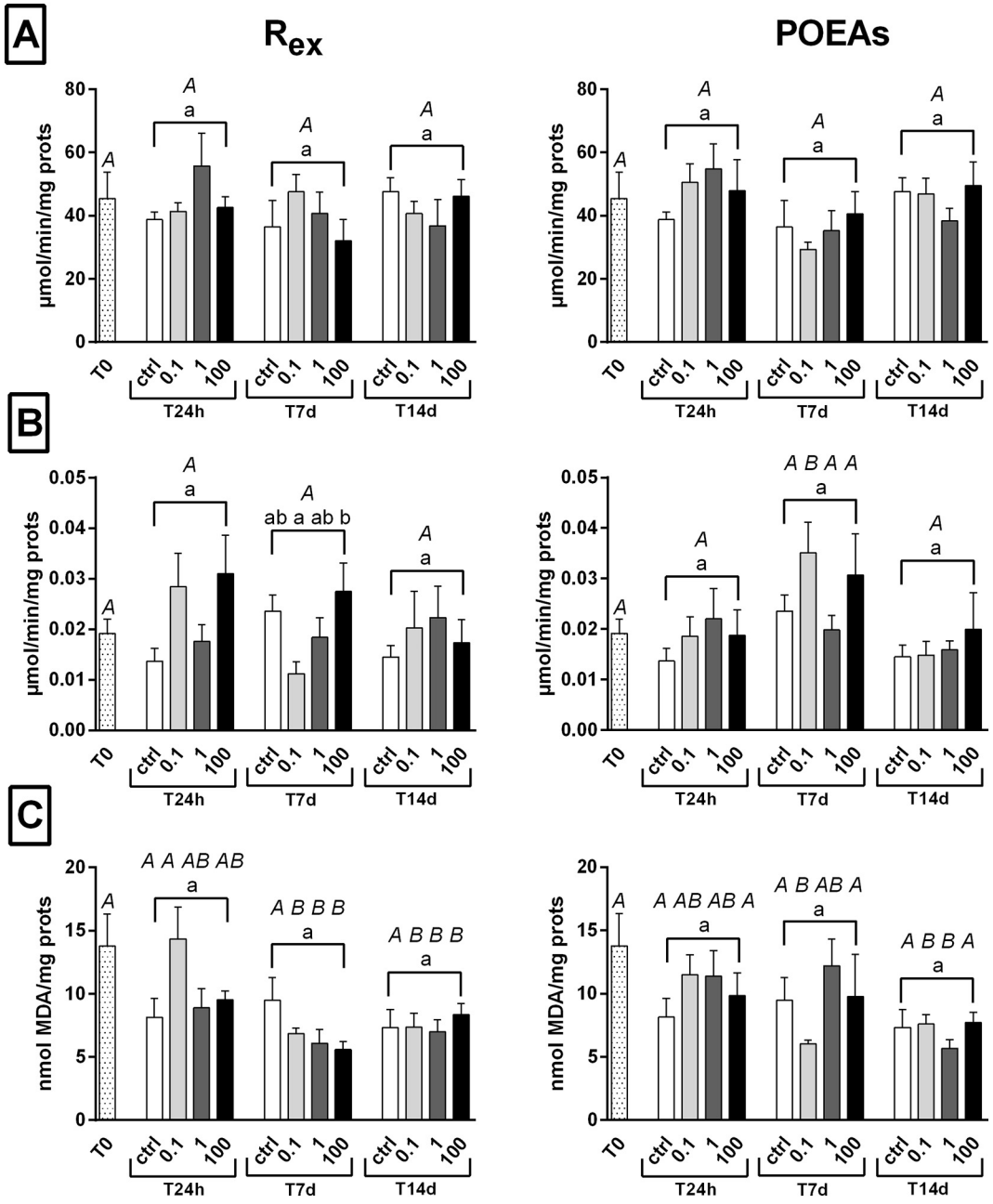

Fig. 4. Oxidative stress biomarkers (mean ± SEM) studied at T0 and in control individuals and oysters exposed to three concentrations of Roundup Express® (R_{EX}) or polyethoxylated long-chain alkylamines (POEAs) at T24h, T7d and T14d. A: catalase (CAT) activities; B: glutathione-*S*-transferase (GST) activities and C: malondialdehyde (MDA) content. The number of studied oysters was n = 6 at each sampling date for CAT and GST and 5 for MDA content. Groups that do not share a common letter are significantly different. Letters in lowercase illustrate significant differences ($p < 0.05$) between experimental conditions at a given sampling date; letters in uppercase and italics illustrate significant differences ($p < 0.05$) between sampling dates for a given experimental condition.

recorded at the beginning of the experiment (T0) and after 24 h of exposure to 0.1 µg L^{-1} R_{EX}. Before the beginning of the experiment, the oysters were maintained during a 1-week acclimatisation, but the high level of MDA content at T0 suggests that this acclimatisation time was not long enough. The overall decrease in MDA levels at the end of the experiment could be explained by a better metabolisation by the oysters and thus non-detection at that time. In *C. gigas* exposed to the same concentrations of glyphosate, no effects of this molecule were also observed on the MDA content (Mottier et al., 2015). CAT activities and MDA content (level of TBARs) in liver of the silver catfish (*Rhamdia quelen*) did not vary during a 96-h exposure to R_{EX} up to 400 µg L^{-1} (Glusczak et al., 2007). In the neotropical fish *Prochilodus lineatus*, exposures to high concentrations of R_{EX} (7.5 and 10 mg L^{-1}) did not induce changes in CAT activities (and only a slight increase in GST activities at 10 mg L^{-1} of R_{EX}) (Langiano and Martinez, 2008). However, low levels of lipid peroxidation are often associated with a high rate of CAT activities in the literature (e.g. Canesi et al., 2008; Damiens et al., 2007). This result was not confirmed in the present study because of the globally low variations of both biomarkers. In the bivalve *Donax trunculus* living at two sites differently affected by pollution, both higher CAT activities and TBAR levels were recorded at the polluted site but at only two sampling dates of six (Tlili et al., 2010). In contrast, in the freshwater snail *Biomphalaria alexandrina* exposed for 4 weeks to LC_{10} of R_{EX} (0.84 ppm), CAT activities were reduced by 23.4%, whereas lipid peroxidation was increased by 36.9% in comparison with control snails (Barky et al., 2012).

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Over a period of 14 days, GST activities did not show significant fluctuations in juvenile *C. gigas* exposed to glyphosate (Mottier et al., 2015) and R_{EX} (present study), whereas these activities were significantly increased after 7 days of exposure to POEAs but only at the lowest concentration (0.1 μg L^{-1}). Nevertheless, when considering the data at a given date, POEAs and glyphosate (Mottier et al., 2015) did not significantly affect GST activities, although they were significantly diminished at T7d in juvenile oysters exposed to 0.1 μg L^{-1} R_{EX} in comparison with the highest concentration. Anderson (1997) showed a decrease in GST activities when the cellular defences spillover, but in the present study, this hypothesis appears unlikely in view of the low effective concentration. In the literature, studies have reported conflicting evidence regarding the effects of pesticides on enzymatic activities. For example, Looise et al. (1996) emphasised the high individual variations in GST activities measured in the whole body of the bivalve *Sphaerium corneum* exposed for 7–8 days to pesticides (dieldrin up to 29 μg L^{-1} and lindane up to 173 μg L^{-1}); these authors did not find significant induction of GST activities, and finally, there was the issue of the relevance of this biochemical biomarker for ecotoxicological tests conducted over few days. In the mussel *Anodonta cygnea* caged at three different exposure sites, CAT activities varied as a function of abiotic factors but showed no relationship to pesticide levels, whereas GST activities (also related to abiotic factors) decreased with increases in total pesticide levels (Robillard et al., 2003). Exposures to 0.01 ppm carbofuran (carbamate biocide) for 96 h did not change GST activities in the gills of the mangrove oyster *C. rhizophorae*, whereas CAT activities increased by 9% (Alves et al., 2002). In *C. gigas* larvae, GST activities significantly increased as a function of carbofuran exposure (both 100 and 1000 μg L^{-1}) but were not affected by exposure to malathion, which is also an insecticide (100 and 300 μg L^{-1}) (Damiens et al., 2004). Iummato et al. (2013) also reported higher GST activities in the mussel *Limnoperna fortunei* exposed for 26 days to glyphosate but at a relative high concentration (1 mg L^{-1}).

Although the enzymatic biomarkers (i.e. CAT and GST) have been considered to be sensitive endpoints after the exposure of bivalves to herbicides, no effects or only very slight effects of R_{EX} and POEAs were recorded in the present study even at the highest concentration (100 μg L^{-1}). In future experiments, it would thus be interesting to study the temporal variations in CAT and GST activities and lipid peroxidation during very short times because these biochemical biomarkers can be considered early ones. If so, they could be inappropriate in the frame of chronic or sub-chronic exposures. Variations in individual antioxidants can be sensitive in revealing a stressful condition but difficult to predict, and different responses can be expected according to tissue, time and intensity of exposures (Regoli and Giuliani, 2014). For example, Vidal-Liñán and Bellas (2013) showed that in *Mytilus galloprovincialis*, GST activities were the highest in gills, whereas the highest values of CAT activities were recorded from the digestive gland.

4.2. Sub-lethal effects of glyphosate, glyphosate-based formulations and POEAs on individual and histological biomarkers

Our study combined biochemical endpoints and individual biomarkers to test the sub-lethal effects of R_{EX} and POEAs on juvenile oysters. Compared to biochemical biomarkers, few studies investigated the effects of glyphosate-based herbicides and adjuvants on biological parameters such as those regarding growth and reproduction in aquatic animals including molluscs. This is partly due to the lower number of chronic or sub-chronic studies, which are particularly challenging in comparison with acute assays. In the present study, control oysters showed the highest mean weight, but no significant differences were found when comparisons with exposed oysters were made. Growth constitutes an integrated and late biomarker, and significant differences might occur if the experiment would be longer than 35 days. In addition to that, the control oysters slightly grew in shell length during the 35-day experiment, and the daily feed ration thus appeared more convenient than that provided to the oysters in our previous study (Mottier et al., 2015). In contrast, no shell growth was observed in exposed individuals, and the results at the end of the study revealed significant differences in shell length between control and exposed oysters, except those exposed to 1 μg L^{-1} R_{EX}. The lack of shell growth might be due to a direct effect of pollutants on shell growth process. However, an acidification caused by glyphosate could not be involved as no differences in pH values were noticed between the water of control and exposed tanks irrespective of the chemical and the concentration tested. This lack of shell growth might also be in relation to energy allocation as already reported by Nicholson and Lam (2005) who highlighted that energy reserves are channelled into energy-consuming detoxification processes in mussels exposed to stressful conditions. Finally, the lack of growth of the oysters exposed to POEAs might also be linked to a decrease in filtration rate as Ostroumov and Widdows (2006) reported that the three classes of surfactants tested induced an inhibition of suspension feeding in marine mussels. Exposures of the clam *R. decussatus* to R_{EX} (purity of 48% and 52% of inert ingredients) at half the concentration of LC_{50} (1.1 μL L^{-1}) for 60 days induced significant behavioural and physiological responses (El-Shenawy et al., 2003); indeed, these authors recorded a decrease in valve activity and respiration and excretion rates, illustrating a decline in metabolism and excretion. In juvenile freshwater mussels *Lampsilis siliquoidea*, a reduction in growth occurred in individuals exposed to high concentrations (from 600 μg L^{-1}) of adjuvants (MON 0818), which was revealed to be the most toxic of the five chemicals tested (Bringolf et al., 2007). A negative effect of glyphosate-based herbicides or adjuvants on organism's growth was also mentioned in crustacean species belonging to the genus *Daphnia*, which shows the advantage of having a short life cycle. Indeed, in *D. magna* exposed to a range of concentrations of glyphosate (in the form of IPA) and R_{EX} (from 0.05 to 4.05 mg L^{-1} active ingredient equivalent), the no observed effect concentration (NOEC) values were similar for both chemicals (0.45 mg L^{-1}); however, at the highest concentration, growth decrease occurred at day 24 for glyphosate and day 6 for R_{EX}, suggesting a more harmful effect of the commercial formulation (Cuhra et al., 2013). In the same species, the deleterious effect of POEAs on growth was significantly demonstrated at concentrations ranging from 100 to 500 μg L^{-1} (Brausch et al., 2007).

Biometric parameters allow the calculation of CIs, which illustrate the physiological status (e.g. level of energetic reserves) and reproductive status (gametogenesis stage) of bivalves. During the 35-day experiment, we recorded an increase in CI for oysters under all conditions. Nevertheless, this increase was significant only for control and oysters exposed to R_{EX} but not for individuals exposed to the three concentrations of POEAs. On the basis of the literature (Howe et al., 2004; Pérez et al., 2011), the concentration of POEAs in formulated herbicides ranges from 10% to 20%. Considering a percentage of 15%, the amount of POEAs in R_{EX} exposures from this study should be close to 0.015, 0.15 and 15 μg L^{-1} (for 0.1, 1 and 100 μg L^{-1} of R_{EX}, respectively). It is noticeable that the results concerning the CIs of oysters exposed to R_{EX} and POEAs at equivalent adjuvants concentrations are not consistent, and this could be due to interactions between molecules in the tested commercial formulation. Nonetheless, a slightly higher effect of POEAs (i.e. POE-15) by comparison with R_{EX} was recorded at the tested concentrations. Quite surprisingly because of their acquisition facility, few ecotoxicological studies deal with CIs in bivalves. Nicholson and Lam (2005) highlighted that mussels inhabiting polluted waters often show retarded growth and poor tissue condition. Low condition indices were also reported in *Mya arenaria*, *Mytilus edulis* (McDowell et al., 1999), *C. virginica* (Scott et al., 2002) and *C. gigas* (Séguin et al., 2016) at the polluted sites. Regarding reproduction, the gametogenesis stages observed at the beginning of the present study were consistent with what is expected for oysters from Normandy (Costil et al., 2005; Royer et al.,

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2008). Then, experimental conditions allowed the oysters to progress during gametogenesis, and after the 35-d exposure, the oysters were in stage I or stage (beginning or more advanced) II. However, this progress during gametogenesis was significant only for control and individuals exposed to the highest concentration (100 μg L^{-1}) of R_{EX} and POEAs and non-significant for oysters exposed to low (0.1 μg L^{-1}) and intermediate (1 μg L^{-1}) concentrations of both chemicals. To our knowledge, the underlying mechanisms of action of these pollutants on the reproduction of non-target organisms remain uninvestigated, but our results suggest a delay of gametogenesis in individuals exposed to environmental and sub-environmental concentrations of R_{EX} and POEAs. Further studies are needed to deeply examine this important issue in terms of ecological relevance. Moreover, it is noticeable that the oysters exposed to glyphosate in our previous study appeared slightly more advanced in gametogenesis than the control oysters (Mottier et al., 2015). Similar results observed in *M. arenaria* were attributed to an increase in the rate of metabolism for the maintenance of homeostasis and integrity (Gagné et al., 2007; Greco et al., 2011). The effect of glyphosate-based herbicides has rarely been investigated in reproductive parameters. However, in *D. magna*, NOEC levels for fecundity were 0.45 and 0.15 mg L^{-1} for glyphosate IPA and R_{EX}, respectively, and the few clutches spawning at 1.35 mg L^{-1} of R_{EX} contained ~100% of aborted embryos (Cuhra et al., 2013). Regarding vertebrates, no changes in fecundity and gonadosomatic index were observed in rainbow trout exposed to concentrations of glyphosate and R_{EX} up to 2 mg L^{-1} (Folmar et al., 1979). Howe et al. (2004) showed that commercial formulations (Roundup®) were more hazardous for frog reproduction (abnormalities of gametogenesis and intersex) than glyphosate. The susceptibility of *C. gigas* and the higher toxicity of commercial formulations in relation to adjuvants have already been demonstrated by considering embryo-larval development and metamorphosis, which also show a high ecological relevance. Indeed, 24- to 48-h EC_{50} values were as low as 0.26 and 3.03 mg L^{-1} for embryo-larval development and metamorphosis processes, respectively, after an exposure to POEA surfactant system (Genamin T-200®) (Mottier et al., 2014); corresponding values were 1.01 mg L^{-1} and 6.37 mg L^{-1} after exposures to R_{EX} and 28.32 mg L^{-1} and >100 mg L^{-1} after exposures to glyphosate (Mottier et al., 2013).

Histologic approach is useful to assess the extent of pathologies at cellular and tissue levels. The index relative to the atrophies of the wall of the digestive tubules was low regardless of the experimental conditions, and the maximum value was recorded at T0. At T35d, some significant differences were noticed, but the associated with pollutant concentration could not be stated, and we could not conclude to a negative effect of R_{EX} and POEAs on the digestive tubule wall of the tested oysters. The observed differences could be due to inter-individual variations during the digestive cycle (Morton, 1977). At the beginning of the study and after 35 days of exposure, the level of haemocytic infiltration was low to moderate, and no significant differences were calculated between all experimental conditions. From a histopathological point of view, the studied oysters were globally in good condition, but as histopathologic parameters correspond to relatively long-term biomarkers, it cannot be excluded that some differences would be significant if the exposure was longer than 35 days. After short exposures to R_{EX} but at high concentrations (7.5 and 10 mg L^{-1}), several pathological changes in the liver of the fish *P. lineatus* were observed (Langiano and Martinez, 2008). In molluscs, Manduzio et al. (2005) noticed that 'the observation of pathologies does not correspond to a major axis of ecotoxicological studies', and presently this assertion remains true. However, regarding the effect of glyphosate-based herbicides on marine bivalves, the study on *R. decussatus* by El-Shenawy et al. (2009) can be cited. This work showed that vacuolation and distortion of gill filaments occurred after 90 days of exposure to 1.1 μg L^{-1} of R_{EX}.

4.3. Lethal effects of glyphosate, glyphosate-based formulations and POEAs and comparative toxicity of these substances

A total of 19 individuals including two control oysters died during the 35-day experiment. In our previous study dealing with glyphosate, no mortalities occurred in control and oysters exposed to the same concentrations of herbicide during 56 days of exposure (Mottier et al., 2015). This comparison suggests that the oyster batch investigated in the present study was a bit more sensitive and/or that the molecules tested were more deleterious for juvenile oyster survival. The higher sensitivity could partly be due to the viral load (OsHV-1 μVar), which was, however, 100-folds lesser than the threshold that induces oyster mortalities (Oden et al., 2011). Nevertheless, we cannot exclude the sub-lethal effects of viral load acting on *C. gigas* physiology. We calculated both daily and cumulative mortality rates, but these two kinds of mortality rates were not strictly related because of a fluctuating total number of oysters as samplings were made; in this context, a dead oyster at the end of the experiment (when the total number of individuals was low) carried greater weight than a dead oyster at the beginning of the experiment. By considering the total number of dead oysters and not the mortality rates, it appeared that a higher number of oysters died when they were exposed to POEAs (13 individuals of 19). Probably because of the database including low numbers of dead oysters, no significant differences were observed. The present results suggest that exposure to POEAs (even at low concentrations) induces an increase in oyster mortality, but further studies are needed to definitely conclude.

The harmful effects of formulated herbicides and adjuvants compared to active ingredient have been demonstrated in various organisms and at different levels of organisation. For instance, the cytotoxicity of glyphosate, different commercial formulations and adjuvants including the same mixture of POEAs used in the present study were tested after 24 h of exposures by using the MTT assay in three human cell lines; all formulations were more toxic than glyphosate, and POE-15 was clearly the most toxic substance (Mesnage et al., 2013). However, the genotoxic effects of not only R_{EX} and POEAs but also glyphosate were demonstrated in the blood cells of the European eel by Guilherme et al. (2012). In tadpole anuran species, acute (48 or 96 h) exposures to various glyphosate formulations and POEAs showed that adjuvants contribute the majority of the toxicity to the herbicide formulations (Howe et al., 2004; Moore et al., 2012). Such a conclusion was also drawn by Tsui and Chu (2003) who tested the acute toxicity of R_{EX} and POEAs on a bacterium (*Vibrio fischeri*), microalgae, protozoa and crustaceans, and these authors ordered the chemical toxicity as follows: POEAs > R_{EX} > glyphosate acid > IPA salt of glyphosate; in the daphnia *Ceriodaphnia dubia*, the corresponding LC_{50} values were 1.15, 5.39, 147 and 415 mg L^{-1}, respectively. Tsui and Chu (2003) concluded that POEAs account for >86% of R_{EX} toxicity, except in microalgae, revealing a species-dependent toxicity. Other studies used crustaceans such as the fairy shrimp *Thamnocephalus platyurus* and *D. magna* as ecotoxicological models to determine LC_{50} values after exposures to glyphosate-based formulations and/or adjuvants (Brausch and Smith, 2007; Brausch et al., 2007; Székács et al., 2014). Moreover, Krogh et al. (2003) and Tush et al. (2013) reviewed the effects of various surfactants in different kinds of organisms including crustaceans, but Tush et al. (2013) indicated that toxicity data on alkylamine ethoxylates (ANEOs including POEAs) are relatively scarce. The review by Krogh et al. (2003) particularly focuses on alcohol ethoxylates, whereas the effect of ANEOs on organisms is practically not documented. In summary, the exposure of the fairy shrimp to three formulations of POEAs was highly toxic, with 48-h LC_{50} as low as 2.01 μg L^{-1} for Surfonic® T-15 surfactant (Brausch and Smith, 2007). In *D. magna* exposed to POEAs, 48-h LC_{50} values ranged from to 97 to approximately 850 μg L^{-1} depending on the number of alkyl and oxide carbons (Brausch et al., 2007). In contrast, exposure of *D. magna* to 3 mg L^{-1} 'surfactants including POEA' (without further specification) or 20 mg L^{-1} R_{EX} did not induce significant mortality (Székács et al., 2014). These discrepancies could be due to the kind of

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additives tested. By using a multivariate quantitative structure-activity relationship (M-QSAR), Uppgård et al. (2000) reported that surfactant toxicity tends to increase with increasing alkyl chain lengths. In their review about the effects of the herbicide glyphosate and glyphosate-based formulations on aquatic ecosystems, Pérez et al. (2011) concluded that R_{EX} showed between 3 and 76 times higher toxicity than glyphosate itself on non-target aquatic invertebrates, but most of the studies were conducted in arthropods.

Finally, in molluscs, Bringolf et al. (2007) reported that the surfactant tested (MON 0818) was more toxic to early life stages and juvenile unionid bivalve, *Lampsilis siliquoidea*, than glyphosate and its formulations (48-h LC_{50} of 0.5 and 3.8 mg L^{-1} in glochidia and juvenile individuals, respectively). Lower 48-h LC_{50} values were also calculated for pediveliger larvae of *C. gigas* exposed to two glyphosate-based formulations including R_{EX} (approximately 8.5 mg L^{-1}) compared to larvae exposed to glyphosate and AMPA (>100 mg L^{-1}) (Mottier et al., 2013). It was noticeable that early life stages are generally more susceptible to contaminants than older stages (Mohammed, 2013). It is difficult to compare results from acute exposures (e.g. 48 h) and chronic or sub-chronic exposures (35 days in the present study). Nevertheless, the LC_{50} values previously mentioned are much higher than the highest concentration tested in the present study (100 μg L^{-1}), and it is thus not surprising that no mass mortalities occurred in the exposed oysters during the 35-day exposure. In *R. decussatus* exposed for 60 days to R_{EX}, LC_{50} values were as low as 16.9 (at 7 days), 12.1 (at 35 days) and, finally, 2.2 μg L^{-1} (at the end of the experiment) (El-Shenawy et al., 2003). In contrast, in the same species, shorter (96 h) exposures to four concentrations of glyphosate and R_{EX} from 10 μg L^{-1} to 10 mg L^{-1} did not induce mortalities (Elandalloussi et al., 2008). However, in a batch of clams infected by *Perkinsus olseni*, the susceptibility to the parasite was increased by exposures to R_{EX} at concentrations of 10 and 25 mg L^{-1}: 7 and 28 dead individuals out of 30, respectively, at T96h, whereas only one control clam died after 120 h (Elandalloussi et al., 2008). This study suggested that the exposure to glyphosate-based formulations pose additional stress to bivalves. In this context, water quality is especially important for *C. gigas* production, which is threatened by mass mortalities due to epizooties occurring from 2008 in Europe and around the world (Dégremont et al., 2015).

5. Conclusions

In addition to an experiment that had tested the effect of sub-chronic exposures to glyphosate (Mottier et al., 2015), the present study aimed to assess the toxicity of (1) a commercial formulation (R_{EX} in which adjuvants are added to glyphosate) and (2) these adjuvants (POEAs) on a second batch of juvenile oysters. Exposures to R_{EX} and POEAs did not lead to high mortality rates, but a higher number of dead oysters was recorded after exposures to POEAs. At the low concentrations tested, biochemical endpoints did not reveal significant effects of both chemicals on the level of lipid peroxidation and the activities of enzymes involved in oxidative stress defences and detoxification. In contrast, individual biomarkers (e.g. shell growth, CI and reproduction) revealed to be more informative and should be preferred when studying chronic exposures and biomonitoring. The most important result was that low (0.1 μg L^{-1}) and intermediate (1 μg L^{-1}) concentrations of both chemicals appeared to induce slight but more changes than the highest concentration one (100 μg L^{-1}). Such a result is ecologically important, and further experiments should be performed to definitely conclude and precisely investigate the mechanisms of action of adjuvants on oysters, which are non-target organisms.

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