Aquatic Toxicology 128–129 (2013) 67–78

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

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

Effects of glyphosate-based herbicides on embryo-larval development and metamorphosis in the Pacific oyster, *Crassostrea gigas*

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ARTICLE INFO

Article history:
Received 13 July 2012
Received in revised form
22 November 2012
Accepted 2 December 2012

Keywords:
Crassostrea gigas
Glyphosate
Roundup®
Embryo-larval development
Metamorphosis

ABSTRACT

Pesticides may be involved in oyster summer mortality events, not necessarily as a single causative agent but as an additional stressor. In this context, the present study aimed to assess the toxicity of glyphosate, its by-product, aminomethylphosphonic acid (AMPA) and two commercial formulations, Roundup Express® (R_{EX}) and Roundup Allées et Terrasses® (R_{AT}), containing glyphosate as the active ingredient, on the early life stages of the Pacific oyster, *Crassostrea gigas*. The embryotoxicity of these chemicals were quantified by considering both the rates of abnormalities and the arrested development or types of abnormalities in D-shaped larvae after 48 h exposure. The success of metamorphosis was examined in pediveliger larvae exposed for 24 h. Experiments involving both endpoints included range finding experiments for herbicide concentrations ranging from 0.1 to 100,000 $\mu g\,L^{-1}$. This range was then narrowed down in order to determine precise EC_{50} values. Actual concentrations of the herbicide were determined at the beginning and after 48 h (embryotoxicity) and 24 h (metamorphosis) to evaluate the potential temporal variation in the concentrations. During embryo-larval development, no mortalities were recorded at any of the concentrations of glyphosate and AMPA, whereas no embryos or D-shaped larvae could be observed after exposure to 10,000 $\mu g\,L^{-1}$ of R_{EX} or R_{AT}. Compared with the controls, no effects on embryo-larval development were recorded between 0.1 and 1000 $\mu g\,L^{-1}$, regardless of the chemical tested. Above a threshold, which varied according to the chemical used, the gradient of herbicide concentrations correlated with a gradient of severity of abnormality ranging from normal larvae to arrested development (an "old embryo" stage). The EC_{50} values were 28,315 and 40,617 $\mu g\,L^{-1}$ for glyphosate and its metabolite, respectively, but much lowered values of 1133 and 1675 $\mu g\,L^{-1}$ for R_{EX} and R_{AT}, respectively. Metamorphosis tests also revealed a significant difference between molecules, as the EC_{50} values exceeded 100,000 $\mu g\,L^{-1}$ for glyphosate and AMPA but were as low as 6366 and 6060 $\mu g\,L^{-1}$ for the commercial formulations, which appeared relatively more toxic. Overall, the embryo-larval development of *C. gigas* was more sensitive to glyphosate-based herbicides compared to various endpoints studied in regulatory model organisms, and embryos and D-shaped larvae were more sensitive compared to pediveliger larvae.

1. Introduction

In Europe, aquatic environments are continuously subjected to various contaminants originating from domestic, industrial and agricultural activities. The English Channel is especially affected by chronic contamination resulting from heavy shipping traffic, industrialisation (including nuclear industry) and high population density along the coastlines. For example, in the early 1980s, the Seine River, which flows into the English Channel, was considered one of the most contaminated rivers in the world (Carpentier et al., 2002). Today, the situation in the Seine Estuary and the Channel remains worrying with respect to their ecological status and the quality of their marine resources (Cachot et al., 2006; Schnitzler et al., 2011). In addition to recent European legislations, which aim to maintain the high ecological status of the hydrosphere, such considerations have led to the launch of a European Interreg IVA program named "Chronexpo". Under this initiative, the "Chronexpo" project aims to study the effects of chronic exposure

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http://dx.doi.org/10.1016/j.aquatox.2012.12.002

to various contaminants on ecologically and economically important marine organisms inhabiting the English Channel (e.g. annelids and molluscs).

The Pacific oyster, *Crassostrea gigas* (Thunberg), was introduced into France in 1967 and now represents an important commercial bivalve species. With annual production varying from 126,000 to 138,000 t during 1996–2006, France ranked top in Europe (and fourth in the world) for oyster production (CNC, 2012). Despite the effects of a current viral infection, France remains a leader in oyster farming producing 82,800 t in 2010 (CNC, 2012). In Normandy and north Brittany, oysters are farmed in the sandy intertidal zones of the Channel, and this production represented, on average 20% and 24%, respectively, of total French production in 2009 (CNC, 2012). Nevertheless, in addition to spat mortalities due to epizootics involving viruses and bacteria, French oyster basins can sporadically experience significant mortality events (>30%) in summer which threaten commercial production (Royer et al., 2007; Soletchnik et al., 2007). The summer mortality syndrome does not appear to result from a single cause but may result from a combination of several extrinsic and intrinsic factors including elevated temperature, low dissolved oxygen (hypoxia), xenobiotics and physiological stresses related to reproduction (Samain et al., 2007). Climatic and hydrological surveys have shown that oyster mortalities occur when the water temperature exceeds 19 °C. In Normandy, these mortalities have been especially high during years of high rainfall and in areas under estuarine influences (compared to zones exclusively under marine influence) (Costil et al., 2005; Ropert et al., 2008). In this context, terrestrial inputs including pesticides may be involved in oyster mortality events, not as a single causative agent, but as additional stressors.

Ecotoxicological studies involving marine organisms often address contaminants such as metals, polychlorobiphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and less frequently, pesticides. Moreover, most of the studies concerning pesticides have focused on the particularly toxic organophosphate and organochlorine insecticides (e.g., Scott et al., 2002; De Mora et al., 2005). Many of these compounds are banned in France, which was the primary European country for pesticide use (62,700 t of active substances used in 2011) (UIPP, 2012). Among pesticides, glyphosate-based herbicides are the most widely used in France and across the world (Baylis, 2000; Woodburn, 2000). In Normandy, more than 1000 t of these active substances were sold in 2009 (Agence de l'Eau Seine Normandie, personal communication). Glyphosate [N-(phosphonomethyl)glycine; $C_3H_8NO_5P$] is a broad-spectrum aminophosphonate-type herbicide used in agricultural and non-agricultural activities; it inhibits plant growth by interfering with 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), which is a key enzyme for the production of essential aromatic amino acids (Amrhein et al., 1980). Humans and animals cannot be considered as target organisms because they do not have this biosynthetic pathway. Since its first commercialisation in 1974, glyphosate has often been considered relatively non-toxic for humans and fauna (Williams et al., 2000; Giesy et al., 2000). It has also been regarded as an environmentally friendly pesticide because of its rapid biodegradation and strong adsorption to soil particles (Koc = 884–60,000 L kg^{-1} depending on soil types) (Agritox, 2012; PPDB, 2012). However, glyphosate is highly water-soluble (10–15.7 g L^{-1}; Battaglin et al., 2005) and is present in surface waters worldwide. In Europe, this herbicide and its metabolite aminomethylphosphonic acid (AMPA) are thus frequently detected in aquatic ecosystems, sometimes at high concentrations (Botta et al., 2009; SOS, 2010; Puértolas et al., 2010).

In France, coastal environments are far less monitored in comparison to freshwater ecosystems, and little data exist describing contamination of marine or coastal waters by herbicides. In Normandy, 6 of the 15 herbicides under study (excluding glyphosate) were detected at low tide in seawater; the highest concentration was recorded for diuron (0.132 μg L^{-1}) (Buisson et al., 2008). In the bay of Arcachon (South West France), 5 herbicides were detected in seawater following tests for a total of 6 herbicides, and the maximum concentration was measured for irgarol (0.066 μg L^{-1}) (Auby et al., 2007). Because of analytical difficulties, the glyphosate/AMPA compounds remain particularly poorly documented in coastal environments (Munaron, 2004). Burgeot et al. (2008) have reported maximum concentrations of glyphosate reaching 0.10 μg L^{-1} and 1.20 μg L^{-1} in spring of 2003 and 2004, respectively, in the seawater of the basin of Marennes-Oléron (South West France). In the past, studies of the impacts of pesticides on organisms focused on active substances, whereas few studies have addressed commercial formulations. In the case of glyphosate, many commercial formulations are used in domestic and agricultural weed control. The brand Roundup® contains glyphosate formulated as an isopropylamine (IPA) salt, and a surfactant, polyoxyethylene tallow amine (POEA), is added to enhance the efficacy of the herbicide (Tsui and Chu, 2003). Tsui and Chu showed that Roundup® or the surfactant used are more toxic than glyphosate alone in bacteria (*Vibrio fischeri*) and in different aquatic plant and animal species. Such a result has also been demonstrated in freshwater oligochaetes (Contardo-Jara et al., 2009), amphibians (Howe et al., 2004; Hedberg and Wallin, 2010) and mammals, including humans (Peixoto, 2005; Pieniążek et al., 2004).

The sensitivity of organisms to contaminants depends on many factors, including the life cycle stages; early stages are often considered particularly sensitive (e.g., Giesy and Graney, 1989; Jha et al., 2000). In marine bivalves (oysters and mussels), often considered as sentinel organisms for coastal environments, embryo-larval stages are used in ecotoxicological studies. Some previous studies have examined larval growth or condition index (Brereton et al., 1973; Geffard et al., 2007); the activities of different enzymes, including those implied in oxidative stress regulation (Damiens et al., 2004; Quiniou et al., 2007); and the integrity of DNA (Jha et al., 2000; Hagger et al., 2005; Cheung et al., 2006; Wessel et al., 2007). Finally, many of the ecotoxicological studies using marine bivalve larvae have mainly evaluated the embryotoxic effects of various pollutants (Robert et al., 1986; His et al., 1999; Nice et al., 2000; Lyons et al., 2002; Libralato et al., 2008; Cachot et al., 2006; Akcha et al., 2012). In this context, the study by Akcha et al. (2012) is especially interesting as it aimed to determine the potential embryotoxicity of two herbicides: diuron and glyphosate but the study tested a highest concentration of 5.0 μg L^{-1}. By comparison, the metamorphosis success of bivalve larvae has rarely been used as a biological response to study the impact of contaminants in the sensitive life stages (His et al., 1997). Studies of marine bivalve metamorphosis have especially addressed the impacts of hormones such as epinephrine (Coon and Bonar, 1987; García-Lavandeira et al., 2005; Wang et al., 2006). It was therefore important to optimise the protocol suggested by Coon and Bonar (1987) to compare the results with those obtained on embryotoxicity evaluation. The aim of the present study was to assess the impacts of glyphosate, AMPA and two commercial formulations (Roundup Express® and Roundup Allées et Terrasses®) on the embryo-larval development of the Pacific oyster, *C. gigas*, by considering abnormality rates and the types of abnormalities. The second objective was to study the effects of the 4 substances on the success of metamorphosis of pediveliger larvae. The hypothesis of a higher toxicity of commercial formulations based on available literature was thus tested by observing two larval stages. In both cases, broad ranges of concentrations (0.1–100,000 μg L^{-1}) were first tested because of a lack of data in the literature, and then, narrower ranges were used to more precisely determine the effective concentration that induces an effect on 50% of the population (EC_{50}). Finally, the present study aimed to assess the usefulness of embryo-larval development and

metamorphosis as toxicity endpoints in marine ecotoxicology, and to contribute to the assessment of glyphosate-based herbicide toxicity. Such an evaluation is particularly useful because glyphosate and AMPA are listed among "the substances subject to review for possible identification as priority substances or priority hazardous substances" (Official Journal of the European Union, 2008).

2. Materials and methods

2.1. Chemical compounds

Glyphosate, the active ingredient in commercial Roundup® herbicides, is highly water soluble (Battaglin et al., 2005). It is considered a non-persistent molecule in soils (half-life, DT50 = 12 days) but relatively persistent in water 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, 2012; PPDB, 2012). Glyphosate metabolises to AMPA which is highly water-soluble and persistent in soil (DT_{50} = 151 days). In this study, the effects of two commercial Roundup® herbicides were also assessed, Roundup Express® (R_{EX}) and Roundup Allées et Terrasses® (R_{AT}) (Monsanto Company, St. Louis, MO, USA), which contain 7.2 and 4.4 g L^{-1} of glyphosate, respectively. In these formulations, adjuvants are used to aid or modify the action of the principal active ingredient (Tu et al., 2001). In Roundup® formulations, the most widely used adjuvants are poly-ethoxylated tallow amines (POEA). In the present study, glyphosate (97% purity) and AMPA (97.5% purity) were obtained from Dr. Ehrenstorfer GmbH® (Augsburg, Germany). The two types of Roundup® were purchased from a garden centre. All concentrations given in this study are expressed in glyphosate equivalents. All of the solutions of herbicides used were prepared with natural sterilised open seawater (0.22 μm, Steritop® Millipore).

For both endpoints, the nominal concentrations corresponding to 0.1, 1, 100 and 10,000 μg L^{-1} of the chemicals (i.e. glyphosate and AMPA) were verified (in duplicate) by ultraperformance liquid chromatography (UPLC) and fluorometric detection (in accordance with NF ISO 21458) using UPLC Acquity with FLR detector (Waters) and a column Acquity BEH C18 −2.1 mm × 150 mm, 1.7 μm. Moreover, the analyses were performed at the beginning and at the end of the exposures to verify the variation in the tested concentrations during the period of the experiment. These analyses were performed once without embryos or larvae to avoid the interaction between the physico-chemical and biological processes. Finally, the concentrations of glyphosate and AMPA in both commercial formulations (R_{EX} and R_{AT}) were determined (5 replicates) to verify the values indicated on the labels by the Monsanto Company.

2.2. Embryotoxicity bioassay and experimental design

Embryo-larval toxicity tests were performed on oyster embryos exposed to herbicides using the standardised AFNOR procedure (AFNOR XP-T90-382) published in 2009. Four experiments were conducted, and for each experiment, herbicide concentrations were tested in triplicate. For the first two experiments, herbicide concentrations ranged from 0.1 to 100,000 μg L^{-1} with a factor of 10× between each two consecutive concentrations (7 concentrations in total + control). Then, embryos were exposed to additional concentrations of herbicides corresponding to narrower ranges to more precisely determine the EC_{50} values. For glyphosate and AMPA, the narrowed range was from 10,000 to 30,000 μg L^{-1}, and 9 and 11 different concentrations (+control) were tested, respectively (Fig. 3A and B). For each commercial formulation, the narrowed range was between 1000 and 2000 μg L^{-1}, and a total of 18 different concentrations (+control) were tested (Fig. 3C and D). Apart from the herbicide exposures and the control (0 μg L^{-1}), $CuSO_4{\cdot}5H_2O$ (Alfa Aesar GmbH®; Karlsruhe, Germany) was used as a positive control, with concentrations ranging from 20 to 100 μg L^{-1} (5 concentrations).

Conditioned oysters were purchased from the Guernsey Sea Farm Ltd. hatchery (Guernsey, UK). Male and female gametes were obtained by thermal stimulation of 3 pairs of genitors (successive baths at 16 °C or 28 °C). After spawning, gametes from the different genitors were observed under a light microscope to select genitors with the best cell qualities: highly concentrated and motile spermatozoa and even, pear-shaped oocytes. The spermatozoa and oocytes of the selected parents were passed through, respectively, 40 and 100 μm screens to remove debris. Female and male gametes (1:6) were then mixed and gently agitated. The occurrence of fertilisation was verified with light microscope observations, and 20-min post-fertilisation, the embryos were distributed into glass pillboxes containing 25 mL of natural sterilised seawater (0.22 μm, Steritop® Millipore). Embryos were exposed at a density of 60,000 L^{-1} (corresponding to 1500 embryos per pillbox) without feeding, aeration and light. After 48 h at 22 ± 1 °C, embryos or D-shaped larvae were fixed using 0.5 mL of an 8% formalin solution.

An average of 100 larvae was counted per replicate using an inverted binocular microscope at 400× magnification (Leica® DM IRB). Observations allowed the calculation of rates of abnormality and the discrimination among types of abnormalities; 5 categories could be distinguished: shell and/or hinge abnormality, mantle abnormality (hypertrophies), shell and/or hinge + mantle abnormality, late arrested development and early arrested development (when cells could be distinguished and counted) (Fig. 1). The results of embryo-larval development in exposed organisms were expressed as net percentages of normal development, NPN_e (adjusted for the controls) (AFNOR, 2009) as follows:

$$NPN_e = 100 - \left[\frac{PA_e - \overline{PA_c}}{100 - \overline{PA_c}} \times 100\right]$$

where

$$PA_e = \frac{NA_e \times 100}{T_e}$$

NA_e = number of abnormal larvae in a given exposure replicate; T_e = total number of larvae in a given exposure replicate.

$$\overline{PAc} = \text{mean of } PA_c \quad \text{(for a given experiment)}$$

$$PA_c = \frac{NA_c \times 100}{T_c}$$

NA_c = number of abnormal larvae in a given control replicate; T_c = total number of larvae in a given control replicate.

2.3. Metamorphosis bioassay and experimental design

The aim of this endpoint was to assess the metamorphosis rate of pediveliger larvae (ready for metamorphosis) exposed to herbicides. Experiments were performed 4 times, and each herbicide concentration was tested at least in triplicate. For the four experiments using glyphosate and AMPA, herbicide concentrations tested ranged from 0.1 to 100,000 μg L^{-1} (broad range). For the Roundup® formulations, the two first experiments were conducted on the broad range of concentrations; then, pediveliger larvae were exposed to a narrow range of concentrations between 1000 and 10,000 μg L^{-1} (19 concentrations + control) (Fig. 5B and C). Twenty-one-day-old pediveliger larvae were purchased from the SATMAR (Société ATlantique de MARiculture) hatchery (Barfleur, France). Larvae were exposed in multiwell plates (12-wells, NUNC®; Penfield, New York, USA) in a final volume of 1.5 mL of natural sterilised

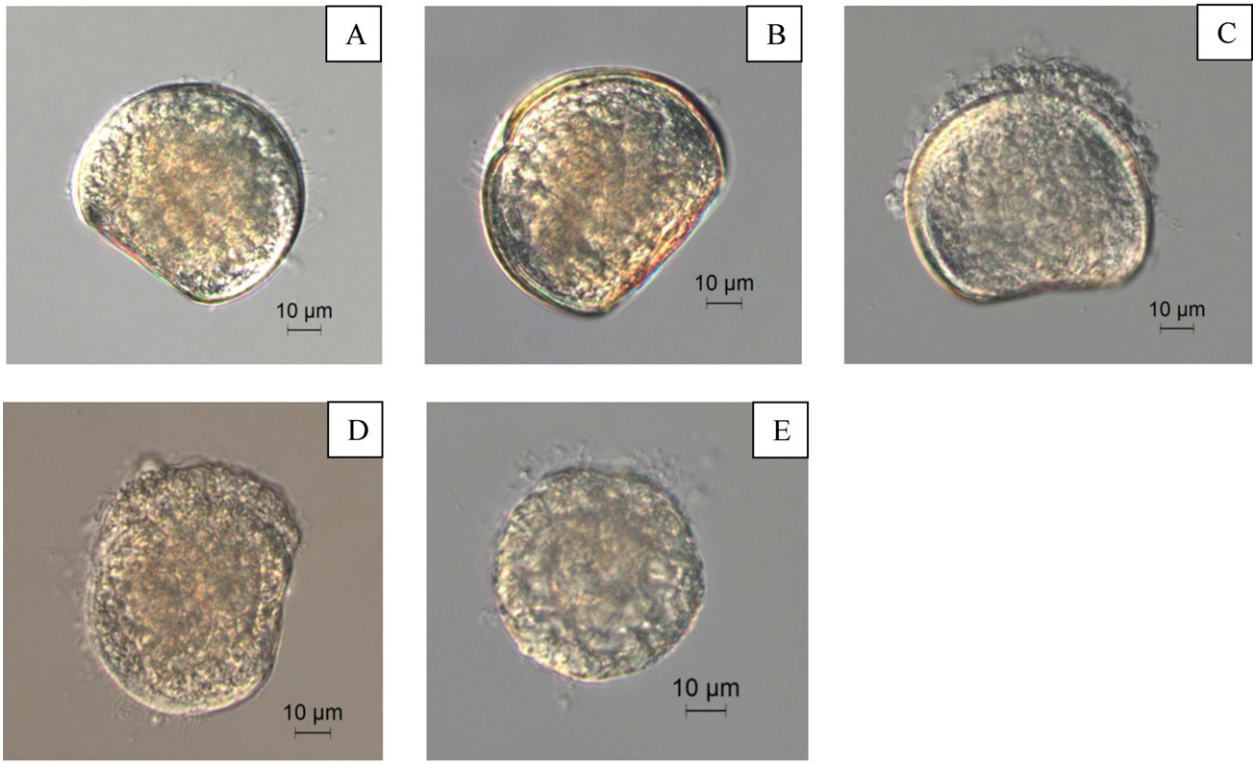

Fig. 1. Light microscopy images (400×) showing the morphology of the developmental stages of *Crassostrea gigas* at 48 h: (A) normal D-shaped veliger; (B) D-shaped veliger showing abnormal shell; (C) D-shaped veliger presenting hypertrophied mantle; (D) D-shaped veliger exhibiting both shell/hinge and mantle abnormalities; (E) embryo showing arrested development at the "old embryo" stage.

seawater (0.22 µm, Steritop® Millipore). Larval density was set between 50 and 80 larvae per well. To promote metamorphosis, epinephrine (Sigma–Aldrich®) was added at a final concentration of 10^{-4} M (Coon and Bonar, 1987). Experiments were conducted for 24 h at 22 °C without feeding, aeration and light.

After 24 h, exposed larvae were observed using an inverted binocular microscope at 100× magnification (Leica® DM IRB) to count dead larvae that showed no movement and/or tissue degradations. Following this first count, larvae were fixed using an 8% formalin solution. The metamorphosis rate was evaluated by counting metamorphosed versus non-metamorphosed larvae. A larva was considered metamorphosed when it presented an obvious loss of its velum, new shell growth and well-developed gills (Fig. 2). Aside from metamorphosis processes, we also examined mortality, considering the latter "more serious" than a lack of metamorphosis. Metamorphosis rates were thus calculated by considering both the percentages of non-metamorphosed and dead larvae versus metamorphosed ones. The results of the metamorphosis test in exposed organisms were expressed as net percentages of metamorphosis, NPM_e (adjusted for the controls) as follows:

$$NPM_e = 100 - \left[\frac{PNM_e - \overline{PNM_c}}{100 - \overline{PNM_c}} \times 100\right]$$

where

$$PNM_e = \frac{NNM_e \times 100}{T_e}$$

NNM_e = number of non-metamorphosed larvae in a given exposure replicate; T_e = total number of larvae in a given exposure replicate.

PNM_c = mean of PNM_c (for a given experiment)

$$PNM_c = \frac{NNM_c \times 100}{T_c}$$

NNM_c = number of non-metamorphosed larvae in a given control replicate; T_c = total number of larvae in a given control replicate.

2.4. Statistical analysis

The comparison of herbicide concentrations at the beginning of the experiments and after 24 h (metamorphosis test) or 48 h (embryotoxicity) exposure was performed using Mann–Whitney tests. Non-linear regressions (using the Hill equation) on data obtained from the two endpoints allowed us to calculate various EC_X (effective concentration for an effect on *X*% of the individuals tested) values for each contaminant. These regressions were conducted using the Excel® macro REGTOX (Vindimian, 2012). Data related to the effects of glyphosate and AMPA on metamorphosis were statistically tested by one-way ANOVAs, as they met the assumptions of parametric tests (normal distribution and homogeneity of variances). On the other hand, the data on the impact of commercial formulations on metamorphosis and all of the data concerning embryotoxicity (even transformed) did not meet these assumptions; thus, Kruskal–Wallis tests were employed to make conclusions about the significance of the differences between both the controls and the various herbicide concentrations and the herbicide concentrations themselves. When ANOVA or Kruskal–Wallis tests revealed significant differences, multiple comparison tests (Student–Newman–Keuls; SNK or SNK modified for Kruskal–Wallis test) were then undertaken to distinguish among different groups (Scherrer, 1984). All of the analyses were conducted using STATISTICA 8.0 software (Statsoft®, Tulsa, OK, USA).

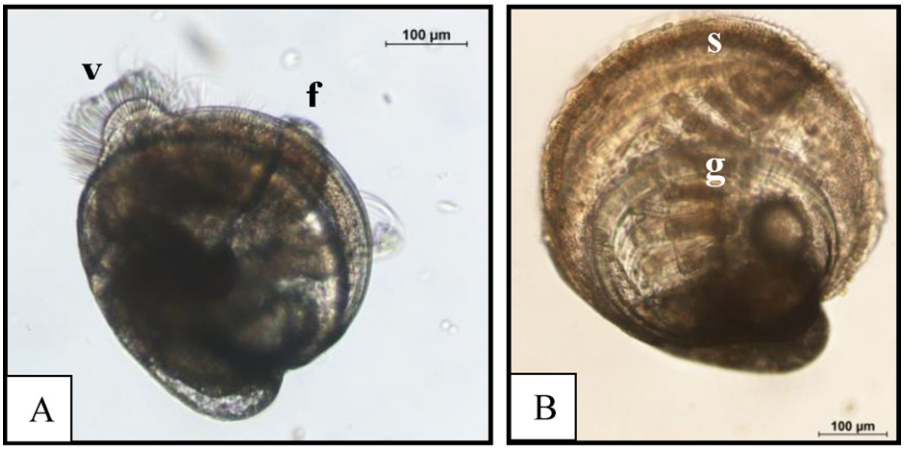

Fig. 2. (A): A 21-day-old *C. gigas* pediveliger larva showing velum (v) and feet (f), versus (B): a 21-day-old metamorphosed larva showing developed gills (g) and a shell growth (s).

3. Results

3.1. Analyses of the tested molecules

For the glyphosate and AMPA exposures, the measured concentrations in general were slightly higher than the nominal concentrations (Table 1); these differences were greater for the lowest concentrations (1 and above all 0.10 $\mu g\,L^{-1}$) and reached a maximum of 40% (0.14 $\mu g\,L^{-1}$ instead of 0.10 $\mu g\,L^{-1}$) for the embryotoxicity test at T0h. However, the measured concentrations were globally close to the nominal concentrations. The glyphosate concentrations of Roundup Express® (R_{EX}) and Roundup Allées et Terrasses® (R_{AT}) averaged 9.52 $g\,L^{-1}$ (±1.23) instead of 7.20 $g\,L^{-1}$, and 6.27 $g\,L^{-1}$ (±1.63) instead of 4.40 $g\,L^{-1}$, respectively. It thus appeared that the commercial formulations were overdosed by +32.22% for R_{EX} and +42.50% for R_{AT}. For the exposures of embryos and larvae to Roundup®, the various solutions were prepared based on the advertised concentrations (i.e., 7.2 and 4.4 $g\,L^{-1}$); therefore, the true concentrations exceeded the nominal ones for both endpoints (Table 1). The maximal difference attained was 50% for the embryotoxicity test with R_{EX} at T0h and T48h (0.15 $\mu g\,L^{-1}$ instead of 0.10 $\mu g\,L^{-1}$), but generally, the excess did not reach the proportion of 32.22% for R_{EX} and 42.50% for R_{AT}, especially for the highest concentration tested (10,000 $\mu g\,L^{-1}$).

When the values recorded at the beginning of the experiments were compared with those measured after 24 or 48 h of exposure, no significant differences were calculated (Mann–Whitney tests, $p > 0.05$), and it could be concluded that the organisms were exposed to constant concentrations during the two types of experiments.

3.2. Effects of glyphosate-based herbicides on embryo-larval development

For each experiment, the results of the embryo-larval bioassay revealed very high levels of fecundation, as almost no oocytes were recorded and the observed organisms were embryos and normal or abnormal D-larvae. Moreover, all of the embryotoxicity tests presented in this study could be validated because they respected the two validation conditions required by the standardised procedure (AFNOR, 2009): in controls, the rate of normal larvae must reach at least 80%, and the EC_{50} Cu^{2+} must fall between 6 and 16 $\mu g\,L^{-1}$. Indeed, these parameters ranged, respectively, from 82.94% (SEM: ±2.51%) to 92.08% (SEM: ±1.48%), and from 8.47 to 12.43 $\mu g\,L^{-1}$ in our experiments.

Exposures to glyphosate (and AMPA) and to commercial formulations gave different results in terms of organism survival and embryo-larval development. No mortalities were observed

Table 1
Results (mean values in $\mu g\,L^{-1}$ ± SEM) of the herbicide analyses performed for both endpoints at the beginning of the experiment and after 24 h or 48 h of exposure to glyphosate, aminomethylphosphonic acid (AMPA) and two commercial formulations: Roundup Express® and Roundup Allées et Terrasses®.

	Nominal concentrations	Embryotoxicity		Metamorphosis rate	
		T0h	T48h	T0h	T24h
Glyphosate ($\mu g\,L^{-1}$)	0.1	0.12 ± 0.01	0.12 ± 0.02	0.12 ± 0.01	0.12 ± 0.01
	1	1.20 ± 0.07	1.20 ± 0.08	1.09 ± 0.01	1.15 ± 0.05
	100	114.1 ± 3.1	114.5 ± 3.9	109.7 ± 7.1	113.0 ± 1.4
	10,000	10,025 ± 113	9833 ± 244	10,407 ± 166	11,055 ± 155
AMPA ($\mu g\,L^{-1}$)	0.1	0.14 ± 0.00	0.14 ± 0.03	0.10 ± 0.00	0.14 ± 0.01
	1	1.09 ± 0.05	1.07 ± 0.01	1.04 ± 0.02	1.08 ± 0.00
	100	108.7 ± 2.8	103.4 ± 0.04	102.8 ± 0.1	104.1 ± 0.6
	10,000	10,552 ± 187	9957 ± 67	9805 ± 7	10,392 ± 39
R_{EX} ($\mu g\,L^{-1}$)	0.1	0.15 ± 0.02	0.15 ± 0.01	0.11 ± 0.01	0.12 ± 0.02
	1	1.21 ± 0.01	1.29 ± 0.00	1.07 ± 0.02	1.00 ± 0.00
	100	108.7 ± 2.6	122.6 ± 15.9	119.6 ± 16.5	110.9 ± 0.2
	10,000	10,625 ± 403	10,745 ± 49	10,455 ± 14	10,757 ± 272
R_{AT} ($\mu g\,L^{-1}$)	0.1	0.13 ± 0.00	0.14 ± 0.00	0.12 ± 0.02	0.11 ± 0.01
	1	1.25 ± 0.00	1.32 ± 0.01	1.25 ± 0.03	1.25 ± 0.03
	100	136.6 ± 4.8	135.6 ± 0.6	138.1 ± 12.7	116.3 ± 3.0
	10,000	10,652 ± 286	10,662 ± 11	12,140 ± 827	11,845 ± 537

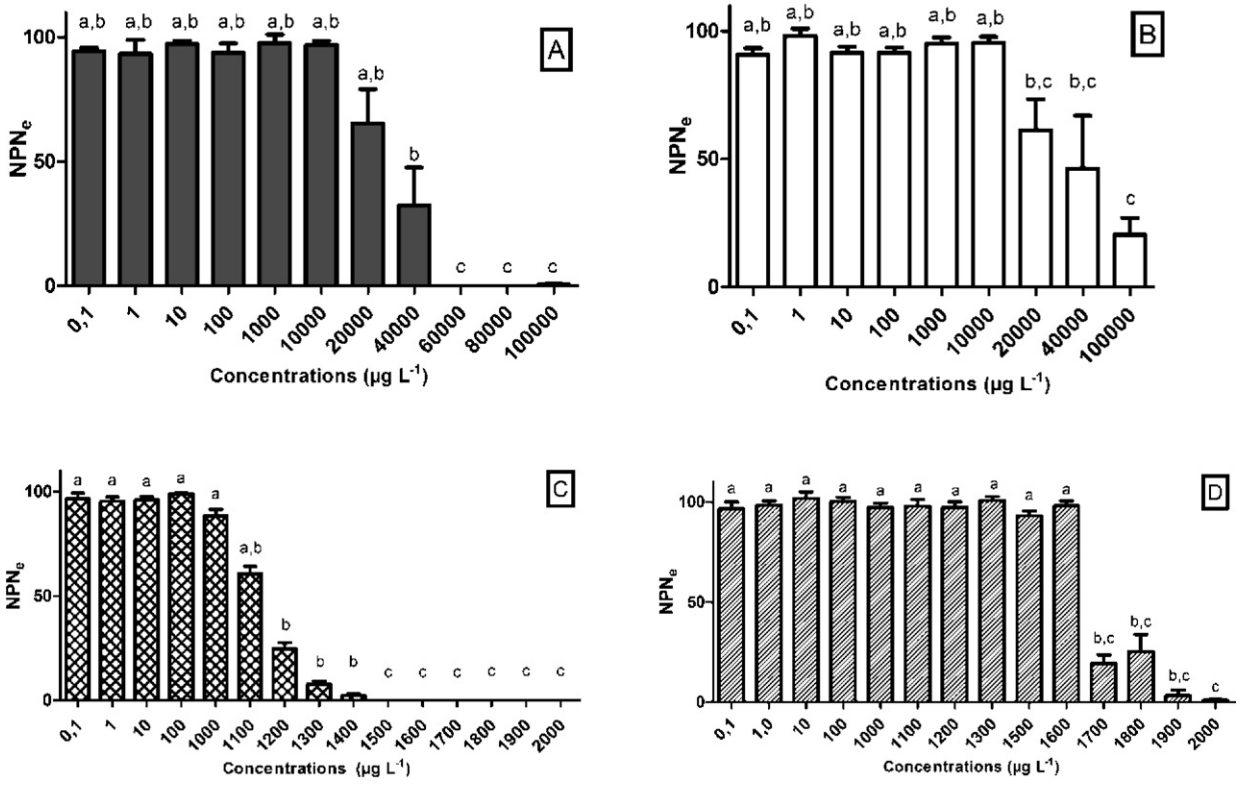

Fig. 3. Net percentages of normal development (NPN_e) (±SEM) in *C. gigas* embryo-larvae observed after 48 h of exposure to herbicides at concentrations ranging from 0.10 to 100,000 µg L^{-1} for glyphosate (A) and AMPA (B) or 2000 µg L^{-1} for Roundup Express® (R_{EX}) (C) and Roundup Allées et Terrasses® (R_{AT}) (D). Since no normal larvae were observed at the tested concentrations of 4000, 6000 and 8000 µg L^{-1} for R_{EX} and R_{AT}, they are therefore not represented. The concentrations that do not share a letter are significantly different; by convention, the controls belong to group a. For R_{AT}, NPN_e data lacking at 1400 µg L^{-1} due to a technical problem.

at any of the concentrations of glyphosate and AMPA tested, even at 100,000 µg L^{-1}; however, at this highest concentration, only embryos and abnormal D-shaped larvae were present. In contrast, at 10,000 µg L^{-1} of R_{EX} and R_{AT}, no embryos or D-larvae were observed, and mortality rates of 100% were thus recorded.

In comparison to controls, no effects on embryo-larval development were recorded between 0.1 and 1000 µg L^{-1} regardless of the chemical studied (Fig. 3). A drastic impact of herbicide exposure was observed between 1000 µg L^{-1} and 10,000 µg L^{-1} for both Roundup® formulations (Fig. 3C and D), whereas this hazardous effect occurred between 10,000 µg L^{-1} and 100,000 µg L^{-1} for glyphosate (Fig. 3A) and AMPA (Fig. 3B). From 20,000 µg L^{-1} of glyphosate or AMPA, the rates of normal D-shaped larvae decreased, but compared to the lower concentrations, the differences were not significant (Kruskal–Wallis and SNK tests, $p > 0.05$) due to a high degree of heterogeneity (Fig. 3A and B). Compared to glyphosate, AMPA showed a higher EC_{50} value: 40,617 µg L^{-1} (versus 28,315 µg L^{-1}) (Table 2). Moreover, at the highest concentration (100,000 µg L^{-1}), glyphosate appeared to be slightly more toxic than AMPA because the net percentages of normal development (NPN_e) were 0.52% (SEM: ±0.45%) and 20.45% (SEM: ±6.88%), respectively. For the exposures to commercial formulations, no normal larvae were observed at the tested concentrations of 4000, 6000 and 8000 µg L^{-1}. This conclusion led us to narrow the range of tested concentrations of R_{EX} and R_{AT} again, to between 1000 and 2000 µg L^{-1}. From the exposure to 1000 µg L^{-1} R_{EX}, NPN_e progressively decreased up to the concentration of 1500 µg L^{-1}, which did not allow normal larval development in *C. gigas* (Fig. 3C). For R_{AT} exposure, the NPN_e profile differed because no significant effects were observed up to 1600 µg L^{-1}, and a sharp decrease was then noted (Fig. 3D). Considering both the EC_{10} and the EC_{50} values (Table 2), the tested commercial formulations were more toxic than the active matter and its metabolite, and among the Roundup® formulations, R_{EX} was the most toxic with an EC_{50} of 1133 µg L^{-1} (versus 1672 µg L^{-1} for R_{AT}).

When abnormal embryo-larval development was observed, different types of abnormalities could be distinguished (Fig. 4A–D). If we consider the controls and the range of herbicide concentrations where no effects were recorded regardless of the chemical used (i.e., 0.1 to 1000 µg L^{-1}), it appeared that the most frequent abnormalities (~4.3–6.3%) were arrested development at the "old embryo" stage (EMB) and abnormalities affecting the shell and/or hinge (SHEL). The percentages of abnormalities affecting the mantle (MANT) or both the shell and the mantle (MASH) significantly increased from approximately 1000 µg L^{-1} for the commercial formulations (Fig. 4C and D) and 10,000 µg L^{-1} for glyphosate and AMPA (Fig. 4A and B). Exposures to R_{EX} could be distinguished by the frequency of these abnormalities. At the highest concentrations, frequent arrest of development at the "old embryo" stage was observed. The level of 50% "old embryos" was not attained for AMPA and occurred at ~40,000 µg L^{-1} for glyphosate, ~1850 µg L^{-1} for R_{AT} and ~1550 µg L^{-1} for R_{EX}.

3.3. Effects of glyphosate-based herbicides on larval metamorphosis

Aside from metamorphosis rates, mortality rates were also considered because mortality processes are "more serious" than a lack of metamorphosis. For the four metamorphosis tests performed, the mortality rate of the controls was 1.73% (SEM: ±0.47%). For the exposures to glyphosate and AMPA, mortality rates were low whatever the concentration tested; they ranged, respectively, from 1.68% (SEM: ±0.71%; 10 µg L^{-1}) to 7.49% (SEM: ±2.76%;

Table 2
Ecotoxicological parameters calculated for (1) the embryotoxicity tests (rates abnormalities in D-shaped larvae) and (2) the rates of pediveliger larvae mortality and metamorphosis after 48 h exposures to 4 herbicide substances: glyphosate (GLY), AMPA, Roundup Express® (R_{EX}) and Roundup Allées et Terrasses® (R_{AT}). EC_X = effective concentration (in $\mu g\,L^{-1}$) which induces an effect on *X*% of the population (10 or 50%).

Endpoints	Parameters	GLY	AMPA	R_{EX}	R_{AT}
Abnormality rates in D-shaped larvae	EC_{10}	13,457	10,299	1006	1628
	EC_{50}	28,315	40,617	1133	1672
Mortality rates of pediveliger larvae	EC_{10}	>100,000	>100,000	6601	4991
	EC_{50}	>100,000	>100,000	8502	7934
Metamorphosis rates of pediveliger larvae	EC_{10}	>100,000	>100,000	5215	4150
	EC_{50}	>100,000	>100,000	6366	6060

100,000 $\mu g\,L^{-1}$) and from 1.22% (SEM: ±0.61%; 10 $\mu g\,L^{-1}$) to 2.84% (SEM: ±1.03%; 1000 $\mu g\,L^{-1}$). In both cases, the EC_{50} value was higher than 100,000 $\mu g\,L^{-1}$ (Table 2). By contrast, the tests applied to the commercial formulations revealed far higher mortality rates; EC_{50} values were as low as 8502 and 7934 $\mu g\,L^{-1}$ for R_{EX} and R_{AT}, respectively.

For the 4 experiments, the metamorphosis rate of the controls reached 78.03% (SEM: ±1.50%). By comparison with mortality rates, the metamorphosis rates of pediveliger larvae showed similar differences between herbicide substances. Indeed, glyphosate and AMPA (Fig. 5A) appeared far less toxic than the two commercial formulations (Fig. 5B and C), and for the former substances, the wide range tested (up to 100,000 $\mu g\,L^{-1}$) did not allow us to determine the EC_{50} values (Table 2). Although no significant differences were found among the different concentrations of AMPA (ANOVA, $p = 0.08$), the highest concentration of glyphosate induced a slight but significant decrease in the metamorphosis rate to 80.15% (SEM: ±5.50%) (ANOVA, $p < 0.001$; SNK, $p < 0.01$). The exposures to R_{AT} showed a significant decrease of the metamorphosis rate with increases in the R_{AT} concentration above 4000 $\mu g\,L^{-1}$ (Kruskal & Wallis, $p < 0.001$) (Fig. 5C). Two rather low concentrations (1 and 10 $\mu g\,L^{-1}$) led to slight decreases in the metamorphosis rate, which did not differ significantly from the rate recorded at 4000–6800 $\mu g\,L^{-1}$ (group b); however, for these two low concentrations, the standard errors were particularly high. The profile of metamorphosis rates recorded for R_{EX} exposures (Fig. 5B) was similar to that of R_{AT} exposures, and from 5000 $\mu g\,L^{-1}$, the metamorphosis rate significantly decreased with an increasing concentration of R_{EX} (Kruskal & Wallis, $p < 0.001$). Finally, for the two types of Roundup®, the EC_{50} showed similar values: 6366 $\mu g\,L^{-1}$ and 6060 $\mu g\,L^{-1}$ for R_{EX} and R_{AT}, respectively (Table 2).

4. Discussion

4.1. Concentrations of chemicals and their dynamics

In the present study, glyphosate-based herbicides were examined because of their very important use in agricultural and non-agricultural activities throughout the world (Baylis, 2000; Woodburn, 2000). Before evaluating the potential effects of these herbicides on the Pacific oyster, it was important to assess the contaminant's fate and concentration dynamics in the glass pillboxes (embryotoxicity) and in the multiwell plates (metamorphosis) during experiments lasting 48 and 24 h, respectively. In the absence of larvae, analyses of glyphosate and AMPA showed no significant

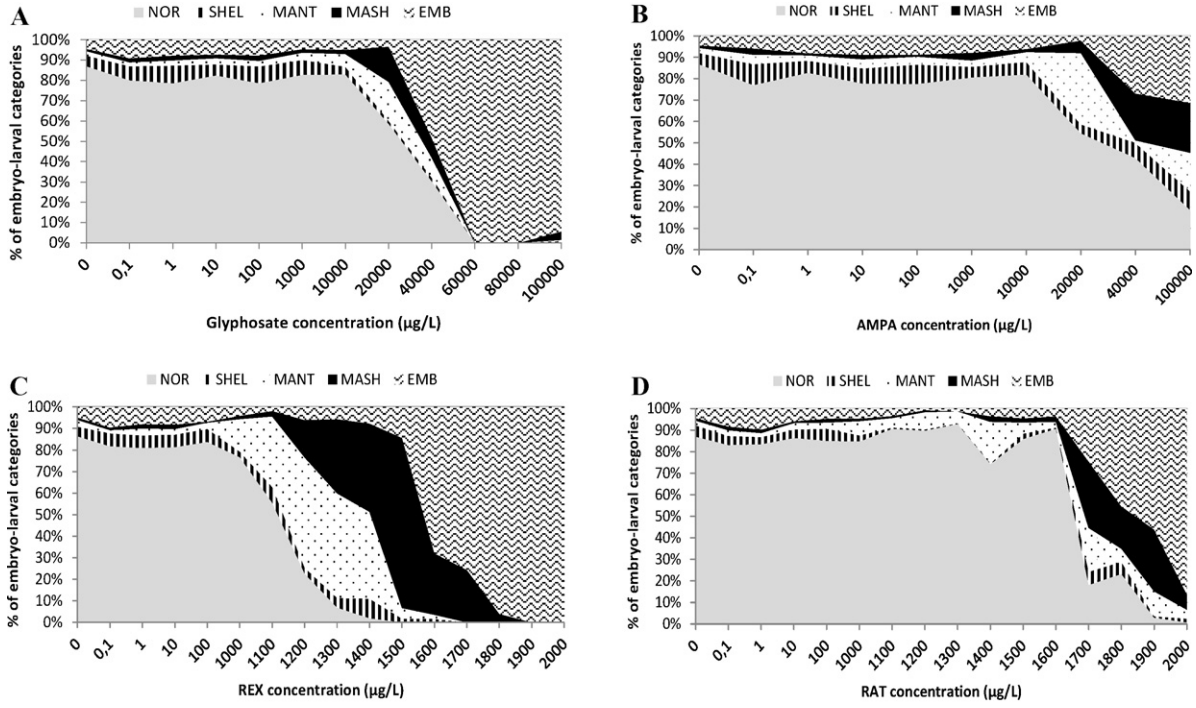

Fig. 4. The occurrence of the various types of abnormalities affecting embryo-larval development in *C. gigas* after 48 h of herbicide exposure in relation to the concentrations of 4 chemicals: (A) glyphosate; (B) AMPA; (C) Roundup Express® and (D) Roundup Allées et Terrasses®. NOR: normal D-shaped larvae; SHEL: D-shaped larvae exhibiting shell and/or hinge abnormalities; MANT: D-shaped larvae showing a hypertrophied mantle; MASH: D-shaped larvae presenting an abnormality affecting both shell/hinge and mantle; EMB: "old embryo".

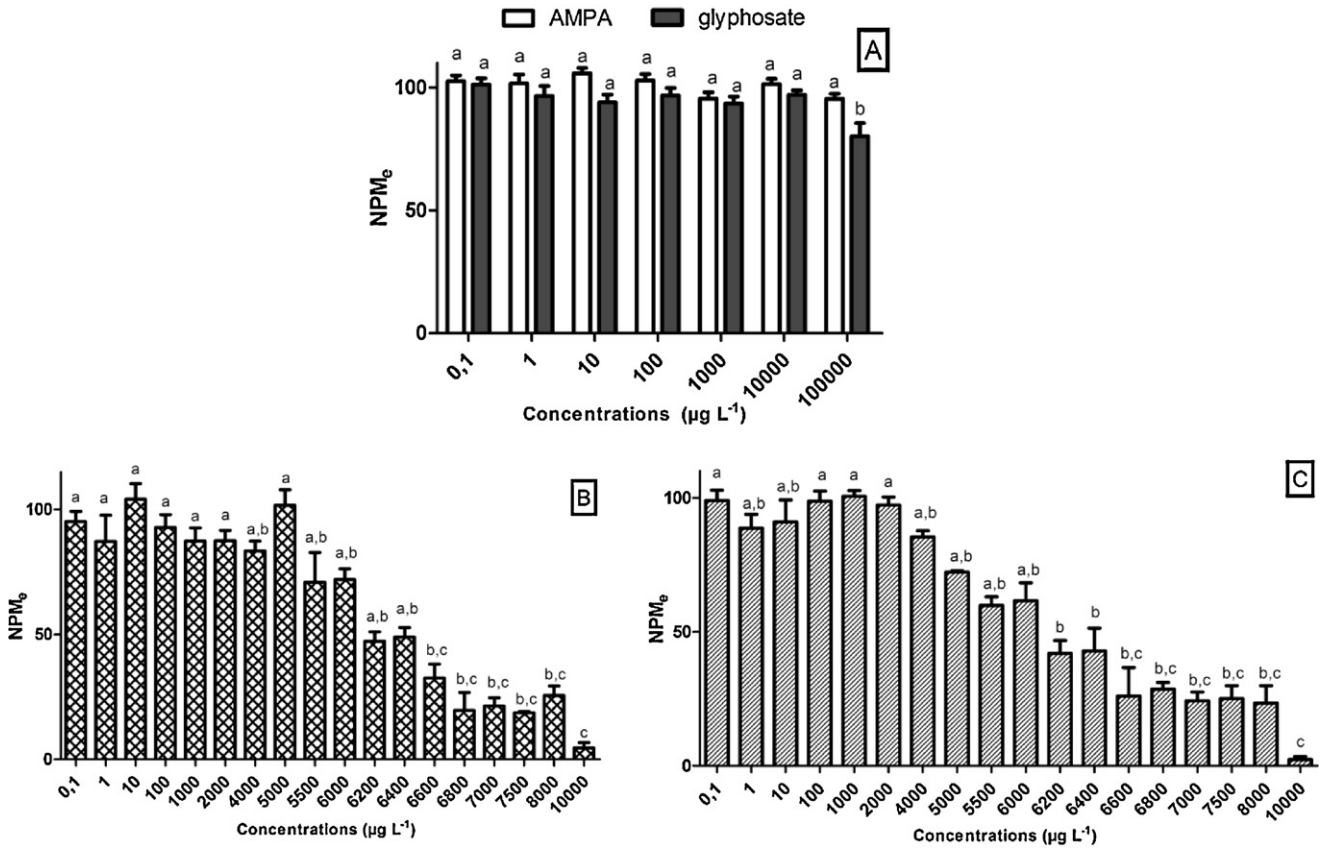

Fig. 5. Net percentages of metamorphosed larvae (NPM_e) (±SEM) observed after 24 h of exposure to herbicides at concentrations ranging from 0.1 to 100,000 µg L^{-1} for glyphosate and AMPA (A) or 10,000 µg L^{-1} for Roundup Express® (R_{EX}) (B) and Roundup Allées et Terrasses® (R_{AT}) (C). Since no metamorphosis was observed at the tested concentration of 100,000 µg L^{-1} for R_{EX} and R_{AT}, the data are therefore not presented. The concentrations that do not share a letter are significantly different; by convention, the controls belong to group a.

differences between the values measured at the beginning and the end of the experiments. This result suggested that no hydrolysis occurred in our experimental conditions; photolysis could also be excluded because the embryos and larvae were incubated in total darkness. The materials used for the embryotoxicity (glass) and metamorphosis tests (polystyrene including the treatment Surface Nunclon™ Δ) also allowed us to avoid a significant adsorption of tested chemicals onto the experimental surfaces. The embryos and larvae were thus exposed to constant herbicide concentrations. The herbicide analyses were also performed to compare the nominal concentrations with the true concentrations in our experimental conditions. At the beginning and end of the experiments, the measured concentrations were generally higher than the nominal ones, and the factor varied according to the concentrations (Table 1). For glyphosate and AMPA, the differences between the nominal and measured concentrations were most likely due to experimental handling and/or changes that occurred during the analyses. For the commercial formulations, the potential differences due to handling remains valid, but another source of difference can also be put forward. Indeed, the commercial formulations were overdosed: 6.27 g L^{-1} (instead of 4.40 g L^{-1}) for Roundup Allées et Terrasses® (R_{AT}) and 9.52 g L^{-1} (instead of 7.20 g L^{-1}) for Roundup Express® (R_{EX}). Regarding the differences between the nominal and measured concentrations, the values of EC_{50} for both endpoints were re-calculated by considering the measured concentrations (Table 3). For herbicide concentrations not analysed, theoretical measured concentrations were calculated from the concentrations analysed (i.e. 0.1; 1; 100 and 10,000 µg L^{-1}) by fitting polynomial equations, the fitness of which was evaluated by the values of R^2. For the embryotoxicity tests, the corrected values of EC_{50} were higher: +3.15% for R_{EX}, +13.51% for AMPA and +19.67% for R_{AT}, except for glyphosate (−4.02%). For the metamorphosis tests, the corrected EC_{50} values were also higher, especially for R_{AT} (+24.59%) compared to R_{EX} (+9.00%) (Table 3). Nevertheless, the values of EC_{50} and corrected EC_{50} were close for each compound and both endpoints.

Table 3
Ecotoxicological parameters corrected by considering the measured concentrations of herbicides (instead of the nominal concentrations; for comparison, see Table 2 for the embryotoxicity tests and the rates of pediveliger larvae metamorphosis). GLY = glyphosate; AMPA = glyphosate metabolite; R_{EX} = Roundup Express® and R_{AT} = Roundup Allées et Terrasses®. EC_X = effective concentration (in µg L^{-1}) that induces an effect on *X*% of the population (10 or 50%).

Endpoints	Parameters	GLY	AMPA	R_{EX}	R_{AT}
Abnormality rates in D-shaped larvae	EC_{10}	13,347	11,032	1037	1951
	EC_{50}	27,175	46,105	1168	2001
Metamorphosis rates of pediveliger larvae	EC_{10}	>100,000	>100,000	5778	5244
	EC_{50}	>100,000	>100,000	6940	7550

4.2. Effects of glyphosate-based herbicides on larval metamorphosis versus embryo-larval development

It has already been reported that early life stages of the organisms are especially sensitive to toxicological injury (e.g., Giesy and Graney, 1989; Jha et al., 2000), and the present study suggested that among the youngest stages, the embryos and 48 h D-shaped larvae were more sensitive than 21 days larvae. The sensitivity of the very early stages can be linked with the very essential processes occurring in this period; morphological axes are being formed, cells are being rearranged, their fate is specified, and multilayered axes are being formed (Gilbert, 2003). By comparison, some morphological and anatomical changes occur during metamorphosis, but they cannot be considered so important. In *C. gigas*, Baker and Mann (1994) distinguished 2 primary metamorphosis stages (prodissoconch post-larvae and dissoconch post-larvae), where the main changes are the progressive degeneration of the velum, the eyespots and then the foot; the gradual development of the gills; and the growth of the shell beyond the margin of the prodissoconch. These changes involve the histolysis of larval tissues, differentiation and proliferation of adult tissues along with associated biochemical and physiological modifications. The mechanisms that control these concomitant changes have been investigated by Coon and Bonar (1987) and García-Lavandeira et al. (2005). GABA and epinephrine induced both settlement and metamorphosis in 4 different marine bivalves, including the oyster *Ostrea edulis* (García-Lavandeira et al., 2005). By studying metamorphosis in *C. gigas*, Coon and Bonar (1987) demonstrated for the first time the existence of alpha$_1$-adrenoceptors in molluscs and suggested 10^{-4} M epinephrine to promote metamorphosis. Compared to natural substrates or substrates used in hatcheries, multiwell plates did not offer an optimal surface for pediveliger larvae to metamorphose. We performed tests without adding epinephrine, but they did not allow metamorphosis to take its course, whereas tests conducted on the same batch of pediveliger larvae led to metamorphosis when epinephrine was added. The effect of herbicides on metamorphosis was assessed by comparing the rates of exposed larvae with those of controls, but an interaction between epinephrine and the tested herbicides cannot be excluded. To avoid the addition of epinephrine, it could be interesting in the future to elaborate a protocol that allows both the use of a more convenient substrate and the possibility of distinguishing metamorphosed larvae from non-metamorphosed individuals. Nevertheless, the metamorphosis test, which is quite inexpensive and easy to apply, provides interesting and ecologically relevant results, and this test can be recommended for ecotoxicological evaluation of contaminants. Quite surprisingly, metamorphosis in bivalves has been rarely employed as an endpoint in ecotoxicology in comparison with the use of embryo-larval development. His et al. (1997) studied the impact of sediment contaminated by PAHs on the metamorphosis of *C. gigas*. They concluded that unfiltered and filtered elutriates drastically reduced larval metamorphosis and showed a dose-response.

4.3. Effects of various contaminants on the embryo-larval development of marine bivalve species

The embryo-larval development of various invertebrates constitutes a useful microscopic endpoint in ecotoxicology. However, in most of the published literature dealing with embryotoxicity, the qualitative nature of abnormalities is not specified, and the results are expressed only in terms of rates of abnormality. In the present study, distinctions between the different types of abnormalities were made, and shell abnormalities appeared to be less severe compared with arrested development (occurring earlier) or abnormalities affecting the mantle (Fig. 4). It would be interesting to determine whether shell abnormalities (showing different degrees of severity) are viable or lethal by studying the course of larval development of the affected larvae. The answer will allow a better standardisation of the results obtained by different research teams. The impacts of various contaminants on embryo-larval development have been studied in different bivalve species, and embryotoxicity is especially well documented in the Pacific oyster. (e.g. His et al., 1999; Lyons et al., 2002; Cachot et al., 2006; Wessel et al., 2007; Libralato et al., 2008; Akcha et al., 2012).

When the concentrations inducing embryotoxicity in *C. gigas* are compared for different types of chemicals, it appears that glyphosate-based herbicides are relatively weakly toxic in terms of their effect on embryo-larval development at a minimum concentration of 1000 $\mu g\,L^{-1}$ (for R_{EX}). Nice et al. (2000) reported a delay in the differentiation of D-shaped larvae exposed to 0.1 $\mu g\,L^{-1}$ 4-nonylphenol; nevertheless, after 48 h, the number of D-shaped larvae in the 3 lowest concentrations (0.1, 1 and 10 $\mu g\,L^{-1}$) did not differ significantly from the controls, whereas less than 10% of the D-shaped larvae were normal at 100 $\mu g\,L^{-1}$. PAHs are well known for their toxicity, and concentrations as low as 2.5 $\mu g\,L^{-1}$ (benzo[a]pyrene) and 100 $\mu g\,L^{-1}$ (pyrene) induced abnormal embryo-larval development (Lyons et al., 2002). Wessel et al. (2007) observed embryotoxicity of benzo[a]pyrene from the lowest concentration tested (0.05 $\mu g\,L^{-1}$), and the threshold of 50% of abnormalities reached at 50 $\mu g\,L^{-1}$; by contrast, ethinylestradiol (a compound in contraceptive pills) had no effect on larval development for the range of tested concentrations (up to 0.425 $\mu g\,L^{-1}$). Compared to the glyphosate-based herbicides tested in the current study, metals were revealed to also be more toxic, and the EC_{50} values reported by His et al. (1999) for Cl_2Hg and $CuSO_4$ were 12.3 and 37 $\mu g\,L^{-1}$, respectively. This toxicity justified the use of $CuSO_4$ as a positive control. Among pesticides, atrazine and simazine (herbicides that are now banned in several European countries) exerted no deleterious effect for concentrations $\leq$1000 $\mu g\,L^{-1}$ (abnormality rates <5%), a slight effect at 2500 and 5000 $\mu g\,L^{-1}$ (~15–20%) and a drastic effect at 10,000 $\mu g\,L^{-1}$ (75%) (Robert et al., 1986). These authors added that in the field, herbicides may also play an indirect role in the development of oyster larvae by curbing the growth of plankton. Aside from metals, His et al. (1999) tested 3 pesticides, methiocarb (molluscicide), dinoterb and glyphosate (herbicides), and they concluded that dinoterb was the only pesticide that was toxic in the range of tested concentrations (up to 200 $\mu g\,L^{-1}$), with an EC_{50} of 72.2 $\mu g\,L^{-1}$. Endosulfan is an insecticide that was investigated by Wessel et al. (2007), who demonstrated an increase in the number of abnormal D-shaped larvae according to a gradient of concentrations; this increase became statistically significant at the highest concentration of 122 $\mu g\,L^{-1}$. With regards to glyphosate, His et al. (1999) found no embryotoxic effects of this herbicide (tested up to 200 $\mu g\,L^{-1}$) on the embryo-larval development of either *C. gigas* or the sea urchin *Paracentrotus lividus*. More recently, Akcha et al. (2012) tested the potential embryotoxicity of low concentrations of two herbicides: diuron (banned in France since December 2008; 0.05–0.5 $\mu g\,L^{-1}$) and glyphosate, plus its commercial formulation, Roundup Express® (R_{EX}) (0.5 to 5 $\mu g\,L^{-1}$). In the narrow and low range of concentrations tested, these authors demonstrated a significant toxic effect of diuron on embryo-larval development from 0.05 $\mu g\,L^{-1}$. By contrast, they found no evidence of embryotoxicity for glyphosate in 2 experiments (corresponding to 2 couples of genitors) and toxicity from a concentration as low as 2.50 $\mu g\,L^{-1}$ in another experiment, which was most likely performed with genitors that were especially sensitive. Indeed, the controls of this third experiment showed an abnormality rate that was too high (~30%) to validate this experiment according to the standardised AFNOR procedure. Finally, Akcha et al. (2012) indicated no embryotoxic effect of R_{EX} and found a higher level of abnormal D-larvae after exposure to glyphosate versus R_{EX}. It

has already been reported that abnormal embryo-larval development could be explained by the accumulation of unrepaired DNA lesions. In this context, Wessel et al. (2007) conducted a study to determine both embryotoxicity and genotoxicity in the same batch of fecundated embryos exposed to pollutants. These authors found a positive and significant correlation between genotoxicity (assessed by the comet assay) and embryotoxicity. In adult oysters exposed to diuron (0.30 and 3 $\mu g\,L^{-1}$), Bouilly et al. (2007) showed a significant increase of the aneuploidy level and a transgenerational effect. Exposures of spat and adult oysters to diuron (Bouilly et al., 2007; Luna-Acosta et al., 2012) or to a mixture of pesticides including glyphosate (Gagnaire et al., 2007) induced significant changes in immunological parameters. Compared to juveniles or adults, studying the defence mechanisms of very early life stages is far more difficult to assess, and this topic is poorly documented in bivalves. Nevertheless, Thomas-Guyon et al. (2009) indicated that pro-phenoloxidase (proPO) activity, which plays an important function in non-shelf recognition, was expressed early in *C. gigas* (from 6 h embryos) and reached a maximum at 21 h. Damiens et al. (2004) investigated the capability to regulate oxidative stress in D-shaped larvae of *C. gigas* exposed to 2 insecticides: carbofuran (100 and 1000 $\mu g\,L^{-1}$) and malathion (100 and 300 $\mu g\,L^{-1}$). These authors concluded that 100 $\mu g\,L^{-1}$ of carbofuran could induce increases in both catalase and glutathione S-transferase (GST) activities, whereas this increase only concerned catalase activity with 300 $\mu g\,L^{-1}$ malathion. Regardless of the insecticide and the concentrations used, the TBARS level (expression of the lipid peroxidation) did not differ significantly between the control and exposed larvae; this result suggested that D-shaped larvae have means of regulating oxidative stress.

4.4. Sensitivity of various aquatic species to glyphosate and AMPA

Among aquatic organisms, the biological models that have classically been used to guide European legislations on contaminants including pesticides are algae (*Pseudokirchneriella subcapitata* and *Navicula pelliculosa* in freshwater; *Skeletonema costatum* in seawater), crustaceans (*Daphnia magna* in freshwater and *Americamysis bahia* in seawater) and fishes (*Cyprinus carpio* in freshwater and *Oncorhynchus mykiss*, which is an amphidromous species) (Agritox, 2012; PPDB, 2012). In the present study, no EC_{50} values could be calculated for the metamorphosis tests, and no comparisons could thus be made with biological models used in regulatory studies. By contrast, the EC_{50} value computed for the embryotoxicity tests with exposures to glyphosate (28.315 $mg\,L^{-1}$) was lower than the values reported for *O. mykiss* (38 $mg\,L^{-1}$), *D. magna* (40 $mg\,L^{-1}$), *A. bahia* (40 $mg\,L^{-1}$), *N. pelliculosa* (42 $mg\,L^{-1}$) and *C. carpio* (115 $mg\,L^{-1}$) (Agritox, 2012; PPDB, 2012). For AMPA, limited EC_{50} data are available, but it appears that the EC_{50} value calculated for embryotoxicity in *C. gigas* (40.617 $mg\,L^{-1}$) was again lower than that indicated for *O. mykiss* (520 $mg\,L^{-1}$) and *D. magna* (690 $mg\,L^{-1}$). Glyphosate and AMPA thus appeared to be more toxic to the Pacific oyster compared to the regulatory model organisms. It could therefore be suggested that the embryotoxicity test *C. gigas* could be a sensitive endpoint for ecotoxicological evaluation. Unfortunately, no comparisons could be made for R_{EX} and R_{AT} because the ecotoxicological data needed to evaluate the effects of commercial formulations on regulatory species are lacking.

4.5. Comparison of the effects of glyphosate/AMPA and Roundup® formulations

In the present study, both R_{EX} and R_{AT} appeared to be far more toxic than glyphosate and its metabolite for the two endpoints studied. In fact, the EC_{50} values computed for the embryos exposed to R_{AT} were 16.93- and 24.29-fold lower by comparison with those calculated for glyphosate and AMPA, respectively. The corresponding factors for R_{EX} compared to glyphosate and AMPA reached 24.99 and 35.85, respectively. Using 7 test organisms (i.e. *marine bacterium*, 2 species each of microalgae, ciliate and planktonic crustacea), Tsui and Chu (2003) highlighted the toxicity of the main adjuvant in Roundup®, POEA, and ranked the following toxicity order: POEA > Roundup® > glyphosate acid > IPA salt of glyphosate. Except in microalgae, POEA accounted for more than 86% of Roundup®'s toxicity, and the toxicity contribution of POEA was shown to be species-dependent. Howe et al. (2004) compared the potential toxicity of glyphosate, POEA and 6 glyphosate-based commercial formulations to 4 amphibian species (*Rana* ssp. and *Bufo americanus*). The toxicity of commercial formulations varied with both species and developmental stages, and in *Rana clamitans*, for example, no significant acute toxicity was observed for glyphosate while POEA was found to be the most toxic. In the blackworm, *Lumbriculus variegatus*, exposure to Roundup Ultra® led to a significant increase in both soluble glutathione S-transferase (sGST) and superoxide dismutase (SOD); exposure to glyphosate led to an elevation of sGST but to a lesser extent (Contardo-Jara et al., 2009). In melanophores from *Xenopus laevis*, Hedberg and Wallin (2010) reported that Roundup® formulations inhibit intracellular transport through the disassembly of the cytoskeleton, possibly by interfering with intracellular Ca^{2+}-balance. According to Peixoto (2005), glyphosate alone did not impact the bioenergetics of isolated rat liver mitochondria, in contrast to Roundup formulation products. Pieniążek et al. (2004) concluded that Roundup Ultra® induced more changes in the function of human erythrocytes than its active substance, which is most likely a result of the properties of its additives. In the backdrop of above information, it would be interesting to test the effect of POEA on the embryo-larval development and metamorphosis of *C. gigas* in future studies.

4.6. Level of contamination of aquatic ecosystems by glyphosate-based herbicides and perspectives

Some authors have reported relatively high environmental concentrations of glyphosate, with peaks reaching 137 $\mu g\,L^{-1}$ in Mediterranean river ecosystems (Puértolas et al., 2010). In hydrographical systems located in Normandy (France), the glyphosate and AMPA concentrations recorded in 2006 never exceeded 1.43 $\mu g\,L^{-1}$ and 1.19 $\mu g\,L^{-1}$, respectively (Agence de l'Eau Seine Normandie, personal communication). Glyphosate analyses in seawater are especially difficult to achieve because of the presence of salts, but the limited data available suggest concentrations of glyphosate of 1.20 $\mu g\,L^{-1}$ in shellfish production areas (Burgeot et al., 2008). Buisson et al. (2008) demonstrated that pesticides such as diuron could be found in seawater at low tide with maximum concentrations of 0.132 $\mu g\,L^{-1}$. The results provided by the current study showed that calculated EC_{50} values were much higher than environmental concentrations. We chose to test ranges of concentrations that could be considered environmentally unrealistic but that also included potential environmental concentrations (0.1 and 1 $\mu g\,L^{-1}$). Besides the EC_X values, it is also interesting to consider the PNEC (predictive no effect concentration). In the frame of the European water framework directive, environmental quality standards (EQSs) are required for substances falling under Annex VIII like glyphosate (Official Journal of the European Union, 2008). In 2010, the UK Technical Advisory Group proposed PNECs values calculated for both fresh and saltwater and both long-term and short-term experiments (Maycock et al., 2012). These values calculated by considering available ecotoxicity data for glyphosate in saltwater are potentially in the range 33 to 398 $\mu g\,L^{-1}$. These values are higher (33–398×) than the concentrations usually recorded in rivers of Normandy; unfortunately, this calculation cannot be performed for Roundup®, which was revealed to be more toxic.

Additional long-term exposures (>1 month) of spat to low doses of pesticides are planned that will allow us to study the effects of these types of contaminants in a more realistic way. Finally, this study focused only on the effects of individual contaminants (glyphosate and AMPA) or commercial formulations (glyphosate and additives), whereas contamination by pesticides is generally multiple in the environment. Further studies should therefore include mixtures of pesticides which could interact differently to induce the biological effects.

5. Conclusion

Our study provides the first data for both embryotoxicity and metamorphosis tests conducted in a marine bivalve. The two endpoints studied appeared to be convenient to determine the potential harmful effects on a non-target species, *C. gigas*. For example, the EC_{50} values computed for the embryotoxicity tests with glyphosate and AMPA were lower than the values reported for regulatory model organisms. In the Pacific oyster, the embryotoxicity test appeared more sensitive but also a little more difficult to assess compared to the metamorphosis assay. In addition to the rates of embryo-larval abnormalities, the distinction of the types of abnormalities could allow a better standardisation of the method in order to avoid subjectivity. In terms of methodology, it is also important to verify the true concentrations, and to compare them to the nominal concentrations.

In comparison to the active ingredients, fewer ecotoxicological data are generally available about the by-products and the commercial formulations. In the current study, this is especially unfortunate because R_{EX} and R_{AT} appeared to be far more toxic for the two endpoints studied. According to the toxicity classification for aquatic species (Giesy et al., 2000), glyphosate and AMPA can be considered as "slightly toxic" (embryotoxicity) or "practically nontoxic" (metamorphosis assay) whereas the two commercial formulations can be classified among the "moderately toxic" molecules for both endpoints. Further studies including chronic exposures on spat and adults to definitely conclude on the toxicity of glyphosate-based herbicides to the Pacific oyster are required.

Acknowledgements

We gratefully acknowledge the staff of the laboratory CNRS-INEE FRE BioMEA and all of the colleagues of the "Chronexpo scientific community", especially Professor Geoffrey Millward, Plymouth University, UK. This study was supported by the Region Basse-Normandie and the European Program Interreg IVA 'Chronexpo' coordinated by Dr. Bruno Fievet from IRSN (Institut de Radioprotection et de Sureté Nucléaire) of Cherbourg-Octeville. Finally, we would like to thank the anonymous reviewers for their constructive comments for improving the manuscript.

References

AFNOR, 2009. Bio indicateur de la toxicité potentielle de milieu aqueux. XP-T90-382. AFNOR, La Plaine-Saint-Denis, France, p. 19.

Agritox, 2012. http://www.dive.afssa.fr/agritox/php/fiches.php (accessed October 2012).

Akcha, F., Spagnol, C., Rouxel, J., 2012. Genotoxicity of diuron and glyphosate in oyster spermatozoa and embryos. Aquatic Toxicology 106–107, 104–113.

Amrhein, N., Deus, B., Gehrke, P., Steinrücken, H.C., 1980. The site of the inhibition of the shikimate pathway by glyphosate. Plant Physiology 66, 830–834.

Auby, I., Bocquene, G., Quiniou, F., Dreno, J.P., 2007. Etat de la contamination du bassin d'Arcachon par les insecticides et les herbicides sur la période 2005–2006. Impact environnemental. Ifremer report, p. 108.

Baker, S.M., Mann, R., 1994. Feeding ability during settlement and metamorphosis in the oyster *Crassostrea virginica* (Gmelin, 1791) and the effects of hypoxia on post-settlement ingestion rates. Journal of Experimental Marine Biology and Ecology 181, 239–253.

Battaglin, W.A., Kolpin, D.W., Scribner, E.A., Kuivila, K.M., Sandstrom, M.W., 2005. Glyphosate herbicides, and transformation products in midwestern streams, 20021. Journal of the American Water Resources Association 41, 323–332.

Baylis, A.D., 2000. Why glyphosate is a global herbicide: strengths, weaknesses and prospects. Pest Management Science 56, 299–308.

Botta, F., Lavison, G., Couturier, G., Alliot, F., Moreau-Guigon, E., Fauchon, N., Guery, B., Chevreuil, M., Blanchoud, H., 2009. Transfer of glyphosate and its degradate AMPA to surface waters through urban sewerage systems. Chemosphere 77, 133–139.

Brereton, A., Lord, H., Thornton, I., Webb, J.S., 1973. Effect of zinc on growth and development of larvae of the Pacific oyster *Crassostrea gigas*. Marine Biology 19, 96–101.

Bouilly, K., Bonnard, M., Gagnaire, B., Renault, T., Lapègue, S., 2007. Impact of diuron on aneuploidy and hemocyte parameters in Pacific oyster, *Crassostrea gigas*. Archives of Environment Contamination and Toxicology 52, 58–63.

Buisson, S., Bouchart, V., Guerlet, E., Malas, J.P., Costil, K., 2008. Level of contamination and impact of pesticides in cupped oyster, *Crassostrea gigas*, reared in a shellfish production area in Normandy (France). Journal of Environment Science and Health, Part B Pesticides 43, 655–664.

Burgeot, T., Gagnaire, B., Renault, T., Haure, J., Moraga, D., David, E., Boutet, I., Sauriau, P.G., Malet, N., Bouchet, V., Le Roux, F., Lapègue, S., Bouilly, K., Le Moullac, G., Arzul, I., Knoery, J., Quiniou, F., Bacher, C., Soletchnik, P., 2008. Les risques associés au stress environnemental. In: Samain, J.F., McCombie, H. (Eds.), Summer Mortality of Pacific Oyster *Crassostrea gigas*. The Morest Project. Ifremer/Quae, Versailles, France, pp. 95–139.

Cachot, J., Geffard, O., Augagneur, S., Lacroix, S., Le Menach, K., Peluhet, L., Couteau, J., Denier, X., Devier, M.H., Pottier, D., Budzinski, H., 2006. Evidence of genotoxicity related to high PAH content of sediments in the upper part of the Seine estuary (Normandy France). Aquatic Toxicology 79, 257–267.

Carpentier, S., Moilleron, R., Beltran, C., Hervé, D., Thévenot, D., 2002. Quality of dredged material in the river Seine basin (France). II. Micropollutants. Science of the Total Environment 299, 57–72.

Cheung, V.V., Jha, A., Owen, R., Depledge, M.H., Galloway, T.S., 2006. Development of the in vivo chromosome aberration assay in oyster (*Crassostrea gigas*) embryo-larvae for genotoxicity assessment. Marine Environment Research 62 (Suppl. 1), S278–S282.

CNC (Comité National de la Conchyliculture), 2012. http://www.cnc-france.com/La-Production-francaise.aspx (accessed October 2012).

Contardo-Jara, V., Klingelmann, E., Wiegand, C., 2009. Bioaccumulation of glyphosate and its formulation Roundup Ultra in *Lumbriculus variegatus* and its effects on biotransformation and antioxidant enzymes. Environmental Pollution 157, 57–63.

Coon, S.L., Bonar, D.B., 1987. Pharmacological evidence that alpha$_1$-adrenoceptors mediate metamorphosis of the pacific oyster, *Crassostrea gigas*. Neuroscience 23, 1169–1174.

Costil, K., Royer, J., Ropert, M., Soletchnik, P., Mathieu, M., 2005. Spatio-temporal variations in biological performances and summer mortality of the Pacific oyster *Crassostrea gigas* in Normandy (France). Helgoland Marine Research 59, 286–300.

Damiens, G., His, E., Gnassia-Barelli, M., Quiniou, F., Roméo, M., 2004. Evaluation of biomarkers in oyster larvae in natural and polluted conditions. Comparative Biochemistry and Physiology C: Comparative Pharmacology 138, 121–128.

De Mora, S., Fowler, S.W., Tolosa, I., Villeneuve, J.-P., Cattini, C., 2005. Chlorinated hydrocarbons in marine biota and coastal sediments from the Gulf and Gulf of Oman. Marine Pollution Bulletin 50, 835–849.

Gagnaire, B., Gay, M., Huvet, A., Daniel, J.Y., Saulnier, D., Renault, T., 2007. Combination of a pesticide exposure and a bacterial challenge: *in vivo* effects on immune response of Pacific oyster, *Crassostrea gigas* (Thunberg). Aquatic Toxicology 84, 92–102.

García-Lavandeira, M., Silva, A., Abad, M., Pazos, A.J., Sánchez, J.L., Luz Pérez-Parallé, M., 2005. Effects of GABA and epinephrine on the settlement and metamorphosis of the larvae of four species of bivalve molluscs. Journal of Experimental Marine Biology and Ecology 316, 149–156.

Geffard, A., Geffard, O., Amiard, J.C., His, E., Amiard-Triquet, C., 2007. Bioaccumulation of metals in sediment elutriates and their effects on growth, condition index, and metallothionein contents in oyster larvae. Archives of Environment Contamination and Toxicology 53, 57–65.

Giesy, J.P., Graney, R.L., 1989. Recent developments in and intercomparisons of acute and chronic bioassays and bioindicators. Hydrobiologia 188–189, 21–60.

Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecological risk assessment for Roundup® herbicide. Reviews of Environment Contamination and Toxicology 167, 35–120.

Gilbert, S.F., 2003. Developmental Biology. Sinauer Associates, Sunderland.

Hagger, J.A., Atienzar, F.A., Jha, A.N., 2005. Genotoxic, cytotoxic, developmental and survival effects of tritiated water in the early life stages of the marine mollusc, *Mytilus edulis*. Aquatic Toxicology 74, 205–217.

Hedberg, D., Wallin, M., 2010. Effects of Roundup and glyphosate formulations on intracellular transport, microtubules and actin filaments in *Xenopus laevis* melanophores. Toxicology in Vitro 24, 795–802.

His, E., Budzinski, H., Geffard, O., Beiras, R., 1997. Action d'un sédiment pollué par les hydrocarbures sur la métamorphose de l'huître japonaise, *Crassostrea gigas* (Thunberg). Comptes Rendus de l' Academie des Sciences Serie III: Sciences de la Vie 320, 797–803.

His, E., Heyvang, I., Geffard, O., de Montaudouin, X., 1999. A comparison between oyster (*Crassostrea gigas*) and sea urchin (*Paracentrotus lividus*) larval bioassays for toxicological studies. Water Research 33, 1706–1718.

Howe, C.M., Berrill, M., Pauli, B.D., Helbing, C.C., Werry, K., Veldhoen, N., 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environmental Toxicology and Chemistry 23, 1928–1938.

Jha, A.N., Hagger, J.A., Hill, S.J., Depledge, M.H., 2000. Genotoxic, cytotoxic and developmental effects of tributyltin oxide (TBTO): an integrated approach to the evaluation of the relative sensitivities of two marine species. Marine Environment Research 50, 565–573.

Libralato, G., Losso, C., Arizzi Novelli, A., Citron, M., Della Sala, S., Zanotto, E., Cepak, F., Volpi Ghirardini, A., 2008. Ecotoxicological evaluation of industrial port of Venice (Italy) sediment samples after a decontamination treatment. Environmental Pollution 156, 644–650.

Luna-Acosta, A., Renault, T., Thomas-Guyon, H., Faury, N., Saulnier, D., Budzinski, H., Le Menach, K., Pardon, P., Fruitier-Arnaudin, I., Bustamante, P., 2012. Detection of early effects of a single herbicide (diuron) and a mix of herbicides and pharmaceuticals (diuron, isoproturon, ibuprofen) on immunological parameters of Pacific oyster (*Crassostrea gigas*) spat. Chemosphere 87, 1335–1340.

Lyons, B., Pascoe, C., McFadzen, I.R., 2002. Phototoxicity of pyrene and benzo[a]pyrene to embryo-larval stages of the Pacific oyster *Crassostrea gigas*. Marine Environment Research 54, 627–631.

Maycock, D., Crane, M., Atkinson, C., Johnson, I., 2012. Proposed EQS for Water Framework Directive Annex VIII Substances: Glyphosate (For Consultation). Water Framework Directive – United Kingdom Technical Advisory Group (WFD-UKTAG)/SNIFFER, p. 121 http://www.wfduk.org/sites/default/files/Media/Glyphosate%20-%20UKTAG.pdf (accessed October 2012).

Munaron, D., 2004. Etude des apports en herbicides et en nutriments par la Charente: modélisation de la dispersion de l'atrazine dans le bassin de Marennes-Oléron. Ph.D. thesis. Univ. Paris VI, 340 pp.

Nice, H., Thorndyke, M., Morritt, D., Steele, S., Crane, M., 2000. Development of *Crassostrea gigas* larvae is affected by 4-nonylphenol. Marine Pollution Bulletin 40, 491–496.

Official Journal of the European Union, 2008. Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on Environmental Quality Standards in the Field of Water Policy, Amending and Subsequently Repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and Amending Directive 2000/60/EC of the European Parliament and of the Council, 14 pp.

Peixoto, F., 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosphere 61, 1115–1122.

PPDB (Pesticide Properties DataBase), 2012. http://sitem.herts.ac.uk/aeru/projects/ppdb/index.htm (accessed October 2012).

Pieniążek, D., Bukowska, B., Duda, W., 2004. Comparison of the effect of Roundup Ultra 360 SL pesticide and its active compound glyphosate on human erythrocytes. Pesticide Biochemistry and Physiology 79, 58–63.

Puértolas, L., Damásio, J., Barata, C., Soares, A.M.V.M., Prat, N., 2010. Evaluation of side-effects of glyphosate mediated control of giant reed (*Arundo donax*) on the structure and function of a nearby Mediterranean river ecosystem. Environmental Research 110, 556–564.

Quiniou, F., Damiens, G., Gnassia-Barelli, M., Geffard, A., Mouneyrac, C., Budzinski, H., Roméo, M., 2007. Marine water quality assessment using transplanted oyster larvae. Environment International 33, 27–33.

Robert, R., His, E., Maurer, D., 1986. Toxicité d'un desherbant, l'atrazine-simazine, sur les jeunes stades larvaires de *Crassostrea gigas* et sur deux algues fourrages, *Isochrysis aff-galbana* et *Chaetoceros calcitrans*. Haliotis 15, 319–325.

Ropert, M., Mazurié, J., Bédier, E., Le Coz, F., Soletchik, P., 2008. Evaluation of summer mortality risk factors in shellfish farming ecosystems. In: Samain, J.F., McCombie, H. (Eds.), Summer Mortality of Pacific oyster *Crassostrea gigas*. The Morest Project. Ifremer/Quae, Versailles, France, pp. 1–61.

Royer, J., Ropert, M., Costil, K., 2007. Spatio-temporal changes in mortality, growth and condition of the Pacific oyster, *Crassostrea gigas*, in Normandy (France). Journal of Shellfish Research 26, 973–984.

Samain, J.F., Dégremont, L., Soletchnik, P., Haure, J., Bédier, E., Ropert, M., Moal, J., Huvet, A., Bacca, H., Van Wormhoudt, A., Delaporte, M., Costil, K., Pouvreau, S., Lambert, C., Boulo, V., Soudant, P., Nicolas, J.L., Le Roux, F., Renault, T., Gagnaire, B., Geret, F., Boutet, I., Burgeot, T., Boudry, P., 2007. Genetically based resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*) and its relationship with physiological, immunological characteristics and infection processes. Aquaculture 268, 227–243.

Scherrer, B., 1984. Biostatistique. Gaëtan Morin, Montreal.

Schnitzler, J.G., Thomé, J.P., Lepage, M., Das, K., 2011. Organochlorine pesticides, polychlorinated biphenyls and trace elements in wild European sea bass (*Dicentrarchus labrax*) of European estuaries. Science of the Total Environment 409, 3680–3686.

Scott, G.I., Fulton, M.H., Wirth, E.F., Chandler, G.T., Key, P.B., Daugomah, J.W., Bearden, D., Chung, K.W., Strozier, E.D., DeLorenzo, M., Sivertsen, S., Dias, A., Sanders, M., Macauley, J.M., Goodman, L.R., LaCroix, M.W., Thayer, G.W., Kucklick, J., 2002. Toxicological studies in tropical ecosystems: an ecotoxicological risk assessment of pesticide Runoff in South Florida estuarine ecosystems. Journal of Agricultural and Food Chemistry 50, 4400–4408.

Soletchnik, P., Ropert, M., Mazurié, J., Gildas Fleury, P., Le Coz, F., 2007. Relationships between oyster mortality patterns and environmental data from monitoring databases along the coasts of France. Aquaculture 271, 384–400.

SOS (Service de l'Observation et des Statistiques), 2010. Les pesticides dans les milieux aquatiques; données 2007. Tregouet B, La Défense, 48 pp.

Thomas-Guyon, H., Gagnaire, B., Bado-Nilles, A., Bouilly, K., Lapègue, S., Renault, T., 2009. Detection of phenoloxidase activity in early stages of the Pacific oyster *Crassostrea gigas* (Thunberg). Developmental and Comparative Immunology 33, 653–659.

Tsui, M.T.K., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52, 1189–1197.

Tu, M., Hurd, C., Randall, J.M., The Nature Conservancy, 2001. Weed Control Methods Handbook: Tools & Techniques for Use in Natural Areas, 218 pp.

UIPP (Union des Industries de la Protection des Plantes), 2012. Rapport d'activité UIPP 2011/2012, 43 pp. http://www.uipp.org/Actualites/Legislation-et-reglementation/Actualite-francaise/Rapport-d-activite-UIPP-2011-2012-consultable-en-ligne (accessed October 2012).

Vindimian, E., 2012. MSExcel Macro Regtox.7.0.6 Freely Available from Eric Vindimian. IRSTEA, France http://www.normalesup.org/~vindimian/fr_index.html (accessed October 2012).

Wang, G., Liu, B., Tang, B., Zhang, T., Xiang, J., 2006. Pharmacological and immunocytochemical investigation of the role of catecholamines on larval metamorphosis by β-adrenergic-like receptor in the bivalve *Meretrix meretrix*. Aquaculture 258, 611–618.

Wessel, N., Rousseau, S., Caisey, X., Quiniou, F., Akcha, F., 2007. Investigating the relationship between embryotoxic and genotoxic effects of benzo[a]pyrene, 17alpha-ethinylestradiol and endosulfan on *Crassostrea gigas* embryos. Aquatic Toxicology 85, 133–142.

Williams, G.M., Kroes, R., Munro, I.C., 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regulatory Toxicology and Pharmacology 31, 117–165.

Woodburn, A.T., 2000. Glyphosate: production, pricing and use worldwide. Pest Management Science 56, 309–312.