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In vitro effects of glyphosate-based herbicides and related adjuvants on primary culture of hemocytes from *Haliotis tuberculata*

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Abstract

Glyphosate-based herbicides are among the most produced and widely-used herbicides. Studies have shown that commercial formulations and adjuvants may be more toxic to non-target organisms than the active ingredients alone, but the mechanisms of action of these chemicals remain unclear. The aim of this study was to investigate the *in vitro* effects of glyphosate, a commercial formulation and adjuvant alone using primary culture of hemocytes from the European abalone *Haliotis tuberculata*, a commonly farmed shellfish. Glyphosate was found to have negligible effects on viability, phagocytic activities and lysosome stability even with very high doses (i.e. 100 mg L^{-1}). By contrast, greater effects on viability were observed for the commercial formulation and adjuvant alone, with EC_{50} values of 41.42 mg L^{-1} and 1.85 mg L^{-1}, respectively. These results demonstrate that the toxic sublethal effects (i.e. phagocytic activity and destabilization of lysosomal membranes) of formulated glyphosate came from adjuvants and suggest they may be related to cell and organelle membrane destabilization.

1. Introduction

The quality of coastal waters greatly depends on human activities in the upstream areas. Among the various contaminants that may be relevant, pesticides from agricultural and domestic activities can be carried by freshwater *via* run-off and leaching processes and subsequently contaminate marine coastal areas [1-3]. Herbicide formulations containing glyphosate as the active ingredient are among the most commonly used pesticides in the world [4]. In these commercial formulations, adjuvants are used to promote the penetration of the active ingredient into plant cuticles [5]. The most common adjuvants in glyphosate-based herbicides are polyethoxylated tallow amines (POEAs) that can be formulated in an oxide:tallow-amine ratio ranging from 5:1 to 25:1. The half-life of POEAs in water has been

estimated from 21 to 42 days suggesting that this adjuvant is relatively persistent in water [6]. For glyphosate itself, hydrolysis (>30 days for pH ranging from 5 to 9) and photolysis times in water (69 and 77 days, for pH of 7 and 9, respectively) also reveal its relative persistence (e.g. [7]). Furthermore, studies have shown that glyphosate is detectable in rivers [8-11] with maximum concentrations higher than 100 µg L^{-1} [12], in contrast to around 1 µg L^{-1} in coastal waters [3,13]. Data from literature have indicated that commercial formulations appeared more toxic for a large panel of non-target organisms compared to glyphosate alone [4,14-16] which may indicate toxicity of adjuvants alone or additive or synergistic toxicity of adjuvants with other(s) component(s) of the formulated compounds. Indeed, the toxicity of POEAs has already been demonstrated in different taxonomic groups such as amphibians [4], freshwater crustaceans [17,18] and molluscs [19,20]. For example, the embryotoxicity of POEAs was quantified after 36 h of exposure considering both arrested development and abnormalities in D-shaped larvae of the Pacific oyster *Crassostrea gigas* [20]; the results suggested that POEAs could be considered very toxic to embryo larval development according to the European toxicity classification [6].

The abalone, *Haliotis tuberculata*, is a marine gastropod species, which can be found in the Northeast Atlantic from Senegal to Ireland. Abalone have been used as sensitive species to assess pollution of coastal areas [21,22] or the potential toxicity of chemical compounds [23-26]. In molluscs, the hemocytes are key components of the immune system, responsible for various mechanisms of defense, such as phagocytosis, pathogen hydrolysis or Reactive Oxygen Species (ROS) production [27-30]. In bivalves, such as *Crassostrea gigas*, many types of hemocytes have been described, including eosinophilic, basophilic and an intermix between granulocytes, vesicular and blast-like cells [31]. By contrast in *H. tuberculata*, Travers et al. [30] have described only one type of hemocytes, i.e. hyalinocytes which can be separated into to two sub-types: blast-like and large cells. As hemocytes play an essential role

in mollusc immunity, the effects of contaminants on these cells could lead to adverse effects for the whole animal [32,33]. Indeed, experiments conducted in the gastropod *Biomphalaria glabrata* by de Monte et al [34] showed that infection with the platyhelminth *Echinostoma paraensei* caused a decrease of circulating hemocytes, and also that association between infection and exposure to the Roundup® (concentration equivalent to 36 mg L^{-1} of glyphosate) greatly increased the percentage of non-viable cells, making the snails more vulnerable to parasitic infections.

In vitro studies are useful tools to assess the potential risks induced by anthropogenic contaminants in the aquatic environments. Indeed, these tools provide good alternatives to animal experimentation and take ethical issues into consideration [35]. *In vitro* methodologies also allow assessment of the effects of multiple contaminant concentrations on the cells of a limited number of individuals, thereby reducing variability, and are easily reproducible. Although the use of *in vitro* tools gives a limited view of the physiological processes that occur at the *in vivo* level, cell culture provides precious information on the mechanisms of toxicity [24]. While several studies have been published which focused on the effects of various contaminants on bivalve hemocytes *in vitro* (e.g. [36-38]), few ecotoxicological investigations have been performed on *Haliotis spp.* hemocytes. However, in the 2010s, these limited numbers of studies have increased, including those demonstrating the adverse effects of metals [24,26], antibacterial agents [23] and antidepressants [39] on hemocytes in *H. tuberculata*. However, there is still a lack of data concerning the effects of herbicides on the hemocytes of gastropods including *H. tuberculata.*

The aims of this study were to assess the *in vitro* effects of (1) glyphosate; (2) a commercial formulation containing glyphosate as the active ingredient (Roundup Express®); and (3) POEA adjuvants on hemocytes; in *H. tuberculata* after 72h exposure by using 3 established biomarkers: viability assessment (MTT assay), phagocytosis (fluorescent beads)

and lysosomal stability (neutral red retention assay). Phagocytosis by hemocytes is the cornerstone of the molluscan immune system [40]. Lysosomes, cellular organelles, are essential components of the humoral immune response in mollusc species. Lysosomes content is released after phagocytosis in order to digest foreign material.

2. Materials and methods

2.1. Hemocyte primary culture

Adult abalone (8-10 cm) were bred and provided by France Haliotis® (Plougerneau, France). The animals were maintained in the Centre de Recherche en Environnement Côtier (CREC; Luc-sur-Mer, Normandy, France) in natural sea water with aeration and *ad libitum* algae supply (*Laminaria sp. and Palmaria sp.*) for a minimum of 2-weeks acclimation.

Primary cell culture of abalone hemocytes has been previously described [24,26,41,42]. Briefly, hemocytes were sampled from the adductor muscle of *H. tuberculata*. Hemolymph was withdrawn from a medio-lateral incision using a syringe fitted with a 25 gauge needle. In order to avoid any cell aggregation, the syringe was moisturized with an Alsever solution (115 mM glucose; 27 mM sodium citrate; 11.5 mM EDTA; 382 mM NaCl). Hemolymph was transferred to a 15 mL centrifuge tube and diluted 1:4 with Alsever solution. Hemocytes were counted in triplicate by using a Thoma cell counting chamber. Cells were plated in 12-well culture plates (NUNC®; Penfield, New York, USA) at a density of 5×10^5 cells per well (MTT assay and phagocytosis analyses) or in 96-well culture plates (neutral red retention assay) at a density of 1×10^5 cells per well. Hemolymph was diluted 1:4 (v/v) with sterile artificial sea water (ASSW). After 1 h, ASSW was removed and replaced with 500 µL (12-well plates) or 200 µL (96-well plates) of sterile modified Hank's 199 medium (250 mM NaCl, 10 mM KCl, 25 mM MgSO4, 2.5 mM CaCl2 and 10 mM Hepes, 2 mM l-glutamine,

100 µg mL^{-1} streptomycin, 60 µg mL^{-1} penicillin G and 2 mM concanavalin; pH: 7.4). Cells were incubated for 24 h before beginning any pesticide exposure. Cells were then exposed for 72 h to the different chemicals and all the cultures were performed in an incubator at 17°C without extra CO_2.

2.2. Exposures to chemicals

In the present study, glyphosate acid (97% purity, CAS number: 1071-83-6) and the POEA mixture were obtained from Dr. Ehrenstorfer GmbH® (Augsburg, Germany) whereas Roundup Express® (R_{EX}) was purchased from a garden centre. For R_{EX}, all the concentrations given in this study were expressed in glyphosate equivalents. All solutions of chemical compounds used were prepared with sterile Hank's M199 medium. Three different endpoints were studied: viability (MTT assay), lysosomal stability (neutral red retention assay) and phagocytic activities (fluorescent beads). The tested concentrations are provided as supplementary data (S1 Table). For the MTT assay, all the concentrations (from 0.1 to 100,000 µg L^{-1}) were tested at least with the cells of four abalones and in triplicate ($N \geq 12$). For neutral red retention assay (NRRA) and phagocytic activities the tested concentrations were chosen according to previous MTT results for R_{EX} and POEAs: No Observed Effect Concentration (NOEC) observed from MTT assay, EC_{20} calculated from MTT, EC_{50} calculated from MTT and finally EC_{80} calculated from MTT. Glyphosate tested concentrations for NRRA and phagocytic activities corresponded to one low and two high concentrations. For the NRRA and the phagocytic activities, all the concentrations were tested at least with the cells of three abalones and in triplicate ($N \geq 9$).

2.3. Studied endpoints

2.3.1. MTT assay

Cell viability was estimated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay. This test was adapted to molluscan cell cultures by Domart-Coulon et al. [43]. It measures the capacity of mitochondrial succinyl dehydrogenase in living cells to convert MTT (yellow) into formazan (dark blue). Briefly, 50 µL of a stock solution (5 mg mL^{-1} MTT in PBS) was added to culture plates (10% v/v). After 24 h incubation, 50 µL of acidified isopranol (0.04 N HCl) was added to each well in order to dissolve neo-formed formazan. Absorbance was measured at 570 nm with a 630 nm reference, and results were expressed as percentages of viability relative to absorbance of the negative control group.

2.3.2. Phagocytic activity

The phagocytic rate of hemocytes was assessed by flow cytometry as described previously [24,26,39]. Briefly, the medium was removed and replaced with 500 µL of pesticide-free medium containing fluorescent latex beads (100 carboxylate-modified FluoroSpheres®/hemocyte, yellow-green fluorescence, 1 µm diameter, Molecular Probes®). After 1 h incubation, the medium was removed and cells were rinsed and then gently scraped into 500 µL MPS (molluscan physiological saline). The hemocyte samples were centrifuged for 10 min at 500 *g* and the resulting pellet was delicately fixed in 300 µL of 3% formaldehyde for further analysis. Hemocytes were analyzed by using a Gallios™ flow cytometer (Beckman Coulter®). A minimum of 20,000 events was considered for each sample. The level of fluorescence was evaluated using FL1 channel. The percentage of phagocytic cells was evaluated as the percentage of hemocytes that had engulfed at least three beads (i.e. immunoefficiency).

2.3.3. Neutral red retention assay (NRRA)

The stability of lysosomal membranes was assessed by the neutral red retention assay (NRRA) as previously described [39] with modifications. Hank's 199 medium was removed and replaced by 300 µL of neutral red working solution (2.10^{-2} M neutral red in MPS: 400 mM NaCl ; 100 mM $MgSO_4$; 20 mM HEPES ; 10 mM CaCl2 ; 10 mM KCl. After 3 h of incubation in the dark, neutral red solution was removed, cells were gently rinsed with MPS and 200 µL of elution solution (1:50:49 v/v/v of acetic acid, absolute ethanol, ultrapure water) was added to each well. Plates were then gently agitated for 30 min in the dark. Finally, the optical density of each well was read using a multiplate reader (FlexStation 3®, Molecular Devices LLC.; Chicago, USA) at 540 and 650 nm as a reference.

2.4. Statistical analyses

As the data from MTT assay and neutral red retention do not meet the assumption of normality and homoscedasticity for an ANOVA, these data were analyzed using non-parametric Kruskal-Wallis (K-W) tests (k > 2) or Mann-Whitney tests (k = 2) for independent samples. In case of H_0 rejection after a K-W test, *post-hoc* Dunn tests were used in order to detect differences among the different concentrations. The data for phagocytic activities and NRRA fulfilled requirements for analysis by one way ANOVAs followed by *a posteriori* Student-Newman-Keuls (SNK) tests or t-tests (k = 2). The statistical analyses were performed using STATISTICA 8.0 software (Statsoft®, Tulsa, OK, USA). EC_{50} values were computed with non-linear regressions (Hill equation) using Excel® macro REGTOX [44].

3. Results

3.1. MTT viability assay

Glyphosate did not induce any significant decrease in MTT activities even at very high concentrations (100,000 µg L^{-1}) (Fig. 1A). No differences from the negative control were found for R_{EX} concentrations lower than 20,000 µg L^{-1} (Fig. 1B). However, exposure to 40,000 µg L^{-1} R_{EX} caused a highly significant decrease of MTT values ($p < 0.001$) to 52.30% (± 18.78) of the control viability. The viability declined to 6.84% (± 4.75) of the control viability at 100,000 µg L^{-1}. The concentration leading to 50% mortality (EC_{50}) was 41,420 µg L^{-1}. After POEA exposure a significant ($p < 0.001$) decrease of hemocyte viability was observed from 1280 µg L^{-1} (67.27 ± 13.74% of the control viability) to 6400 µg L^{-1} (3.42 ± 1.93% of the control viability) (Fig. 1C). Finally, an EC_{50} of 1855 µg L^{-1} was calculated for exposures to POEAs.

3.2. Phagocytic activity

Under our experimental conditions, the percentage of hemocytes that engulfed three beads or more was 33.90 ± 7.91 for the controls. After glyphosate exposure, no significant changes in phagocytic activities were observed (Fig. 2A). Phagocytic activities significantly decreased ($p < 0.001$) after exposures to R_{EX} from 10,000 (NOEC MTT) to 68,000 µg L^{-1} (EC_{80} MTT) (Fig. 2B). Interestingly, the NOEC MTT concentration decreased phagocytic activity (53.52 % ± 16.43 of the control phagocytosis). Exposure to POEAs led to a significant decrease ($p < 0.001$) in hemocytes phagocytosis at concentrations of 1024 µg L^{-1} (59.76 % ± 15.57 of the control phagocytosis), 1920 µg L^{-1} (61.09 % ± 25.82 of the control phagocytosis) and 3200 µg L^{-1} (53.55 %) (Fig. 2C). An overall comparison between the 3 chemicals tested at the NOEC MTT concentrations revealed significant differences ($p < 0.05$) with a more pronounced effect of R_{EX} compared to glyphosate (the concentration of 100,000 being considered as the NOEC MTT; $p < 0.05$) and POEAs ($p < 0.01$) (Fig. 2). Whereas the effects of R_{EX} and POEAs on phagocytosis efficiency significantly differed when exposed at

EC_{20} MTT concentrations ($p < 0.05$) it was no longer the case when exposed at EC_{50} MTT ($p = 0.49$) and EC_{80} MTT ($p = 0.48$) concentrations.

3.3. Neutral red retention assay (NRRA)

Low doses of glyphosate (i.e. 0.1 µg L^{-1}) induced a slight but significant increase (×1.29) of neutral red retention (NRR) in lysosomes compared to control group ($p < 0.05$). In contrary, the two highest doses of glyphosate (10,000 µg L^{-1} and 100,000 µg L^{-1}) did not lead to any significant changes in lysosomal stability (Fig. 3A) ($p = 0.0256$). A trend toward an increase of neutral red retention in lysosomes was also observed in hemocytes exposed to the lowest doses of $R_{EX}^{®}$ (i.e. 0.1 µg L^{-1}) but was not statistically significant (Fig. 3B). Nevertheless, at higher doses of $R_{EX}^{®}$, NRR values were significantly ($p < 0.001$) lower compared to the control and ranged from 12.03% (± 4.80%) at 10,000 µg L^{-1} (NOEC MTT) to 2.74% (± 5.81%) at 68,000 µg L^{-1} (EC_{80} MTT). Finally, POEA exposure did not induce significant modification of neutral red retention up to 1024 µg L^{-1} (EC_{20} MTT) compared to the control (Fig. 3C). However, large significant losses of lysosome neutral red were observed at 1920 (EC_{50} MTT) and 3200 µg L^{-1} (EC_{80} MTT), with relative values of 1.61% (± 4.37) and 0.54% (± 3.52), respectively. The overall comparison between the 3 chemicals tested at the NOEC MTT or the 3 EC concentrations for NRR led to similar results than those recorded for phagocytic activity: at the NOEC MTT concentrations, a higher effect of R_{EX} compared to glyphosate ($p < 0.001$) and POEAs ($p < 0.001$), and only at EC_{20} MTT concentrations, a more marked effect of R_{EX} by comparison to POEAs ($p < 0.001$).

4. Discussion

Hemocytes have a predominant role in the immune response in molluscs and adverse effects to these cells could lead to fatal consequences for the whole animal. Effects on

hemocytes could be lethal and reflected by biomarkers such as MTT activities or sublethal by affecting the phagocytic activity and/or the lysosomal system.

At the range of concentrations tested, glyphosate did not lead to significant changes in hemocyte MTT activities while exposures to 1024 mg L^{-1} POEAs and 25,000 µg L^{-1} R_{EX} induced a significant effect of 20% (EC_{20}). Beyond these values, cellular viability sharply decreased and a dose-response curve was recorded. In the freshwater snail *Biomphalaria glabrata*, *in vivo* 24h-exposure to Roundup original® at the concentration of 36 mg L^{-1} induced a significant increase of the number of dead hemocytes assessed by trypan blue vital dye exclusion assay [34]. Viability tests based on mitochondrial activity (such as MTT assay) are also sensitive endpoints that have been previously used to assess the toxicity of different chemical compounds in marine molluscs. Domart-Coulon et al. [43] have shown the toxicity of Mexel-432® (anti-fouling compound) on the heart cells of *Crassostrea gigas* and gill cells from the clam *Ruditapes decussatus.* In *H. tuberculata* hemocytes, viability tests using mitochondrial activities were used to assess the effect of zinc [26], cadmium [24], triclosan [23] and antidepressants [39]. To our knowledge, no data are available on the cytotoxicity of the compounds studied in this work by using *in vitro* mollusc cell cultures. However, *in vitro* toxicity of commercial formulations and adjuvants has been previously reported in different mammalian cell types and gives interesting points of comparison with our results. Mesnage and his collaborators [45] tested the viability of hepatic (HepG2), embryonic (HEK293) and placental (JEG3) cell lines after 24h exposure to glyphosate or different commercial glyphosate-based formulations and adjuvants. These authors have demonstrated the very low toxicity of glyphosate which was non-toxic on Hep G2 cells and slightly toxic on HEK293 and JEG3 cell lines with EC_{50} values of 19,300 mg L^{-1} and 11,192 mg L^{-1}, respectively. In our study, the maximum tested concentration (100 mg L^{-1}) of glyphosate had no effect on hemocyte viability. This result was in accordance with the results from Mesnage and

collaborators [45] which correspond to concentration values 100 times higher than those tested in this study. Exposure of hemocytes to R_{EX} and POEAs led to the same patterns than those observed by Mesnage et al. [45]: glyphosate-based commercial formulations expressed a higher toxicity compared to glyphosate alone and adjuvants were the most toxic compounds. Furthermore, the EC_{50} values for abalone hemocytes were comparable with the EC_{50} values calculated for JEG3 cell line by Mesnage et al. [45]. Indeed, the exposure to Roundup Grands Travaux® led to an EC_{50} of 32 mg L^{-1} (41.42 mg L^{-1} for abalone hemocytes exposed to R_{EX}) whereas the POE-15 (one of the ethoxylated amine in adjuvant formulations) induced 50% of cell mortality at the concentration of 1 mg L^{-1} (1.86 mg L^{-1} for abalone hemocytes exposed to POEAs). Our results and data from the literature suggest that the toxicity of glyphosate-based compounds is not specific to cell types but seems to be similar between mammalian's cell lines and cells from mollusc primary culture. All these results suggest that the toxicity mechanisms are not specific to a particular metabolic pathway but rather act on common targets for different cell types.

Phagocytosis has been shown to be impaired by a large panel of contaminants [46]. Consequently, the phagocytic activity of marine and freshwater bivalves after *in vitro* exposures is a sensitive endpoint to assess the effects of pollutants at sublethal concentrations [38]. After glyphosate exposure, a slight decrease of phagocytosis occurred at the concentration of 100,000 µg L^{-1} while no decreases in hemocyte viability were observed at this concentration. Similarly, inhibition of phagocytic activities was recorded after R_{EX} exposure from a concentration that did not lead to the inhibition of mitochondrial succinyl deshydrogenase (i.e. 10,000 µg L^{-1}). A similar result was also observed by Bado-Nilles et al. [36] who have shown the inhibition of phagocytosis in *C. gigas* exposed *in vitro* to pyrene and fluorene without any decrease of cell viability. Likewise, Luna-Acosta et al. [47] observed

pronounced inhibition of phagocytosis in *C. giga*s hemocytes after *in vivo* exposure of spat to a mixture of pesticides and pharmaceuticals (5 µg L^{-1} diuron, 5 µg L^{-1}isoproturon and 5 µg L^{-1} ibuprofen) but no decrease in cell viability was recorded. In addition, the exposure of abalone hemocytes to clomipramin, citalopram, and paroxetine decreased phagocytosis whereas amitryptilin induced a dose-related increase of phagocytosis [39]. Authors hypothesized that the increase in phagocytosis could be explained by the interaction of amitryptilin with a variety of receptors (e.g. histaminic, cholinergic, serotonin and adrenergic receptors) even if all of these receptors have not been yet evidenced in abalone hemocytes. Such increases were not observed after glyphosate, R_{EX} or POEA exposures suggesting again that the mechanisms of toxicity are not specific to a particular metabolic pathway. Our results on phagocytosis and those reported by the different authors highlight the potential in studying biomarkers such as phagocytosis which could reveal early effects on cells before any loss of viability. In the Chinese crab *Eriocheir sinensis* exposed to a range of glyphosate concentrations (from 4.4 to 98 mg L^{-1}) up to 96h, Hong et al. [48] studied phagocytic activity by observing cells that had incorporated fluorescent beads under an inverted fluorescence microscope. After 6h exposure to all glyphosate concentrations including 4.4 mg L^{-1}, these authors recorded a significant decrease of phagocytic activity that tended to be at the lowest level at 96h. Therefore, the comparison with the results recorded here in *H. tuberculata* (no significant change for exposure to 10 mg L^{-1}) suggest a higher sensibility of the species *E. sinensis* and/or *in vivo* exposure. The EC_{50} values for cellular tests were higher than the corresponding values for *Daphnia magna* and fish, indicating that the cellular tests (i.e. cell viability) are less sensitive than whole organisms [49].

Lysosomes are able to sequester and accumulate a large number of different contaminants [50]. Literature reported that the lysosomal system could be a target site for the

toxic effects of different type of xenobiotics. Pollutants could not only cause lysosome membrane destabilization but also change the structure, the dynamic and the composition of lysosomal system. Neutral red retention (NRR) is considered as a sensitive biomarker of exposure to chemicals and contaminated areas and it has been used in various mollusc species (e.g. [24,51,52). In the present study, the three chemicals tested showed significant effects on lysosome membrane but in different ways. Although glyphosate did not affect cell mortality at any tested concentration, this molecule seemed to stimulate lysosomal system at low doses suggesting a like-hormesis effect. Such results have been previously observed by various authors: Canty et al. [53] who have reported a significant increase of NRR after 1 h and 24 h in hemocytes of mussels (*Mytilus edulis*) exposed *in vivo* to the organophosphate pesticide azamethiphos; Bado-Nilles et al. [36] in *C. gigas* hemocytes exposed *in vitro* to dibenzo-[a,h]-anthracene, and Braunbeck and Appelbaum [54] in *Cyprinus carpio* intestinal epithelium exposed *in vivo* to ultra-low doses of the insecticide endosulfan. As abalone hemocytes are non-proliferative cells, the increase in NRR can be considered as an increase of the number and/or the size of lysosomes in the exposed hemocytes. Lysosomes size and number increases have been reported in mollusc for a wide range of contaminants (e.g. [55,56]) and could correspond to an unspecific sign of stress after exposure to a contaminant. The R_{EX} and POEA exposures did not induce a significant increase of NRR even if some trends could be observed at the lowest dose of contaminants. Conversely, these two chemicals compounds led to a drastic decrease of the NRR from exposures to NOEC MTT concentration for R_{EX} and to EC_{50} MTT concentration for POEAs. It is interesting to note that this decrease appeared at R_{EX} concentrations which did not affect the cell viability (i.e. 10,000 µg L^{-1}). Moreover, at the concentration of POEAs that inhibited 50% of cell viability, the NRR value was near zero. Such effects cannot be explained by cell lysis but very probably by a specific effect of R_{EX}, and to lesser extent POEAs, on the lysosomal system. This type of effect has been previously

observed in the Haliotidae. In *Haliotis rubra* exposed *in vivo* to air, Song et al. [57] have observed the early response of lysosomal system of hemocytes before any mortalities of exposed abalone. In addition, NRR values drastically decreased when hemocytes from *H. tuberculata* were exposed *in vitro*, for 48, h to different antidepressants [39]. More precisely, the decrease was observed at the concentration equivalent to EC_{10} MTT for amitryptiline, and EC_{20} MTT for paroxetine and citalopram. Likewise, a reduction in lysosome membrane stability has been reported in mussel and oysters exposed to heavy metal and this response has been proposed as an indicator of cell damage [50].

It is important to note that the adverse effects of the three chemical compound and mixtures tested in the present study were observed at non-realistic environmental concentrations (> 1 µg L^{-1} for glyphosate). Furthermore, the bioavailability of all the chemicals was maximized by the experimental design because of the direct exposure of targeted cells. The major disadvantage of *in vitro* methods is the difficulty of extrapolating the results to *in vivo* situations but they also have the advantages of being easy to use and reproducible [39]. However, the results provided by the three endpoints, and particularly the values of NOEC and EC determined with the MTT assay, allowed us to classify the toxicity of the chemicals as follows: glyphosate <R_{EX}< POEAs and other studies have already reported this ranking (e.g. [4,16,58]). Further studies should also include *in vivo* exposures in *H. tuberculata* and other immune parameters should be investigated. Indeed, parameters such as the hemocyte concentration in hemolymph and the volume of hemocytes should be considered because significant changes have been recorded after *in vivo* exposures to AMPA or glyphosate for 7 days, respectively, from concentrations as low as 1 µg L^{-1} in *Mytilus galloprovincialis* [59] and 10 µg L^{-1} in *R. philippinarum* [60]. From a case study in *M. galloprovincialis* in which both cellular and biochemical parameters had been affected,

Matozzo and his collaborators [61] concluded to a potential risk of glyphosate for aquatic invertebrates. By a transcriptional study conducted in the Pacific oyster, Mottier et al. [62] showed that the level of gene expression significantly increased after sub-chronic exposures to glyphosate from 0.1 µg L^{-1} (mutli-xenobiotic resistance) or 100 µg L^{-1} (GST and metallothioneins). In digestive gland from *M. galloprovincialis*, Milan et al. [63] demonstrated a significant effect of the exposure to 10 µg L^{-1} glyphosate for 21 days on the expression of 111 genes including some involved in endoplasmic reticulum stress response.

Mechanisms of POEA toxicity remain not fully explained but several authors have suggested that non-ionic surfactants could interact with the lipid bilayer and membrane proteins altering fluidity and oxygen transport [64-66]. The present results also suggest this mode of action since the effects of Roundup® and POEAs seem to be related to the lysosomal membrane stability and cytoplasmic membrane deformation (phagocytosis).

5. Conclusion

This study presents the first results on the effects of glyphosate-based herbicides in *H. tuberculta* hemocytes and provided important information to compare the toxicity of the active ingredient with one of its commercial formulations and its associated adjuvants. While no effect on cell viability appeared with any tested concentration of glyphosate or at concentrations below 20 mg L^{-1} of R_{EX}, effects on cell membranes have been suggested at sublethal concentrations, thus clearly demonstrating the utility of multi-biomarker approaches and *in vitro* exposures. The adjuvants seemed to be mainly responsible for the toxicity of commercially formulated glyphosate. However, despite the scarcity or lack of data about the contamination of coastal environments, the toxicity of the tested molecules was most likely at much higher level of magnitude of concentrations than those observed in the environment.

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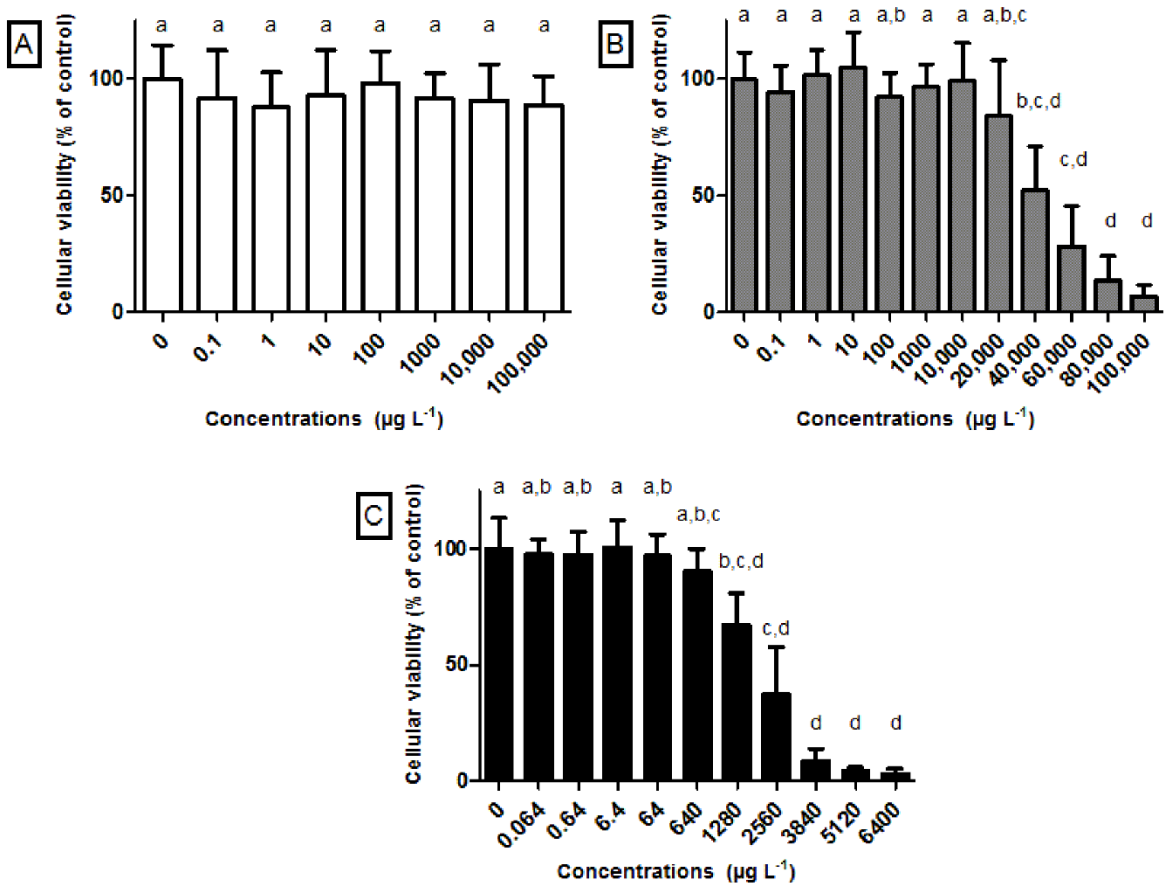

Fig. 1. Cell viability ± standard deviation (in % of values recorded for control group) after 72h exposure to glyphosate (A) Roundup Express® (R_{EX}) (B) and POEAs (C). For each exposure condition, hemocytes came from the hemolymph of at least four abalones and each one's cells were used in triplicate. The concentrations that do not share a letter are significantly different.

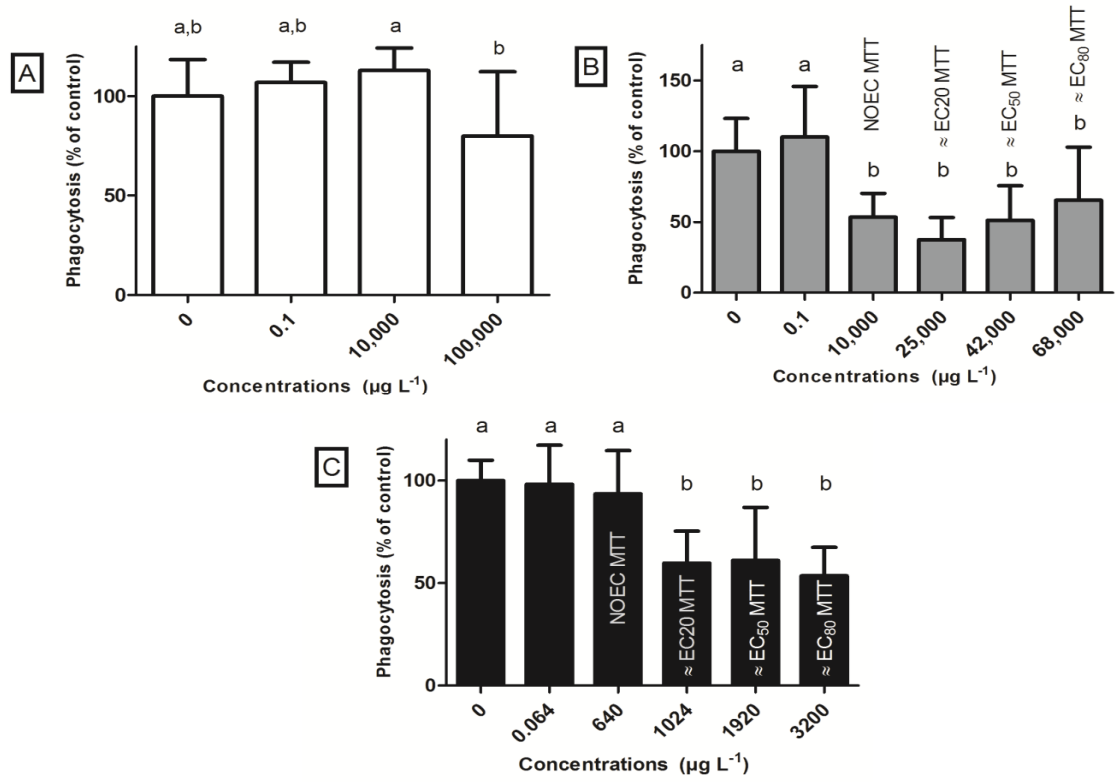

Fig. 2. Phagocytic activity expressed as % of hemocytes that had phagocytosed 3 beads or more ± standard deviation (in % of values recorded for control group) after 72 h exposure to glyphosate (A) Roundup Express® (R_{EX}) (B) and POEAs (C). For each exposure condition, hemocytes came from the hemolymph of at least three abalones, and a minimum of 20,000 events was considered for each sample. The concentrations that do not share a letter are significantly different.

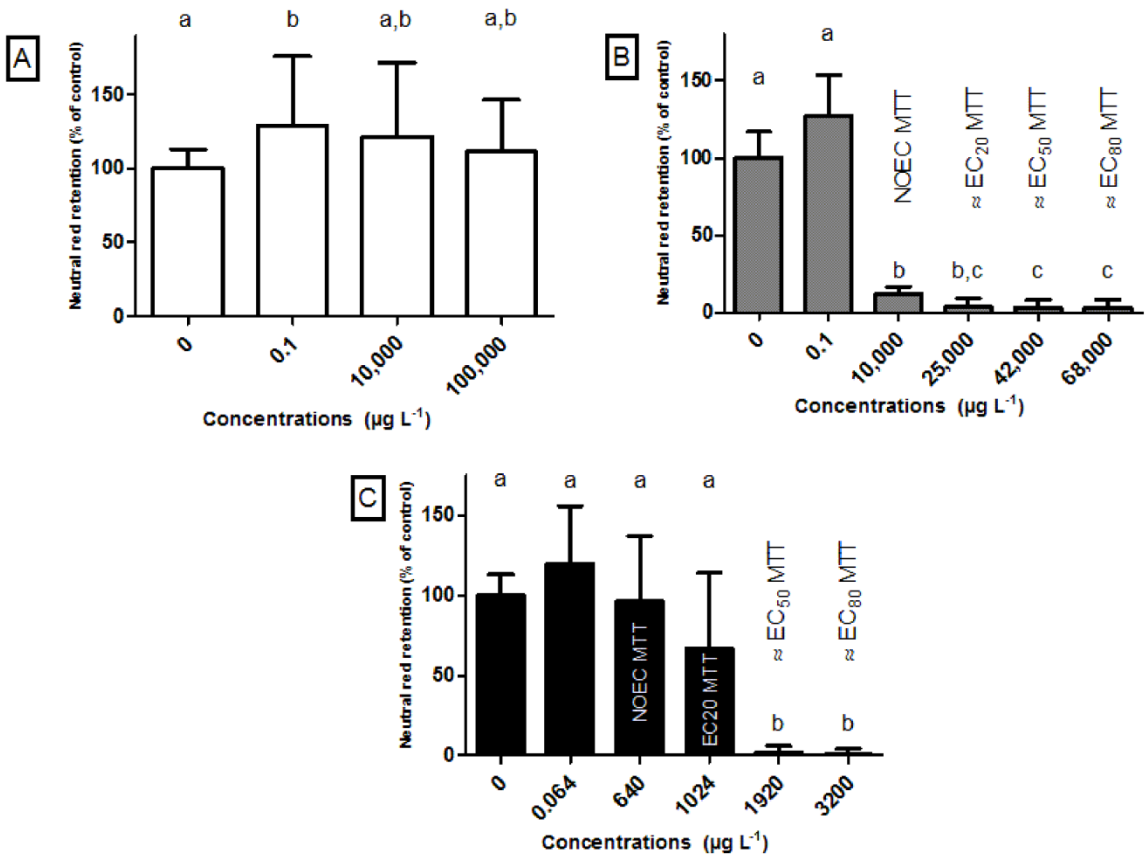

Fig. 3. Neutral red retention ± standard deviation (in % of values recorded for control group) after 72h exposure to glyphosate (A) Roundup Express® (R_{EX}) (B) and POEAs (C). For each exposure condition, hemocytes came from the hemolymph of at least three abalones and each one's cells were used in triplicate. The concentrations that do not share a letter are significantly different.

Highlights

- Glyphosate by-itself is practically non-toxic on *Haliotis tuberculata* hemocytes
- Toxicity of glyphosate based herbicides comes from adjuvants
- Sublethal effects occur at non-realistic environmental concentrations
- The mechanisms of toxicity seem to be linked to biological membrane destabilization