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

3

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16

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18

19 **Keywords:** hemocytes; *Haliotis tuberculata*; glyphosate; POEAs; viability assay;  
20 phagocytosis; neutral red retention assay.

21

## 22 **Abstract**

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

## 35 **1. Introduction**

36 The quality of coastal waters greatly depends on human activities in the upstream areas.  
37 Among the various contaminants that may be relevant, pesticides from agricultural and  
38 domestic activities can be carried by freshwater *via* run-off and leaching processes and  
39 subsequently contaminate marine coastal areas [1-3]. Herbicide formulations containing  
40 glyphosate as the active ingredient are among the most commonly used pesticides in the world  
41 [4]. In these commercial formulations, adjuvants are used to promote the penetration of the  
42 active ingredient into plant cuticles [5]. The most common adjuvants in glyphosate-based  
43 herbicides are polyethoxylated tallow amines (POEAs) that can be formulated in an  
44 oxide:tallow-amine ratio ranging from 5:1 to 25:1. The half-life of POEAs in water has been  
45 estimated from 21 to 42 days suggesting that this adjuvant is relatively persistent in water [6].

46 For glyphosate itself, hydrolysis (>30 days for pH ranging from 5 to 9) and photolysis times in  
47 water (69 and 77 days, for pH of 7 and 9, respectively) also reveal its relative persistence (e.g.  
48 [7]). Furthermore, studies have shown that glyphosate is detectable in rivers [8-11] with  
49 maximum concentrations higher than  $100 \mu\text{g L}^{-1}$  [12], in contrast to around  $1 \mu\text{g L}^{-1}$  in coastal  
50 waters [3,13]. Data from literature have indicated that commercial formulations appeared more  
51 toxic for a large panel of non-target organisms compared to glyphosate alone [4,14-16] which  
52 may indicate toxicity of adjuvants alone or additive or synergistic toxicity of adjuvants with  
53 other(s) component(s) of the formulated compounds. Indeed, the toxicity of POEAs has already  
54 been demonstrated in different taxonomic groups such as amphibians [4], freshwater  
55 crustaceans [17,18] and molluscs [19,20]. For example, the embryotoxicity of POEAs was  
56 quantified after 36 h of exposure considering both arrested development and abnormalities in  
57 D-shaped larvae of the Pacific oyster *Crassostrea gigas* [20]; the results suggested that POEAs  
58 could be considered very toxic to embryo larval development according to the European  
59 toxicity classification [6].

60 The abalone, *Haliotis tuberculata*, is a marine gastropod species, which can be found in the  
61 Northeast Atlantic from Senegal to Ireland. Abalone have been used as sensitive species to  
62 assess pollution of coastal areas [21,22] or the potential toxicity of chemical compounds [23-  
63 26]. In molluscs, the hemocytes are key components of the immune system, responsible for  
64 various mechanisms of defense, such as phagocytosis, pathogen hydrolysis or Reactive Oxygen  
65 Species (ROS) production [27-30]. In bivalves, such as *Crassostrea gigas*, many types of  
66 hemocytes have been described, including eosinophilic, basophilic and an intermix between  
67 granulocytes, vesicular and blast-like cells [31]. By contrast in *H. tuberculata*, Travers et al.  
68 [30] have described only one type of hemocytes, i.e. hyalinocytes which can be separated into  
69 to two sub-types: blast-like and large cells. As hemocytes play an essential role in mollusc  
70 immunity, the effects of contaminants on these cells could lead to adverse effects for the whole

71 animal [32,33]. Indeed, experiments conducted in the gastropod *Biomphalaria glabrata* by de  
72 Monte et al [34] showed that infection with the platyhelminth *Echinostoma paraensei* caused a  
73 decrease of circulating hemocytes, and also that association between infection and exposure to  
74 the Roundup<sup>®</sup> (concentration equivalent to 36 mg L<sup>-1</sup> of glyphosate) greatly increased the  
75 percentage of non-viable cells, making the snails more vulnerable to parasitic infections.

76 *In vitro* studies are useful tools to assess the potential risks induced by anthropogenic  
77 contaminants in the aquatic environments. Indeed, these tools provide good alternatives to  
78 animal experimentation and take ethical issues into consideration [35]. *In vitro* methodologies  
79 also allow assessment of the effects of multiple contaminant concentrations on the cells of a  
80 limited number of individuals, thereby reducing variability, and are easily reproducible.  
81 Although the use of *in vitro* tools gives a limited view of the physiological processes that occur  
82 at the *in vivo* level, cell culture provides precious information on the mechanisms of toxicity  
83 [24]. While several studies have been published which focused on the effects of various  
84 contaminants on bivalve hemocytes *in vitro* (e.g. [36-38]), few ecotoxicological investigations  
85 have been performed on *Haliotis spp.* hemocytes. However, in the 2010s, these limited numbers  
86 of studies have increased, including those demonstrating the adverse effects of metals [24,26],  
87 antibacterial agents [23] and antidepressants [39] on hemocytes in *H. tuberculata*. However,  
88 there is still a lack of data concerning the effects of herbicides on the hemocytes of gastropods  
89 including *H. tuberculata*.

90 The aims of this study were to assess the *in vitro* effects of (1) glyphosate; (2) a  
91 commercial formulation containing glyphosate as the active ingredient (Roundup Express<sup>®</sup>);  
92 and (3) POEA adjuvants on hemocytes; in *H. tuberculata* after 72h exposure by using 3  
93 established biomarkers: viability assessment (MTT assay), phagocytosis (fluorescent beads)  
94 and lysosomal stability (neutral red retention assay). Phagocytosis by hemocytes is the  
95 cornerstone of the molluscan immune system [40]. Lysosomes, cellular organelles, are essential

96 components of the humoral immune response in mollusc species. Lysosomes content is released  
97 after phagocytosis in order to digest foreign material.

98

## 99 2. Materials and methods

### 100 2.1. Hemocyte primary culture

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

105 Primary cell culture of abalone hemocytes has been previously described [24,26,41,42].  
106 Briefly, hemocytes were sampled from the adductor muscle of *H. tuberculata*. Hemolymph was  
107 withdrawn from a medio-lateral incision using a syringe fitted with a 25 gauge needle. In order  
108 to avoid any cell aggregation, the syringe was moisturized with an Alsever solution (115 mM  
109 glucose; 27 mM sodium citrate; 11.5 mM EDTA; 382 mM NaCl). Hemolymph was transferred  
110 to a 15 mL centrifuge tube and diluted 1:4 with Alsever solution. Hemocytes were counted in  
111 triplicate by using a Thoma cell counting chamber. Cells were plated in 12-well culture plates  
112 (NUNC®; Penfield, New York, USA) at a density of  $5 \times 10^5$  cells per well (MTT assay and  
113 phagocytosis analyses) or in 96-well culture plates (neutral red retention assay) at a density of  
114  $1 \times 10^5$  cells per well. Hemolymph was diluted 1:4 (v/v) with sterile artificial sea water  
115 (ASSW). After 1 h, ASSW was removed and replaced with 500  $\mu$ L (12-well plates) or 200  $\mu$ L  
116 (96-well plates) of sterile modified Hank's 199 medium (250 mM NaCl, 10 mM KCl, 25 mM  
117 MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub> and 10 mM HEPES, 2 mM l-glutamine, 100  $\mu$ g mL<sup>-1</sup> streptomycin, 60  
118  $\mu$ g mL<sup>-1</sup> penicillin G and 2 mM concanavalin; pH: 7.4). Cells were incubated for 24 h before

119 beginning any pesticide exposure. Cells were then exposed for 72 h to the different chemicals  
120 and all the cultures were performed in an incubator at 17°C without extra CO<sub>2</sub>.

## 121 2.2. Exposures to chemicals

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

## 138 2.3. Studied endpoints

### 139 2.3.1. MTT assay

140 Cell viability was estimated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-  
141 diphenyltetrazolium bromide (MTT) reduction assay. This test was adapted to molluscan cell  
142 cultures by Domart-Coulon et al. [43]. It measures the capacity of mitochondrial succinyl

143 dehydrogenase in living cells to convert MTT (yellow) into formazan (dark blue). Briefly, 50  
144  $\mu\text{L}$  of a stock solution ( $5 \text{ mg mL}^{-1}$  MTT in PBS) was added to culture plates (10% v/v). After  
145 24 h incubation,  $50 \mu\text{L}$  of acidified isopropanol ( $0.04 \text{ N HCl}$ ) was added to each well in order to  
146 dissolve neo-formed formazan. Absorbance was measured at  $570 \text{ nm}$  with a  $630 \text{ nm}$  reference,  
147 and results were expressed as percentages of viability relative to absorbance of the **negative**  
148 control group.

### 149 2.3.2. Phagocytic activity

150 The phagocytic rate of hemocytes was assessed by flow cytometry as described  
151 previously [24,26,39]. Briefly, the medium was removed and replaced with  $500 \mu\text{L}$  of pesticide-  
152 free medium containing fluorescent latex beads ( $100$  carboxylate-modified  
153 FluoroSpheres<sup>®</sup>/hemocyte, yellow-green fluorescence,  $1 \mu\text{m}$  diameter, Molecular Probes<sup>®</sup>).  
154 After  $1 \text{ h}$  incubation, the medium was removed and cells were rinsed and then gently scraped  
155 into  $500 \mu\text{L}$  MPS (**molluscan physiological saline**). The hemocyte samples were centrifuged for  
156  $10 \text{ min}$  at  $500 g$  and the resulting pellet was delicately fixed in  $300 \mu\text{L}$  of  $3\%$  formaldehyde for  
157 further analysis. Hemocytes were analyzed by using a Gallios<sup>™</sup> flow cytometer (Beckman  
158 Coulter<sup>®</sup>). A minimum of  $20,000$  events was considered for each sample. The level of  
159 fluorescence was evaluated using FL1 channel. **The percentage of phagocytic cells was**  
160 **evaluated as the percentage of hemocytes that had engulfed at least three beads (i.e.**  
161 **immunoefficiency).**

### 162 2.3.3. Neutral red retention assay (NRRA)

163 The stability of lysosomal membranes was assessed by the neutral red retention assay  
164 (NRRA) as previously described [39] with modifications. Hank's 199 medium was removed  
165 and replaced by  $300 \mu\text{L}$  of neutral red working solution ( $2.10^{-2} \text{ M}$  neutral red in **MPS**;  $400 \text{ mM}$   
166  $\text{NaCl}$ ;  $100 \text{ mM}$   $\text{MgSO}_4$ ;  $20 \text{ mM}$  HEPES;  $10 \text{ mM}$   $\text{CaCl}_2$ ;  $10 \text{ mM}$   $\text{KCl}$ . After  $3 \text{ h}$  of incubation

167 in the dark, neutral red solution was removed, cells were gently rinsed with MPS and 200  $\mu\text{L}$   
168 of elution solution (1:50:49 v/v/v of acetic acid, absolute ethanol, ultrapure water) was added  
169 to each well. Plates were then gently agitated for 30 min in the dark. Finally, the optical density  
170 of each well was read using a multiplate reader (FlexStation 3<sup>®</sup>, Molecular Devices LLC.;  
171 Chicago, USA) at 540 and 650 nm as a reference.

#### 172 2.4. Statistical analyses

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

182

### 183 3. Results

#### 184 3.1. MTT viability assay

185 Glyphosate did not induce any significant decrease in MTT activities even at very high  
186 concentrations (100,000  $\mu\text{g L}^{-1}$ ) (Fig. 1A). No differences from the negative control were found  
187 for  $R_{EX}$  concentrations lower than 20,000  $\mu\text{g L}^{-1}$  (Fig. 1B). However, exposure to 40,000  $\mu\text{g L}^{-1}$   
188  $R_{EX}$  caused a highly significant decrease of MTT values ( $p < 0.001$ ) to 52.30% ( $\pm 18.78$ ) of  
189 the control viability. The viability declined to 6.84% ( $\pm 4.75$ ) of the control viability at 100,000  
190  $\mu\text{g L}^{-1}$ . The concentration leading to 50% mortality ( $EC_{50}$ ) was 41,420  $\mu\text{g L}^{-1}$ . After POEA

191 exposure a significant ( $p < 0.001$ ) decrease of hemocyte viability was observed from 1280  $\mu\text{g}$   
192  $\text{L}^{-1}$  ( $67.27 \pm 13.74\%$  of the control viability) to 6400  $\mu\text{g}$   $\text{L}^{-1}$  ( $3.42 \pm 1.93\%$  of the control  
193 viability) (Fig. 1C). Finally, an  $\text{EC}_{50}$  of 1855  $\mu\text{g}$   $\text{L}^{-1}$  was calculated for exposures to POEAs.

### 194 3.2. Phagocytic activity

195 Under our experimental conditions, the percentage of hemocytes that engulfed three  
196 beads or more was  $33.90 \pm 7.91$  for the controls. After glyphosate exposure, no significant  
197 changes in phagocytic activities were observed (Fig. 2A). Phagocytic activities significantly  
198 decreased ( $p < 0.001$ ) after exposures to  $R_{\text{EX}}$  from 10,000 (NOEC MTT) to 68,000  $\mu\text{g}$   $\text{L}^{-1}$  ( $\text{EC}_{80}$   
199 MTT) (Fig. 2B). Interestingly, the NOEC MTT concentration decreased phagocytic activity  
200 ( $53.52 \% \pm 16.43$  of the control phagocytosis). Exposure to POEAs led to a significant decrease  
201 ( $p < 0.001$ ) in hemocytes phagocytosis at concentrations of 1024  $\mu\text{g}$   $\text{L}^{-1}$  ( $59.76 \% \pm 15.57$  of  
202 the control phagocytosis), 1920  $\mu\text{g}$   $\text{L}^{-1}$  ( $61.09 \% \pm 25.82$  of the control phagocytosis) and 3200  
203  $\mu\text{g}$   $\text{L}^{-1}$  ( $53.55 \%$ ) (Fig. 2C). An overall comparison between the 3 chemicals tested at the NOEC  
204 MTT concentrations revealed significant differences ( $p < 0.05$ ) with a more pronounced effect  
205 of  $R_{\text{EX}}$  compared to glyphosate (the concentration of 100,000 being considered as the NOEC  
206 MTT;  $p < 0.05$ ) and POEAs ( $p < 0.01$ ) (Fig. 2). Whereas the effects of  $R_{\text{EX}}$  and POEAs on  
207 phagocytosis efficiency significantly differed when exposed at  $\text{EC}_{20}$  MTT concentrations ( $p <$   
208  $0.05$ ) it was no longer the case when exposed at  $\text{EC}_{50}$  MTT ( $p = 0.49$ ) and  $\text{EC}_{80}$  MTT ( $p = 0.48$ )  
209 concentrations.

### 210 3.3. Neutral red retention assay (NRRA)

211 Low doses of glyphosate (i.e. 0.1  $\mu\text{g}$   $\text{L}^{-1}$ ) induced a slight but significant increase ( $\times 1.29$ )  
212 of neutral red retention (NRR) in lysosomes compared to control group ( $p < 0.05$ ). In contrary,  
213 the two highest doses of glyphosate (10,000  $\mu\text{g}$   $\text{L}^{-1}$  and 100,000  $\mu\text{g}$   $\text{L}^{-1}$ ) did not lead to any  
214 significant changes in lysosomal stability (Fig. 3A) ( $p = 0.0256$ ). A trend toward an increase of

215 neutral red retention in lysosomes was also observed in hemocytes exposed to the lowest doses  
216 of R<sub>EX</sub><sup>®</sup> (i.e. 0.1 µg L<sup>-1</sup>) but was not statistically significant (Fig. 3B). Nevertheless, at higher  
217 doses of R<sub>EX</sub><sup>®</sup>, NRR values were significantly ( $p < 0.001$ ) lower compared to the control and  
218 ranged from 12.03% ( $\pm 4.80\%$ ) at 10,000 µg L<sup>-1</sup> (NOEC MTT) to 2.74% ( $\pm 5.81\%$ ) at 68,000  
219 µg L<sup>-1</sup> (EC<sub>80</sub> MTT). Finally, POEA exposure did not induce significant modification of neutral  
220 red retention up to 1024 µg L<sup>-1</sup> (EC<sub>20</sub> MTT) compared to the control (Fig. 3C). However, large  
221 significant losses of lysosome neutral red were observed at 1920 (EC<sub>50</sub> MTT) and 3200 µg L<sup>-1</sup>  
222 (EC<sub>80</sub> MTT), with relative values of 1.61% ( $\pm 4.37$ ) and 0.54% ( $\pm 3.52$ ), respectively. The  
223 overall comparison between the 3 chemicals tested at the NOEC MTT or the 3 EC  
224 concentrations for NRR led to similar results than those recorded for phagocytic activity: at the  
225 NOEC MTT concentrations, a higher effect of R<sub>EX</sub> compared to glyphosate ( $p < 0.001$ ) and  
226 POEAs ( $p < 0.001$ ), and only at EC<sub>20</sub> MTT concentrations, a more marked effect of R<sub>EX</sub> by  
227 comparison to POEAs ( $p < 0.001$ ).

228

#### 229 4. Discussion

230 Hemocytes have a predominant role in the immune response in molluscs and adverse  
231 effects to these cells could lead to fatal consequences for the whole animal. Effects on  
232 hemocytes could be lethal and reflected by biomarkers such as MTT activities or sublethal by  
233 affecting the phagocytic activity and/or the lysosomal system.

234 At the range of concentrations tested, glyphosate did not lead to significant changes in  
235 hemocyte MTT activities while exposures to 1024 mg L<sup>-1</sup> POEAs and 25,000 µg L<sup>-1</sup> R<sub>EX</sub>  
236 induced a significant effect of 20% (EC<sub>20</sub>). Beyond these values, cellular viability sharply  
237 decreased and a dose-response curve was recorded. In the freshwater snail *Biomphalaria*  
238 *glabrata*, *in vivo* 24h-exposure to Roundup original<sup>®</sup> at the concentration of 36 mg L<sup>-1</sup> induced

239 a significant increase of the number of dead hemocytes assessed by trypan blue vital dye  
240 exclusion assay [34]. Viability tests based on mitochondrial activity (such as MTT assay) are  
241 also sensitive endpoints that have been previously used to assess the toxicity of different  
242 chemical compounds in **marine** molluscs. Domart-Coulon et al. [43] have shown the toxicity of  
243 Mexel-432<sup>®</sup> (anti-fouling compound) on the heart cells of *Crassostrea gigas* and gill cells from  
244 the clam *Ruditapes decussatus*. In *H. tuberculata* hemocytes, viability tests using mitochondrial  
245 activities were used to assess the effect of zinc [26], cadmium [24], triclosan [23] and  
246 antidepressants [39]. To our knowledge, no data are available on the cytotoxicity of the  
247 compounds studied in this work by using *in vitro* mollusc cell cultures. However, *in vitro*  
248 toxicity of commercial formulations and adjuvants has been previously reported in different  
249 mammalian cell types and gives interesting points of comparison with our results. Mesnage and  
250 his collaborators [45] tested the viability of hepatic (HepG2), embryonic (HEK293) and  
251 placental (JEG3) cell lines after 24h exposure to glyphosate or different commercial glyphosate-  
252 based formulations and adjuvants. These authors have demonstrated the very low toxicity of  
253 glyphosate which was non-toxic on Hep G2 cells and slightly toxic on HEK293 and JEG3 cell  
254 lines with EC<sub>50</sub> values of 19,300 mg L<sup>-1</sup> and 11,192 mg L<sup>-1</sup>, respectively. In our study, the  
255 maximum tested concentration (100 mg L<sup>-1</sup>) of glyphosate had no effect on hemocyte viability.  
256 This result was in accordance with the results from Mesnage and collaborators [45] which  
257 correspond to concentration values 100 times higher than those tested in this study. Exposure  
258 of hemocytes to R<sub>EX</sub> and POEAs led to the same patterns than those observed by Mesnage et  
259 al. [45]: glyphosate-based commercial formulations expressed a higher toxicity compared to  
260 glyphosate alone and adjuvants were the most toxic compounds. Furthermore, the EC<sub>50</sub> values  
261 for abalone hemocytes were comparable with the EC<sub>50</sub> values calculated for JEG3 cell line by  
262 Mesnage et al. [45]. Indeed, the exposure to Roundup Grands Travaux<sup>®</sup> led to an EC<sub>50</sub> of 32  
263 mg L<sup>-1</sup> (41.42 mg L<sup>-1</sup> for abalone hemocytes exposed to R<sub>EX</sub>) whereas the POE-15 (one of the

264 ethoxylated amine in adjuvant formulations) induced 50% of cell mortality at the concentration  
265 of 1 mg L<sup>-1</sup> (1.86 mg L<sup>-1</sup> for abalone hemocytes exposed to POEAs). Our results and data from  
266 the literature suggest that the toxicity of glyphosate-based compounds is not specific to cell  
267 types but seems to be similar between mammalian's cell lines and cells from mollusc primary  
268 culture. All these results suggest that the toxicity mechanisms are not specific to a particular  
269 metabolic pathway but rather act on common targets for different cell types.

270

271 Phagocytosis has been shown to be impaired by a large panel of contaminants [46].  
272 Consequently, the phagocytic activity of marine and freshwater bivalves after *in vitro* exposures  
273 is a sensitive endpoint to assess the effects of pollutants at sublethal concentrations [38]. After  
274 glyphosate exposure, a slight decrease of phagocytosis occurred at the concentration of 100,000  
275 µg L<sup>-1</sup> while no decreases in hemocyte viability were observed at this concentration. Similarly,  
276 inhibition of phagocytic activities was recorded after R<sub>EX</sub> exposure from a concentration that  
277 did not lead to the inhibition of mitochondrial succinyl deshydrogenase (i.e. 10,000 µg L<sup>-1</sup>). **A**  
278 **similar result** was also observed by Bado-Nilles et al. [36] who have shown the inhibition of  
279 phagocytosis in *C. gigas* exposed *in vitro* to pyrene and fluorene without any decrease of cell  
280 viability. Likewise, Luna-Acosta et al. [47] observed pronounced inhibition of phagocytosis in  
281 *C. gigas* hemocytes after *in vivo* exposure of spat to a mixture of pesticides and pharmaceuticals  
282 (5 µg L<sup>-1</sup> diuron, 5 µg L<sup>-1</sup> isoproturon and 5 µg L<sup>-1</sup> ibuprofen) but no decrease in cell viability  
283 was recorded. In addition, the exposure of abalone hemocytes to clomipramin, citalopram, and  
284 paroxetine decreased phagocytosis whereas amitriptylin induced a dose-related increase of  
285 phagocytosis [39]. Authors hypothesized that the increase in phagocytosis could be explained  
286 by the interaction of amitriptylin with a variety of receptors (e.g. histaminic, cholinergic,  
287 serotonin and adrenergic receptors) even if all of these receptors have not been yet evidenced  
288 in abalone hemocytes. Such increases were not observed after glyphosate, R<sub>EX</sub> or POEA

289 exposures suggesting again that the mechanisms of toxicity are not specific to a particular  
290 metabolic pathway. Our results on phagocytosis and those reported by the different authors  
291 highlight the potential in studying biomarkers such as phagocytosis which could reveal early  
292 effects on cells before any loss of viability. In the Chinese crab *Eriocheir sinensis* exposed to a  
293 range of glyphosate concentrations (from 4.4 to 98 mg L<sup>-1</sup>) up to 96h, Hong et al. [48] studied  
294 phagocytic activity by observing cells that had incorporated fluorescent beads under an inverted  
295 fluorescence microscope. After 6h exposure to all glyphosate concentrations including 4.4 mg  
296 L<sup>-1</sup>, these authors recorded a significant decrease of phagocytic activity that tended to be at the  
297 lowest level at 96h. Therefore, the comparison with the results recorded here in *H. tuberculata*  
298 (no significant change for exposure to 10 mg L<sup>-1</sup>) suggest a higher sensibility of the species *E.*  
299 *sinensis* and/or *in vivo* exposure. The EC<sub>50</sub> values for cellular tests were higher than the  
300 corresponding values for *Daphnia magna* and fish, indicating that the cellular tests (i.e. cell  
301 viability) are less sensitive than whole organisms [49].

302

303 Lysosomes are able to sequester and accumulate a large number of different  
304 contaminants [50]. Literature reported that the lysosomal system could be a target site for the  
305 toxic effects of different type of xenobiotics. Pollutants could not only cause lysosome  
306 membrane destabilization but also change the structure, the dynamic and the composition of  
307 lysosomal system. Neutral red retention (NRR) is considered as a sensitive biomarker of  
308 exposure to chemicals and contaminated areas and it has been used in various mollusc species  
309 (e.g. [24,51,52]). In the present study, the three chemicals tested showed significant effects on  
310 lysosome membrane but in different ways. Although glyphosate did not affect cell mortality at  
311 any tested concentration, this molecule seemed to stimulate lysosomal system at low doses  
312 suggesting a like-hormesis effect. Such results have been previously observed by various  
313 authors: Canty et al. [53] who have reported a significant increase of NRR after 1 h and 24 h in

314 hemocytes of mussels (*Mytilus edulis*) exposed *in vivo* to the organophosphate pesticide  
315 azamethiphos; Bado-Nilles et al. [36] in *C. gigas* hemocytes exposed *in vitro* to dibenzo-[a,h]-  
316 anthracene, and Braunbeck and Appelbaum [54] in *Cyprinus carpio* intestinal epithelium  
317 exposed *in vivo* to ultra-low doses of the insecticide endosulfan. As abalone hemocytes are non-  
318 proliferative cells, the increase in NRR can be considered as an increase of the number and/or  
319 the size of lysosomes in the exposed hemocytes. Lysosomes size and number increases have  
320 been reported in mollusc for a wide range of contaminants (e.g. [55,56]) and could correspond  
321 to an unspecific sign of stress after exposure to a contaminant. The R<sub>EX</sub> and POEA exposures  
322 did not induce a significant increase of NRR even if some trends could be observed at the lowest  
323 dose of contaminants. **Conversely**, these two chemicals compounds led to a drastic decrease of  
324 the NRR from exposures **to NOEC MTT concentration for R<sub>EX</sub> and to EC<sub>50</sub> MTT concentration**  
325 **for POEAs**. It is interesting to note that this decrease appeared at R<sub>EX</sub> concentrations which did  
326 not affect the cell viability (i.e. 10,000 µg L<sup>-1</sup>). Moreover, at the concentration of POEAs that  
327 inhibited 50% of cell viability, the NRR value was near zero. **Such effects cannot be explained**  
328 **by cell lysis but very probably by a specific effect of R<sub>EX</sub>, and to lesser extent POEAs, on the**  
329 **lysosomal system**. This **type of effect** has been previously observed in the Haliotidae. In  
330 *Haliotis rubra* exposed *in vivo* to air, Song et al. [57] have observed the early response of  
331 lysosomal system of hemocytes before any mortalities of exposed abalone. In addition, NRR  
332 values drastically decreased when hemocytes from *H. tuberculata* were exposed ***in vitro***, for  
333 48, h to different antidepressants [39]. More precisely, the decrease was observed at the  
334 concentration equivalent to EC<sub>10</sub> MTT for amitryptiline, and EC<sub>20</sub> MTT for paroxetine and  
335 citalopram. Likewise, a reduction in lysosome membrane stability has been reported in mussel  
336 and oysters exposed to heavy metal and this response has been proposed as an indicator of cell  
337 damage [50].

338

339 It is important to note that the adverse effects of the three chemical compound and  
340 mixtures tested in the present study were observed at non-realistic environmental  
341 concentrations ( $> 1 \mu\text{g L}^{-1}$  for glyphosate). Furthermore, the bioavailability of all the chemicals  
342 was maximized by the experimental design because of the direct exposure of targeted cells. The  
343 major disadvantage of *in vitro* methods is the difficulty of extrapolating the results to *in vivo*  
344 situations but they also have the advantages of being easy to use and reproducible [39].  
345 However, the results provided by the three endpoints, and particularly the values of NOEC and  
346 EC determined with the MTT assay, allowed us to classify the toxicity of the chemicals as  
347 follows: glyphosate  $< R_{EX} <$  POEAs and other studies have already reported this ranking (e.g.  
348 [4,16,58]). Further studies should also include *in vivo* exposures in *H. tuberculata* and other  
349 immune parameters should be investigated. Indeed, parameters such as the hemocyte  
350 concentration in hemolymph and the volume of hemocytes should be considered because  
351 significant changes have been recorded after *in vivo* exposures to AMPA or glyphosate for 7  
352 days, respectively, from concentrations as low as  $1 \mu\text{g L}^{-1}$  in *Mytilus galloprovincialis* [59] and  
353  $10 \mu\text{g L}^{-1}$  in *R. philippinarum* [60]. From a case study in *M. galloprovincialis* in which both  
354 cellular and biochemical parameters had been affected, Matozzo and his collaborators [61]  
355 concluded to a potential risk of glyphosate for aquatic invertebrates. By a transcriptional study  
356 conducted in the Pacific oyster, Mottier et al. [62] showed that the level of gene expression  
357 significantly increased after sub-chronic exposures to glyphosate from  $0.1 \mu\text{g L}^{-1}$  (mutli-  
358 xenobiotic resistance) or  $100 \mu\text{g L}^{-1}$  (GST and metallothioneins). In digestive gland from *M.*  
359 *galloprovincialis*, Milan et al. [63] demonstrated a significant effect of the exposure to  $10 \mu\text{g}$   
360  $\text{L}^{-1}$  glyphosate for 21 days on the expression of 111 genes including some involved in  
361 endoplasmic reticulum stress response.

362 Mechanisms of POEA toxicity remain not fully explained but several authors have  
363 suggested that non-ionic surfactants could interact with the lipid bilayer and membrane proteins

364 altering fluidity and oxygen transport [64-66]. The present results also suggest this mode of  
365 action since the effects of Roundup® and POEAs seem to be related to the lysosomal membrane  
366 stability and cytoplasmic membrane deformation (phagocytosis).

367

## 368 **5. Conclusion**

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

379

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386

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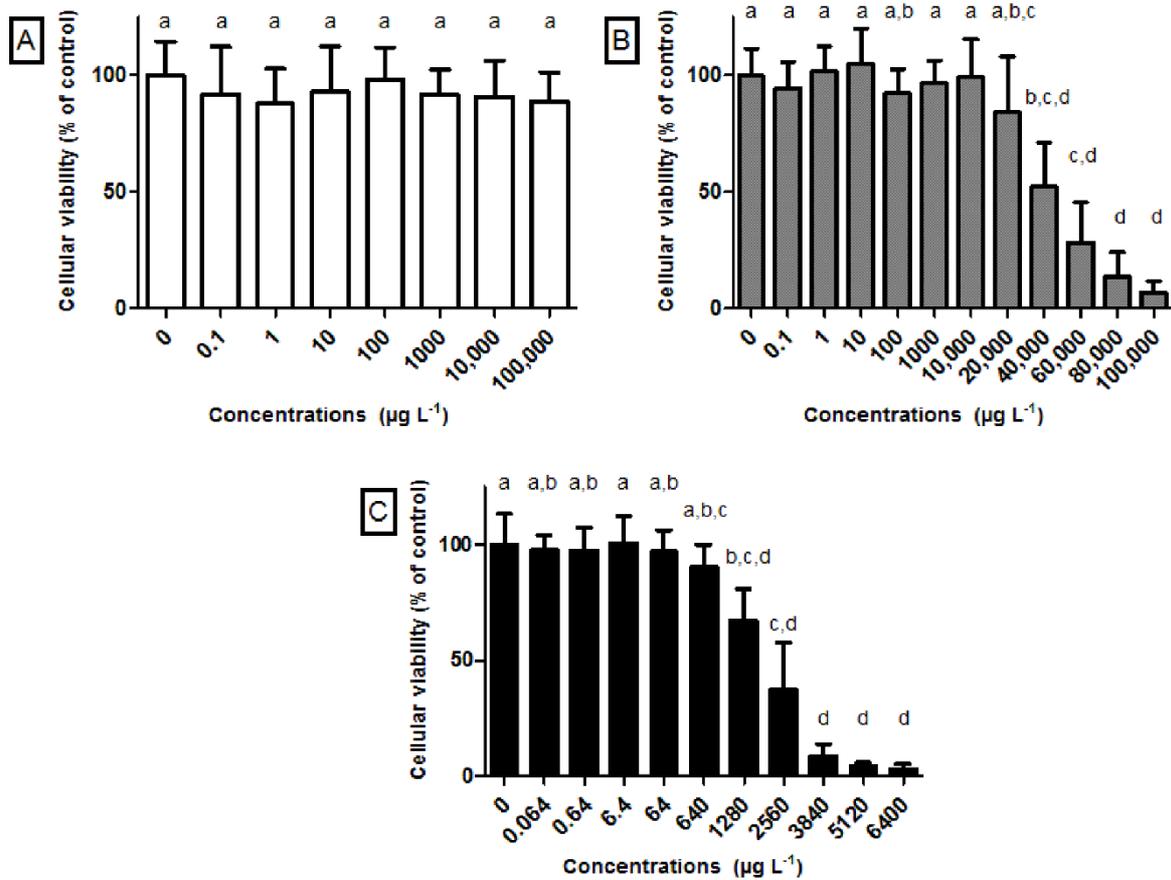
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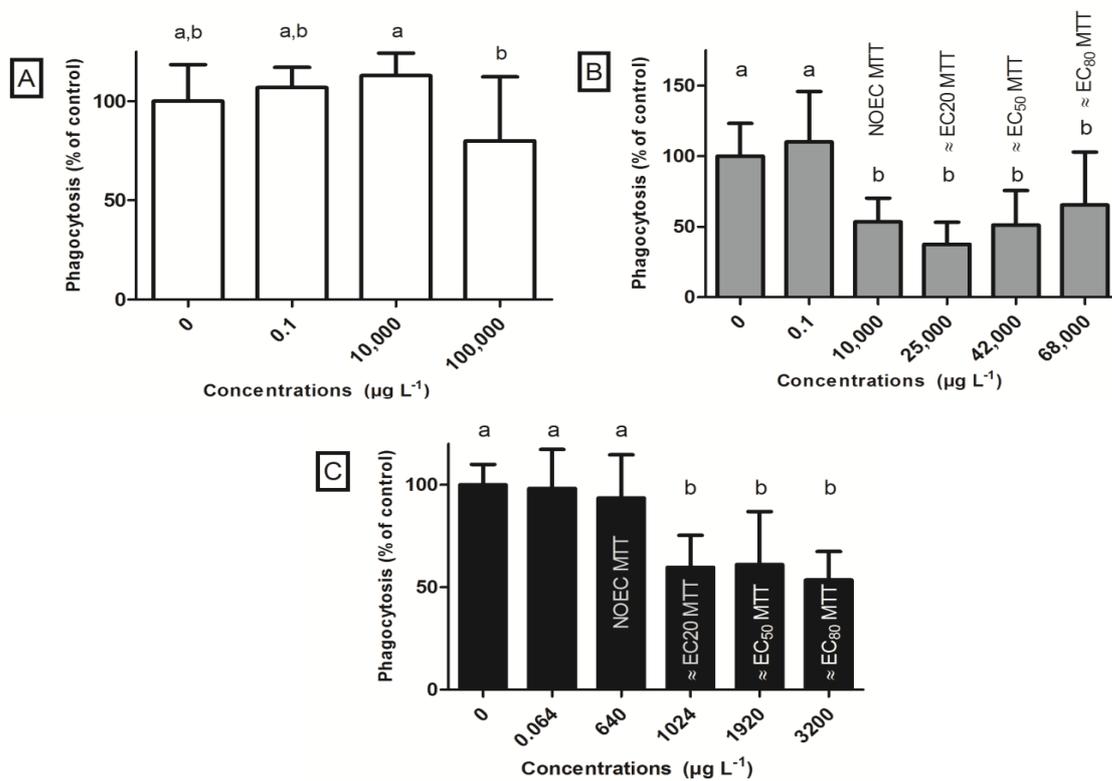
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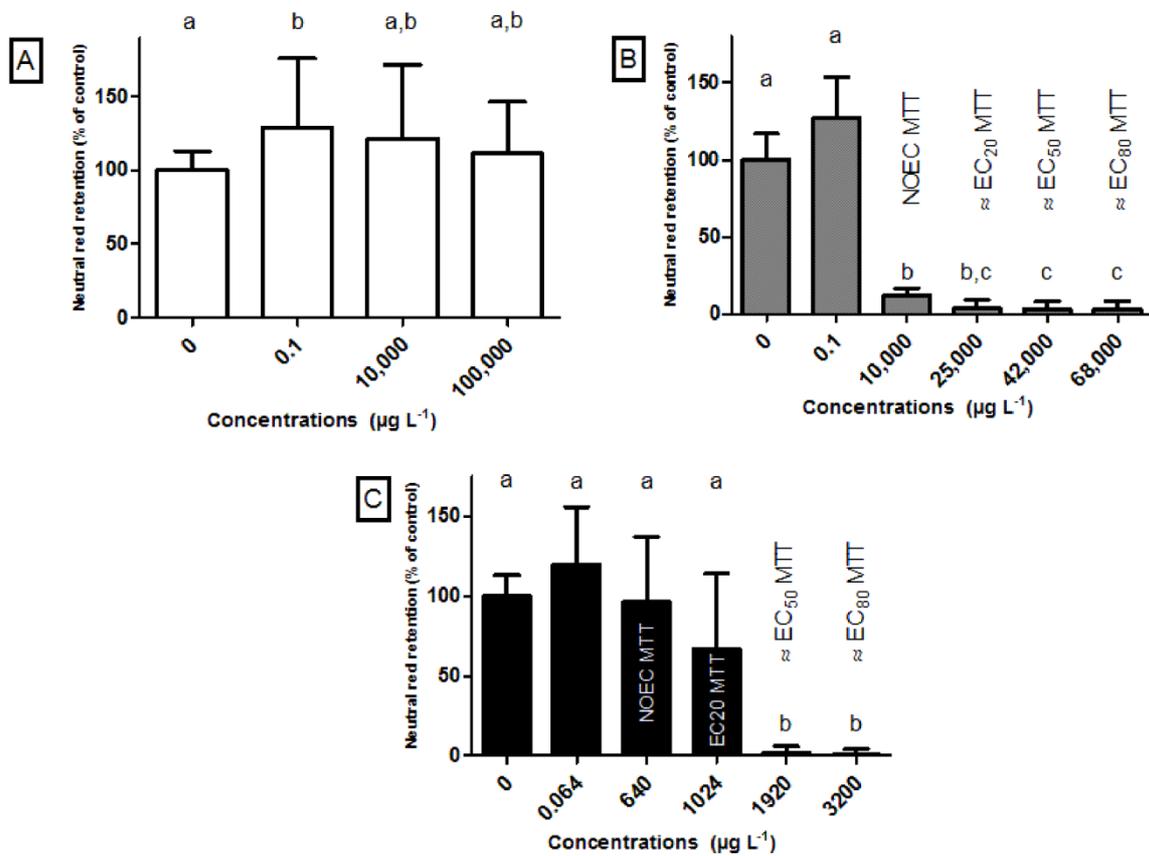
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**Fig. 1.** Cell viability  $\pm$  standard deviation (in % of values recorded for control group) after 72h exposure to glyphosate (A) Roundup Express<sup>®</sup> (R<sub>EX</sub>) (B) and POEAs (C). For each exposure condition, hemocytes came from 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  $\pm$  standard deviation (in % of values recorded for control group) after 72 h exposure to glyphosate (A) Roundup Express® (R<sub>EX</sub>) (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  $\pm$  standard deviation (in % of values recorded for control group) after 72h exposure to glyphosate (A) Roundup Express® (R<sub>EX</sub>) (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.