Toxicological evaluation of the herbicide glyphosate in the cultured oyster *Crassostrea gasar*

**Abstract**

Glyphosate is the most widely used herbicide in the world. *Crassostrea* spp. Oyster farming is being developed in Northeast Brazil, and one of its most important threats is infection of oysters by *Perkinsus* protozoans. Host microbiota, including those in the gastrointestinal tract, are implicated in promoting host health and preventing infections and diseases. The effect of glyphosate on the oyster *Crassostrea gasar* was evaluated. Oysters were exposed for 7 days to Termifin, a commercial formulation of glyphosate (0.5mgL⁻¹). Additionally, defence cells (haemocytes) were exposed for 4h to Termifin (0.5, 8.5, 16.9, 42.3, 84.5 and 169mgL⁻¹) and technical grade glyphosate at equivalent molar concentrations. The toxicity of glyphosate was assessed by evaluating the total haemocyte count (THC), viability, production of reactive oxygen species (ROS), phagocytosis of haemocytes, total heterotrophic bacteria from the gastrointestinal tract and *Perkinsus* infection. Oysters exposed to Termifin had reduced THC and an increased haemocyte ROS level, but neither alteration of phagocytosis nor cell death was observed. In contrast, haemocytes had decreased viability, increased bacterial load and decreased phagocytosis when treated with Termifin (169 and 1,000mgL⁻¹). Termifin exposure caused an increase in total heterotrophic bacteria but did not change *Perkinsus* spp. infection. We conclude that the glyphosate-based commercial formulation and its active ingredient are toxic in oysters, affecting two major mechanisms of defence and altering the balance of microbiota, which consequently compromises the ability of oysters to defend themselves against infectious agents.

**Keywords:** bacteria, haemocytes, *Perkinsus*, phagocytosis, reactive oxygen species, toxic chemical

**Introduction**

The oyster *Crassostrea gasar* is a native species of commercial interest in northeast Brazil. *C. gasar* is naturally found in estuarine environments, settling mangrove roots, sediment bottoms and rocky shores. This species is primarily cultured in the north and northeast regions and shows a higher growth rate than *C. rhizophorae*.¹

Threats to bivalve production systems involve protozoan parasites, notably those of the genus *Perkinsus.*² Oysters infected by this parasite occur in northeast Brazil, where the disease has been studied. Several natural and cultured populations of native *Crassostrea* spp. oysters are affected by *Perkinsus* spp. with prevalences (5 to 100%) and intensities of infection (very light to heavy) that vary widely according to the region and environment.³⁻¹⁰ Therefore, this parasite may represent a risk to the development of oyster culture.

Another threat to bivalve farming is the contamination of marine and estuarine ecosystems by agrochemicals. Glyphosate is the most widely used herbicide in the world, and its use has been greatly intensified after the production of genetically modified herbicide-tolerant crops (soybean, maize and cotton) especially in the United States, Brazil and Argentina.¹¹ In Brazil, with the increasing use of tolerant crop species, glyphosate use rates per hectare per crop year have risen sharply; for example, in soybeans, the rate increased from 250µg to 590µg in 1995 to 4.5kg/ha in 2014.¹² Sugarcane, for sugar and ethanol production, is a traditional crop in Brazil, which became the world’s largest producer. Glyphosate is used on sugarcane to control ratoon crops and emerging weeds and is also used as a ripener.¹² In the state of Paraíba, the production of *C. gasar* oysters occurs only in the estuary of the Mamanguape River, which undergoes effluent interferences from sugarcane crops.¹³,¹⁴ Consequently, the river receives high concentrations of agrochemicals, including glyphosate, which is the main herbicide used in Brazil.¹¹ In Brazil, glyphosate is classified as having low toxicity by the National Animal Surveillance Agency (ANVISA). However, following the classification of glyphosate in March 2015 as “probably carcinogenic to humans” by the International Agency for Research on Cancer (IARC), new toxicity analyses are underway in Brazil and are expected to be completed by 2019.

Studies that evaluate the behaviour, fate and amount of pesticides in soils, seawater and freshwater in tropical regions are limited compared to those performed in temperate regions (Annett et al. 2014).¹⁵ Concentrations of glyphosate in aquatic environments are variable; examples include 0.20µg/L in stream water from Switzerland,¹⁶ 59µg/L in the Ruhr River in Germany,¹⁷ 41µg/L in surface water from Ontario,¹⁸ 54µg/L in drainage systems after sugarcane irrigation in Australia,¹⁹ and 100–700µg/L in water sampled near soybean cultivation in Argentina.²⁰ Glyphosate acts by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), thus stopping the sixth step in the shikimate pathway, which is required for the synthesis of aromatic amino acids and secondary compounds that have defence functions in plants and many microorganisms.²¹ The main metabolite of glyphosate biotransformation is amino methyl phosphonic acid (AMPA).²² Thus, it was believed that glyphosate would not pose a risk to non-target organisms. However, several reports have shown teratogenic effects in animals and humans²³ and adverse effects on aquatic animals²⁴,²⁵ and seawater.²⁶ Glyphosate also induces changes in fresh water quality and on pico and phytoplankton communities.²⁷
Bivalves are sentinel organisms and little is known about the effect of glyphosate-based herbicides on them.\textsuperscript{30} Some studies have indicated physiological damage including negative effects on gametogenesis, alteration of the antioxidant enzymes of the gills and digestive glands, and DNA damage.\textsuperscript{31–33} Nevertheless, few studies have evaluated the functional activities of haemocytes from bivalves exposed to pesticides.\textsuperscript{34–37}

The objective of this study was to evaluate the effect of glyphosate herbicide on different aspects of \textit{C. gasar} oyster biology, including immune defence responses, gastrointestinal tract microbiota and \textit{Perkinsus} spp. infection.

**Materials and methods**

**Exposure of oysters to the commercial herbicide glyphosate**

Adult oysters (>7 cm) of the species \textit{Crassostrea gasar} were collected in May 2017 from a culture system located in the Mamanguape River estuary (S 06°47′08.2″; W 34°59′46.7″). Oysters were cleaned of fouling organisms, washed in running water, and then were distributed in 6 tanks (15 oysters / tank), with 10L of sea water, a salinity of 20, and constant aeration. After 2 days of acclimation, seawater was changed and 3 tanks received daily doses of glyphosate herbicide (Termifin, Dexter Latina, Brazil; 1%) at 500µgL\textsuperscript{-1} for 7 days; the other 3 tanks received no treatment (control). This concentration was determined by considering the doses found in aquatic environments, which vary from 0.59-700µgL\textsuperscript{-1},\textsuperscript{16-20,38} and was at least 100x higher than what is considered environmentally relevant 4µgL\textsuperscript{-1}.\textsuperscript{39} The seawater in the tanks was changed twice and new additions of the herbicide were made in the treated group.

After 7 days, oysters were removed from tanks and prepared for different studies. For the immunocellular parameter analyses and \textit{Perkinsus} spp. diagnosis, 5 oysters per tank (\textit{N}=15/treatment) were used. For the microbiological analysis, five oysters per tank were pooled and 2 pools were prepared for each tank, for a total of 6 pools/treatment. The experiment was repeated 3 times (Figure 1).

Figure 1 Experimental design.
Microbiological analysis

The gastrointestinal tract (GT) of the 5 oysters from the same tank were aseptically excised, pooled, homogenized in filtered seawater (1:10, w:v), and serially diluted (1/10 to 1/100,000). The suspensions (1mL) were distributed (in triplicate or duplicate) in Petri dishes containing plate count agar medium (PCA, M091A, HIMEDIA). Plates were incubated for 48h at 30°C for bacterial growth. Colony forming units (CFU) were counted and the total cultivable heterotrophic bacteria was estimated as CFU/mL of tissue.

Analysis of the presence and intensity of Perkinsus infection

Five oysters per tank (N=15/treatment) were opened by cutting the adductor muscle. The gills were removed and placed individually into 15mL tubes containing Ray’s fluid thioglycollate medium (RFTM) supplemented with penicillin G, streptomycin and nystatin (final concentrations of 100U/mL, 100μgmL and 100μmL, respectively). Samples were maintained at room temperature and in the dark for seven days. Then, gills were macerated and stained with Lugol (4%).

The intensity of the infection was determined according to the scale described by Mackin et al. and adapted by da Silva et al. Null infection (0): no Perkinsus spp. hypnospores detected in the whole slide (100x); Very light infection (1): up to 10 Perkinsus spp. hypnospores observed in the whole slide (100x); Light infection (2): 11–100 Perkinsus spp. hypnospores observed in the whole slide (100x); Moderate infection (3): up to 40 Perkinsus spp. hypnospores observed in 10 random fields (400x) scattered throughout the preparation; Heavy infection (4): more than 40 Perkinsus spp. hypnospores observed in 10 random fields (400x) scattered throughout the preparation.

For each tank, the prevalence of Perkinsus spp. was calculated as the number of infected oysters, divided by the total number of oysters and multiplied by 100. The Perkinsus spp. prevalence of each treatment (control and herbicide) was estimated as the mean prevalence of the 3 tanks. For each tank, the intensity of infection by Perkinsus spp. was calculated as the sum of infection levels of the infected animals (levels 1 to 4) divided by the total number of infected oysters. Perkinsus intensity of each treatment (control and herbicide) was estimated as the mean intensity of the 3 tanks.

Haemolymph sampling

Haemolymph was withdrawn from the adductor muscle of each oyster with a 21G needle attached to a 1mL syringe and deposited into a cooled micro tube on crushed ice. The evaluation of immune cell parameters for in vivo (oysters) exposure was performed with haemolymph from individual oysters per replicated tank (N=15 per treatment). For in vitro (haemocytes) exposure, haemolymph from 2 oysters was pooled (N=4 pools per treatment) to obtain a higher volume of haemolymph (2mL) to test various herbicides concentrations at the same time. For the in vivo exposure assay, an aliquot of the haemolymph (100μL) of each oyster was immediately fixed in formaldehdye (final concentration 2%) for determination of the total haemocyte count (THC).

Exposure of haemocytes to commercial and purified glyphosate herbicide

The pooled haemolymph was distributed in flow cytometry tubes containing glyphosate (treatment) or filtered sterilized seawater (ESSW) (control) (Figure 1). For in vitro assay, it was possible to test concentrations higher than that used in the in vivo assay (0.5 mg L⁻¹). Eight concentrations of the herbicide were tested separately. For the commercial glyphosate Terminiflora, concentrations included 0.5, 5, 8.5, 16.9, 42.3, 84.5 and 169mgL⁻¹, and for purified glyphosate (N-(phosphonomethyl) glycine; CAS no. 1071-83-6; MW: 169.07; ESTANAL®, Sigma), concentrations were equivalent in μM (30, 50, 100, 250, 500, 1000). The flow cytometry tubes containing the haemocyte suspensions were kept at 20°C in the dark for a total period of 4h, which was estimated as sufficient to generate an acute response (Hégaret et al. 2011). The experiment was performed once.

Flow cytometry analysis

Fluorescent markers were added separately to the cell suspensions after 3h of incubation. The protocols used for the flow cytometry measurements were adapted from Hégaret et al. and were previously tested with C. gasar. The protocols are briefly described below.

Haemocyte viability was determined using double staining with Sybr Green I (in vitro, final concentration: 10-30µl from the original solution at 10,000x) and propidium iodide (PI, Sigma, final concentration: 10µg mL⁻¹). The results are presented as the percentage of PI-unstained cells (dead) to Sybr Green I-stained cells (dead and live). Fixed haemolymph was also analysed with Sybr Green I to estimate the THC (cells mL⁻¹). Phagocytosis was examined using fluorescent latex beads (Fluoresbrite® Yellow Green Microspheres, 2µm, Polysciences, Inc, 1x10⁶particles mL⁻¹) with a ratio of approximately 1:100 haemocytes: beads. The phagocytosis rate was estimated as the percentage of haemocytes that engulfed one or more fluorescent beads. Production of reactive oxygen species was determined using 2’,7’-dichlorofluorescein diacetate (DCFH-DA, Sigma, final concentration:10μM). The results are expressed as arbitrary units (U.A.) of fluorescence. The compound tert-butyl hydroperoxide (TBHP, Sigma, 10mM) was used as an ROS inducer for the in vivo assay.

All haemocyte suspensions were incubated for 1h at 20°C with the fluorescent markers. The suspensions were immediately analysed using the flow cytometer FACs Calibur (BD Biosciences, San Jose, California, USA). Cells were run in high flow and 10,000 events were measured, except for the THC, in which samples were run for 30s to estimate the cell concentration. Flow cytometer data were processed using Flowing software (version 2.5.1, Turku, Finland).

Statistical analysis

Normality and homogeneity of variance were checked before comparisons to choose the most appropriate test. The percentage data were arcsin transformed prior to analysis. For the oyster exposure assay, the effect of the treatment (Terminiflora 0.5mgL⁻¹) was analysed using t-tests when the data were normal, or by the Mann-Whitney test when the data were not normal. For the haemocyte exposure assay, the effect of the two herbicide formulations (commercial and purified glyphosate) and the various concentrations were analysed by a Two-way ANOVA. When differences were observed a One-way ANOVA was applied, followed by the LSD post hoc test. Differences were considered significant when P<0.05. Data are presented as the mean and standard error (SE). All statistical analyses were performed using Statgraphics Centurion Software, version XVII.
Results

Exposure of oysters to commercial glyphosate Termifin (0.5mgL⁻¹) did not cause dead to haemocytes, which maintained high viability, as observed in oysters from the control group (Table 1). Similarly, haemocyte phagocytosis capacity was not altered (Table 1). In contrast, changes in two immunological parameters were detected after Terminif exposure; production of induced ROS significantly increased and the THC was significantly reduced (Table 1).

For the in vitro assays, the viability of the haemocytes was analysed after exposure to a wide range of concentrations of the two formulations of glyphosate. There was a difference in haemocyte viability between the two glyphosate formulations, but not among concentrations (Two-way ANOVA, P=0.0095 and P=0.7995, respectively). Purified glyphosate induced haemocyte mortality at 250µM (One-way ANOVA, P=0.0072), whereas no mortality was induced with the commercial formulation (Figure 2a).

ROS production and the phagocytosis rate of haemocytes was analysed with herbicide concentrations higher than 8.5mgL⁻¹(50µM). ROS production was not modified by any glyphosate formulation or concentration (Two-way ANOVA, P=0.1560 and P=0.2011, respectively) (Figure 2b). The haemocyte phagocytosis rate did not vary between glyphosate formulations but was affected by concentration (Two-way ANOVA, P=0.1594 and P=0.0245, respectively); phagocytosis decreased to 84.5/500 and 169/000 (mgL⁻¹/µM) (Figure 2c).

Parasitic indices of Perkinsus spp. on the gills of oysters exposed to the commercial glyphosate Terminif did not change in relation to the control oysters (Table 2). In contrast, the amount of total heterotrophic bacteria increased in the gastrointestinal tract of oysters exposed to glyphosate (Table 2).

Table 1 Results of immune cellular parameters of Crassostrea gasar oyster after exposure to commercial glyphosate Terminif (0.5mgL⁻¹) or not (control). Data are reported as mean±SE. N: total number of oysters individually analysed in three independent experiments

<table>
<thead>
<tr>
<th>Glyphosate</th>
<th>N</th>
<th>Control</th>
<th>N</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive oxygen species (Fluorescence A.U.)</td>
<td>54.8±6.48</td>
<td>45</td>
<td>30.9±4.56</td>
<td>45</td>
</tr>
<tr>
<td>Total haemocyte count (cellsx10⁶/mL⁻¹)</td>
<td>1.8±2.1</td>
<td>45</td>
<td>3.1±2.6</td>
<td>45</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>94.2±0.61</td>
<td>45</td>
<td>95.1±0.47</td>
<td>45</td>
</tr>
<tr>
<td>Phagocytosis (%)</td>
<td>34.7±2.08</td>
<td>45</td>
<td>33.8±0.94</td>
<td>45</td>
</tr>
</tbody>
</table>

*Mann-Whitney; t test.

Table 2 Results of parasitic indices of Perkinsus spp. on gills and amount of total heterotrophic bacteria from the gastrointestinal tract of oysters exposed to commercial glyphosate Terminif (0.5mgL⁻¹) and not (control). Data are reported as mean±SE. N: total number of pools of haemolymph analysed in three independent experiments

<table>
<thead>
<tr>
<th>Glyphosate</th>
<th>N</th>
<th>Control</th>
<th>N</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence (%)</td>
<td>65.6±11.26</td>
<td>9</td>
<td>78.5±5.88</td>
<td>9</td>
</tr>
<tr>
<td>Intensity of infection (1-4)</td>
<td>1.65±0.32</td>
<td>9</td>
<td>2.22±0.21</td>
<td>9</td>
</tr>
<tr>
<td>Amount of bacteria (UFC g⁻¹x10⁵)</td>
<td>3.2±0.24</td>
<td>18</td>
<td>2.4±0.26</td>
<td>18</td>
</tr>
</tbody>
</table>

*t test.

Discussion

In the present study, the effect of glyphosate in the commercial formulation Terminif (1%) and its purified form were tested in a sessile and feed-filtered animal model, the oyster C. gasar, which is subject to environmental adversities and therefore, is considered a good bioindicator of environmental contamination.46 The response of oysters to the environment can be monitored in different ways; in this study, we chose to evaluate changes in haemocytes because these cells, in addition to acting in defence mechanisms against pathogens, are also implicated in innumerable physiological functions.47 Moreover, haemocytes are an excellent model for in vitro studies with contaminants.48

The exposure of oysters to commercial glyphosate was examined to understand effects of the herbicide on the animal. The in vitro approach using haemocytes was used to reveal the degree of cytoxicity of glyphosate. C. gasar oysters exhibited an increase in haemocyte ROS production in response to Terminif exposure. Reactive oxygen intermediates are produced through internal cellular processes that consume oxygen, such as respiration and photosynthesis. Due to their chemical properties, these radicals interact and damage organic molecules; in contrast, antioxidant enzymes control the amount of these free radicals in cells.49 Herbicides, including glyphosate, are known to inhibit antioxidant enzymes and increase cellular oxidative stress.50 Catalase is an antioxidant enzyme and its activity was reduced after exposure of C. gigas to glyphosate (0.1, 1 and 100µgL⁻¹) for 24 hours.51 Dos Santos & Martinez52 examined the effect of Roundup (10 ppm) on the freshwater shellfish Corbicula fuminea and found an increase in lipid peroxidation, which was consistent with changes in the activity of various antioxidant enzymes in the gills and digestive glands, including catalase, which had reduced activity. Therefore, the increase of ROS in the haemocytes can be explained by this effect, corroborating the previous literature.

C. gasar oysters exposed to Terminif exhibited a decrease in the number of circulating haemocytes. A similar study in mussels (Mytilus galloprovincialis) exposed for 7 days to purified glyphosate at concentrations of 10, 100 and 1,000µgL⁻¹ also presented a decrease in the amount of circulating haemocytes.53 The decrease in THC could be explained by the migration of haemocytes to the tissues to repair damage or to aid in the biotransformation of toxic compounds at the digestive gland level.54 Significant haemocytic infiltration into the connective tissue and digestive glands in addition to changes in its architecture have been reported in juvenile C. gigas oysters after exposure to purified glyphosate at a concentration of 0.1µgL⁻¹.52

Toxicological evaluation of the herbicide glyphosate in the cultured oyster Crassostrea gasar

a much lower dose than that used in the present study (500μgL⁻¹). Moreover, Gagnaire et al., 35 hypothesized that the up-regulation of haemocyte defence genes in C. gigas oysters exposed to a combination of pesticides/herbicides following bacterial challenge caused tissue damage.

C. gasar oysters exposed to Termifin did not modify phagocytosis capacity, in contrast to the haemocytes at the two highest concentrations tested. Phagocytosis is one of the most important cellular defence mechanisms in bivalves33 and is also one of the major biomarkers of immune-toxicity.30,34 C. gigas oyster exposed to a mixture of eight xenobiotics, including glyphosate (0.7μgL⁻¹), for seven days at environmentally relevant concentrations caused a reduction in the phagocytosis capacity of the haemocytes,35,36 whereas the other three cellular parameters (viability, quantity of esterase and ROS) did not change.35 The differences in results between the in vivo and in vitro assays may be in the concentrations tested, which were much lower for the in vivo exposure. Additionally, the commercial formulation used in the present study (Termifin) likely contains a complex mixture of adjuvants and surfactants, which in higher doses (in vitro) produced major effects on haemocytes. It is known that commercial formulations of glyphosate-based herbicides can be more harmful to organisms than their isolated active principles.26 Therefore, we hypothesize that if oysters had been exposed to Termifin at a higher concentration, inhibition of phagocytic capacity would have been observed.

The toxicity of glyphosate and its adjuvants were tested in larval and juvenile phases of the freshwater bivalve Lampsilis siliquoidea and it was demonstrated that Roundup® and its surfactant MON 0818 were more toxic than technical grade glyphosate.26,29 Similarly, Mottier et al.,27 tested two glyphosate commercial formulations, Roundup Express® (REX) and Roundup Allées et Terrasses® (RAT) during the embryo-larval development and metamorphosis of C. gigas oysters, and found that both were more toxic than isolated glyphosate and its metabolite AMPA. Mottier et al.,28 examined the same biological model and life stages and observed high toxicity of the surfactant polyethoxylated tallow amine (POEA, Genamin T200®). These reports highlight the need to characterize the toxicity of the adjuvants and not only the active ingredients of the herbicides.

Oysters exposed to Termifin did not undergo mortality. This result demonstrates that, despite the haemocyte oxidative stress observed, molecular mechanisms of cell repair and cell survival must be active, allowing for proper immune and physiological functions. Two in vitro studies on C. gigas haemocytes did not report cell mortality from the herbicide atrazine (9.3, 93, 930μM) or a mixture of 14 contaminants (herbicides, fungicides, insecticides and molluscicides). et al.,30 In contrast, among the eight herbicides tested (metolachlor, alachlor, terbutylazine, glyphosate, diuron, atrazine, 2,4-dichlorophenoxyacetic acid (2,4D), only the 2,4D caused mortality of C. virginica haemocytes after 4h of exposure, without modulating other haemocyte parameters (ROS, phagocytosis, esterases, lysosomes); glyphosate did not cause any change in haemocytes at the tested concentration (12μmolL⁻¹).30 These results suggest that the mode of action of herbicide formulations on oyster haemocytes are different and require investigation.

Milan et al.,31 suggested modification in some physiological pathways by glyphosate on the digestive glands of the mussel Mytilus galloprovincialis. This group found (1) down-regulation of calcium- and calmodulin-dependent phosphodiesterase type 1, which interferes with calcium signalling, (2) down-regulation of ABC transporters proteins, which impairs the transport of deleterious compounds out of the cell, and (3) down-regulation of phosphoinositide phosphatase, which alters the degradation of phosphoinositides, a group of key signalling molecules involved in many cellular processes.
It is important to point out that the defence response of bivalves to infectious agents may be compromised due to cellular effects caused by xenobiotics. This was demonstrated in a study with *C. gigas* oysters that were challenged with pathogenic bacterial strains (*Vibrio splendidus*, LGP31 and LGP32) following exposure to a mixture of pesticides and herbicides, including glyphosate. Exposed and challenged oysters suffered higher mortality than untreated oysters. Haemoctye phagocytosis decreased and 19 selected genes were down-regulated, indicating that the contaminants may have altered the overall health of the animal, rendering the oysters more susceptible to vibrios and death.

Considering that the immunological status of the *C. gasar* oysters exposed to Temifin was changed, we inferred a weaknesses of the oysters’ ability to defend against the *Perkinsus* parasite, which would result in an increase of parasitic indices. Nevertheless, we observed a decrease of the *Perkinsus* parasitological indices, although not significant, that could suggest a direct toxicity effect of the herbicide against *Perkinsus*. Elandalloussi et al., observed a reduction in *P. olseni* prevalence when *Ruditapes decussatus* was exposed to Roundup® at 25 mg L⁻¹ for 5 days. The dose tested in the present study was 50 times lower (0.5 mg mL⁻¹) than that used by the authors. This treatment caused a reduction of approximately 17% in parasite prevalence, suggesting that Temifin could have a similar effect to Roundup® when used in high concentrations. Elandalloussi et al., also directly confirmed glyphosate cytotoxicity in *P. olseni* cells, revealing that Roundup® was 10 times more potent (IC₅₀: 0.4 mM) than its isolated active principle (IC₅₀: 3.4 mM). This hypothesis can be tested in the future with *P. marinus* isolates from Brazil, a species that occurs in *C. gasar* oysters of the coast of Paraíba, together with the species *P. berheiainensis*. The present study also investigated whether exposure to Temifin affected the amount of total heterotrophic bacteria in the gastrointestinal tract of *C. gasar*. A 33% increase in the mean CFUs observed in oysters exposed to the herbicide indicates an impact to this parameter, suggesting an imbalance of bacterial communities. Significant effects on oyster health would be expected, since gastrointestinal microbiota play an important role in oyster health, as reported for several other aquatic invertebrates and humans. Recent studies have shown a complex and metabolically versatile of the resident gastrointestinal microbiota in oysters. It is also possible that some bacterial taxa prevail over others in the treated animals. Indeed, a study on poultry microbiota confirmed that glyphosate caused a disturbance in the normal bacterial community, altering the balance between pathogenic and beneficial resident bacteria. The authors observed that pathogenic bacteria (*Clostridium botulinum, Salmonella Typhimurium*) exposed to glyphosate in vitro were resistant to the herbicide, whereas others considered beneficial (*Bacillus badius, Lactobacillus spp.*) were susceptible. If similar selectivity occurred in oysters treated with Temifin in the present study, it is possible that treatment would favour opportunistic and pathogenic bacteria. Thus, glyphosate is a concern to aquaculture, because *Vibrio* are common bacteria in seawater and in the gastrointestinal tracts of bivalves, and are pathogenic to bivalves. The identification of the bacteria present after exposure to the herbicide would confirm this hypothesis.

We conclude that the herbicide glyphosate in its commercial formulation and its active ingredient are toxic to oysters, affecting two major mechanisms of defence and consequently compromising the ability of oysters to defend against infectious agents. In contrast, our data suggests that the gastrointestinal tract microbiota is sensitive to the commercial formulation of glyphosate, which could make oysters more susceptible to pathogenic bacterial strains. Overall, these results increase our understanding of the impact of the herbicide glyphosate on a non-target species, the oyster *C. gasar*.

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**Conflicts of interest**

Author declare that there is no conflicts of interest.

**References**


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Toxicological evaluation of the herbicide glyphosate in the cultured oyster Crassostrea gasar


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