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Effect of the Herbicide Roundup® on *Perkinsus olseni* in vitro Proliferation and in vivo Survival when Infecting a Permissive Host, the Clam *Ruditapes decussatus*

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Abstract Coastal habitats are increasingly being exposed to herbicide contamination from urban and agricultural catchments. Data on its toxicity on aquatic ecosystems, especially those based on sediment, are relatively scarce. This study aimed at investigating whether the susceptibility of an aquatic filter-feeding organism, the carpet-shell clam (*Ruditapes decussatus*) to the parasite *Perkinsus olseni* was influenced by the herbicide Roundup® and its active ingredient glyphosate. The effect of Roundup® and glyphosate on *P. olseni* in vitro proliferation was also evaluated and appeared to confirm the higher toxicity of Roundup when compared with technical grade glyphosate.

Keywords *Perkinsus olseni* · Carpet-shell clam · Glyphosate · Roundup®

Roundup® is a glyphosate-based herbicide widely used in weed control (Worthing 1987). Bivalves are filter-feeding organisms, a characteristic that allows them to accumulate chemicals in concentrations high above those found in the surrounding waters. There is growing evidence that contaminants may be partially responsible for the observed increase in diseases affecting marine organisms (Harvell et al. 1999). Mass mortalities of the clam *Ruditapes decussatus*, a filter-feeding bivalve living in sediments, have been observed for the past 20 years in the southern coast of Portugal (Ruano and Cachola 1986; Leite et al. 2004). This mortality has been attributed to the infection by a protozoan parasite, *Perkinsus olseni* (Azevedo 1989). This parasite has been demonstrated to be sensitive to glyphosate (Elandalloussi et al. 2005b). However, the effect of glyphosate or its commercial formulation Roundup® on *Perkinsus*-infected clams is unknown. Our results appear to confirm the higher toxicity of Roundup when compared with technical grade glyphosate.

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Materials and Methods

The in vitro clonal culture of *P. olseni* isolated from *R. decussatus* clams collected in the Ria Formosa was cultured as previously described (Robledo et al. 2002). Parasites were maintained in continuous culture in DMEM:Ham's F12 (1:2) medium supplemented with 5% fetal bovine serum (FBS) and incubated at 28°C with no added CO_2. *Perkinsus olseni* trophozoites were harvested for experiments while they were in log-phase growth. For in vitro sensitivity testing of *P. olseni*, cells were seeded in 96-well plates in quadruplicate for each concentration of glyphosate (N-phosphomethyl glycine, Sigma) or Roundup (Roundup® *Forte*, Monsanto) used. The concentrations for both chemicals ranged from 25 μM to 50 mM. The concentration of glyphosate in Roundup was calculated taking in consideration that ammonium glyphosate is 68% w/w in Roundup formula. Experiments were repeated at least three times with a concentration of 5×10^6 cells/mL in 100 μL of medium containing the different chemical concentrations to be tested. Dose–response relationships were evaluated after

72 h incubation at 28°C using the cell proliferation assay to determine parasite viability (Elandalloussi et al. 2005a). Cell proliferation assay reagents were mixed according to the manufacturer's instructions (Promega, CellTiter 96® Aqueous Non radioactive Cell Proliferation Assay) and 20 µL of the resulting solution added to microplate wells containing 100 µL cell suspensions, yielding assay concentrations of 318 µg/mL MTS (3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium) and 25 µM PMS (phenazine methosulfate). Assay plates were incubated for 1 h at 28°C in the dark, and absorbance was read at 490 nm with a microplate reader. The percent viability obtained from proliferation tests is relative to that of the control (untreated cells).

Hatchery-reared *R. decussatus* grown to a 25 mm size were obtained from IPIMAR-INIAP, Tavira. All groups were produced and reared under similar conditions, and within each batch, all clams were of the same age. Ten clams were sacrificed initially and examined for the presence of *P. olseni* using a modified Ray's Fluid Thioglycollate Medium diagnosis assay (Leite et al. 2004). Prior to the experiments, clams were acclimatised for at least 1 week at 22°C and 30 ppt salinity. Clams were held in the CCMar laboratory in 5 L aquaria in filtered seawater.

A 96 h acute toxicity test was conducted using hatchery-reared *R. decussatus* free of *P. olseni* obtained from IPIMAR in Tavira, Portugal. The experimental design consisted of duplicate treatments of 5 clams per 2 L tanks. At the start of the experiment, 10 clams were individually assayed for the presence of the parasite. Clams were exposed for 96 h to four daily renewal baths of glyphosate and Roundup at the following concentrations: 0.01, 0.1, 1, and 10 mg/L. Clam mortality was monitored throughout the experimental period.

Initially, two procedures for in vivo clam infestation were tested, the feeding procedure and the adductor muscle injection. In the feeding procedure, hatchery-reared clams were fed with the normal diet, supplemented with the equivalent of 1×10^6 *P. olseni* trophozoites per clam. In the second procedure, clams were challenged with 1×10^6 *P. olseni* trophozoites obtained from an in vitro culture, by direct injection into the adductor muscle. To prevent the clams from ejecting the inoculum, they were kept for the 24 h immediately following injection in a humid chamber created using wet filter paper, prior to being returned to the aquaria. Six weeks later clams were sacrificed to monitor infection.

Prior to in vivo exposure experiments, clams were challenged with in vitro cultured *P. olseni* and 6 weeks later randomly distributed into three groups of 30 individuals, each one subdivided into six subgroups of five clams each. Clams were kept in 5 L of salt water at 30 ppt, at room temperature with daily water changes. During the in vivo exposure experiment, clams were fed daily with their algal diet and exposed either to vehicle (control group), 10 or 25 mg L^{-1} Roundup for 5 days. Mortality was monitored throughout the experiment and dead clams were processed to determine their level of infection. At the end of the experiment, all clams were sacrificed and both prevalence and infection intensity were determined using the whole clam wet tissue technique (Leite et al. 2004). Briefly, whole clams were individually incubated in fluid thioglycollate medium (Difco) at room temperature in the dark for 7 days. Sodium hydroxide (2 M) was added to each sample and incubated for 3 h at 50°C to lyse the tissues. Samples were then washed three times with filtered seawater and stained with Lugol's iodine solution. The number of prezoosporangium in three different 100 µL aliquots of each sample was counted under the microscope.

Data analysis of results from in vitro and in vivo experiments was performed using the computer program GraphPad Prism® v4.0 (GraphPad software). A two-tailed student's *t*-test was used to compare differences between treatments and to identify those that differed significantly from control. The level of significance was set at $p < 0.05$. Data for dose–response curves was fitted using this program and the concentration of drug responsible for 50% inhibition of parasite growth (IC_{50}) was determined from the curve.

Results and Discussion

The herbicide Roundup was tested for in vitro inhibition of *P. olseni* growth using the in vitro cell proliferation assay to determine parasite viability. A typical dose-dependent chemical-response curve was obtained in the presence of Roundup (Fig. 1) with an IC_{50} of 0.41 ± 0.01 mM. For comparison, glyphosate was also tested for its in vitro inhibitory properties and found to display an IC_{50} of 3.4 mM. Therefore, Roundup was 10 times more toxic to the parasite cells than its active ingredient glyphosate. These results suggest that either Roundup adjuvant enhanced glyphosate bioavailability and/or bioaccumulation or the surfactant used in the formulation (ethoxylated tallowamine) displayed cytotoxic properties.

To assess the influence of dosing methods on in vivo clam infestation, hatchery-reared clams were challenged with in vitro cultured parasites via direct injection into the adductor muscle. Six week post-infection, parasite burdens were determined using the FTM assay and revealed that, while no infections could be detected in fed clams, heavy infections ($35{,}571 \pm 6{,}576$ *Perkinsus* g^{-1} clam tissue) occurred after adductor muscle injection. These findings are in agreement with previous studies showing that experimental challenges of oysters with *P. marinus* via

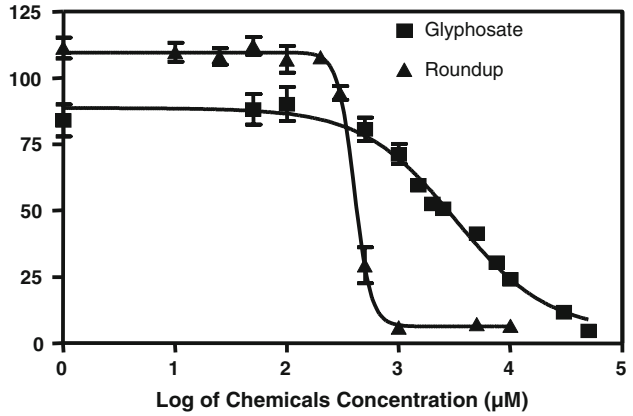

Fig. 1 Glyphosate and Roundup dose–response curves for in vitro-cultured *P. olseni* after a 72-h drug exposure. Error bars absence indicates a standard deviation below 5%

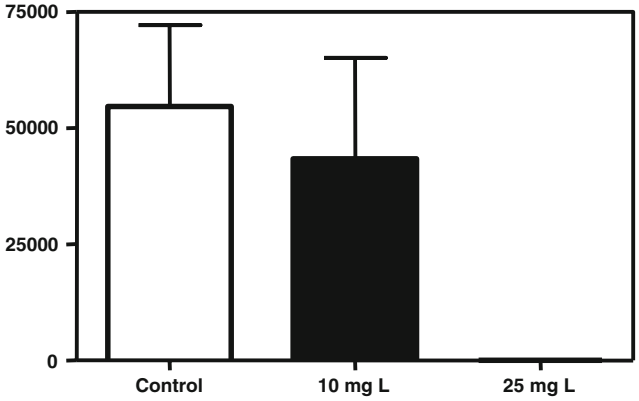

Fig. 2 Intensity of *Perkinsus* infection detected on carpet-shell clam exposed to Roundup for 5 days. Error bars indicate the standard deviation in analyzed samples

feeding produced none or little infections when compared to shell cavity and adductor muscle injection trials (Chintala et al. 2002; Bushek et al. 1997). This suggests that the latter method might permit the entry of parasites directly into the bivalve tissues.

Acute toxicity experiments using *Perkinsus*-free clams revealed that no mortality was observed after 96-h treatments with either glyphosate or Roundup (data not shown). On the basis of these results, in vivo experiments have been set up to determine whether glyphosate and its commercial formulation have an effect on the progression of the disease in infected clams. The influence of glyphosate was previously tested under controlled conditions using *Perkinsus*-free clams grown in a closed hatchery and challenged with 1×10^6 trophozoites per clam. No significant effect ($p > 0.05$) could be detected in heavily infected clams treated with glyphosate compared with controls without treatment (data not shown). On the other hand, all 30 clams died after a 5 day treatment with daily renewal baths of 25 mg L^{-1} Roundup and seven out of 30 clams died at the end of the treatment with 10 mg L^{-1} Roundup (Table 1). In all clams that died with the 25 mg L^{-1} Roundup treatment, no parasites could be detected by FTM analysis but when Roundup concentration was decreased to 10 mg L^{-1} the infection level did not change significantly when compared to levels observed in non treated group ($p = 0.6846$, Fig. 2). Since no additional deaths were detected in clams treated with the same dose for additional 24 h (Table 1), this results suggest that the lower dose used is not toxic for clams.

Exposure of oysters to contaminants has been demonstrated to enhance pre-existing infections caused by *P. marinus* as well as prevalence of experimentally induced infections (Anderson et al. 1995; Fisher et al. 1995). However, our results suggest that the exposure to Roundup did not enhance disease expression in the clams. Results may reflect the cytotoxic effect of this herbicide on the parasite. Similarly to early data from Anderson et al. (1995), we observed a higher mortality of the experimentally infected clams indicating that combination of the two stresses (parasite infection and chemical treatment) cause greater mortalities than either treatment alone. Contaminants have been hypothesized to increase the severity of disease by affecting bivalve immune system (Wootton et al. 2003; Pipe and Coles 1995). On the other hand, glyphosate exposure did not affect the progression of the disease nor caused mortalities in infected clam. These results appeared to confirm the higher toxicity of Roundup when compared with technical grade glyphosate. Glyphosate is believed to be rather specific, targeting the

Table 1 Mortality of carpet-shell clam exposed to Roundup and intensity of *Perkinsus* infection determined on dead clams

Concentration (mg L^{-1})	0 h	72 h		96 h		120 h (end of experiment)		
	No. of live clams	No. of dead clams	No. of *Perkinsus* cells/g^{-1} tissue	No. of dead clams	No. of *Perkinsus* cells/g^{-1} tissue	No. of dead clams	No. of *Perkinsus* cells/g^{-1} tissue	No. of clams at the end of experiment
Control	30	0	–	0	–	1	208	29
10	30	0	–	7	451 ± 330	0	–	23
25	30	2	0	28	0	–	–	0

shikimate pathway in plants (Steinrucken and Amrhein 1980).

However, Roundup has previously been found to be more toxic to fish and benthic organisms than its active ingredient glyphosate (Folmar et al. 1979; Abdelghani et al. 1997; Tsui and Chu 2004), and therefore, Roundup surfactant has been hypothesised to be responsible for this observed toxicity (Mitchell et al. 1987; Servizi et al. 1987). Recent studies in the freshwater mussel *Utterbackia imbecillis* (Conners and Black 2004) revealed a LC_{50} for Roundup exposure close to the maximum value tested for *Ruditapes decussatus*, consolidating the data obtain in our observations.

In conclusion, our findings together with available information suggest that surfactant composition should be taken into consideration when evaluating toxicity and emphasize the need to assess the toxicity of the commercial formulation and not solely of the active ingredients. Such data provide crucial information for aquatic management and for establishing limits for use of herbicides near aquatic ecosystems.

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