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Towards the development of an embryotoxicity bioassay with terrestrial snails: Screening approach for cadmium and pesticides

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ABSTRACT

Currently no bioassays are available to assess the embryotoxicity of chemicals with terrestrial soil invertebrates. We therefore presented a new method for embryotoxicity testing with snail eggs: a relevant biological material that incubates in soil and that can be exposed to contaminants from leachates and soil solution. The effects of aqueous solutions of two herbicide formulations, Reglone® (active ingredient (a.i.), diquat) and Roundup® or its a.i., glyphosate, of a surfactant (Agral® 90, a.i., nonylphenol polyethoxylates) and of cadmium (Cd) were studied. Endpoints were the hatching success and observations of embryo abnormalities after exposure. Roundup® was found to be more toxic than its a.i. alone ($EC50_{a.i.}$ = 18 mg/l and about 1300 mg/l, respectively). Reglone® ($EC50_{a.i.}$ = 0.72 mg/l) and Agral® ($EC50_{a.i.} \approx 50$ mg/l) were also tested together, revealing that Reglone® accounted for more than 99% of the mixture's toxicity. An antagonistic interaction between the two substances was found. For Cd (EC50 = 3.9 mg/l), a significant transfer from exposure medium to eggs was emphasized, particularly affecting the albumen. Abnormalities of embryogenesis in non-hatched embryos depended on the substance and the concentration considered.

1. Introduction

To assess the hazards involved in the action of chemicals on soil organisms, several biological methods have been developed on nematodes, earthworms, collembolans and snails [1]. The toxicity endpoints of most of the bioassays are survival, growth and reproduction [2–5]. To our knowledge, no standardized bioassay specifically concerns the effects of pollutants on embryonic development of any of the terrestrial organisms that make up the soil fauna. Yet, soil is often the first receptor of contaminants and hatching success is crucial for the stability of populations [6]. Moreover, on the basis of data on aquatic organisms, the embryos are generally found to be more sensitive than adults [7–10].

Thus, our aim was to lay down the bases of a method to assess the embryotoxicity of chemicals on *Helix aspersa*. This terrestrial gastropod is already the subject of a standardized test based on the effects of pollutants on survival and growth of juveniles [2] and can be adapted for reproduction [11].

Snails lay their eggs in the topsoil (2–5 cm depth) and can be exposed to contaminants deposited on the ground and then leached downwards. Thus, we chose here to expose eggs to aqueous solutions of contaminants, i.e. in a liquid phase bioassay.

The toxicity of inorganic (cadmium) and organic (pesticides) compounds on embryo development and hatching success, of *H. aspersa* was assessed. While embryotoxicity data for Roundup® and Reglone® exist for aquatic organisms [12–14], they are not available for this life stage in terrestrial invertebrates. Although glyphosate, which is among the most widely used pesticides in the world [15], was found to be less toxic alone than its formulation (e.g. Roundup®) [16–19] on different organisms as well as on human cells, there is no available information on that point for land snails. Reglone®, which contains the a.i. diquat, is often used with adjuvants, for instance with nonylphenol polyethoxylates (NPEOs). It was tested alone and in mixtures with Agral® 90, a commercial formulation of a mixture of NPEOs. Cd was chosen as a reference substance known for its high embryotoxicity [20,21]. To document the permeability of egg membranes to metal and internal concentration–effect relationships, Cd concentrations were measured in different parts of the exposed eggs. The embryogenesis of unexposed and exposed embryos was compared.

2. Materials and methods

2.1. Chemicals

Reglone® 2 (200 g/l diquat dibromide; Syngenta Agro S.A.S., Velizy-Villacoublay, France), Agral® 90 (945 g/l NPEOs; Syngenta Agro) and Roundup® Biovert 360 (360 g/l glyphosate; Monsanto Europe S.A.) were used. Aqueous solutions of Cd and glyphosate

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Table 1
Range of nominal concentrations used for each contaminant and corresponding pH.

Solution	Replicate number	Test number	Range of concentrations	pH
Glyphosate (mg/l)	3	Test 1	225–450–900–1800–3600	2.3 < pH < 3.1
	3	Test 2	225–450–900–1800–3600	
	4	Test 3	1000–1400–1960–2740–3840	
Roundup® (mg/l of glyphosate)	5	Test 1	1.8–9–45–225	4.9 < pH < 6.2
Reglone® (mg/l of diquat)	6	Test 1	0.375–0.75–1.5–3–6	6.4 < pH < 6.6
Agral® (mg/l of NPEOs)	3	Test 1	12.5–25–50–100–200	6.4 < pH < 6.9
	3	Test 2	12.5–25–50–100–200	
	4	Test 3	39–54.7–76.5–107.1–150–210	
Mixture (mg/l of diquat) [a]	4	Test 1	0.4–0.6–0.8–1.1–1.5–2.1–3	6.1 < pH < 6.5
Cd (mg/l)	4	Test 1	2–4–6–8–10	5.8 < pH < 6.3

[a] Proportion of Reglone® in mixture: 0.68, according to the recommendations of Syngenta Agro S.A.S.

were prepared with solid $CdCl_2$ (99.99%, Sigma Chemical Co., St. Louis, MO; C-2544) and solid glyphosate (99%, Sigma–Aldrich Chimie SARL, Lyon, France, CAS no. 1071-83-6). All dilutions were prepared with demineralised water (pH 6.2), which also served as control. Concentrations of final tests are presented in Table 1. For glyphosate and Agral®, the repeatability (test 2 with the same concentrations) and reproducibility (test 3 with other concentrations) were tested.

2.2. Snails and clutches

Adult *Helix aspersa aspersa* Müller (*syn. Cantareus aspersus aspersus* Müller, 1774 or *Cornu aspersum*) snails (aged between four months and one year) came from our standardized laboratory rearing [2]. To isolate the clutches, 125-ml glass pots filled with damp horticultural compost (SEM NF 44-551, with fertiliser; organic matter: 82%, pH: 6.5) were placed in snail cages the evening. The next morning, the glass pots were isolated to identify the laying snail and to be sure that there was only one clutch per glass pot (Fig. 1). The duration of egg-laying is between 24 and 32 h. After snails have laid their eggs, the clutches can be removed from the compost. In each clutch, eggs were counted (between 70 and 150 per clutch), washed with tap water at 20 °C to remove soil particles and then deposited on damp blotting paper in Petri dishes (Sterilin, 90 mm × 14.2 mm, crystal polystyrene, triple vent) until the experiments.

2.3. Exposure device

Rapidly after egg-laying, each clutch was separated into groups of 8–10 eggs which were placed in Petri dishes (Greiner Bio-one, 35 mm × 10 mm, crystal polystyrene, triple vent). Four layers of paper (Quantitative filter paper grade 40 ashless, Whatman) dampened with 0.8 ml of control or contaminated solutions (Table 1) were laid on the bottom of the Petri dishes (this volume is enough to keep with the humidity until hatching). The eggs were incubated in these dishes at 20 ± 2 °C, 18 h light per day and humidity of 80–90% until hatching (about 14 days for controls). For each compound, between 3 and 6 clutches were exposed (Table 1). Twenty days after the beginning of exposure (to be sure not to omit late hatchlings), the mean hatching success for each concentration was calculated. Results were considered valid if the hatching success of controls was higher than or equal to 70% (average value observed in our laboratory rearing for controls).

As the range of pH values for the different solutions was high (Table 1), the sensitivity of egg development to the pH of the medium was assessed using a range of seven pH values (1, 2, 4, 6, 8, 10, and 12) with 6 replicates each, in demineralised water adjusted with HCl or NaOH.

Fig. 1. Laying snails in glass pots allowing easy sampling of fresh alive eggs.

2.4. Sample preparation for Cd analyses

Two clutches were divided into two parts each; the first parts of each clutch (46 eggs, 261.2 mg dry mass, DM and 57 eggs; 301.9 mg of DM) were used as controls and the second parts (50 eggs, 269.3 mg of DM and 62 eggs, 334.9 mg of DM) were exposed to 4 and 8 mg Cd/l, respectively, for 7 days. After, the eggs were washed with water and the eggshell separated from the albumen. After drying in an oven (60 °C), samples of eggshell or albumen were digested in 50% HNO_3 (2.5 ml for about 100–150 mg of DM) for 40 h at 60 °C. Then 7 ml ultrapure water were added and Cd concentrations were determined using a furnace atomic absorption spectrophotometer (220Z, Varian, Les Ulis, France). The reliability of the analysis was assessed with standard reference material (TORT-2, lobster hepatopancreas, from NRCC-CNRL, Canada).

2.5. Embryo observation

The main steps of embryogenesis were described in unexposed embryos according to Fol's typology [22]. As the eggs are opaque, the eggshell must be removed to see the embryo, which is enclosed in transparent albumen. Embryos were observed with an inverted microscope (Nikon Eclipse TE300) or a binocular microscope equipped with a camera (Nikon Digital Camera DXM1200). After exposure to the various chemicals studied, non-hatched eggs were examined in the same way to determine the stage at which their embryonic development was interrupted.

2.6. Statistical analyses

The null hypothesis of independence between exposure and hatching success was tested using the Kruskal–Wallis rank test. When the null hypothesis was rejected, multiple comparisons were performed on ranks using Tukey's Honestly Significant Difference test. All statistics were performed with *R* (2.9.2) (R Development Core Team, 2004).

The dose-dependent curves and the EC50 values were determined with Hill's model using the macro Excel Regtox free version EV6.1.

Mixture toxicity data were analysed with the CA (Concentration Addition) model which is commonly used to predict the toxicity of a mixture and is the most pessimistic for risk assessment [23,24]. Following Loewe's CA theory [25], the curve of the predicted mixture effect [26] (given by Eq. (1)) of Reglone® and Agral® was built and compared to the curve of the observed mixture effect.

$$\mathrm{ECx}_{\mathrm{mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{\mathrm{ECx}_i} \right)^{-1} \quad (1)$$

where $\mathrm{ECx}_{\mathrm{mix}}$ is the predicted effect for the mixture for a given concentration, P_i, the proportion of component i in the mixture and ECx_i, the calculated concentration effect of individual component i (for Agral®, results of the test 1 + 2 + 3 were used, see Table 2). To calculate the relative contribution (RC) of each compound in the mixture [24], toxic units (TUs), defined as the quotient C_i/ECx_i, where C_i is the concentration of component i in the mixture, were used. For instance for the effect of a 50% mixture:

$$\mathrm{RC}_{\mathrm{Reglone}} = \frac{\mathrm{TU50}_{\mathrm{Reglone}}}{(\mathrm{TU50}_{\mathrm{Reglone}} + \mathrm{TU50}_{\mathrm{Agral}})} \times 100$$
$$\mathrm{RC}_{\mathrm{Agral}} = \frac{\mathrm{TU50}_{\mathrm{Agral}}}{(\mathrm{TU50}_{\mathrm{Reglone}} + \mathrm{TU50}_{\mathrm{Agral}})} \times 100. \quad (2)$$

3. Results

3.1. Hatching success

The influence of pH on hatching success was not significant except for the extreme values, 1 and 12, for which the percentage of hatching was 0% (Fig. 2), with the eggs becoming reddish brown. As pH values of tested solutions were in the range 2.3–6.9 (Table 1), it is likely that the effects observed on eggs are more due to the chemicals than to low pH.

The toxicity of chemicals decreased in the following order: Reglone® (most toxic) > Cd > Roundup® > Agral® > glyphosate (Table 2 and Fig. 3). Hatching was completely inhibited by Cd from 8 mg/l (Fig. 3a). Roundup®, at the same concentration as glyphosate, was almost 100-fold more toxic than glyphosate alone (Table 2). Indeed, Fig. 3b shows that, from 225 mg glyphosate/l in Roundup®, hatching success was totally inhibited whereas, for glyphosate alone at the same concentration, hatching success was equivalent to that of the controls.

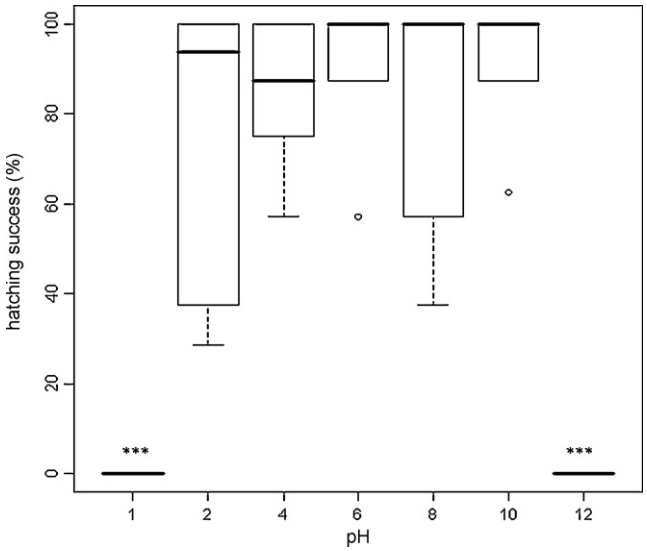

Fig. 2. Hatching success of snail eggs exposed to increasing pH. Significant differences ($p < 0.001$) are indicated by ***.

Reglone® was about 100-fold more toxic than Agral® with a noteworthy variability between the three tests of Agral® (Table 2 and Fig. 3d). Fig. 4 compares the calculated and predicted toxicity of the mixture Reglone®–Agral® determined at concentrations recommended for agricultural use. A significant difference between the two curves (no overlapping of 95% confidence interval for the respective EC50 values) indicates an antagonistic interaction between the two products with a higher contribution of Reglone® (99.45%) in the mixture.

3.2. Transfer of Cd

Table 3 presents the transfer of Cd from exposure medium to eggs which increased with the Cd concentrations. Albumen contained more Cd than eggshell in terms of dry mass for the two

Table 2
Toxicity (EC10 and EC50) of the different contaminants on the hatching success of snail embryos and recommended concentrations for agricultural use.

Contaminant	Test	NOEC	EC10 (CI 95%)	EC50 (CI 95%)	Agricultural recommended concentrations
Glyphosate (mg/l)	Test 1	1800	1262 (830–1822)	1840 (1210–2140)	3600
	Test 2	1400	732 (438–1387)	1190 (910–1530)	
	Test 3	1800	1451 (912–1500)	1580 (1020–1640)	
	Test 1 + 2 + 3[a]	1000	854 (685–1348)	1324 (1087–1574)	
Roundup® (mg/l of glyphosate)	Test 1	45	1 (0.02–31)	18 (2.8–66)	3600
Reglone® alone (mg/l of diquat)	Test 1	0.375	0.51 (0.30–0.68)	0.72 (0.60–0.79)	2000
Agral® alone (mg/l of NPEOs)	Test 1	50	55.7 (31.9–84)	85.6 (68.1–97.3)	945
	Test 2	54.7	22.3 (11.3–52.6)	46.45 (33.53–68.57)	
	Test 3	200	23.3 (8.4–37.9)	26.34 (17.80–39.22)	
	Test 1 + 2 + 3[a]	54.7	22.2 (12.4–43.5)	47.63 (36.34–59.52)	
Mixture (mg/l of diquat)	Test 1	1.5	0.93 (0.62–1.38)	1.31 (1.10–1.51)	2000
Cd (mg/l)	Test 1	2	2.62 (1.90–3.72)	3.94 (3.43–4.38)	

[a] Tests 1 + 2 + 3 were run with all exposure concentrations of each test and all replicates summed.

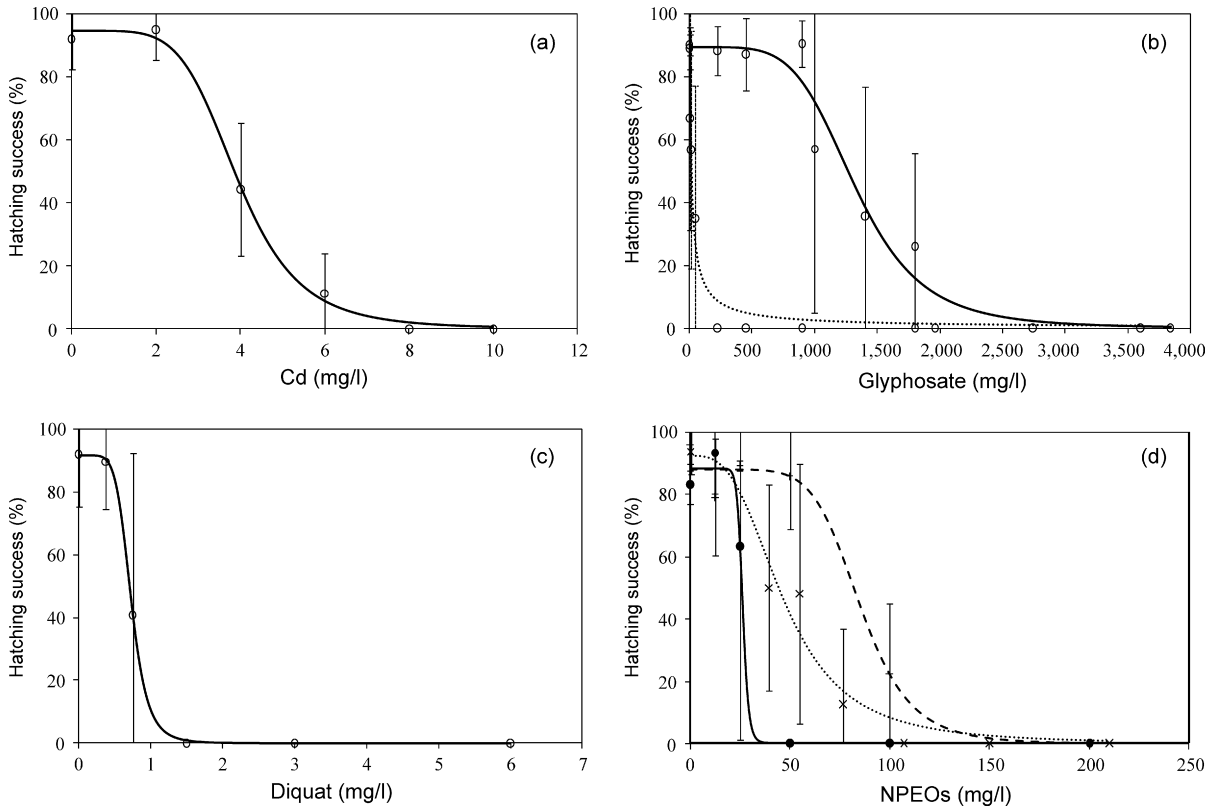

Fig. 3. Concentration–response curves obtained by Hill's model after 14 days of exposure of *Helix aspersa* eggs to Cd (a), glyphosate (test 1 + 2 + 3, solid line; (b) and Roundup® (dotted line; (b), Reglone® (c) and Agral® (test 1: dashed line; test 2: dotted line and test 3: solid line; (d). Plots represent the mean (with 95% confidence interval).

Table 3
Transfer of Cd from exposure media to eggshell and albumen.

Nominal exposure concentrations (mg Cd/l)	Measured exposure concentrations (mg Cd/l)	[Eggshell (μg/g)]		[Albumen (μg/g)]	
		Fresh mass	Dry mass	Fresh mass	Dry mass
0	–	0.006	0.020	0.001	0.008
4	3.9	2.52	7.5	2.66	22.1
8	8.2	6.79	29.1	5.97	39.6

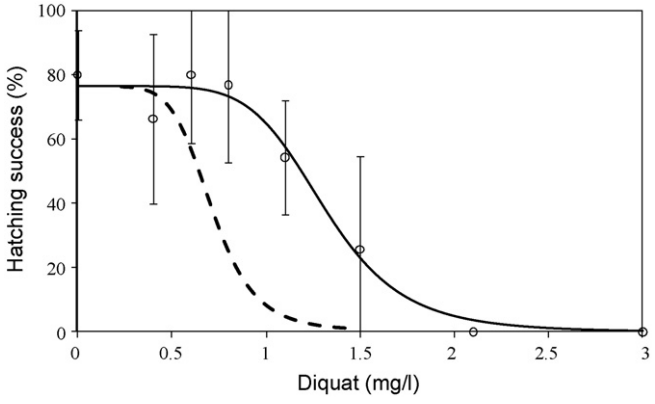

Fig. 4. Concentration–response curve obtained by Hill's model after 14 days of exposure of *Helix aspersa* eggs to mixture Reglone®–Agral® (solid line) compared with the predicted concentration–response of the mixture determined by the CA model (dashed line). Concentration is estimated in diquat. Plots represent the mean (with 95% confidence interval).

concentrations of exposure, showing Cd was able to cross the eggshell.

3.3. Normal and disrupted embryogenesis

The main stages of embryogenesis are shown in Fig. 5. Embryos exposed to glyphosate were blocked late in their development, in a larval stage corresponding to 12 day's development in controls (Fig. 5F). Embryos exposed to Reglone® at 0.75 and 1.5 mg diquat/l were blocked either at the morula stage or at a larval stage (Fig. 6A): the eyes were present but not the tentacles. When embryos were exposed to higher concentrations, they were blocked earlier in their development, corresponding to a one-cell stage or morula in controls (Fig. 5A and 5C). All non-hatched embryos exposed to Agral® stopped developing at early stages of embryogenesis corresponding to 3–4 days after fertilization for controls (Fig. 5C and 5D). The development of embryos exposed to Cd was stopped at different stages depending on the concentration of exposure. We also observed variations for eggs exposed to the same concentration. For instance, at 4 mg Cd/l, some embryos were blocked at the gastrula stage (Fig. 6B) whereas others continued their development until metamorphosis (Fig. 6C). These embryos

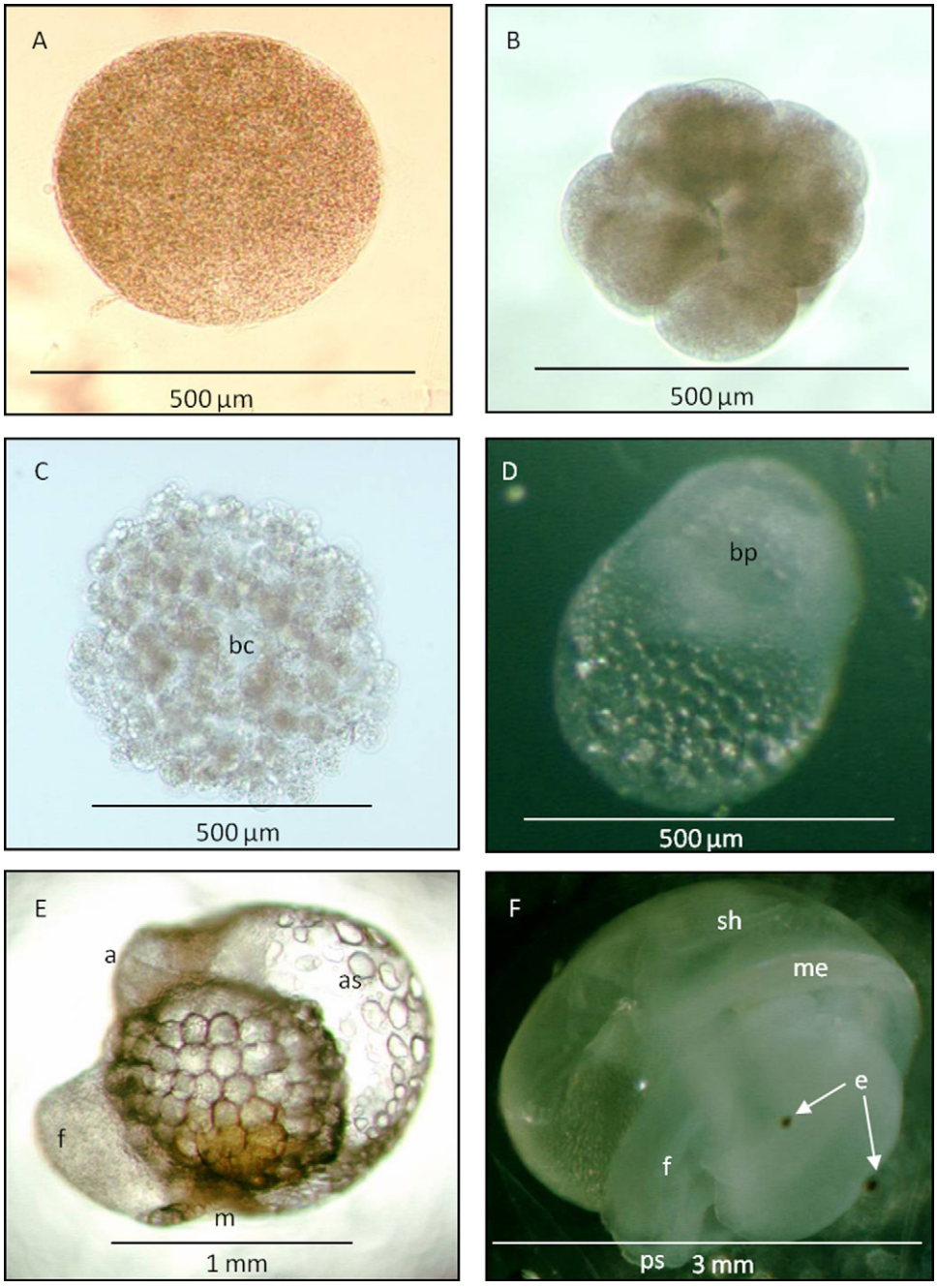

Fig. 5. Embryos of non-exposed *Helix aspersa* eggs at the one-cell stage (A, 0–3 h after fertilization), 8 cells (B, 8–12 h after fertilization), morula (C, 2–3 days after fertilization), gastrula (D, 4 days after fertilization) and larvae (E, 7 days and F, 12 days after fertilization). Legend: a: anus; as: anterior sac; bc: blastocoel; bp: blastopore; e: eye-spot of the anterior tentacles; f: foot; m: mouth; me: mantle edge; ps: pedal sinus; sh: shell.

were still alive, but did not hatch at the end of the metamorphosis period; their eggshell remained white and thick instead of becoming thin and transparent to allow the snail to hatch. Malformations were observed, e.g. at 6 mg Cd/l (Fig. 6D): the cephalic region was not individualized and the eye-spots were not visible. An abnormal vesicle was visible and the pedal sinus was still present due to a late invagination whereas, for controls at 12 days, it was strongly reduced (Fig. 5F). At 8 and 10 mg Cd/l, stages ranged from one cell to larvae but they were small and malformed and never hatched.

4. Discussion

4.1. Herbicide and mixture toxicity

Diquat and glyphosate and their formulations or their associated adjuvants were toxic to snail embryos at lower concentrations than the recommended application concentrations for agriculture.

In comparison with aquatic organisms, which are exposed by complete immersion in contaminated solutions, snail embryos are relatively sensitive. For instance, $LC50_{a.i.}$ values for frog embryos

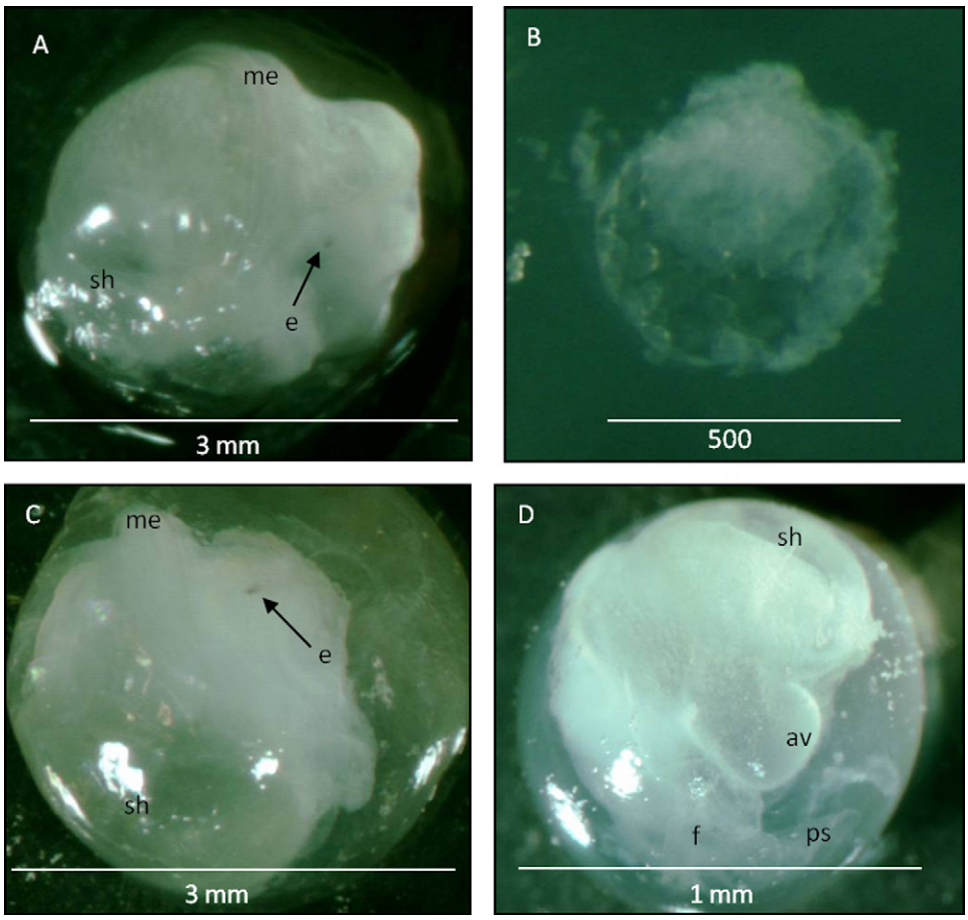

Fig. 6. Non-hatched embryos of eggs after 14 days exposure to Reglone® (A, 1.5 mg a.i./l) and Cd (B and C, 4 mg/l; D, 6 mg/l). Legend: av: abnormal vesicle; e: eye-spot; f: foot; me: mantle edge; ps: pedal sinus; sh: shell.

[12] or juvenile silver catfish [27] were respectively 9.3 mg/l and 7.3 mg/l Roundup® whereas in this study, the $EC50_{a.i.}$ was 18 mg/l. For the Reglone®, diquat disturbed the growth of *Lymnaea stagnalis* [14] (from 0.22 mg/l) whereas we found an EC50-hatchability of *H. aspersa* of 0.72 mg/l. Glyphosate has been shown to be toxic to animals, e.g. by causing oxidative stress and increasing the activity of an antioxidant enzyme, superoxide dismutase, as demonstrated in the aquatic worm, *Lumbricus variegatus* [28] and it has been proved that Roundup can affect acetylcholinesterase activity in fish [29,30].

At equal concentrations of glyphosate, Roundup® was more toxic than glyphosate alone. Two mechanisms could be evoked: a higher toxicity of glyphosate in the Roundup® due to potentialisation of its effects by adjuvants and/or the toxicity of the adjuvants. A study on an aquatic oligochaete showed that it accumulated more glyphosate when it was exposed to Roundup® Ultra than to glyphosate alone [28]. The authors hypothesized that the surfactant polyoxyethylene amine (POEA, also called MON 818 [13,31]) contained in Roundup®, improved the transfer of glyphosate, by interacting with the plasma membrane. Another hypothesis is that the POEA is in fact the compound mainly responsible for the toxicity of Roundup® [32,33] and could even be more toxic than the Roundup® itself [12,13,17,31]. Tsui and Chu [16] also determined that POEA accounted for more than 86% of Roundup® toxicity for bacteria, protozoa and crustaceans. So, in contrast with a study which reported no difference of toxicity between glyphosate and Roundup® on several stages of a nematomorph worm [34], the present data confirm, on snail embryos, the higher toxicity of Roundup® compared to its a.i.

The results for the mixture Reglone®/Agral®, revealed an antagonistic effect between the two products on the embryonic development of the snail with a major contribution of Reglone® (99.45%). This is in agreement with Coutellec et al. [14] who found that Reglone® had an effect on the embryogenesis of *L. stagnalis* but this effect was decreased in the mixture Reglone®–Agral®, suggesting an antagonistic interaction.

Whatever the mixture considered (Roundup® with its integrated adjuvant or Reglone® with a recommended adjuvant such as Agral®), it appears necessary to assess the risk of the final product (which will be applied to crops) and not only of the active ingredient individually. More data on the ecotoxicity are also needed for adjuvant, for which ecotoxicological information is scarce or absent from databases.

4.2. Effect of Cd and transfer to eggs

Our study showed that Cd was toxic to terrestrial snail eggs. Toxicity is lower than for aquatic snails: for example no hatching were observed at 0.02 mg/l for *Stagnicola elodes* [35], 0.1 mg/l for *Biomphalaria glabrata* [36] and 0.4 mg/l for *L. stagnalis* [7] versus 8 mg/l for *H. aspersa*, but is higher than for zebrafish embryos where the 48h-LC50 is 30.1 mg/l [21] versus 3.94 mg/l for *H. aspersa*. After 7 days of exposure at the concentration that caused 50% embryo mortality, Cd in the albumen reached 2.66 μg/g fresh mass. Brasfield et al. [20] also showed strong uptake of Cd by lizard embryos. Our results indicate that uptake increased with exposure concentrations, as also observed for zebrafish eggs by Burnison et al. [37]. Dry mass concentrations were higher in the albumen than in the

eggshell whereas, for zebrafish eggs, concentrations were higher in the chorion [37], that is the external membrane of eggs. This difference of permeability and adsorption capacities of these external membranes (eggshell and chorion) may be the result of different composition and structure between snail and zebrafish and also of the exposure route to contaminants in their environment.

4.3. Disruption of embryogenesis

The effects of Agral may be attributed to nonylphenol (NP) which is its main component (according to its Material Security Data Sheet). NP was plainly recognized as an endocrine disrupter and particularly for estrogenic effects [38]. Several studies found that NP has an effect on embryogenesis of aquatic organisms, like the microcrustacean *Daphnia magna*, where development of embryos stopped at a high exposure concentration (EC50 = 738 μg/l, [39]). Concerning studies on aquatic snails, Oliveira-Filho et al. [40] found a LC50 for *Biomphalaria tenagophila* embryos between 29.25 and 640.25 mg/l following exposure duration and Lalah et al. [41] showed that the branched NP isomer (4(3′,6′-dimethyl-3′-heptyl)-phenol at an average concentration of 0.105 mg/l) slowed the embryonic growth and reduce hatching success of *L. Stagnalis*; both authors suspected that this low observed effect on embryos is probably due to a reduced penetration of NP in the lengthwise encapsulating jelly strand that surrounds the eggs [40,41]. NP also induces concentration-dependent malformations of sea urchin embryos [42] and these authors find, as we do, that embryos are blocked at early stage.

Effects of glyphosate appeared later in the embryonic development just before hatching. Disturbance of hatching in relation with a hatching enzyme has been evidenced in urchin embryos [17]: Roundup® inhibited the global transcription of DNA after fertilization at the 16-cell stage, including transcription of the hatching enzyme which is normally secreted at blastula stage and allows the embryo to hatch by digesting its fertilization envelope.

The time of appearance of the effects of diquat on embryos is concentration-dependent. Indeed, the higher the concentration, the earlier the development of the embryo is blocked.

Cd effect also is concentration-dependent. Several authors demonstrated a similar effect for aquatic mollusc embryos [7,35]. But we also observed that the stage of blocking also varied for a given concentration. Other authors have shown that the effect of Cd is "stage-dependent", suggesting that the earlier the embryos are exposed, the greater the effect [17,43]. As embryos of a single clutch were not at exactly the same stage when the exposure to a chemical started (due to the duration of egg-laying which varied from 24 to 32 h), this could explain the variations in the effects of exposure to a given concentration.

Cd can interact with calcium (Ca) by competition for uptake sites [44]. Ca is forwarded from adults to eggs in the eggshell and is then used to build the shell of the juvenile snails [45]. Thus, due to possible competition with Cd, Ca metabolism in the embryos could be affected, making the eggshell remain thick, preventing the snail from hatching.

4.4. Utility and limits of an embryotoxicity bioassay with snail eggs

The bioassay we presented here to study the embryotoxicity of various chemicals on snail eggs proved to be relevant for some pesticides and a metal, and is easy to perform with simple and cost effective equipment. The biological material can easily be obtained, and, as one clutch contains from 70 to 150 eggs, the quantities available for testing do not constitute a restraint.

One limitation of this test, which also exists with most bioassays, involves the toxicity parameters themselves. NOEC and EC50 values are determined using different statistical methods, which explain that, for several of our tests, NOEC was higher than EC50. The concept of NOEC has often been criticized because the value depends strongly on the dataset and the chosen concentrations [46,47]. However, as this parameter still serves as a reference for risk assessment, we chose to use both NOEC and EC50 values. This limitation also depends on the variability of the response, which could be high (e.g. for NPEO, Fig. 3d), due to the stage of development of the eggs at the beginning of exposure, as discussed previously for Cd (see Section 4.3).

Embryotoxicity bioassays proved the advantage of this early stage of the life cycle, as shown with urchins [48] or oyster embryos [8]. Indeed, it is generally recognised that early stages of fish, and notably zebrafish, offer practicable and highly sensitive bioassays [9,49,50]. Only a study on a freshwater snail (*B. tenagophila*) found that embryos are less sensitive than juveniles or adults [40]. However, all these examples concern the aquatic environment because terrestrial bioassays with soil invertebrates are rare: we found only one experiment on a pest slug, reporting the high sensitivity of the embryos to metal salts [51]. Methods on this part of the life cycle would complete existing bioassays for the assessment of chemicals on snail survival, growth and reproduction [2,11,52] and, more generally, terrestrial bioassays used for risk assessment, such as tests on nematodes [5], earthworms [53], honeybee larvae [54,55] or birds [56].

Until they become free-feeding larvae, embryos are considered like *in vitro* systems [57]. Thus, a land snail embryotoxicity bioassay could be considered as an *in vitro* test which therefore responds to current expectations, notably to alternative testing methods [58]. The present results and methodology enlarge the relatively restricted panel of available tests for assessing the embryotoxicity of chemicals towards terrestrial invertebrates.

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References

[1] ISO Website, www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_tc_browse.htm?commid=54366&published=on, 2010.
[2] ISO 15952, Effects of pollutants on juvenile land snails (Helicidae), Determination of the effects on growth by soil contamination, International Organization for Standardization, Geneva, Switzerland, 2006.
[3] M.C.K. Leung, P.L. Williams, A. Benedetto, C. Au, K.J. Helmcke, M. Aschner, J.N. Meyer, *Caenorhabditis elegans*: an emerging model in biomedical and environmental toxicology, Toxicol. Sci. 106 (2008) 5–28.
[4] J.-Y. Roh, Y.-K. Park, K. Park, J. Choi, Ecotoxicological investigation of CeO2 and TiO2 nanoparticles on the soil nematode *Caenorhabditis elegans* using gene expression, growth, fertility, and survival as endpoints, Environ. Toxicol. Pharmacol. 29 (2010) 167–172.
[5] ISO 10872, Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda), 2010.
[6] R.E. Ricklefs, G.L. Miller, Ecology, fourth ed., W.H. Freeman, 1999.
[7] A. Gomot, Toxic effects of cadmium on reproduction, development, and hatching in the freshwater snail *Lymnaea stagnalis* for water quality monitoring, Ecotoxicol. Environ. Saf. 41 (1998) 288–297.
[8] O. Geffard, H. Budzinski, E. His, The effects of elutriates from PAH and heavy metal polluted sediments on *Crassostrea gigas* (Thunberg) embryogenesis, larval growth and bio-accumulation by the larvae of pollutants from sedimentary origin, Ecotoxicology 11 (2002) 403–416.
[9] M. Strmac, A. Oberemm, T. Braunbeck, Effects of sediment eluates and extracts from differently polluted small rivers on zebrafish embryos and larvae, J. Fish Biol. 60 (2002) 24–38.
[10] M. Schirling, A. Bohlen, R. Triebskorn, H.R. Köhler, An invertebrate embryo test with the apple snail *Marisa cornuarietis* to assess effects of potential developmental and endocrine disruptors, Chemosphere 64 (2006) 1730–1738.
[11] F. Gimbert, A. de Vaufleury, F. Douay, M. Coeurdassier, R. Scheifler, P.M. Badot, Long-term responses of snails exposed to cadmium-contaminated soils

in a partial life-cycle experiment, Ecotoxicol. Environ. Saf. 70 (2008) 138–146.
[12] P.J. Perkins, H.J. Boermans, G.R. Stephenson, Toxicity of glyphosate and triclopyr using the frog embryo teratogenesis assay—*Xenopus*, Environ. Toxicol. Chem. 19 (2000) 940–945.
[13] R.B. Bringolf, W.G. Cope, S. Mosher, M.C. Barnhart, D. Shea, Acute and chronic toxicity of glyphosate compounds to glochidia and juveniles of *Lampsilis siliquoidea* (Unionidae), Environ. Toxicol. Chem. 26 (2007) 2094–2100.
[14] M.A. Coutellec, G. Delous, J.P. Cravedi, L. Lagadic, Effects of the mixture of diquat and a nonylphenol polyethoxylate adjuvant on fecundity and progeny early performances of the pond snail *Lymnaea stagnalis* in laboratory bioassays and microcosms, Chemosphere 73 (2008) 326–336.
[15] EPA Website, http://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=200005X7.txt, 2010.
[16] M.T.K. Tsui, L.M. Chu, Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors, Chemosphere 52 (2003) 1189–1197.
[17] J. Marc, M. Le Breton, P. Cormier, J. Morales, R. Belle, O. Mulner-Lorillon, A glyphosate-based pesticide impinges on transcription, Toxicol. Appl. Pharmacol. 203 (2005) 1–8.
[18] S. Richard, S. Moslemi, H. Sipahutar, N. Benachour, G.E. Seralini, Differential effects of glyphosate and roundup on human placental cells and aromatase, Environ. Health Perspect. 113 (2005) 716–720.
[19] L.M. Elandalloussi, R.B. Leite, P.M. Rodrigues, R. Afonso, M.L. Cancela, Effect of the herbicide Roundup® on *Perkinsus olseni* in vitro proliferation and in vivo survival when infecting a permissive host, the clam *Ruditapes decussatus*, Bull. Environ. Contam. Toxicol. 80 (2008) 512–515.
[20] S.M. Brasfield, K. Bradham, J.B. Wells, L.G. Talent, R.P. Lanno, D.M. Janz, Development of a terrestrial vertebrate model for assessing bioavailability of cadmium in the fence lizard (*Sceloporus undulatus*) and in ovo effects on hatchling size and thyroid function, Chemosphere 54 (2004) 1643–1651.
[21] A.V. Hallare, M. Schirling, T. Luckenbach, H.-R. Köhler, R. Triebskorn, Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos, J. Therm. Biol. 30 (2005) 7–17.
[22] H. Fol, Studies on the molluscan development. Third memoir: on the development of pulmonate gastropods, Arch. Zool. Exp. Gen. 8 (1879–1880) 102–232.
[23] K. Syberg, A. Elleby, H. Pedersen, N. Cedergreen, V.E. Forbes, Mixture toxicity of three toxicants with similar and dissimilar modes of action to *Daphnia magna*, Ecotoxicol. Environ. Saf. 69 (2008) 428–436.
[24] O. Adam, P.M. Badot, F. Degiorgi, G. Crini, Mixture toxicity assessment of wood preservative pesticides in the freshwater amphipod *Gammarus pulex* (L.), Ecotoxicol. Environ. Saf. 72 (2009) 441–449.
[25] S. Loewe, H. Muischnek, Effect of combinations: mathematical basis of the problem, Arch. Exp. Pathol. Pharmacol. 114 (1926) 313–326.
[26] M. Faust, R. Altenburger, T. Backhaus, H. Blanck, W. Boedeker, P. Gramatica, V. Hamer, M. Scholze, M. Vighi, L.H. Grimme, Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants, Aquat. Toxicol. 56 (2001) 13–32.
[27] L.C. Kreutz, L.J.G. Barcellos, T.O. Silva, D. Anziliero, D. Martins, M. Lorenson, A. Marteninghe, L.B. Da Silva, Acute toxicity test of agricultural pesticides on silver catfish (*Rhamdia quelen*) fingerlings, Cienc. Rural 38 (2008) 1050–1055.
[28] V. Contardo-Jara, E. Klingelmann, C. Wiegand, Bioaccumulation of glyphosate and its formulation Roundup Ultra in *Lumbriculus variegatus* and its effects on biotransformation and antioxidant enzymes, Environ. Pollut. 157 (2009) 57–63.
[29] J. Salbego, A. Pretto, C.R. Gioda, C.C. de Menezes, R. Lazzari, J. Radünz Neto, B. Baldisserotto, V.L. Loro, Herbicide formulation with glyphosate affects growth, acetylcholinesterase activity, and metabolic and hematological parameters in piava (*Leporinus obtusidens*), Arch. Environ. Contam. Toxicol. (2010) 1–6.
[30] K.A. Modesto, C.B.R. Martinez, Roundup® causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*, Chemosphere 78 (2010) 294–299.
[31] L.C. Folmar, H.O. Sanders, A.M. Julin, Toxicity of the herbicide glyphosate and several of its formulations to fish and aquatic invertebrates, Arch. Environ. Contam. Toxicol. 8 (1979) 269–278.
[32] R.M. Mann, J.R. Bidwell, The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs, Arch. Environ. Contam. Toxicol. 36 (1999) 193–199.
[33] D.W. Sparling, C. Matson, J. Bickham, P. Doelling-Brown, Toxicity of glyphosate as Glypro® and LI700 to red-eared slider (*Trachemys scripta elegans*) embryos and early hatchlings, Environ. Toxicol. Chem. 25 (2006) 2768–2774.
[34] C.L. Achiorno, C. de Villalobos, L. Ferrari, Toxicity of the herbicide glyphosate to *Chordodes nobilii* (Gordiida, Nematomorpha), Chemosphere 71 (2008) 1816–1822.
[35] M. Pietrock, T. Meinelt, D.J. Marcogliese, Effects of cadmium exposure on embryogenesis of *Stagnicola elodes* (Mollusca, Gastropoda): potential consequences for parasite transmission, Arch. Environ. Contam. Toxicol. 55 (2008) 43–48.
[36] M. Ansaldo, D.E. Nahabedian, C. Di Fonzo, E.A. Wider, Effect of cadmium, lead and arsenic on the oviposition, hatching and embryonic survival of *Biomphalaria glabrata*, Sci. Total Environ. 407 (2009) 1923–1928.
[37] B.K. Burnison, T. Meinelt, R. Playle, M. Pietrock, A. Wienke, C.E. Steinberg, Cadmium accumulation in zebrafish (*Danio rerio*) eggs is modulated by dissolved organic matter (DOM), Aquat. Toxicol. 79 (2006) 185–191.
[38] A. Soares, B. Guieysse, B. Jefferson, E. Cartmell, J.N. Lester, Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters, Environ. Int. 34 (2008) 1033–1049.
[39] L. Zhang, R. Gibble, K.N. Baer, The effects of 4-nonylphenol and ethanol on acute toxicity, embryo development, and reproduction in *Daphnia magna*, Ecotoxicol. Environ. Saf. 55 (2003) 330–337.
[40] E.C. Oliveira-Filho, B.R. Geraldino, C.K. Grisolia, F.J. Paumgartten, Acute toxicity of endosulfan, nonylphenol ethoxylate, and ethanol to different life stages of the freshwater snail *Biomphalaria tenagophila* (Orbigny, 1835), Bull. Environ. Contam. Toxicol. 75 (2005) 1185–1190.
[41] J.O. Lalah, G.F. Severin, K.W. Schramm, D. Lenoir, A. Behechti, K. Guenther, A. Kettrup, Effects of a branched p-nonylphenol isomer (4(3′,6′-dimethyl-3′-heptyl)-phenol) on embryogenesis in *Lymnae stagnalis* L, Arch. Environ. Contam. Toxicol. 52 (2007) 104–112.
[42] O.C. Arslan, H. Parlak, R. Oral, S. Katalay, The effects of nonylphenol and octylphenol on embryonic development of sea urchin (*Paracentrotus lividus*), Arch. Environ. Contam. Toxicol. 53 (2007) 214–219.
[43] R. Pennati, S. Groppelli, G. Zega, M. Biggiogero, F. De Bernardi, C. Sotgia, Toxic effects of two pesticides, Imazalil and Triadimefon, on the early development of the ascidian *Phallusia mammillata* (Chordata, Ascidiacea), Aquat. Toxicol. 79 (2006) 205–212.
[44] A. Craig, L. Hare, A. Tessier, Experimental evidence for cadmium uptake via calcium channels in the aquatic insect *Chironomus staegeri*, Aquat. Toxicol. 44 (1999) 255–262.
[45] A. Beeby, L. Richmond, Variation in the mineral composition of eggs of the snail, *Helix aspersa* between populations exposed to different levels of metal contamination, Environ. Pollut. 101 (1998) 25–31.
[46] M. Crane, M.C. Newman, What level of effect is a no observed effect? Environ. Toxicol. Chem. 19 (2000) 516–519.
[47] J. Baas, T. Jager, S.A.L.M. Kooijman, A model to analyze effects of complex mixtures on survival, Ecotoxicol. Environ. Saf. 72 (2009) 669–676.
[48] J. Bellas, A. Granmo, R. Beiras, Embryotoxicity of the antifouling biocide zinc pyrithione to sea urchin (*Paracentrotus lividus*) and mussel (*Mytilus edulis*), Mar. Pollut. Bull. 50 (2005) 1382–1385.
[49] T. Braunbeck, M. Böttcher, H. Hollert, T. Kosmehl, E. Lammer, E. Leist, M. Rudolf, N. Seitz, Towards an alternative for the acute fish LC50 test in chemical assessment: the fish embryo toxicity test goes multi-species—an update, Altex 22 (2005) 87–102.
[50] A.V. Hallare, T. Kosmehl, T. Schulze, H. Hollert, H.-R. Köhler, R. Triebskorn, Assessing contamination levels of Laguna Lake sediments (Philippines) using a contact assay with zebrafish (*Danio rerio*) embryos, Sci. Total Environ. 347 (2005) 254–271.
[51] J. Iglesias, J. Castillejo, R. Parama, R. Mascato, M.J. Lombardia, Susceptibility of the eggs of the pest slug *Deroceras reticulatum* to contact with metal salts, J. Molluscan Stud. 66 (2000) 171–176.
[52] A. de Vaufleury, M. Coeurdassier, P. Pandard, R. Scheifler, C. Lovy, N. Crini, P.M. Badot, How terrestrial snails can be used in risk assessment of soils, Environ. Toxicol. Chem. 25 (2006) 797–806.
[53] ISO 11268-2, Effects of pollutants on earthworms (*Eisenia fetida*) Part 2: determination of effects on reproduction, International Organization for Standardization, Geneva, Switzerland, 1998.
[54] P. Aupinel, D. Fortini, H. Dufour, J.-N. Tasei, B. Michaud, J.-F. Odoux, M.-H. Pham-Delègue, Improvement of artificial feeding in a standard in vitro method for rearing *Apis mellifera* larvae, Bull. Insectol. 58 (2005) 107–111.
[55] P. Aupinel, P. Medrzycki, D. Fortini, B. Michaud, J.-N. Tasei, J.-F. Odoux, A new larval in vitro rearing method to test effects of pesticides on honey bee brood, Redia XC (2007) 91–94.
[56] EEC, COUNCIL DIRECTIVE of 15 July 1991 concerning the placing of plant protection products on the market. 91/414/EEC, Official Journal of the European Union L230 (1991) 1–154.
[57] A.V. Weisbrod, J. Sahi, H. Segner, M.O. James, J. Nichols, I. Schultz, S. Erhardt, C. Cowan-Ellsberry, M. Bonnell, B. Hoeger, The state of in vitro science for use in bioaccumulation assessments for fish, Environ. Toxicol. Chem. 28 (2008) 86–96.
[58] EC, Regulation No. 1907/2006 of The European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No. 793/93 and Commission Regulation (EC) No. 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC, Official Journal of the European Union 49 (2006) 1–849.