Embryogénotoxicité des contaminants chez l'escargot

Ce chapitre est composé de 2 publications scientifiques rédigées en anglais dont une acceptée et une en préparation.

III.2.1. Coupling of Random Amplified Polymorphic DNA Profiles Analysis and High Resolution Capillary Electrophoresis System for the Assessment of Chemical Genotoxicity

Cette partie a fait l'objet d'une publication acceptée en Juillet 2013 dans la revue *Environmental Science and Technology*. Elle porte sur la mise en place de la méthode RAPD sur le modèle d'étude retenu pour cette thèse et le couplage de cette technique avec un système d'électrophorèse capillaire haute résolution. Les auteurs sont Pierre-Emmanuel Baurand, Annette de Vaufleury, Renaud Scheifler et Nicolas Capelli.

Résumé

La technique Random Amplified Polymorphic DNA (RAPD) est dérivée de la Polymerase Chain Reaction (PCR). Elle a le potentiel de détecter un large éventail de dommages à l'ADN. Initialement, cette méthode a été utilisée pour détecter le polymorphisme lors d'études phylogénétiques et plus récemment lors d'études de génotoxicité. Son principe repose sur l'analyse par électrophorèse des profils correspondant aux différents fragments obtenus après amplification aléatoire par PCR d'ADN génomique. Cependant la phase de lecture ces profils reste perfectible car elle est souvent dépendante de l'observateur ou encore de la qualité des images analysées. Le but de notre étude est de proposer un nouvel outil couplant la technique RAPD-PCR et un système d'électrophorèse capillaire haute résolution (SHR) afin d'améliorer les étapes de séparation des produits RAPD et d'analyse de ces profils. Afin de valider notre méthode, nous avons choisi d'évaluer le potentiel génotoxique du cadmium chez des embryons d'escargot terrestre *Helix aspersa* (syn. *Cantareus aspersus*) Des œufs d'escargot ont été exposés à des solutions de Cd (2, 4 et 6 mg/L) au début de leur développement embryonnaire. Par comparaison avec la méthode d'électrophorèse classique sur gel d'agarose, le SHR permet d'améliorer la qualité des images et la résolution des profils obtenus, de diminuer le temps de migration des échantillons. De plus, après paramétrage du logiciel intégré au SHR, l'analyse des profils peut être automatisée et ainsi permettre de

réduire la subjectivité existante lors de la lecture des profils avec la méthode classique. L'analyse des produits RAPD via SHR a montré des différences dans les profils d'amplicons d'embryons exposés et non-exposés à partir de 2 mg/L de Cd. Ces modifications au niveau génomique révèlent un effet génotoxique du Cd sur les stades embryonnaires de l'escargot. Notre étude démontre l'intérêt du couplage RAPD-SHR qui semble un outil prometteur pour un dépistage haut débit rapide et plus efficace des effets des contaminants chimiques sur l'ADN.

Mots clés : *Helix aspersa*, escargots, embryotoxicité, cadmium

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Coupling of Random Amplified Polymorphic DNA Profiles Analysis and High Resolution Capillary Electrophoresis System for the Assessment of Chemical Genotoxicity

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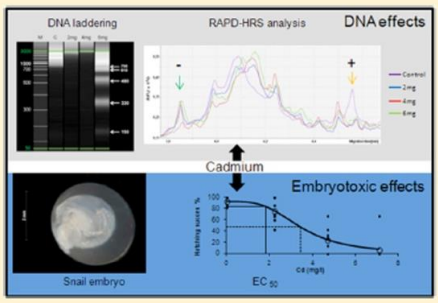
Supporting Information

ABSTRACT: Cadmium (Cd) can be toxic to terrestrial snails, but few data are available about its genotoxic effects on early life stages (ELS). The aim of this study was to investigate the genotoxic potential of Cd in embryos of *Helix aspersa* using a new approach that couples Random Amplified Polymorphic DNA (RAPD) and a high-resolution capillary electrophoresis system (HRS). Clutches of *H. aspersa* were exposed to Cd solutions (2, 4, and 6 mg/L) from the beginning of their embryonic development. In addition to a dose-dependent effect of Cd on hatching rate, DNA fragmentation was observed in embryos that were exposed to 6 mg Cd/L. The analysis of RAPD products with HRS showed differences between the profiles of exposed and nonexposed embryos, starting at 2 mg Cd/L. In comparison to the profiles of the control samples, all profiles from the exposed snails exhibited an additional 270 bp DNA fragment and lacked a 450 bp DNA fragment. These profile modifications are related to the genotoxic effect of Cd on the ELS of *H. aspersa*. Our study demonstrates the efficacy of coupling RAPD and HRS for a rapid and efficient screening of the effects of chemicals on DNA.



III.2.1.1. Introduction

Since embryonic development determines hatching success and, contributes to the dynamic nature of populations, embryotoxicity bioassays have become important tools for environmental hazard risk assessments. During the last 20 years, most of the standardized biological methods have involved aquatic organisms such as zebrafish *Danio rerio* (Hallare et al., 2006 ; Osterauer et al., 2011 ; ISO 12980 ; ISO 15088) or oyster embryos (Geffard et al., 2002 ; XP T90-382). Less data are available concerning the early life stages (ELS) of terrestrial organisms such as soil invertebrates. Among them, landsnails, which are mainly phytophagous and detritivorous organisms living at the soil-plant-air interface, have already been used for ecotoxicological purposes at various life stages. Juvenile or adult *Helix aspersa* (syn. *Cantareus aspersus*) have been used in field studies to assess the bioavailability of metals on contaminated sites (Fritsch et al., 2011) as well as laboratory experiments to study cadmium (Cd) bioaccumulation (Scheifler et al., 2002) and Cd toxic effect on survival, growth, and reproduction (Gomot, 1997 ; Gomot-de Vaufleury and Kerhoas, 2000 ; ISO 15952). Recently, bioassays that use *H. aspersa* eggs to assess the embryotoxicity of various organic (pesticides) or metallic (Cd) contaminants have been developed (Druart et al., 2010, 2012).

Cd is an environmental pollutant, both in terrestrial than aquatic ecosystems (Herber, 2008). This metal is accumulated by many organisms, including the land snail *H. aspersa* (Hispard et al., 2008), and induces various harmful effects. An increased production of reactive oxygen species (ROS) has been reported in the land snail *Theba pisana* collected from urban metal polluted sites showing increased concentrations of Cd, Cu, Pb, and Zn (Radwan et al., 2010). Cd has been recognized as a mutagenic, teratogenic, and embryotoxic agent in the apple snail *Marisa cornuarietis* (Schirling et al., 2006). In addition, some data on Cd toxicity are available for marine organisms. For instance, there are data regarding the ability of Cd to induce the expression of a set of stress proteins (Heat Shock Proteins) in sea urchin embryos *Paracentrotus lividus* (Roccheri et al., 2004). Recently, embryotoxic and genotoxic effects of Cd on the ELS of the Pacific oyster *Crassostrea gigas*, when exposed at environmentally relevant concentrations, have been reported (Mai et al., 2012). Such effects have not been studied in terrestrial molluscs, whose eggs are exposed quite differently than eggs of aquatic invertebrates. Until now, the toxicity of Cd has been demonstrated on the hatching success of *H. aspersa*, and abnormalities or delays in the development of exposed eggs have been described (Druart et al., 2010). However, it is still unknown whether genotoxic effects are involved. Generally, the eventual genotoxicity of chemicals is studied using the comet assay, which allows for the specific detection of DNA breakages, often on

whole nuclei extracted from blood cells. This method has been applied, for example, on coelomocytes of earthworm species (Fourie et al., 2007), on the aquatic mollusc *Littorina littorea* (Noventa et al., 2011) and on embryos of *C. gigas* (Mai et al., 2012) or *M. cornuarietis* (Osterauer et al., 2011), or even on fish embryos such as the *Japanese medaka* (Morin et al., 2011). The advantage of the comet method is that it provides a quantitative approach to genotoxicity at the cellular level. Data obtained using isolated cells from ELS, however, are sometimes criticized (Jha, 2008) because the treatments (e.g., enzymatic, thermic) used for the dissociation of cells can damage DNA material and induce misinterpretations of the comet test results. Another assay, now recognized as one of the most successful to detect genotoxic carcinogens, is the micronucleus test. However, this assay is tricky when working with embryos because isolated cells in division are required to show chromosomal aberrations (Jha, 2004).

For 10 years, the Random Amplified Polymorphic DNA (RAPD) PCR technique has been used to study potential damages in DNA extracts from the whole organism (Atienzar et al., 2000 ; Purohit et al., 2003 ; Rocco et al., 2012). Initially, this method was used to detect polymorphisms in taxonomy and phylogenetic studies (Atienzar and Jha, 2006). RAPD-PCR has the potential to detect a wide range of DNA damages (e.g., DNA adducts, DNA breakage) and mutations (e.g., insertions, rearrangements, deletions). RAPD reactions are performed with a single arbitrary short primer (8-12 nucleotides), and after PCR, the amplified fragments are visualized using agarose gel electrophoresis (De Wolf et al., 2004). One of the main advantages of this method is that no prior knowledge of the genome is necessary (Atienzar and Jha, 2006). This point could be of great importance in experiments with non-model species with little genomic information. RAPD requires only small quantities of DNA and can be considered an efficient method for preliminarily screening populations of genotoxic effects (De Wolf et al., 2004).

RAPD has been used in different studies in ecotoxicology to analyze the genotoxicity of pharmaceutical substances (Rocco et al., 2010, 2011, 2012) or to investigate the effects of Cd and methyl mercury (Cambier et al., 2010, 2012) on the model species zebrafish. Except in plants (Cenkci et al., 2009), examples of using RAPD are rare; as far as we know, this method has never been applied to study the embryogenotoxicity of chemicals in terrestrial invertebrates. For an accurate use of RAPD analysis, an important step is the final visualization of RAPD products after agarose gel electrophoresis and the subsequent comparison of the DNA profiles of controls *versus* exposed organisms (De Wolf et al., 2004). Cambier et al. (2010) developed a method for analyzing profiles using qPCR and a melting curve of RAPD products instead of an agarose gel electrophoresis and band pattern analysis

because the latter approach makes it difficult to interpret the faint differences between control and contaminated DNA at low toxicant doses. However, Cambier et al., (2010) also stated that the resolution between bands in an agarose gel is higher than that in melting curves. The conventional systems currently used for the analysis of profiles are mainly visual and may sometimes be subjective and observer-dependent. Thus, we aimed to improve the RAPD profile analysis by proposing a new method that would give an accurate analysis of RAPD products. For this purpose, a new high-resolution capillary electrophoresis system (HRS) has been used in the present work. This approach involves performing the electrophoresis of samples followed by automatized analyses of product migration. Recent studies have used this HRS in different contexts, e.g., to detect signs of DNA fragmentation in human blood samples (Hughes-Stamm et al., 2011), for epidemiological typing of *Staphylococcus* aureus (McMurray et al., 2010), or even for the identification of parasitoids in agricultural systems (Macfadyen et al., 2009). Only one publication coupling HRS and RAPD for quality control of food is currently available (Wu et al., 2012). To our knowledge, coupling RAPD-PCR and HRS has never been utilized to study the genotoxicity of chemicals.

The aims of our study were to investigate the genotoxic effects of Cd in land snail embryos *H. aspersa* using RAPD-PCR and to evaluate the efficiency of an HRS for RAPD products profile analysis.

III.2.1.2. Materials and Methods

Chemicals

Aqueous solutions of Cd were prepared using solid $CdCl_2$ (99.99%, Sigma Chemical Co., St. Louis, MO; C-2544). All dilutions were prepared with demineralized water (pH 6.2), which was also used as a control solution. The range of nominal exposure concentrations of Cd (0, 2, 4, and 6 mg Cd/L) was chosen to surround the EC_{50} (3.9 mg Cd/L) value found by Druart et al. (2010). The concentrations of Cd were measured using ICP-AES (ICAP 6000 series model radial, Thermo scientific, France). The quality of the results was verified using a certified reference water (Hard Drink Water, ERM- CAO11a, Molsheim, France) Cd-certified at 4.94 µg/L (+/- 0.23 µg/L, average recovery of 93%). The actual verified concentrations were, respectively, 2.2, 4.7, and 7.1 mg Cd/L.

Eggs and exposure device

Eggs were obtained from our standardized laboratory rearing and were exposed using a liquid phase bioassay as previously described (Druart et al., 2010, 2012). Ten clutches were used. Each clutch was separated into groups of 6–9 eggs, which were placed in Petri dishes on

four layers of paper (Quantitative filter paper grade 1 ashless, Whatman) dampened with 0.8 mL of control or Cd solutions. Eggs were exposed rapidly after egg-laying, a maximum of 24 h after fertilization, which corresponds to the 1–8 cell stage (Figure III-9 :A and B). Twenty days after the beginning of the exposure (to avoid omitting late hatchlings), the mean hatching success for each concentration was calculated. The results were considered valid if the hatching success of controls was at least of 70% (the average value observed in our laboratory rearing for controls). Each Petri dish was then kept at -80°C until DNA extractions were performed.

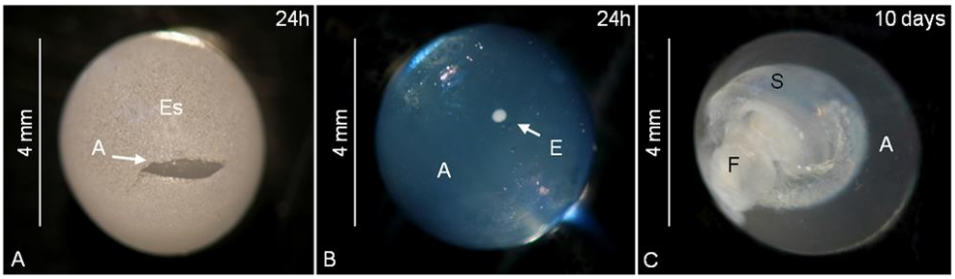


Figure III-9 : Visualization of embryos: eggs with or without eggshell.

(A) Entire snail egg, (B) Egg aged 24 h maximum in albumen (stage: 2 cells), (C) Embryo aged 10 days in albumen. Legend: A: albumen, Es: eggshell, S: shell, F: foot, E: embryo.

DNA extraction

After exposure, DNA isolations were performed on 2 clutches from the 10 used for the Cd exposure. DNA was extracted from eggs at each Cd concentration (i.e., 4 concentrations for 2 clutches and, thus, 8 DNA extracts). To optimize the RAPD technique, the genomic DNA of 5 other unexposed, freshly hatched clutches (which serve as reference samples) was also extracted. Depending on the group (control or exposed to 2, 4, or 6 mg Cd/L), between 10 and 30 mg of biological material was used. Due to low hatching success in the group exposed to 6 mg Cd/L, this material consisted of both hatched embryos and non-hatched eggs (without albumen). Extractions were performed on defrosted embryos using the DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the addition of RNase A (100 mg/mL) in the extraction buffer. DNA samples were kept at -20 °C until RAPD amplifications were performed. The concentrations of the purified genomic DNA were measured at 260 nm, and the purity was estimated by measuring the 260/280 nm absorbance ratio with a Nanodrop Spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Wilmington, USA). DNA concentrations of the 150 µL samples obtained after the elution step were between 20 and 130 ng/µL. The 260/280 nm ratio was between 1.65 and 1.85. The quality of DNA samples was checked on 2% agarose gels stained with

ethidium bromide (EtBr), and a comparison with the HRS using the OM1200 method (with standard parameters) was conducted.

RAPD-PCR procedure

RAPD was performed using the PuRe Taq Ready-To-Go PCR beads kit (GE Healthcare Biosciences, Pittsburgh, PA, USA). For each reaction, we added one bead of the kit (containing dNTPs, BSA and the polymerase AmpliTaqTM), 40 ng of DNA, 5 µL of random primer (5 pmol/µL) and ultra-pure water, for a final volume of 25 µL. The optimal template concentration for the RAPD reaction was determined by testing several amounts of genomic DNA. Then, a screening of 16 decamer primers (Supporting Information Table S1) was performed on the 5 control clutches that were used as references to obtain amplification products with a reasonable number of distinct bands and a good reproducibility. Finally, the RAPD reactions were performed on genomic DNA extracted from samples exposed to various Cd concentrations with the selected primers. Each RAPD reaction was performed twice on each DNA extract. Amplification reactions were performed in a Mastercycler thermal cycler (Eppendorf, Le Pecq, France) as follows: one initial denaturation step of 5 min at 95 °C followed by 45 cycles at 95 °C for 1 min (denaturation), 36 °C for 1 min (annealing), and 72 °C for 2 min (extension). The RAPD reaction products were stored at -20 °C prior to use.

Micro capillary electrophoresis and RAPD profile analysis

RAPD profiles have been analyzed via capillary electrophoresis using a QIAxcel system (http://www.qiagen.com/products/qiaxcelsystem.aspx#Tabs=t1). This system allows an automatic, rapid, and high-resolution migration of the amplification products. The HRS gives a digital gel image and an electropherogram (i.e., a graphic showing the evolution of fluorescence detected during the migration) for fragment analysis. We chose to use the OM500 method (separation sample voltage 5 KV) with a time sample injection of 30 s to obtain a better pattern after migration (clear profile with distinct fragments). The size of the amplified fragments was determined automatically by referring to the fragments obtained with the known size marker QX DNA Size Marker 100 bp-2.5 kbp. Next, the data obtained have been analyzed using the biocalculator software of the system. The QIAxcel software gives the option to adjust 5 parameters. Here, we kept the default values for the positive threshold at 5% (a signal exceeding this value of maximal fluorescence is detected as a peak), the baseline filter at 40 s (determination of the baseline detection), the minimum distance at 0.25 s (the distance for considering 2 peak clusters to be 2 distinct peaks), and the suspend integration at 0.5 min (turns off peak detection for the period selected). To obtain a better

definition of the RAPD products after migration, we tested various smoothing filter values (used to resolved two closed peaks). To allow for a robust analysis of control and exposed sample profiles, the fragments/bands with better reproducibility (present in 100% of cases) along the pattern that was revealed after electrophoresis of the RAPD products in the 5 non-exposed clutches were selected. These selected fragments give the reference profile used for subsequent analysis. The size of these reference fragments (in bp) and their tolerance (in % of bp) were set in the biocalculator software of the HRS. It allows using the binary peak function to automatize fragment detection during profile comparison. The software produces a binary matrix of results, where the presence (1) or absence (0) of the reference bands in the amplification patterns is noted. Digital gel images and electropherograms are used to visually confirm the results given by the binary function. For the detection of new amplified fragments, we first visualized the gel images of RAPD performed in one clutch (1 non-exposed and 3 Cd-exposed samples). Then, we superposed the electropherogram(s) obtained on the electropherogram of the reference profile. If a new fragment is detected after the superposition, parameters of this new peak are entered in the biocalculator software. After reparameterization, the completed/revised reference profile is used to analyze the presence of this new fragment in all exposed samples. To detect the loss of fragments along profiles, each of the RAPD profiles was analyzed after programming the parameters in the binary peak function. The analysis of control *versus* exposed profiles was performed on 4 RAPD profiles for each Cd concentration, i.e., a total of 16 RAPD profiles (2 clutches x 4 Cd concentrations x 2 RAPD profiles for each DNA extract).

Statistical analyses

The null hypothesis of independence between Cd exposure and hatching success was tested using the Kruskal–Wallis rank test. When this hypothesis was rejected, multiple comparisons were performed on ranks using Tukey's Honestly Significant Difference test. All statistics were performed with R (2.13.2) (R Development Core Team, 2004, http://www.R-project.org/). The dose-dependent curves and the EC_{10} and EC_{50} values were determined with the Hill's model using the macro Excel Regtox free version EV6.1.

III.2.1.3. Results

Hatching success

The EC_{10} and EC_{50} values for eggs exposed at the 1–8 cell stage were 1.83 mg/L (Confidence interval (CI) 95%: 1.28–3.76) and 3.47 mg/L (CI 95%: 2.91–4.51) of Cd, respectively. The hatchability of snail eggs exposed rapidly after the end of egg laying is not

significantly reduced at 2.2 mg/L (NOEC) compared to the control. From 4.7 mg/L Cd (LOEC), however, the effect on hatchability is statistically significant.

Genomic DNA analysis

Total DNA isolated from controls and from eggs exposed to 2 and 4 mg/L Cd was of high molecular weight (greater than 23 kpb) and unfragmented (Figure III-10A and B). In contrast, the electrophoresis of DNA extracts reveals an internucleosomal fragmentation (DNA laddering) at 6 mg Cd/L. First, the samples were visualized with conventional agarose gel electrophoresis (Figure III-10A) and with the HRS (Figure III-10B). The HRS-electropherogram provides a detailed picture of the fragmentation and reveals 5 peaks of 150, 330, 480, 610, and 700 bp (Figure III-10B and C) in eggs exposed to 6 mg/L Cd. For the controls, 2 mg Cd/L, and 4 mg Cd/L, no peaks are visible.

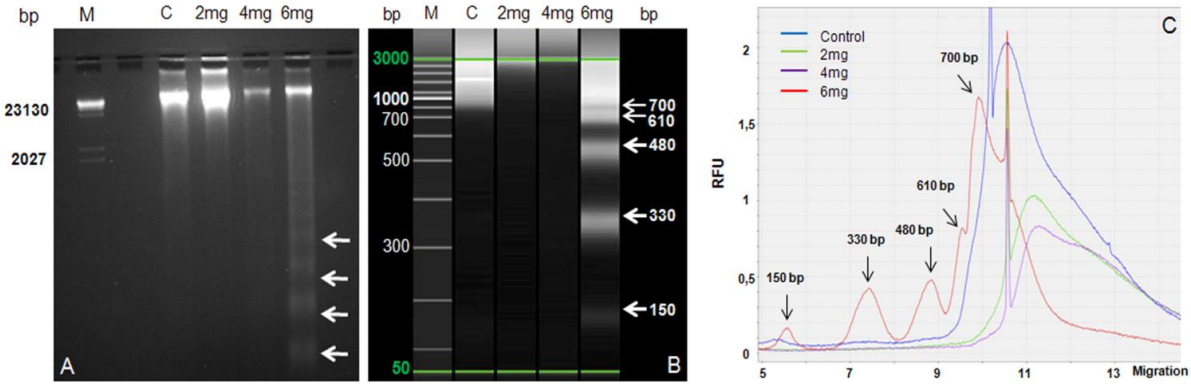


Figure III-10 : The genomic DNA pattern of snails' eggs exposed to Cd (0-24 h after fertilization) after a conventional agarose gel electrophoresis (A) or a High Resolution System (B and C).

C: control, 2 mg, 4 mg and 6 mg: snails exposed to 2, 4, 6 mg Cd/L, respectively. (A) M: Lambda DNA Hind III marker; Genomic bands for C, 2 mg, and 4 mg showed the same size, approximately 23 kbp. (B) M: Marker 100 bp-2.5 kbp; DNA pattern between 50 and 3000 bp obtained using the same samples with the HRS. White arrows show the 5 fragments detected. (C) HRS-electropherograms obtained with HRS. Each curve represents the variation of fluorescence (in RFU: Relative Fluorescence Units) detected by the biocalculator of the HRS as a function of migration time.

RAPD

After testing 3 amounts of DNA template (4, 40, and 400 ng) with the primer Essam-08 (Tableau III-3) in the RAPD reaction, 40 ng was found to be the best quantity to obtain a clear and distinct profile. A comparison of the RAPD products of non-exposed eggs using the classic and HRS methods shows that HRS provides the clearest profile with more distinct bands (Figure III-11).

Tableau III-3 : Sequences of primers used in the RAPD-PCR amplification of *Helix aspersa* embryos' genomic DNA.***** Kit RTG; [A] Armbruster et al. 2007; [B] Gutierrez et al. 2003; [C] Bin Dajem et al. 2011.

Primers	Sequence	Primers	Sequence
P1 *****	5' GGTGCGGGAA-3'	H19 [A]	5'-CTGACCAGCC-3'
P2 *	5'-GTTTCGCTCC-3'	X19 [A]	5'-CCACCGCCAG-3'
P3 *	5'-GTAGACCCGT-3'	OPA-02 [B]	5'-TGCCGAGCTG-3'
P4 *	5'-AAGAGCCCGT-3'	OPB-08 [B]	5'-GTCCACACGG-3'
P5 *	5'-AACGCGCAAC-3'	OPG-05 [B]	5'-CTGAGACGGA-3'
P6 *	5'-CCCGTCAGCA-3'	OPG17 [B]	5'-ACGACCGACA-3'
H18 [A]	5'-GAATCGGCCA-3'	Saad-2 [C]	5'-GTGCTACGTC-3'
Osama-5 [C]	5'-GACCATCGTC-3'	Essam-08 [C]	5'-GCTCGAACTT-3'

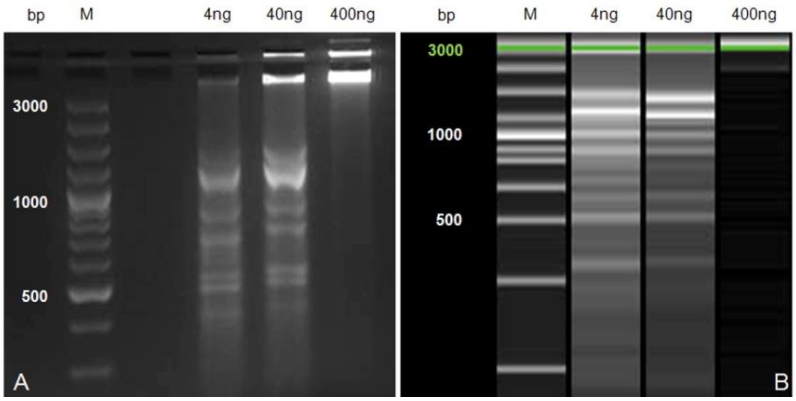


Figure III-11 : Electrophoresis pattern of RAPD products obtained using the Essam-08 primer of DNA of unexposed *Helix aspersa* eggs.

Comparison of the conventional gel electrophoresis method (A) and HRS (B). M: Size marker in bp (100 bp Plus DNA Ladder); 4, 40 and 400 ng: quantities of template tested.

Selection of HRS analysis parameters and analysis of reference profiles

The smoothing filter value retained was 0 point, as it provides better definition. The default values for the other parameters provided the best compromise to detect a reasonable number of peaks in all samples and to minimize the background noise. These values were the same for all comparisons performed with the HRS.

Two of the 16 primers tested were selected to validate the feasibility of the method in our experimental conditions: OPG17 and X19 (Tableau III-3). These 2 primers provided amplification products with a strong reproducibility among DNA of the 5 non-exposed clutches tested. For amplification with OPG17 (Figure III-12A and C), the pattern obtained showed 4 reproducible fragments from 300 to 500 bp, whereas the X19 pattern showed 5 fragments from 550 bp to 1000 bp (Figure III-12B and D). For the primer OPG17, the 4 fragments were called B1 to B4 and were selected to automatize the profile analysis (Figure

III-12C). For the primer X19, the 5 fragments were called B1 to B5 and were selected for the automatized profile analysis (Figure III-12D). Next, the binary peak function parameters were memorized in the software. For each peak obtained, the mean size and tolerance in % were entered. For the peaks of the OPG17 primer, the tolerance was 2% for the 300, 360, and 450 bp fragments and 1.5% for the 320 bp product. For the peaks of the X19 control profiles, the size and tolerance (in brackets) of the B1 to B5 fragments were 544 bp (1.5%), 580 bp (1.8%), 726 bp (2.1%), 852 bp (4.7%), and 1053 bp (2.8%), respectively.

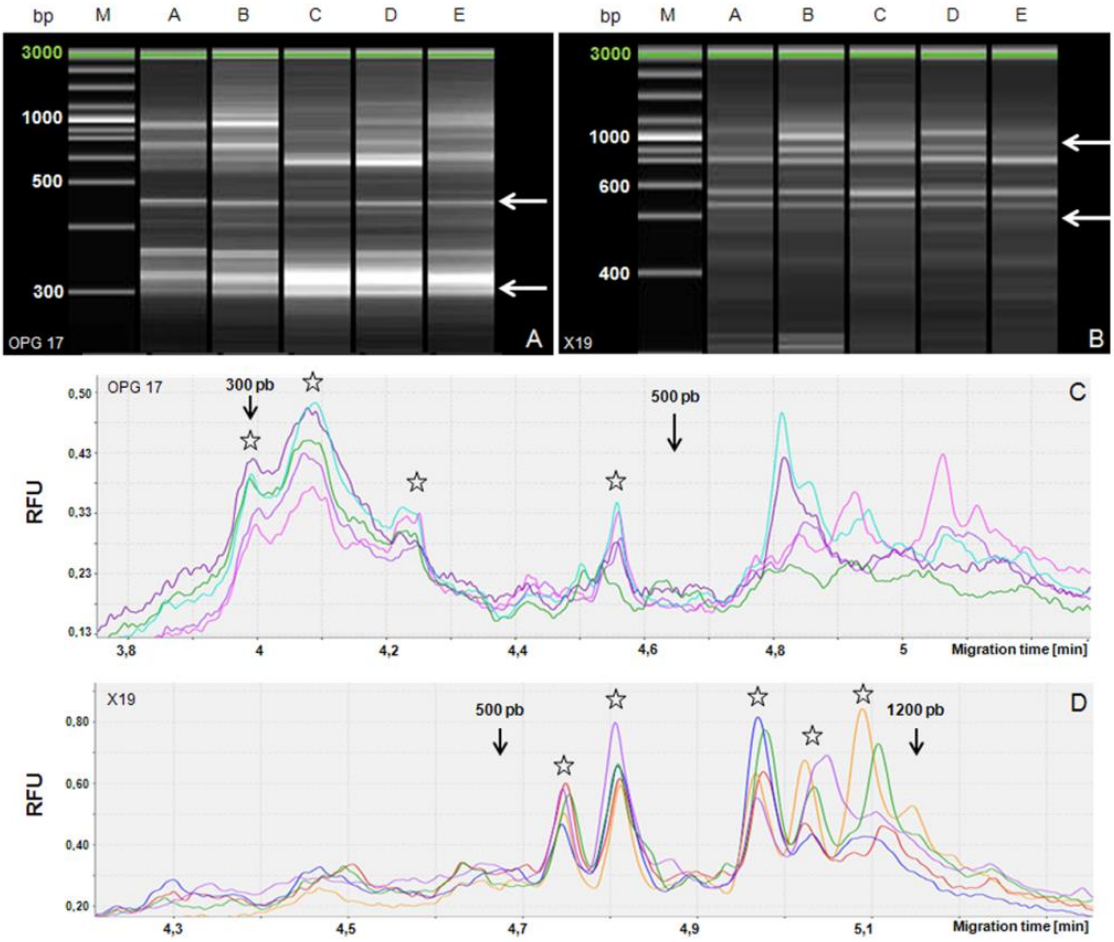


Figure III-12 : Comparison of RAPD profiles and electropherograms obtained with the primer OPG17 (A and C) and with the primer X19 (B and D) used on DNA from 5 controls as a reference (A to E).

M: Marker 100 bp-2.5 kbp. Sizes of fragment are given in base pairs (bp). The white arrows surround the similar pattern obtained. (C) and (D) Each curve represents the variation of fluorescence (in Relative Fluorescence Units) detected by the HRS as a function of migration time. Similar variations with common distinct bands are visible between black arrows. (C) Stars represent the 4 peaks retained for the profile analysis with the primer OPG17 (300, 320, 360 and 450 bp, from left to right). (D) Stars represent the 5 peaks retained for the profile analysis with the primer X19 (approximately 540, 578, 720, 850 and 1050 bp, from left to right).

Comparison of RAPD profiles of control and exposed eggs

The 2 RAPD performed on a same extract demonstrated the reproducibility of analized DNA bands (data not showed). For Cd-exposed samples, the RAPD profiles amplified with OPG17 showed changes in comparison to controls. The 3 selected bands (B1 to B3) were always detected (Figure III-13B, B1 to B3 arrows on the electropherograms). Band 4 was only present in the control profiles (Figure III-13A, box B4) and on the electropherograms (Figure III-13B, peak shown by arrow B4). Band B0 was detected in the 3 Cd-exposed samples, but not in the control sample (Figure III-13A, box B0; Figure III-13B, peak B0). These results were confirmed by the binary matrix of detection (Figure III-13C). For the RAPD profiles obtained with the X19 primer, no changes in the genomic DNA were detected (Figure III-14A, B and C).

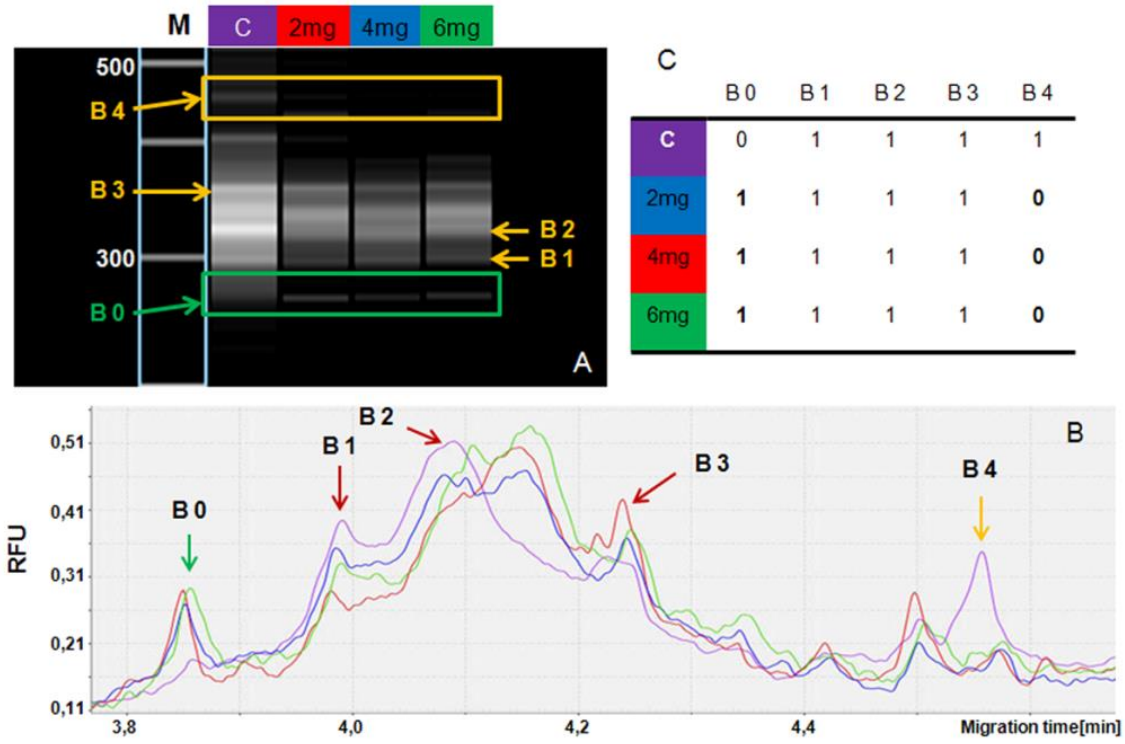


Figure III-13 : Gel image (A), electropherograms (B) and binary matrix (C) of RAPD profiles migration obtained with OPG17.

M: size marker 100 bp-2.5 kbp. C: control; 2, 4, 6 mg: Cd-exposed samples at 2, 4 and 6 mg/L. (A) B1 to B4: bands 1 at 4; B0 band 0.

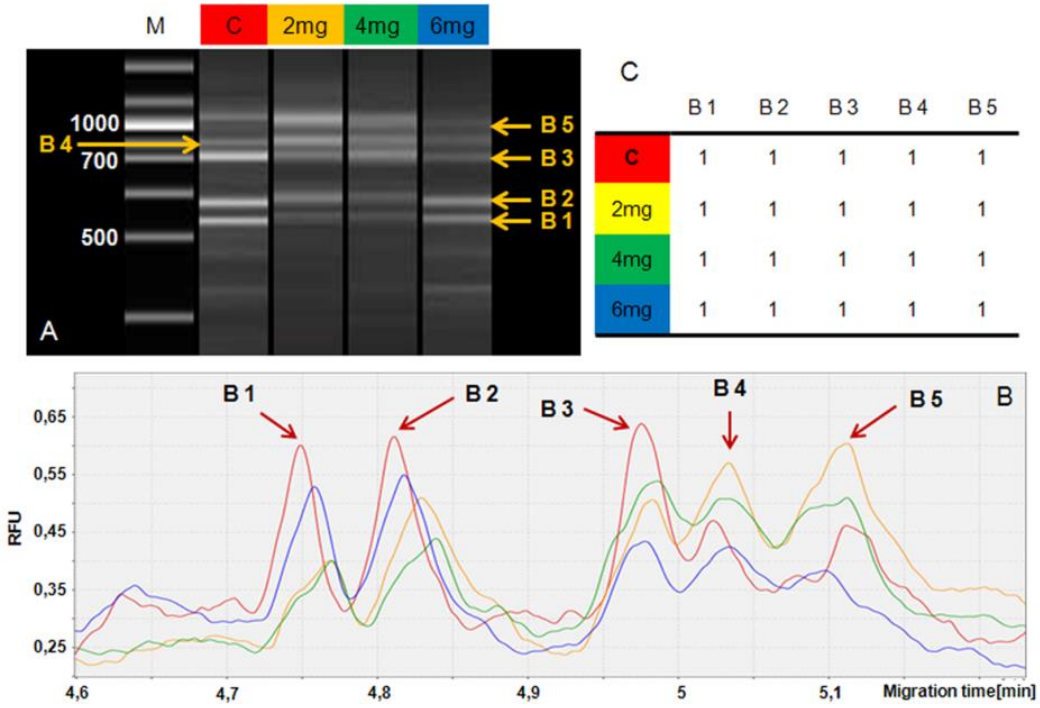


Figure III-14 : RAPD profiles migration obtained with primer X19.

A: Gel image, B: electropherograms, C: binary matrix. M: size marker 100 bp-2.5 kbp. C: control, 2, 4, 6 mg: Cd-exposed samples at 2, 4 and 6 mg/L. (A) and (C): B1 to B5: bands 1 at 5.

III.2.1.4. Discussion

Acute toxicity bioassays using animals are often not well accepted and must respect current animal welfare legislation. Therefore, embryotoxicity testing using eggs that incubate outside of the mother is one of the promising alternatives, at least with fish animal models (Lammer et al., 2009). The fish embryo toxicity assay (FET) using the zebrafish is one of the proposed replacement alternatives to the acute fish test for hazard and risk assessment (Embry et al., 2010). Akagi et al. (2012) confirm that the FET assay emerges as a powerful experimental model in drug discovery and environmental toxicology. For terrestrial organisms, the development and validation of embryotoxicity bioassays are needed to propose such alternative tools. The embryotoxicity test with land snail eggs provided good reproducibility with Cd: indeed the EC_{50} found for the 1–8 cell stage using 10 replicates (3.47 mg Cd/L) is of the same order of magnitude as that found (3.94 mg/L) by Druart et al. (2010). The use of Cd as a known genotoxic agent constitutes a possible positive control to detect the embryo and genotoxic effects of chemicals. Other physical or chemical agents, which are safer for use in routine analysis (e.g., Osterauer et al. (2011) show that UV or H_2O_2 are efficient positive genotoxic agents), could also constitute reference genotoxic stressors but remain to be tested in our conditions. Sublethal effects of Cd on hatchability of the apple snail embryos *M. cornuarietis* were observed at 250 µg/L (Schirling et al., 2006). The lower sensitivity of *H. aspersa* to Cd may result from a specific response and/or the nature of the

exposure, with the eggs of aquatic gastropods being completely and continuously immersed by contaminated liquid, whereas those of terrestrial snails are not. Differences in the composition of the material surrounding the embryos (egg capsules and albumen) may modulate the mobility of Cd from the environment to the embryos and may thus also explain the differences in sensitivity between organisms.

This study is the first one, to our knowledge, that uses various molecular approaches (i.e., an analysis of genomic DNA and RAPD-PCR) to screen the effect of Cd on the ELS of a terrestrial land snail. DNA fragmentation was detected in *H. aspersa* embryos exposed to the highest Cd concentration (6 mg Cd/L). Such elevated concentrations are close to the EC_{50}-hatchability and can be found in industrial effluents, as reported by Lokhande et al. (2012), who found Cd concentrations of up to 28 mg Cd/L in such wastes. The DNA laddering pattern (internucleosomal fragmentation of DNA) observed is characteristic of apoptosis, a type of programmed cell death (PCD). We found that analysis of DNA profiles with HRS provided a more precise determination of the size of ladders/fragments than the conventional agarose gel electrophoresis method (Figure III-10).

The present study is the first to report genotoxic effects based on RAPD-PCR coupled with HRS analysis. RAPD has already been used successfully in different ecotoxicological contexts to demonstrate the genotoxic potential of various physical and chemical stressors: nanoparticles Zn0 and $Ce0_2$ on soybean *Glycine max* (López-Moreno et al., 2010), organic compounds on the zebrafish *D. rerio* (Rocco et al., 2011, 2012 ; Zhiyi and Haowen, 2004), mercury, boron, chromium, and zinc on the common bean *Phaseolus vulgaris* (Cenkci et al., 2009), arsenic on rice *Oryza sativa* (Ahmad et al., 2012), and copper on *Daphnia magna* (Atienzar et al., 2001). In plants, the genotoxicity of Cd has mainly been studied with RAPD in *Arabidopsis thaliana* (Liu et al., 2012), *Hydrilla verticillata*, and *Ceratophyllum demersum* (Gupta and Sarin, 2009). Only a few studies used RAPD-PCR on the ELS of animals: Hagger et al. (2005) have used it on mussel embryos exposed to a range of concentrations (0.37–370 kBq/mL) of tritiated water. Using this method, the genotoxic effect of Cd was studied in the zebrafish (Cambier et al., 2010 ; Orieux et al., 2011), but not in the embryos of other animals. The exposure of *H. aspersa* embryos to Cd confirms the reliability of RAPD-PCR to investigate the genotoxic potential of chemicals but also provides insight into some limits of this technique as discussed below.

RAPD-PCR coupled with HRS allowed for the detection of DNA profile modification in exposed samples, starting at 2 mg Cd/L. Using the OPG17 primer, the disappearance of an amplified fragment of 450 bp (B4) and the presence of new RAPD products (B0) of 270 bp were found in all Cd-exposed samples (2 to 6 mg/L), compared to the controls. Hagger et al.

(2005) underlined the difficulty in deciphering the mechanisms responsible for the changes detected with the RAPD. In our case, modifications of profiles can be related to 3 types of alterations: point mutations, DNA damages (e.g., adducts, breaks), and complex chromosomal rearrangements for the fragment disappearance (Atienzar and Jha, 2006). The appearance of fragments can reveal changes in oligonucleotide priming sites due to point mutations (new annealing events), large deletions (leading to a new annealing site), and/or to homologous recombination/rearrangements (Atienzar and Jha, 2006 ; Ahmad et al., 2012). This method is a qualitative or a semi-quantitative (as RAPD-PCR-HRS does not support quantifying the number of mutations) that provides evidence of genomic modifications (Atienzar and Jha, 2006). The only way to know the exact part of the genome that has been modified would be to sequence the fragment of interest. However, RAPD is useful if the goal is to perform a preliminary screening of the genotoxicity of chemicals. In our experimental conditions, all of the profiles analyzed after RAPD-PCR for the 4 tested modalities (0, 2, 4, and 6 mg/L Cd) presented the same modifications.

In this study, we found modifications of DNA products with only one of the two primers that we used. Usually, several primers are necessary to screen a large part of genomic DNA. For example, by using 6 primers, Zhiyi and Haowen (2004) detected a genotoxic effect of cyclophosphamide and dimethoate on zebrafish. Using 11 primers, Ahmad et al. (2012) showed an increase in the frequency of band loss in RAPD profiles with the increase of As (III) concentrations in rice *Oryza sativa*. To discriminate signs of genotoxicity between the control and exposed samples, the selected primers must give very reproducible profiles. In our study, the primer OPG17 met these two criteria, but the primer X19 did not because it did not enable differentiating between exposed and control profiles. It must also be stressed that if no profile change is detected, this does not mean that no genotoxic effects occurred; that is why the use of several primers is recommended for RAPD studies. On the basis of the present results, it is possible now to enlarge the number of primers to obtained more information on the response of snail eggs (and other organisms) to Cd and other chemicals.

Coupling DNA laddering detection and RAPD analysis gives complementary information. DNA can be fragmented without a detectable mutation. The results obtained with the primer X19 for the Cd-exposed samples illustrate this situation: no signs of genotoxicity were detected by RAPD, whereas the DNA laddering showed a fragmentation at 6 mg Cd/L. This could be explained by the fact that this primer does not match the fragmented DNA, e.g., if the damaged DNA is an AT-rich region. Because RAPD probes generally present a high GC content and allow for mainly screening the CG-rich part of the genome (De Wolf et al., 2004), the DNA fragment will not be recognized by the selected probe. Apoptotic DNA

fragmentation is characterized by endonuclease activation, which cleaves nuclear DNA between nucleosomes, producing a mix of DNA fragments with periodic length, and forms ladders on gel (Gladish et al., 2006). RAPD gives supplementary data on genotoxic effects based on the appearance or disappearance of DNA bands, which can be caused by various DNA modifications.

HRS shows an interesting potential to improve the analysis of DNA profiles with the help of electropherogram superposition and the biocalculator software. After a parameterizing step, this part of the system is able to automatize the profile analysis. The automatization of the detection of peak fluorescence helps reduce the subjectivity of the profile analysis. Indeed, the analysis is conducted with the parameters of detection entered before the start of the migration of the RAPD products from exposed embryos. The detection of new fragments "gained" in the profiles is more difficult because the reference profile does not include the new fragments. For this part of the analysis, an additional supplementary step of reprogramming the reference profile is necessary to include a "0", i.e., absence of a peak in the binary matrix (Figure III-13). This additional step is the only way to gain information about the fragment size and to be able to enter its specificities into the biocalculator software. The conventional analysis of profiles obtained with agarose gel electrophoresis is often based on simple visualization of pictures. However, the lack of precision of RAPD-PCR profiles analysis, which is dependent on the quality of the electrophoretic separation and band staining procedures (De Wolf et al., 2004), remains infrequently discussed in the literature.

Coupling RAPD-PCR and HRS presents several advantages. This association decreases problems that can occur during the migration: for example, the system adjusts non-linear migrations due to the difference in the pH of the analyzed samples. Some recent publications present a final picture with an angle of deviance at the end of the migration between 3 and 5° (Liu et al., 2009 ; Zhou et al., 2011). With the HRS, all of the separated fragments are linear, in comparison to the "U" shape that is often obtained in agarose gel electrophoresis. Recently, Wu et al. (2012) coupled the RAPD with a 2100-bioanalyzer to assess the purity of barley. Like the HRS, the bioanalyzer is able to minimize the electrophoresis conditions that are subject to variation, such as gel concentration, temperature, and buffer composition. They reported that this coupling improved inter-assay reproducibility; however, a step involving loading a maximum of 12 samples and reagents in a chip is necessary before the migration. With the HRS system, 12 to 96 samples can migrate for 12 to 90 minutes with a reduced preparation: the products of amplification can be used for migration directly after RAPD-PCR. Atienzar et al. (2000) highlighted the fact that to render new PCR products visible in conventional agarose gel, a minimum of 10% of mutations may be required at the same locus

in a sufficient number of cells. With HRS, this percentage required for detection may decrease because this new system considerably increases the global quality of the migration (e.g., better resolution and separation, linear migration) and decreases the time needed to obtain a picture of electrophoresis, as we have shown, in comparison with a method using a conventional gel. These improvements can directly influence the interpretation of DNA product profiles, especially the determination of the amplified fragments size. Furthermore, the time-consuming steps involving the preparation, polymerization as well as EtBr staining of the agarose gel are no longer needed. As EtBr is a potentially mutagenic agent that must be handled with extreme caution, the limitation of its use in routine analysis is recommended. HRS requires only low quantities of DNA and low volumes of samples (< 1 µL *versus* 20 µL generally needed for the conventional method) and, thus, considerably increases the amount of remaining sample available for subsequent analyses.

This study is the first to demonstrate that the embryotoxicity of Cd in terrestrial snails *Helix aspersa* is associated with genomic (DNA fragmentation) and genotoxic effects (i.e., DNA mutations causing the gain and/or loss of RAPD fragments). We could speculate that when a mutation reaches a critical level, apoptosis is induced, leading to DNA fragmentation. RAPD-HRS is adapted to indicate DNA alteration after the selection of adapted probes and parameterization of the reading system. The quantification and identification of the type of DNA damages involved constitute limitations of RAPD-HRS. HRS improved the rapidity and the number of samples analyzed as well as the objectivity of the analysis of RAPD products after an accurate calibration of the biocalculator (this step requires some expertise). This innovative method appears promising for a fast and efficient screening of the effects of chemicals on DNA. The *Helix aspersa* embryotoxicity bioassay, combined with RAPD-PCR-HRS, constitutes a new tool that can help in the REACH directive by providing information on the ecotoxic/genotoxic potential of the numerous chemicals for which such data are still not available.

III.2.1.5. Acknowledgments

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III.2.1.6. References

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III.2.2. Genotoxicity assessment of pesticides and Cd on terrestrial snail embryos with Random Amplified Polymorphic DNA

Cette seconde partie du chapitre 1 (présentée sous forme d'un projet de publication rédigé en anglais) s'intéresse à la détection d'effets génotoxiques chez l'embryon d'escargot lors d'expositions continues à 3 pesticides (2 organiques et 1 métallique). Une étude cinétique des potentiels effets génotoxiques d'une exposition de 24 heures au Cd a également été réalisée.

Résumé

Aucune donnée n'est disponible concernant la génotoxicité de formulations commerciales de pesticides (Round Up Flash® (RU®), Corail®, Bouillie Bordelaise (BB)) chez les embryons d'escargots terrestres. De même la génotoxicité pouvant résulter d'une exposition courte au Cd en début de développement n'est pas connue. Le but de ces travaux est d'évaluer la génotoxicité de ces 3 pesticides à des concentrations d'exposition réalistes d'un point de vue environnemental et du Cd après une exposition de 24h. Pour cela, la méthode RAPD-SHR a été utilisée. Des pontes d'escargot *Cantareus aspersus* ont été exposées durant 20 jours à différentes concentrations de pesticides proche de la EC_{50} : 15 à 70 mg/L de RU®; 0,05 mg/L à 0,15 mg/L de Corail® et 1 à 2 g/L de cuivre (Cu) provenant de la BB. Les concentrations de Cd testées en exposition de 24 heures étaient de 5, 10 et 15 mg/L. Les 3 amorces utilisées pour les amplifications RAPD sur les ADN d'embryons exposés aux pesticides ont mis en évidence des effets génotoxiques du RU® (dose-dépendant) et du Corail® mais pas de la BB. Les 6 amorces utilisées lors des amplifications RAPD réalisées sur des embryons en cours de développement (Jour 1, 6, 7 et 12) exposés au Cd durant 24 heures n'ont pas révélé d'effets génotoxiques. Cette étude confirme que de la méthode RAPD-SHR est adaptée au criblage haut débit des effets sur l'ADN de contaminants variés chez l'embryon d'escargot exposé en continu.

Mots clés : *Cantareus aspersus*, formulation commerciale de pesticides, embryotoxicité, glyphosate, tébuconazole, Bouillie Bordelaise

III.2.2.1. Introduction

Random Amplified Polymorphic DNA (RAPD) is a technique derived from the PCR which was originally used in taxonomic and phylogenetic approaches to detect polymorphism (De Wolf et al., 2004). Since one decade, this method is used in ecotoxicology for the detection of different types of DNA damage. RAPD permits to realize a rapid screening of the genotoxic potential of various contaminants after a PCR step with arbitrary short primers. The analysis is based on comparison of amplification profiles of exposed and control samples where each change detected along profiles (disappearance/appearance of fragment, changes in intensity of bands) can be directly related to various types of DNA damages and mutations in the genome studied (Atienzar and Jha, 2006 ; De Wolf et al., 2004).

RAPD was successfully used on many organisms as plants (Liu et al., 2009 ; Korpe and Aras, 2011 ; Gjorgieva et al., 2013), algae (Atienzar et al., 2000 ; Tuney et al., 2007), fishes (Mahrous et al., 2006 ; Mohanty et al., 2009 ; Galindo et al., 2010 ; Lerebours et al., 2013 ; Ben Salem et al., 2014). This technique has been used to detect signs of genotoxicity of various contaminants as heavy metals non essential: cadmium (Cd), bore, arsenic, mercury, nickel, aluminum (Atienzar et al., 2001 ; Cenkci et al., 2009 ; Al-Qurainy et al., 2010 ; Ahmad et al., 2012 ; Erturk et al., 2013) or essential: zinc, sodium, manganese, copper (Cu) (Enan, 2006 ; Tuney et al., 2007 ; Korpe and Aras, 2011 ; Orieux et al., 2011 ; Aydin et al., 2012 ; Gjorgieva et al., 2013). RAPD has also been used to demonstrate genotoxicity of various compounds: physical as the UV A and B (Atienzar et al., 2000), gamma rays (Dhakshanamoorthy et al., 2011), or still radioactivity of uranium and tritium (Hagger et al., 2005 ; Plaire et al., 2013) and emergent pollutant (nanoparticules: ZnO, CuO ; Lee et al., 2013). The genotoxicity of organic compounds like drugs (Rocco et al., 2010, 2011, 2012) or herbicides (Mohanty et al., 2009 ; Doganlar, 2012 ;Aksakal et al., 2013 ; Bozari and Aksakal, 2013) has also been largely studied. However, up to know, the potential of RAPD to detect DNA damages has been little used on embryos and on terrestrial animals. As far as we know, only 2 publications are available concerning the use of the RAPD method on embryos of aquatic invertebrates as urchins *Paracentrotus lividus* (Guida et al., 2010) or mussels *Mytilus edulis* (Hagger et al., 2005). Just one concerns terrestrial vertebrate: the kangaroo rats *Dipodomys merriami* (Theodorakis et al., 2001).

Recently, Baurand et al. (2013) have demonstrated that RAPD is adapted to show that Cd causes embryogenotoxic effect on the terrestrial snail embryos *Cantareus aspersus* (syn. *Helix aspersa*) at least after continuous exposure. They proposed a method to reduce subjectivity during profiles analysis by coupling the RAPD amplifications with a High Resolution electrophoresis System (HRS). This association helps to decrease problems and

time of migrations while increasing their quality and permits also a rapid automatic analysis of amplification profiles. RAPD-HRS has been used to demonstrate DNA damages of a continuous exposure to Cd for 20 days on *C. aspersus* embryos from 2 mg/L. Otherwise, Baurand et al. (2014a) have demonstrated the existence of early capacities of defense against the Cd by studying the expression of 3 isogenes coding metallothioneins (MTs) after 24 hours of exposure to Cd which mimics brief exposure e.g. to contaminated soil leachates. However, the genotoxic potential of such a short exposure to Cd has not been studied yet neither on embryos just after egglaying nor on older embryos.

Few data are available about embryotoxicity of pesticides on *C. aspersus*. EC_{50} values based on hatching success are known for the Round Up flash® (RU® ; ~50 mg/L of glyphosate), the Corail® (~10 mg/L of tebuconazole) and the Bordeaux Mixture (BM ; ~1.5 g/L of Cu) (Druart et al., 2012). The embryogenotoxic potential of these commercial formulation used in vineyard treatment is still unknown. Until now, the couple RAPD-HRS has not been used to assess possible impact of herbicides or fungicides on DNA of snail embryos.

The aims of this work were:

1/ to determine if the couple RAPD-HRS is relevant to screen the genotoxic potential of pesticides (RU®, Corail®, BM) on the terrestrial snail embryos *C. aspersus.* In this purpose continuous exposure of eggs just after egglaying to relevant environmental concentrations of these chemicals was performed.

2/ to assess the influence of the exposure regime (24h vs continuous) on Cd genotoxicity. For that, the same scenario of exposure to Cd (24 h) as used by Baurand et al. (2014a) for MT analysis was applied here. Data obtained will be compare to the results obtained by Baurand et al. (2013) after continuous exposure to Cd.

3/ to determine if the genotoxic response to Cd was modulate by the age of embryos upon exposure. In this purpose, embryos were exposed at 2 keys steps of their development: at the start of embryogenesis (0 day) and organogenesis (6^{th} day).

III.2.2.2. Materials and Methods

Chemicals

The concentrations of pesticides tested were chosen to surround the EC_{50} values found by (Druart et al., 2010, 2012). All dilutions were prepared in demineralized water (pH 6.2), which was also used as a control solution. Solutions of RU® were prepared from commercial

formulation of Round Up Flash® (glyphosate 450 g/L, Monsanto). Nominal concentrations were 15, 30, 50 and 70 mg/L of glyphosate. The solutions of Corail® were prepared with commercial formulation Corail® (tebuconazole 250 g/L, Bayer Cropsciences). The tested nominal concentrations were 0.05, 0.10, 0.15 mg/L of tebuconazole. The solutions of BM were done from powder of Bordeaux Mixture RSR Disperss (20% of Cu, Cerexagri). Nominal exposure concentrations tested were 0.5, 1, 1.5, 2 g/L of Cu.

The solutions of Cd were prepared using solid $CdCl_2$ (99.99%, Sigma Chemical Co., St. Louis, MO; C- 2544). The nominal concentrations were 5, 10 and 15 mg/L as in Baurand et al. (2014b).

The concentrations of Cd and Cu were measured using ICP-AES (ICAP 6000 series model radial, Thermo scientific, France). The quality of the results was verified using a certified reference water (Hard Drink Water UK, ERM- CAO11a, LGC Promochem, Molsheim, France) Cd-certified at 4.94 µg/L (average recovery of 93%) and Cu-certified at 1970 µg/L (average recovery 97%). The actual verified concentrations were, respectively, 3.8, 8.7, and 13.1 mg/L for the Cd and 0.37, 0.76, 1.19, 1.49 g/L for the Cu.

Exposure device

Eggs were obtained from our laboratory rearing and were exposed using the liquid phase bioassay as previously described by Druart et al. (2010) and Baurand et al. (2013, 2014a). Five clutches per substance tested have been used. Each clutch was separated into groups of 10 eggs, which were placed in Petri dishes on four layers of paper (quantitative filter paper grade 1 ashless, Whatman) dampened with 0.8 mL of control or contaminated solution. For the 3 herbicides tested, eggs were exposed rapidly after the end of laying (maximum 24 h). Cadmium exposure was realized according to Baurand et al. (2014a). Eggs were exposed for 24 h to 3 Cd solutions (5, 10, 15 mg/L) at the beginning of their development or at the sixth day of embryogenesis.

After twenty days of development (to avoid omitting late hatchlings), the mean hatching success for each concentration was calculated. The results were considered valid if the hatching success of controls was at least of 70% (the average value observed in our laboratory rearing for controls). The dose-dependent curves and the EC_{10} and EC_{50} values were determined with the Hill's model using the macro Excel Regtox free version EV6.1. Each Petri dish with eggs was then kept at −80 °C until DNA extractions were performed.

DNA extraction

After exposure, DNA extractions were performed on two clutches among the 5 used for exposure to contaminants. The two chosen replicates presented a near response in terms of

hatching success. DNA has been extracted from eggs exposed at each concentration tested for the Corail® and the RU®. For the BM, DNA was extracted for three of the 4 concentrations used (1, 1.5, 2 g/L of Cu). For Cd, extractions were done for two (5 and 15 mg/L) of the 3 concentrations tested and a kinetic study was realized. For eggs exposed at day 0, sampling dates were 1 (end of exposure period), 6 and 12 days. For the samples exposed at 6 days of embryogenesis, the sampling dates were after 7 (end of exposure) and 12 days.

For pesticides, DNA extractions were realized on embryos after 20 days of exposure. To select primers and determine reference profiles for RAPD analysis, the genomic DNA from five other unexposed clutches has been extracted. Six day-old embryos were used to determine the reference profiles needed for RAPD analysis on the developing embryos. Embryos of 20 days (fresly hatched) were used for the reference profile used for RAPD on the samples exposed to pesticides.

All extractions on embryos aged of seven days maximum (or very small embryos which were exposed to the higher concentrations tested) were performed on embryos preliminary isolated from albumen of eggs using the QIAamp® DNA Micro (Qiagen, Hilden, Germany) whereas extractions on embryos aged of 12 days or more were realized with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

For one day-old samples, 15 embryos were pooled whereas for the others samples (6, 7, 12 and 20 days) only three were sufficient. DNA samples were kept at −20 °C until the step of RAPD amplifications. The concentrations of the purified genomic DNA were measured at 260 nm, and the purity was estimated by measuring the 260/280 nm absorbance ratio with a Nanodrop Spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Wilmington, U.S.A.). The 260/280 nm ratio for DNA samples from embryos aged of 6 days or less was between 1.0 and 1.4. The ratio for the DNA samples from embryos aged of 7 and 12 days was between 1.45 and 1.9. The quality of DNA samples was checked on 2% agarose gels stained with ethidium bromide.

RAPD-PCR procedure

The RAPD reactions were performed using the PuRe Taq Ready-To-Go PCR beads kit (GE Healthcare Biosciences, Pittsburgh, PA) according to Baurand et al. (2013). For each reaction, we added one bead of the kit (containing dNTPs, the BSA, and the polymerase AmpliTaqTM), 40 ng of DNA, 5 µL of random primer (5 pmol/µL), and ultrapure water for a final volume of 25 µL.

RAPD was performed, in a first time, on the five DNA extracts of i) 6 days-old non-exposed embryos and ii) 20 days-old embryos (freshly hatched). This procedure was realized

to create references profiles for analysis i) on developing embryos 24h-exposed to Cd and ii) on embryos pesticides-exposed during 20 days. Six primers were retained for RAPD realized developing embryos (for the kinetic study): Osama05 (GACCATCGTC), OPG05 (CTGAGACGGA), Essam08 (GCTCGAACTT), X19 (CCACCGCCAG), OPG17 (ACGACCGACA), Saad02 (GTGCTACGTC) whereas 3 primers were used (X19, OPG17 and Saad02) for amplifications on pesticides-exposed samples.

Selected primers permit obtaining amplification products with a reasonable number of distinct bands and a good reproducibility. For all the chemicals studied, each RAPD reaction was performed at least twice on each DNA extract. Amplification reactions were performed in a Mastercycler thermal cycler (Eppendorf, Le Pecq, France) as follows: one initial denaturation step of 5 min at 95 °C followed by 45 cycles at 95 °C for 1 min (denaturation), 36 °C for 1 min (annealing), and 72 °C for 2 min (extension). The RAPD reaction products were stored at −20 °C.

Micro capillary electrophoresis and RAPD profile analysis

RAPD profiles have been analyzed via capillary electrophoresis using the QIAxcel system (http://www.qiagen.com/products/qiaxcelsystem.aspx#Tabs=t1). The parameters for migration were in the same used by Baurand et al. (2013) (OM500 method with a time sample injection of 30 s). The size of the amplified fragments was determined automatically by referring to the fragments obtained with the known size marker QX DNA Size Marker 100 bp-2.5 kbp. Next, the data obtained have been analyzed using the biocalculator software of the HRS. The standard parameters were kept for automatic analysis of profiles except for the positive threshold (a signal exceeding this value of maximal fluorescence is detected as a peak). We have chosen 6% for OPG17 primer, 5% for X19 and 4% for Saad02.

Estimation of genomic template stability

At each change observed in RAPD profiles (disappearance of bands and/or appearance of new bands) was given the arbitrary score of +1. The average number of changes was calculated for each chemicals with all primers used. The genomic template stability (GTS, %) was calculated for each primer as follows: GTS (%) = (1 – a/n) × 100, where a is the average number of changes in DNA profiles and n the number of bands selected in DNA control profiles.

III.2.2.3. Results

Effect of Cd and pesticides on hatching success

For Cd, the $EC_{50\text{-}24h}$ based on hatching success for embryos exposed at 0 days was 5.05 mg/L (IC 95%: 4.41 – 5.69, Baurand et al., 2014a). For embryos exposed at 6 days, the $EC_{50\text{-}24h}$ was 9.48 mg/L (IC 95%: 7.84 – 11.23).

EC_{50} values for RU®, Corail® and BM were respectively 29.02 mg/L of glyphosate (IC95%: 21.3 – 41.3), 0.087 mg/L of tebuconazole (IC95%: 0.079 – 0.10) and 1.99 g/L of Cu (IC95%: 1.89 – 2.09).

RAPD-PCR on developing embryos exposed to Cd

The 6 primers used for the RAPD amplifications on developing embryos 24h-exposed to Cd generated a total of 24 specific and stable bands. The mean number of fragments amplified per primer was 4 (5 for X19 and OPG05 primer, 4 for OPG17 and Saad02, 3 for Essam08 and Osama05). No change between the profiles of controls and Cd-exposed samples (at 5 and 15 mg/L) was detected neither for embryos exposed at 0 day nor for those exposed at 6 days.

Figure III-15 represents results obtained for 2 of the 6 primers used: Saad02 primer for embryos 15 mg/L-exposed at 0 days (Figure III-15A and B) and Osama05 primer for 5mg/L-exposed embryos at 6 days (Figure III-15C and D).

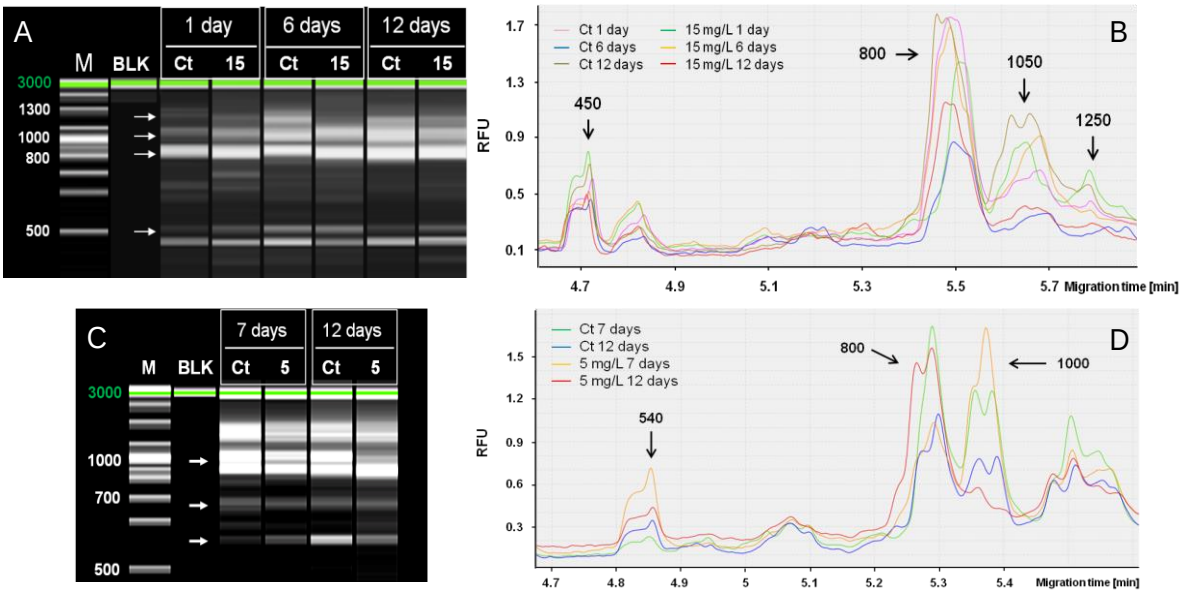


Figure III-15 : Migration pictures and electropherograms otained for 2 of the 6 primers used in RAPD amplifications on Cd-exposed embryos.

Migration pictures (A) and electropherograms (B) obtained with Saad02 primer for Control or 15mg/L Cd-exposed embryos at 0 day and sampled after 1, 6 and 12 days and amplified. Migration pictures (C) and electropherograms (D) obtained with Osama 05 primer for Control or 5mg/L Cd-exposed embryos at 6 days and sampled at 7 and 12. M: size marker in bp; BLK: blank without DNA; Ct: control; 5, 15: 5 and 15mg/L Cd-exposed embryos. Arrows show fragments retained for each profile analysis.

RAPD-PCR on embryos exposed to pesticides

For each pesticides studied, the results presented below concern 1 clutch of the 2 studied showing the highest changes in profiles with one of the three primers used.

Round Up®

The samples exposed to the commercial formulation of glyphosate showed the largest number of changes in amplification profiles from the three pesticides used, with an increase of the modifications in profiles with the increase of concentration tested (Tableau III-4).

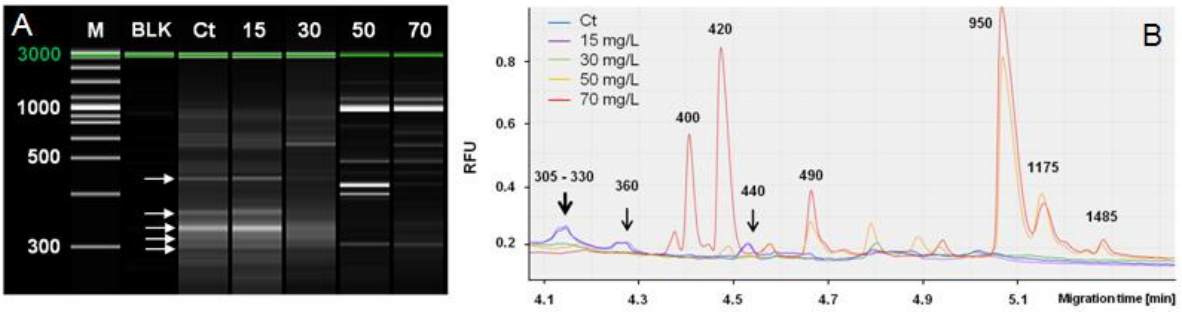
As shown on Figure III-16 presenting the clutch with the highest changes in RAPD profiles, RAPD-PCR with OPG17 primer show clear modifications from 30 mg/L of glyphosate with 2 disappearances of bands of 360 and 440 bp. At 50 and 70 mg/L of glyphosate, amplification profiles are completely modified and presented 6 or 5 new bands and 4 disappearances respectively.

Corail®

One of the 2 clutches exposed to Corail® presents modifications in RAPD profiles from the first concentration tested (0.05 mg/L). For example, amplifications realized with the Saad02 primer (Figure III-17) permit to detect appearance of one band (315 bp) in all Corail® exposed samples whereas 2 bands disappeared (1050 and 1250 bp) respectively in samples exposed at 0.05 and 0.10 mg/L of tebuconazole.

Bordeaux Mixture

No change was detected in all samples amplified with the 3 primers as shown in the Figure III-18 with RAPD profiles obtained after amplifications with the X19 primer.

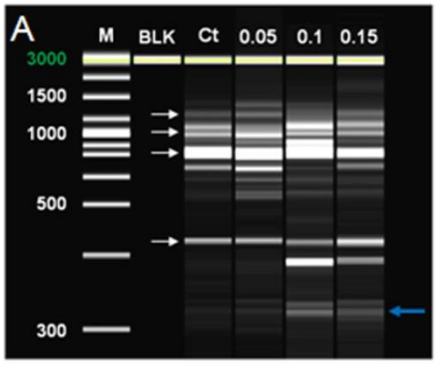


C

	305	320	330	360	400	420	440	490	950	1175	1485
BLK	0	0	0	0	0	0	0	0	0	0	0
Ct	1	1	1	1	0	0	1	0	0	0	0
15	1	1	1	1	0	0	1	0	0	0	0
30	1	1	1	**0**	0	0	**0**	0	0	0	0
50	1	**0**	**0**	**0**	**1**	**1**	**0**	**1**	**1**	**1**	**1**
70	1	**0**	**0**	**0**	0	**1**	**0**	**1**	**1**	**1**	**1**

Figure III-16 : RAPD profiles obtained with OPG17 primer on Round Up®-exposed samples.

A: Gel image, B: electropherograms, C: binary matrix. M: size marker 100 bp to 2.5 kbp. BLK: blank without DNA; Ct: control; 15, 30, 50, 70: samples exposed at 15, 30, 50, 70 mg/L of active ingredient glyphosate. 300 to 1485: size of fragments in bp. Black and white arrows show the 5 fragments retained for profiles analysis.



C

	315	450	760	1050	1250
BLK	0	0	0	0	0
Ct	0	1	1	1	1
0.05	**1**	1	1	**0**	1
0.10	**1**	1	1	1	**0**
0.15	**1**	1	1	1	1

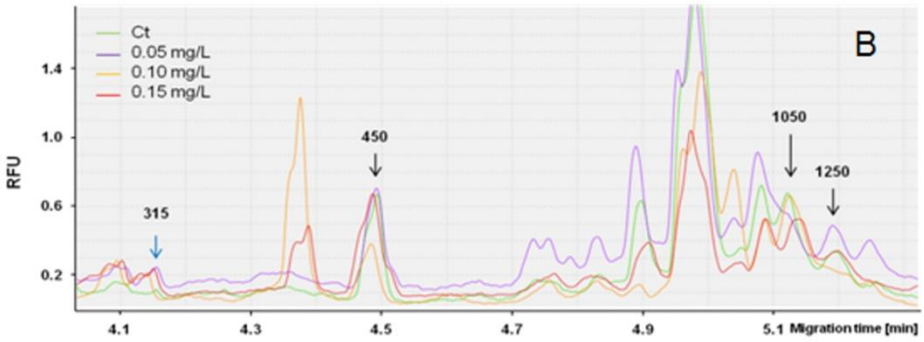
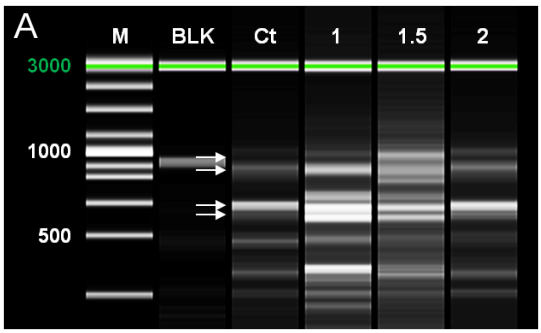


Figure III-17 : RAPD profiles migration obtained with Saad 02 primer on Corail®-exposed samples.

A: Gel image, B: electropherograms, C: binary matrix. M: size marker 100 bp to 2.5 kbp. BLK: blank without DNA; Ct: control; 0.05, 0.1, 0.15: samples exposed at 0.05, 0.10, 0.15 mg/L of active ingredient tebuconazole. 315 to 1250: size of fragments in bp. Black an white arrows show the 4 fragments retained for profiles analysis, the blue one show the new amplified fragment.



C

	540	570	800	1050
BLK	0	0	0	0
Ct	1	1	1	1
1	1	1	1	1
1.5	1	1	1	1
2	1	1	1	1

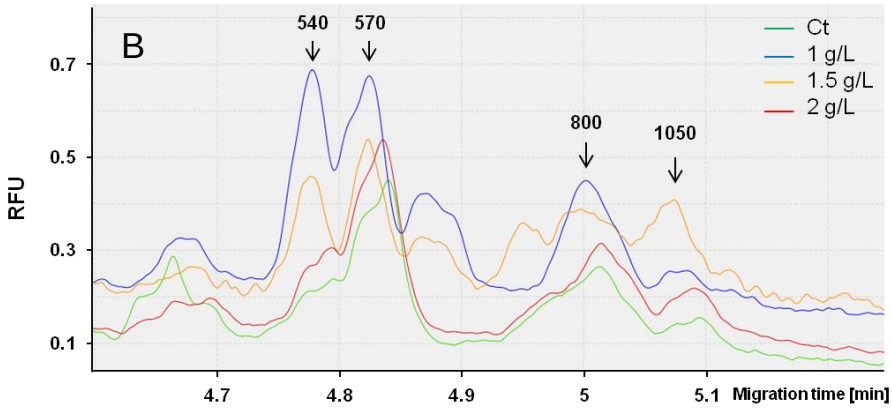


Figure III-18 : RAPD profiles migration obtained with X19 primer on Bordeaux Mixture-exposed samples.

A: Gel image, B: electropherograms, C: binary matrix. M: size marker 100 bp to 2.5 kbp. BLK: blank without DNA; Ct: control; 1, 1.5, 2: samples exposed at 1, 1.5, 2 mg/L of Copper. 540 to 1050: size of fragments in bp. Black and white arrows show the 4 fragments retained for profiles analysis.

Changes in profiles

Tableau III-4 : Summary of changes in RAPD profiles detected on pesticides-exposed embryos.

Number of bands in control samples and appearance (+) or disappearance (−) of bands for the 3 primers used in Round Up® (15 to 70 mg/L of glyphosate), Corail® (0.05 to 0.15 mg/L tebuconazole) and Bordeaux mixture (1 to 2 g/L of Copper) exposed samples.

			RU (mg/L of glyphosate)								Corail (mg/L of tebuconazole)						B. Mixture (g/L of Cu)					
	Clutch	Ctrl	15		30		50		70		0.05		0.10		0.15		1		1.5		2	
			+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Saad	a	4	0	0	0	0	1	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0
	b		1	2	3	2	5	2	3	0	1	0	1	1	1	1	0	0	0	0	0	0
OPG	a	5	0	0	0	0	0	1	6	4	0	0	0	0	0	0	0	0	0	0	0	0
	b		0	0	0	2	7	4	5	4	0	0	0	0	0	0	0	0	0	0	0	0
X19	a	4	0	0	0	0	2	1	5	3	1	0	0	1	0	1	0	0	0	0	0	0
	b		0	0	1	1	6	3	6	3	1	0	0	0	0	1	0	0	0	0	0	0

Tableau III-4 resumes the changes detected in RAPD profiles of the samples exposed to pesticides (by comparisons to the control samples) for the 2 clutches investigated. For RU®-exposed samples, for one primer, the modifications detected after RAPD can be variable depending on the analyzed replicate. For example, Saad02 can detect the first modifications at

50 mg/L of glyphosate on one clutch (a) whereas in the other one, first changes in profiles were revealed at 15 mg/L (b). A huge number of changes in profiles were also detected from 50 mg/L with the 3 primers used. For amplifications with the primers OPG17 and X19, the alterations in profiles appear from 50 mg/L in one case (b) or from 30 mg/L (b) on the other clutch.

For the samples exposed to Corail®, if the primer OPG17 has not permitted to show changes in profiles, RAPD with primer X19 gives globally the same results on the two replicates used. In each case, one band appears from 0.05 mg/L of tebuconazole and one disappears at 0.1 and 0.15 mg/L. The primer Saad02 permits to detect modifications in profiles in one clutch from 0.05 mg/L (b). Globally the number of changes in profiles of samples exposed to the Corail® formulation were lower than the changes detected in samples exposed to RU®.

For the samples exposed to BM, no changes were revealed after RAPD with the 3 primers used.

Genetic templates stability

The value of Genetic templates stability **(**GTS, %) is a measure which reflects changes in RAPD profiles generated with the 3 primers used. The GTS total value was calculated for the 3 pesticides studied (Tableau III-5). For RU® exposure, the value of GTS decreases with the increased of RU® concentration (88.3% at 15 mg/L to 4.2% at 70 mg/L). On the contrary for samples exposed to Corail®, the GTS remain relatively stable (87.5 to 83%) with the increase of concentrations tested whereas for BM exposure, GTS value is not modified (100%).

Tableau III-5 : Genomic template stability in Round Up®, Corail® and Bordeaux Mixture exposed samples.

Genomic template stability for each primer was calculated by the formula GTS (%) = (1−a/n)×100, where a is the average number of changes in DNA profiles and n the number of bands selected in control DNA profiles. a, b: cluch a and b for each pesticides studied.

		RU (mg/L)				Corail (mg/L)			BM (g/L)		
	clutch	15	30	50	70	0.05	0.1	0.15	1	1.5	2
Saad	a	100	100	25	0	100	100	100	100	100	100
	b	25	0	0	25	75	50	50	100	100	100
	mean	**62.5**	**50**	**12.5**	**12.5**	**87.5**	**75**	**75**	**100**	**100**	**100**
OPG	a	100	100	80	0	100	100	100	100	100	100
	b	100	60	0	0	100	100	100	100	100	100
	mean	**100**	**80**	**40**	**0**	**100**	**100**	**100**	**100**	**100**	**100**
X19	a	100	100	25	0	75	75	75	100	100	100
	b	100	50	0	0	75	100	75	100	100	100
	mean	**100**	**75**	**12.5**	**0**	**75**	**87.5**	**75**	**100**	**100**	**100**
mean	**Total**	**88.3**	**68.3**	**21.6**	**4.2**	**87.5**	**87.5**	**83.3**	**100**	**100**	**100**

III.2.2.4. Discussion

Cd short exposure effects on embryos

In order to realize a larger screening of the genome of embryos and thus to improve the chances to detect modifications in RAPD patterns, 6 primers were used in this study instead of the 2 used by Baurand et al. (2013). A kinetic study of apparition of DNA damages and/or mutations was realized during the embryonic development of *C. aspersus*. Surprisingly, no change was detected at the sampling dates investigated from the end of exposure periods (1 day or 7 days) until 12 days of development. This was not expected as Baurand et al. (2013) have shown signs of genotoxicity from 2 mg/L in eggs exposed during 20 days. Several parameters may be responsible of these contrasted results obtained for the two types of scenarios of Cd exposure (24 hours here and continuous exposure in Baurand et al., 2013).

First, in the present study, the later point which has been investigated was 12 days. Thus, the duration of exposure (8 days less here) maybe involved in the case of Cd genotoxic effects become visible only at the end of the development. Agnello et al. (2006) have hypothesized, when studying Cd effect on *Paracentrotus lividus* embryos, the occurrence of toxic effect would depend both on Cd concentration and of the duration of exposure. According to these authors, we can assume that the metallic stress generated by a 24 hours of Cd exposure (from 5 to 15 mg/L) is not the same that the one of a 20 days continuous exposure (at 2, 4 and 6 mg/L) because these 2 factors change (exposure duration and concentration). A differential

diffusion of Cd^{2+} ions between the 2 scenarios of exposure possibly occured as evoked by Baurand et al. (2014b).

Secondly, interventions of DNA reparation systems or of metal-specific systems of defense like metallothioneins (MTs), which are known to have a role in Cd detoxification in developing snail embryos (Baurand et al., 2014a) can be suspected. It has been demonstrated that early life stages of *C. aspersus* are able to respond to a stress induced by the Cd by synthesizing a specific MT from their first day of development. Baurand et al. (2014, *in prep*) has also shown that embryos Cd exposed at the beginning of their organogenesis for 24 hours have higher capacities of *CdMT* gene expression at 12 days than embryos exposed with the same scenario at the start of the embryonic development. Our study show that the difference of sensivity of 0 and 6 days-exposed embryos observed on the EC_{50}-hatchability is not related to a difference in genotoxic effects.

Overall present data demonstrate that the assessment of genotoxicity must be carefully interpreted as many factors can influence the result (e.g. regime of exposure, age and individual sensitivity to chemicals, capacities of detoxification process throughout embryogenesis…). RAPD-HRS system appears as an adapted method to investigate these parameters.

Pesticides exposure effects on embryos

The toxicity values (EC_{50}), based on the hatching success, found in this study for the 3 pesticides investigated are in the same range of sensitivity than found by Druart et al. (2012, 2010) and confirm the good repeatability of the liquid phase bioassay. They have reported EC_{50} of 0.095 mg/L of tebuconazole for the Corail®, 43 and 55 mg/L of glyphosate for the RU® and 1.5 g/L of Cu. All concentrations tested in our study were inferior to the dose preconized for field applications on vineyards (http://e-phy.agriculture.gouv.fr). These recommended values are of 700 mg/L of tebuconazole, 5140 mg/L of glyphosate, and 7.64 g/L of Cu respectively for the the Corail®, RU® and the BM (Druart et al., 2012).

Round up®

The RU® is an herbicide containing glyphosate as active ingredient and also adjuvants like the surfactant polyoxyethylene amine (POEA). The Saad02 primer, in our experimentation, has permitted to highlight genotoxic effects from the first concentration tested (15 mg/L of glyphosate) whereas no significant effect on hatchability was found at this concentration. Present study is the first one reporting genotoxic effects of commercial formulation of glyphosate (Round Up Flash®) on embryos of a terrestrial species on the basis

of RAPD-PCR detection. Among the 3 pesticides studied, the RU® was the one who showed the highest number of alterations in profiles of exposed samples. The screening of effect with the 3 primers used revealed a huge number of modifications (disappearances and appearances of bands) that confirm strong changes at genomic level. The disappearance of a band in a RAPD profile can be related to different events such as DNA damage (single and/or double-strand breaks, DNA adducts, oxidized bases, DNA–protein cross-links), point mutations and/or complex chromosomal rearrangements (Atienzar et al., 1999, 2000). The new amplification products may reveal changes in some oligonucleotide priming sites due to mutations, large deletions (bringing to pre-existing annealing site closer), and/or homologous recombination (juxtaposition of two sequences that match the sequences of primer) (Atienzar et al., 1999).

There is a lack of information concerning the genotoxic potential of RU® on embryos. Using the micronucleus test and comet assays, Poletta et al. (2009) showed the genotoxicity of RU® in erythrocytes of caiman embryos (*Caiman latirostris*) exposed to 500 µg of RU/eggs. In our study a strong genotoxic effect was revealed for exposure to RU® as shown the changes in GTS total (88.3% at 15 mg/L to 4.2% to 70 mg/L). The EC_{50} value based on hatching success of 29 mg/L was associated to a decreased in GTS values near to 30% (100% for control to 68.3% for 30 mg/L exposed embryos).

Corail®

Corail® is a fungicide of the triazol family, used in preventive and curative treatment phytopathogenic fungi like the powdery mildew, the Brenner and the black rot of vine. Its active ingredient is tebuconazole (250 g/L). There is no data available about genotoxicity of Corail®. However, toxic effect has been reported in *Gammarus pulex* with an LD_{50} of 1.6 mg/L of a.i. (Adam et al., 2009) or on zebrafish *Danio rerio* with a LD_{50} of 20 mg/L of a.i. (Sancho et al., 2010). Our study brings evidences concerning the genotoxic potential of the commercial formulations of Corail® which appears at very low concentration (0.05 mg/L) compared to the recommended dose (700 mg/L of tebuconazole). But no concentration effect response was found as shown by the decrease of GTS values about 13% for the 3 tested concentrations. This pesticide was the most toxic of the 3 tested (based on EC_{50} values) but effects are poorly related to genotoxicity in the present study.

Bordeaux Mixture

The BM is a mixture of sulfate of copper ($CuSO_4$) and slaked lime ($Ca(OH)_2$) also used as a fungicide. It is sprayed in vineyards, but also in fruit - farms and gardens to prevent infestations of downy mildew, powdery mildew and other fungi. Genotoxic effects of Cu or

Cu-based compounds have been demonstrated in different organisms. For example, Cu sulfate has been reported as genotoxic after sister chromatid exchange (SCE) tests in lymphocytes of rabbits gavaged by 7.5 mg Cu/kg body weight daily for 6 days (Georgieva et al., 2013). Comet assays as permitted to detect genotoxic effect of Cu in whole blood of mice gavaged for 6 days with 8.5 mg/kg copper (Pra et al., 2008) whereas genotoxic potential of Cu has been demonstrated in larvae of chironomidae (*Chironomus kiiensis*) after a 24 hours exposure (0.2 to 20 mg/L) by Al-Shami et al. (2012).

In our study, the BM doesn't cause genotoxic effect on snails embryos (on the basis of the results obtained with the 3 primers used) whereas this fungicide shows an inhibitory effect on hatchability of *C. aspersus* snails from 2 g/L of Cu. Thus there is not links between toxicity based on hatching success and genotoxic effect in our study. Cu is an essential metal which can be regulated by organisms partly due to the presence of the CuMT that can play a role in homeostasis of Cu. Transcription of the gene coding this MT has been demonstrated in *C. aspersus* embryos (Baurand et al., 2014b). The *CuMT* gene was known to be up-regulated during Cu stress in adults (Höckner et al., 2011). Thus the probable presence of this protein in embryos and its induction possibly explained the low genotoxic potential found for this contaminant in our experimental conditions.

III.2.2.5. Conclusion

Our work demonstrate that the RAPD-HRS is adapted for a rapid and efficient screening of genotoxic effect of various contaminants at relevant environmental concentration. This method appears adapted to genotoxicity detection even in very young embryos (from one day) of the terrestrial snail *C. aspersus*.

On the basis of variation of the GTS % values we have evidenced i) importance of the regime of exposure to Cd (duration of exposure, period of embryogenesis, range of exposure concentrations) during genotoxic potential study of a chemical, and ii) individual sensitivity pesticide-exposed embryos.The most toxic pesticide (Corail) for snail embryos is not the most genotoxic and the toxicity of some toxicants can be i) related to genotoxicity (RU®) or ii) independent of genotoxic effect (for Cu) or iii) in part related to genotoxic effect (Corail®).

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III.2.2.7. References

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III.2.3. Résumé du chapitre 2

- La technique RAPD couplée à un système d'électrophorèse haute résolution permet une détection efficace des effets génotoxiques de contaminants variés chez l'embryon d'escargots exposés en continu.

- Cette méthode permet de détecter des effets génotoxiques du Cd variable selon le régime d'exposition (effets dès 2 mg/L en exposition continue, aucun effet en exposition de 24 h). Elle a également montré des effets au niveau génomique après exposition à des concentrations de Round Up Flash® et de Corail® bien inférieures aux doses recommandées pour les traitements de la vigne.