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# **PHD THESIS**

**TOXIC EFFECTS AND OBSERVATIONAL ASPECTS  
RELATING TO CADMIUM AND COPPER ON  
EXPERIMENTAL MODELS**

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## INTRODUCTION

Although cadmium and copper can be found in nature in small quantities (ppm order), the modernization and multiplication of technological accessories that use electricity has led to the spread and accumulation of toxic heavy metals such as cadmium and copper in different components of electromechanical devices (cables, batteries, circuits, plastics, tires, etc.). The wear of these devices allows important quantities of these metals to pass into the environment, thus contributing to the increase of toxic effects on the soil, vegetation and implicitly of the human.

Since modern instrumental analysis allows the quick and high sensitivity detection of Cd and Cu, determining their polluting effects on the living world remains an open problem. The use of gastropods as a bioindicator of these effects is included in the current research panel. Gastropods such as *Cantareus aspersus* (Müller, 1774; syn. *Cornu aspersum* or *Helix aspersa*) are tolerant of high levels of cadmium via ingestion and accumulate important levels in its body tissues.

Terrestrial pulmonate snails require copper as an essential trace element, but important amounts of Cu can produce toxic effects on snails. The homeostatic regulation of Cu must consequently be an essential aim of terrestrial snails to survive. These mollusks accumulate copper almost equally in most of their organs, with a certain fraction being bound to a Cu-metallothionein (Cu-MT) isoform, at constant concentrations, irrespective of whether the animals had been exposed to physiological or elevated amounts of Cu.

The overall aim of my PhD thesis was to assess the effect of cadmium exposure and copper exposure via different exposure paths on different toxicological models.

The present work is structured in two main parts: the general part and the specific part. The general part contains theoretical information on the environmental and human relevance of cadmium and copper exposures. This part also provides a detailed insight into toxic effects of these trace elements at multiple organismal levels, including the epigenome, as compelling evidence for the usefulness of snails as bioindicators.

To this end, I employed the Brown garden snail, *Cantareus aspersus*, as invertebrate animal model and HT-29 cell human line as human toxicological model. To provide toxicologically-relevant data, I have used broad range of toxicological endpoints, with emphasis on changes occurring at gene level and key mechanisms fine-tuning their functionality (DNA methylation).

By using exposure scenarios with multiple time points, paths and doses, the results of my PhD thesis should provide a new dimension to the understanding of toxic effects related to copper and cadmium contamination/pollution and the molecular changes associated with these events.

### **Study 1. Exposure to Copper- enriched / contaminated soils**

**Objective:** The main aims of this study, were: (i) to show whether land snails take up soil Cu independently of food ingestion and (ii) to assess the toxic potential of soil contamination with copper on land snails (*Pulmonata*). Juvenile specimens of the Brown garden snail, *Cantareus aspersus* (Müller, 1774) were used as an animal model because this species has a well-known biology and is easily reared under both laboratory and field conditions [5]. Therefore, we evaluated Cu transfer from soils to snails as it occurs in nature, that is simultaneously via epithelial contact and soil ingestion. Bioaccumulation (Cu levels in the snail hepatopancreas, foot and shell), morphological (shell size) and lethality (survival rate) endpoints were measured, whereas copper sulfate was used as a source of copper. To provide ecologically-relevant data, semi-field conditions were established for this experiment, with most parameters replicating those encountered in nature (i.e., sunlight, air currents, temperature, photo-period).

**Materials and methods:** This work was performed in the village of Temeresti (Timis county, Romania; 45.8747° lat. N, 22.2117° long. E). To provide a homogeneous test population, 350 juvenile three-month-old *C. aspersus* specimens, were purchased from a snail farm in Romania (S.C. Edimpe Auto S.R.L., Muntenii de Sus, Vaslui county). After being adapted to cage rearing for a six-week pre-exposure period, the snails were fed *ad libitum* a copper-free diet containing fodder chalk (15%), wheat meal (10%), corn meal (7%), soya grits (10%), corn germ grits (10%), sunflower grits (15%), mono calcic phosphate (3%), Inlavit (10%), wheat pollard (10%) and vitamin-mineral premix for piglets (10%).

**Chemical analysis:** Given the abovementioned rationale, pure copper sulfate pentahydrate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 99.99% trace metal basis) was purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) and was used to prepare the following spiking solutions: (i) in the E1 phase: M1, control group; for the Cu1.1. treatment, 30 milligrams per liter (mg/L) Cu; for the Cu2.1. treatment, 60 mg/L Cu; for the Cu3.1. treatment, 160 mg/L Cu; for the Cu4.1. treatment, 300 mg/L; and (ii) the E2 phase: M2, control group; for the Cu1.2. treatment, 60 mg/L Cu; for the Cu2.2. treatment, 120 mg/L Cu; for the Cu3.2. treatment, 320 mg/L Cu; for the Cu4.2. treatment, 600 mg/L Cu. This

approach allowed us to obtain for all Cu-contaminated substrates two-fold higher soil copper concentrations at 60 days as compared to 30 days. Copper concentrations were quantified by flame atomic absorption spectrophotometry with high resolution continuum source (Model ContrAA 300, Analytik Jena, Germany; fig. 12).

**Results:** Copper concentrations of soil correlated highly with those measured in the hepatopancreas ( $r = 0.765$ ,  $p = 0.000$ ) and the foot ( $r = 0.631$ ,  $p = 0.000$ ), but correlated moderately with those determined in the shell ( $r = 0.426$ ,  $p = 0.019$ ). At 30 days, the hepatopancreas reached a plateau concentration, between 38 and 41 mg/kg d. wt for the three highest treatments. The measured values at 60 days increased with soil copper content, but only after showing similar values for the two lowest treatments, approximately 55 mg/kg d. wt.

If the hepatopancreas: soil copper ratios in controls were above 10, in exposed gastropods they were often less than 1 and at least 10-fold lower than those measured in controls. The soil-to-hepatopancreas regressions were significant (E1 phase:  $p = 0.031$ ,  $R^2 = 0.321$ ; E2 phase:  $p = 0.000$ ,  $R^2 = 0.902$ ), but their slopes were similar between the E1 and E2 phase (ANCOVA,  $p = 0.191$ ), whereas the corresponding intercepts were statistically different ( $p = 0.000$ ).

In addition, the hepatopancreatic Cu content of control snails in the E1 phase differed consistently from that measured in the E2 phase (Tukey's HSD test,  $p = 0.035$ ). However, the measured values in the foot at these two time points, were not significantly different (Tukey's HSD test,  $p \geq 0.194$ ). At 30 days, the hepatopancreas copper reached a plateau concentration, between 38 and 41 mg/kg d. wt for the three highest treatments.

The soil-to-hepatopancreas regressions were significant (E1 phase:  $p = 0.031$ ,  $R^2 = 0.321$ ; E2 phase:  $p = 0.000$ ,  $R^2 = 0.902$ ), but their slopes were similar between the E1 and E2 phase (ANCOVA,  $p = 0.191$ ), whereas the corresponding intercepts were statistically different ( $p = 0.000$ ). For all treatments of the E2 phase, post hoc testing revealed significantly lower survival rates in exposed specimens than in control snails (Breslow's test,  $p \leq 0.029$ ).

## **Study 2. Exposure to Cadmium through food consumption for 14, 28 and 112 days**

**Objective:** The present experiment aimed to determine the level of dietary Cd which led to an increase in Cd retention in the snail hepatopancreas. We used a continuous 14, 28 and 112-day exposure and a multiple dose study design using newly-matured *Cantareus aspersus* snails as invertebrate model.

**Materials and methods:** Five experimental doses, with three replicate jars per dose, were used; the nominal concentrations were: 0, 0.02, 0.05, 0.2, and 1 mg/kg dw. Cadmium sulfate ( $\text{CdSO}_4$ , 99.99% trace metal basis, Sigma-Aldrich) was used as a source of cadmium. An artificial fodder (20 g carrot baby food (HiPP, UK), 50 g fortified infant cereals (Nestle Nestum5 – Five Cereals), and 3 mL fungicide (1% methyl paraben solution) was used to achieve an even distribution of cadmium in the snail feeding medium. Hepatopancreas samples to be analyzed for Cd were thawed, oven dried (105 °C, 24 h), and then weighed to the nearest 0.01 mg. The sample was calcinated in a muffle furnace (Nabertherm B150, Lilienthal; 550 °C, 6 h), the resulting ash was submitted to wet acid digestion. Briefly, the ash was treated with 0.5 mL of 65%  $\text{HNO}_3$  (Merck, suprapure), heated to dryness and dis-solved in 20 mL of 0.5 N  $\text{HNO}_3$ . After filtration through ash-free filter paper, the volume of each sample was brought to 30 mL with 10 mL  $\text{HNO}_3$  0.5 N.

**Results:** Eight snails were sampled for each treatment group at each time point. Hepatopancreas Cd levels tended to increase with exposure dose and duration, reaching significant differences compared to controls for the highest treatments (marked with \* for  $p \leq 0.05$ ): at 14 days: for the 0Cd treatment,  $2.23 \pm 0.32$  mg/kg dw Cd; for the 0.02Cd treatment,  $2.16 \pm 0.44$  mg/kg dw Cd; for the 0.05Cd treatment,  $2.01 \pm 0.05$  mg/kg dw Cd; for the 0.2Cd treatment,  $2.37 \pm 0.29$  mg/kg dw Cd; for the 1Cd treatment,  $11.30 \pm 1.49$  \* mg/kg dw Cd; and at 28 days: for the 0Cd treatment,  $2.38 \pm 0.22$  mg/kg dw Cd; for the 0.02Cd treatment,  $2.73 \pm 0.55$  mg/kg dw Cd; for the 0.05Cd treatment,  $3.71 \pm 0.93$  mg/kg dw Cd; for the 0.2Cd treatment,  $6.54 \pm 0.27$  \* mg/kg dw Cd; for the 1Cd treatment,  $13.73 \pm 2.57$  \* mg/kg dw Cd. The median cadmium concentrations measured at 112 days in the hepatopancreas were: for the 0.02Cd treatment, 3.80 mg/kg dw Cd; for the 0.05Cd treatment, 4.85 mg/kg dw Cd; for the 0.2 Cd treatment, 4.98 mg/kg dw Cd; for the 1Cd treatment, 44.34 mg/kg dw Cd; for the 10Cd treatment, 212.14 mg/kg dw Cd; for the 100Cd treatment, 406.36 mg/kg dw Cd. These results suggest the potential existence of threshold level below which these land snails are able to maintain relatively stable cadmium concentrations in the hepatopancreas, which is up to 5 mg/kg d. wt.

### **Study 3. Effect of low-dose dietary cadmium on Copper, Manganese and Iron homeostasis in hepatopancreas**

**Objective:** We have currently investigated the short-term effects of dietary Cd uptake (as cadmium sulfate) on the concentration of Cd, Cu, Mn, and Fe in the hepatopancreas of land

snails. *Cantareus aspersus* (Müller, 1774) was used as a study system because its physiology is well understood and because it is easily reared, both under field, as well as laboratory conditions.

**Materials and methods:** The rearing protocol and corresponding procedures are similar to those described at study 2.

**Results:** Hepatopancreas Cd levels tended to increase with exposure dose and duration. In contrast, no dose-dependent response to low dose Cd feeding was observed for the hepatopancreas copper, manganese, and iron. However, copper levels were higher in the hepatopancreas of Cd-exposed specimens than in controls during the 0–14 days' period and the 15–28 days' period. We also noted elevated concentrations of manganese relative to those found in controls during the first period, whereas no clear trend was evident for hepatopancreas iron.

#### **Study 4. Determination of Cd-MT gene methylation status**

**Objective:** The main aims of this study were determining whether the promotor of the Cd-MT gene of *Helix aspersa* is natively methylated, and whether and to what extent does Cd affect its methylation status

**Materials and method:** Genomic DNA was extracted from hepatopancreatic cells using an QIAamp DNA Micro kit (Qiagen, Germany), in a final volume of 10 mL. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), with the measured concentrations ranging between 120 and 875 ng/mL. Next, the bisulfite conversion of hepatopancreas DNA was performed using an EpiTect Bisulfite Kit (Qiagen, Germany). After the bisulfite conversion, I conducted a Methylation-specific PCR (MS-PCR) using targeted-designed primers for post bisulfite converted DNA. Targeted primers for Cd-MT gene promoter used here were: CdMTbs-Fw1-GGATTTATYGTAGGATATTAATTAAGG promoter, CdMTbs-R1- TAAAAATAAAACCAAATACCAATCCTAC; CdMTbs-R2 CCTTACCACACTTACAACCATC.

**Results:** Analysis of DNA extracted from snails exposed at doses of 0 mg / L Cd, 0.1 mg / L Cd, 0.2 mg / L Cd, 1 mg / L Cd, 5 mg / L Cd, 10 mg / L Cd and 100 mg / L Cd via MS-PCR analysis suggested the lack of promoter methylation for this gene.

#### **Study 5. Effect of cadmium on cell proliferation (BrdU-assay)**

**Objective:** The aims of this study was determining the cytotoxic effect of cadmium on hepatopancreas cells.

**Materials and method:** After 28 days of continuous dietary Cd exposure, I selected six snails from the control group, the 10-ppm group, and the 100-ppm group; and injected them with BrdU (Roche) at a dose of 0.1mg/g wet weight 14 hours prior to their sacrifice. The BrdU solution used was obtained by dissolving under sterile conditions the BrdU powder into Ringer solution (80 mM NaCl, 4 mM KCl, 5 mM MgCl<sub>2</sub>, 7 mM CaCl<sub>2</sub>, 20 mM HEPES buffer, 5 mM galactose, 2 mM trehalose, 5 mM glucose, 1 mM sodium acetate)

**Results:** The present results suggest that cellular proliferation of hepatopancreas cells of adult snails, *Helix aspersa*, is sensitive to cadmium exposure starting from doses of 10 mg/kg dw Cd onward.

### **Study 6. Effect of cadmium on total DNA methylation level**

**Objective:** The aim of the present study was to determine whether and to what extent cadmium influence the global DNA (hydroxy)methylation levels in the hepatopancreas of *C. aspersus* snails.

**Materials and method:** DNA was isolated from the hepatopancreatic tissue of four snails on each replicate using the DNeasy Blood & Tissue kit (Qiagen) according to the standard protocol for animal tissues. The extracted DNA was verified qualitatively (agarose gel migration) and quantitatively (260/280 nm, NanoDrop-2000, Thermo Fisher Scientific Inc., USA);

The total content of 5mC in hepatopancreatic DNA was determined using the colorimetric ELISA method. This method was chosen as it represents a fast, cheap and reliable alternative to the methods currently used to determine global DNA methylation, especially for serial measurements, as was the case with the present experiment

**Results:** 5mC levels in hepatopancreatic DNA ranged from  $0.29 \pm 0.10$  in the 10Cd group to  $0.98 \pm 0.24$  in the 100Cd group. For the snails in the reference group, the level of 5mC was  $0.34 \pm 0.08$ . The measured 5mC values for the highest cadmium dose (100Cd treatment) were significantly elevated compared to controls (test Tukey HSD,  $p \leq 0.001$ ), but not the values determined for the other treatment groups (test Tukey HSD,  $p \geq 0.05$ ).

### **Study 7. Cadmium effect on global methylation and expression of DNMT with RT-PCR**

**Objective:** The overall aim of this was to investigate the effect of Cd exposure on key genes regulating the methylation cycle.

**Materials and method:** The RNA was isolated using a Direct-zol™ RNA MiniPrep kit and quantitatively assessed with a DS-11 Spectrophotometer, with reverse transcription being performed using the Maxima® First Strand cDNA Synthesis Kit. Cycling conditions in a Quant Studio 5 real-time PCR system (Thermo Fisher Scientific, USA) instrument was 95 °C 10 sec followed by 40 cycles of denaturing at 95 °C for 15 s and annealing and extension at 55 °C for 1 min. The primers use to amplify the analyze genes were:

18S (hs) f: 5'GTACCCGTTGAACCCATT3', r:5'CCATCCAATCGGTAGTAGCG3',  
DNMT1: f:5'ACCGCTTCTACTTCCTCGGCCTA3', r:5'GTTGCAGTCCTCGTGAACACTGTGG3',  
DNMT3A: f: 5'CACACAGAAGCATATCCAGGAGT G3', r:5'AGTGGACTGGGAAACCAAATACCC3'.  
DNMT3B f: 5'AATGTGAATCCAGTCAGG3', r: 5'ACTGGATTAACTCCAGGAACCGT3'.

**Results:** The expression of DNMT1 in controls (the 0Cd group) was lower than those quantified for the 0.02Cd snails, the 0.2Cd snails, the 10Cd snails and the 100Cd snails, and higher than those determined in the 0.05Cd snails and the 1Cd snails, but paired *post hoc*



comparisons against controls did not show any statistical differences. Our findings showed that among different DNMT- encoding genes only the candidate gene encoding DNMT1 is functional in the hepatopacres of mature *C. aspersus* snails. After searching with BLAST within the DDBJ Sequence Read Archive (DRA) for the correspondence between the primers for human DNMT1 and DNMT3 and the nucleotide sequences from the transcriptome of *C. aspersus* (accession SRX1058255), we identified only for DNMT1 nucleotide sequences showing a similarity of 64% with the human DNMT1 gene. By connecting the DNMT-encoding candidate genes' expression and cadmium exposure, our findings also significantly expand previous knowledge on potential mechanisms underlying the altered DNA methylation status resulting from exposure to chemicals in toxicologically-relevant invertebrate species.

That is, cadmium might could act via *disruption of normal expression* of DNMT1 leading to changes in genomic 5mC levels. The relationship between dietary cadmium and DNMT1 expression did not reveal a clear dose-reponse curve, but rather a bimodal dose-response pattern, with the distribution of gene transcripts showing two maxima. Based on very limited knowledge of DNA methylation in mollusks and its interaction with abiotic factors, it is difficult to explain the biological relevance of the present findings.

### **Study 8. Exposure to Cd and Cu induces cytotoxic and epigenetic changes in human colorectal cancer cells - HT-29**

**Objective:** The present study was aimed to verify the impact of CdCl<sub>2</sub> and CuSO<sub>4</sub> aqueous solutions on human colorectal carcinoma cells – HT-29 in terms of cells viability, morphology and migration capacity, and DNA methylation.

**Materials and methods:** CuCl<sub>2</sub> and CuSO<sub>4</sub> were acquired from Sigma Aldrich (Germany) as powders of analytical grade purity. The cell culture media: McCoy's 5a Medium Modified and supplements – fetal bovine serum (FBS), penicillin/streptomycin antibiotic mixture. The other reagents used in the present experimental design, as: phosphate saline buffer (PBS), Trypan blue, and Alamar blue and applied as recommended by the manufacturers. The *in vitro* experimental part of the present study was conducted on a human colorectal carcinoma cell line – HT-29 (ATCC® HTB-38™). Total RNA was isolated from HT-29 cells using trizol reagent and The Quick-RNA™ purification kits from Zymo Research. The primers use to amplify the analyze genes were the same like in Study 7.

**Results:** Addition of CdCl<sub>2</sub> solution into HT-29 cells medium and exposure to this compound for 24 h were associated with notable changes in cells' morphology as compared to controls (unstimulated) and solvent treated cells. Thus, the lowest concentrations – 0.05 and 0.2 µg/mL had no impact on cells' shape, confluence or adherence to the culture plate, whereas starting with 1 µg/mL the cells became round, were floating in the medium and at the highest concentration tested – 100 µg/mL, the cells seemed to disintegrate, confirming the viability data and indicating a cytotoxic effect for Cd.

CuSO<sub>4</sub> stimulation (at concentrations of 0.05; 0.2 and 1 µg/mL) for 24 h had no impact on HT-29 cell morphology in terms of shape, adherence or confluence as compared to control cells or solvent-stimulated cells. However, certain changes were observed in the cells exposed to the highest Cu concentrations of 10 and 100 µg/mL. These cells showed a different morphology related to the other groups of cells and were visible as round cells that were detached and floating in the culture medium.

Cadmium chloride-induced expression of DNMT1, DNMT3A and DNMT3B showed a similar pattern of evolution with increasing of Cd concentrations. The determined values decreased from the lowest cadmium treatment to the second highest cadmium treatment only to increase to elevated values for the highest exposure dose.

## **General conclusions.**

1. Terrestrial gastropods can accumulate soil Cu independently from dietary uptake, thus refining our knowledge on the toxicity of copper on soil-dwelling invertebrates.
2. The study confirmed (via Elisa) the presence of 5-mC in *C. aspersus* and that cadmium at high doses can affect the global 5-mC levels in hepatopancreas for this species
3. MS-PCR analysis suggested the lack of promoter methylation for the Cd-MT gene.
4. The candidate gene for Dnmt1 in *C. aspersus* snails appears to be functionally express in the hepatopacres; and Cadmium exposure might affect the activity of this gene.
5. Cadmium cytotoxicity was found to be dependent on dietary Cd dose.
6. CdCl<sub>2</sub> solution induced cytotoxicity on Ht-29 cells (after 24 h stimulation) in a dose-dependent manner and it's characterized by a decrease of cells viability and changes of cell morphology.
7. CuSO<sub>4</sub> exerted a cytotoxic effect at low doses (0.05; 0.2, 1 and 10 µg/mL) by significantly reducing cells viability, whereas at (high dose – 100 µg/mL) a stimulatory effect was observed.