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Toxicity of a fungicide based on a copper oxychloride in the presence of cadmium on snail (*Helix aspersa*) biomarkers

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Abstract

Considerable attention has been paid to the problem of soil contamination by heavy metals. Heavy metal pollutant toxicity to the environment has been gained from tests involving single pollutants. However, multiple metals commonly occur together and they may exert toxicity simultaneously. This work aim to evaluate the synergistic effect of Cadmium and a copper-based fungicide, on biomarker response of a gastropod, *Helix aspersa*. Cd and fungicide were ingested alone or mixed for 15, 30 and 90 days. After each period, Protein, Malonedialdehyde and Glutathione levels were measured in liver while acetylcholine esterase activity was assessed in brain. Obtained results showed that malonedialdehyde levels in the liver of treated snails increased compared to the corresponding control (from +66% to +230%). In addition, the highest increase was seen in snails treated with Cd + Fungicide (+330 and +230% at respectively day 30 and 90). Reduced glutathione content decreased in all treated groups (-34%, -25% and -22% at day 15, 30 and 90 for Cd and fungicide group) and the highest decrease (-43%) was observed at day 90 in snails treated with Cd + Fungicide. In addition, acetylcholine esterase activity also decreased in the three treatment groups (-13% to -63%) and the strongest decrease was seen in snails treated with Cd + Fungicide after 90 days (-63%). These results showed the appearance of oxidative stress, which was much more pronounced when the fungicide was combined with Cd. Based on these results, users of plant protection products are advised to be vigilant and cautious in order to avoid a possible cocktail effect.

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Introduction

The city of Annaba, an important industrial and economic center located in East Algeria, has observed markedly increased levels of pollution in recent decades, most notably heavy metal contamination (Larbaa and Soltani, 2014). In one study demonstrating the extent of this pollution, elevated levels of heavy metals (Cu, Zn, Pb, Cd) were detected in a locally prevalent edible mollusc *Donax trunculus* (Beldi *et al.*, 2006). More generally, various studies had shown that several pollutants considerably contaminated the industrialized coastal regions (Li *et al.*, 2007; Wang *et al.*, 2009). Heavy metals (in particular Cd), fungicides and pesticides represented the main pollutants found. Copper (Cu) is an essential trace metal to all aquatic biota and human beings, but even a small excess amount of it is extremely toxic to aquatic organisms (Banci *et al.*, 2008). It is usually discharged by metal finishing industries, mining process, copper-based products in agriculture as fertilizers, fungicides, herbicides, algaecides and molluscicides into aquatic environment and its deleterious effects on freshwater fauna and flora has been widely studied and well documented (ATSDR, 2004). Among several invertebrates, pulmonate gastropods are known for their ability to accumulate heavy metals from the aquatic environment (Pyatt *et al.*, 2002).The gastropods of the genus *Helix* are easy to find in nature, their acclimatization and manipulation in laboratory conditions present no difficulties. Due to their type of habitat, their low mobility, and their feeding behaviour, snails can provide information on the quality of the environment; in fact, contact with a pollutant can occur through the ingestion of soil and vegetation, contact with the soil, and inhaling air (Sebbio *et al.*, 2014). *Helix aspersa* (Mollusca, Pulmonata, Helicidae; Müller, 1774) is a good bioindicator of metal and organic soil contamination (Scheifler *et al.*, 2002; Gimbert *et al.*, 2006) and is the most abundant and widespread gastropod species in Northeast Algeria (Larba and Soltani 2013).

Therefore, these species are potential sentinels for the bioavailability of contaminants in both soil and air, since they have the capability of accumulating different classes of chemicals. The environmental risk assessment involves the use of biomarkers designed to highlight an early stage of pollution (Jebali *et al.*, 2011, Seraphim *et al.*, 2011). Many biochemical and cellular biomarkers have been studied in aquatic and terrestrial organisms, and particularly in fish and bivalve molluscs (Tlili *et al.*, 2010; Cravot *et al.*, 2012). Acetylcholinesterases (AChE) play an important role in the functioning of the neuromuscular system by preventing continuous muscular contraction (Munari *et al.*, 2014). AChE activity has been proposed as a biomarker of exposure to several chemicals such as organophosphorus compounds (Führer *et al.*, 2012), and also by other contaminants such as metals, synthetic detergents, some components of fuel oils and algal toxins (Oliveira *et al.*, 2007; Tim-Tim *et al.*, 2009). Malondialdehyde, a specific biomarker to oxidative stress, is derived from lipid peroxidation of polyunsaturated fatty acids in cell membranes during oxidative stress (Hamza-Chaffai *et al.*, 2003; Dewes *et al.*, 2006; Oruc *et al.*, 2007) and reduced glutathione (GSH) involved in the antioxidant defence system (Sureda *et al.*, 2006).Traditionally, information on the toxicity of heavy metal pollutants to the environment has been gained from tests involving single pollutants (Qian *et al.*, 2011). So, this study aims to evaluate the synergistic effect of two xenobiotics, Cadmium and a copper-based fungicide,on biomarker response of a bio-indicator gastropod, the snail *Helixaspersa*.

Materials and methods

Adult snails, *Helix aspersa*, (8 ± 1g) were collected from a less polluted area in North East of Algeria and immediately transferred in laboratory. After 14 days of adaptation to laboratory conditions (20 ± 2°C, 18hL/6hO and 80 - 90%hygrometry),snails were divided into four groups (control, Cd-treated, Fungicide-treated and Cd + Fungicide-treated, n=9 for each group), reared in perforated polystyrene boxes (25×13.5×16.5 cm)and fed with fresh lettuce.

Cadmium and Fungicide treatment

Cadmium ($CdCl_2$, Sigma) was used in a solution of 800µg/l of distilled water. The fungicide (2g/l of distilled water) (Vacomyl-plus®) used in this study included two active ingredients: 15% ofmetalaxyl (Residual systemic fungicide) and 35% of copper oxychloride (broad spectrum fungicide). 1ml of $CdCl_2$(0.8µg) or 1ml of fungicide (2mg) or 1ml of mixture ($CdCl_2$ + Fungicide, 1/1, V/V, 0.8µg + 2mg) were dropped on fresh lettuce of respectively Cd, Fungicide, Cd + Fungicide treated groups and 1 ml of distilled water was used for the fourth group as control. These solutions were renewed every week. Lettuce was provided three to four times a week when cleaning boxes.

Experiment was conducted for 15, 30 and 90 days under previously controlled laboratory conditions. After each treatment period, three snails from each group were weighted and sacrificed by decapitation. Liver and brain were removed, weighted and recovered in appropriate buffer. Protein, MDA and GSH content were evaluated in liver and Ach E activity was determined in brain.

Protein analysis

Liver and brain proteins of control and treated snails were quantified according to the method of Bradford (1976), using Blue Brilliant of Coomassie (G250, Merck) as reagent and bovine serum albumin (BSA, Sigma) as standard protein. The absorbance was read at a wavelength of 595 nm.

Malondialdehyde analysis

MDA determination was carried out in control and treated snail livers using the colorimetric method of Draper and Hadley (1990), which is based on the reaction of thiobarbituric acid with MDA. MDA levels were estimated at 532 nm. The concentration of lipid peroxidation in livers was expressed as µg of MDA per mg of proteins.

Glutathione analysis

Content of GSH in control and treated snail livers was quantified according to Weckberger and Cory (1988). Glutathione levels were estimated at 412nm and expressed as µM of glutathione per mg of proteins.

Acetylcholine esterase activity

Activity of AChE in control and treated snail brains was performed using a method described by Ellman *et al.*, (1961) with the use of acetylthiocholine (ASCh) as substrate. The activity rate was measured aschange in absorbance/min at 412 nm (extinction coefficient1. $36x10^4$ $M^{-1}.cm^{-1}$). Activity was expressed as nmol/min/mgprotein.

Statistical tests

Data were expressed as mean ± standard deviation (SD). All statistical calculations were performed with the MINITAB Software (Version 16, Penn State College, PA, USA). Data were tested using two-way analysis of variance (ANOVA) and Tukey' stest. A significant difference was assumed when $p< 0.05$.

Results and discussion

In this study, we had evaluated toxicity of Cd alone, then of a copper-based fungicide and finally of a mixture of Cd + Fungicide on snails during 15, 30 and 90 days (corresponding respectively on acute, sub-acute and chronic toxicity). Content of protein, MDA and GSHin liver and AChE activity in brain were assessed.

Obtained results showed that liver protein concentration (Tab.1) decreased sharply after 15 days of Cd, fungicidal and mixed treatment. This decrease was much more pronounced in snails receiving Cd + Fungicide than in snails receiving Cd or Fungicide alone. However, after 30 and 45 days of treatment, protein levels in treated snails increased compared to the corresponding control. This increase was more important in the group receiving Cd + fungicide. These results suggested that in the beginning of treatment (15 days) there was an oxidative stress, which caused a fall in protein content, because liver proteins were used for biomarker synthesis. After 30 and 90 days of treatment, protein synthesis was

enhanced probably by adaptive mechanism to toxicity. Some studies also showed a significant increase of total protein as a result of chemical stress in various biological models (protists ciliates, rabbits) (Peccini *et al*., 1994; Masaya *et al*., 2002).

The concentrations of MDA, a break down product of the oxidative degradation of cell membrane lipids, increased along the metal gradient. Increased levels of MDA following Cd exposure have been reported in other species (Cossu *et al*., 2000; Guiguere *et al*., 2003; Machreki-Ajmi *et al*.,2008).

Table 1. Protein content (µg/mg of liver) on control and treated snail livers (m±s, n=3; For each treatment period, means followed by the same letter are not significantly different from corresponding control at p<0.05).

Treatment	Treatment duration (days)		
	15	30	90
Control	14.27 ± 0.41**a**	14.76 ± 1.01**a**	14.73 ± 0.96**a**
Cd (0.8 µg)	12.30 ± 1.18**a**	15.81 ± 0.14**a**	16.77 ± 0.77**b**
Fungicide (2 mg)	06.94± 2.87**b**	15.51 ± 1.45**a**	17.20± 0.77**b**
Cd (0.8 µg) + Fungicide (2 mg)	06.70 ± 2.34**b**	18.36 ± 1.63**b**	19.70 ± 0.97**c**

Our results indicated a significant increase of MDA levels (Tab. 2) after cadmium and/or fungicide exposure at only 30 and 90 days of treatment. Moreover, increase of MDA levels was more pronounced with the mixture treatment (Cd + Fungicide) at 30 and 90 days, which indicated lipid peroxidation due to oxidative stress.

An increase in lipid peroxidation was also reported in *Ruditapes decussatus* (Roméo and Gnassia-Barelli, 1997). Similarly, exposure of bivalves to cadmium affected the activation of antioxidant enzymes and increased lipid peroxidation (Geret *et al*., 2002; Khebbeb *et al*., 2010), and cadmium, copper and mercury stimulated lipid peroxidation in the mussel *Mytilus galloprovincialis* (Viarengo *et al*., 1990).

Table 2. Malondialdehyde content (µg/mg of protein) on control and treated snail livers (m±s, n=3; For each treatment period, means followed by the same letter are not significantly different from corresponding control at p<0.05).

Treatment	Treatment duration (days)		
	15	30	90
Control	0.031 ± 0.002a	0.033 ± 0.001a	0.03 ± 0.004a
Cd (0.8 µg)	0.05 ± 0.002b	0.10 ± 0.012b	0.08 ± 0.012b
Fungicide (2 mg)	0.05 ± 0.005b	0.11 ± 0.003b	0.08 ± 0.003b
Cd (0.8 µg) + Fungicide (2 mg)	0.05 ± 0.004b	0.13 ± 0.01c	0.10 ± 0.02c

Glutathione plays a central role in the process of intracellular defence and exists in two forms, oxidized GSSG and reduced GSH. GSH deficiency exposes the cell to a risk of oxidative damage (Sies, 1999), through its ability to bind to heavy metal ions (Adam *et al*., 2005). The glutathione-enzymes include glutathione peroxidase (GSH-Px) and glutathione S-transferase (GST) involved in the detoxification reaction intermediates and oxygen radicals (Yu, 1994). Our results (Tab.3) showed, overall, a decrease of GSH content in all treated groups compared with control group. In addition, the greatest decrease was observed after 90 days in snails treated with Cd + Fungicide. Several studies confirmed these results and helped to better explain the relationship between the decrease in GSH and the level of contamination. This had been observed in mussels, *Crassostrea virginica* (Ringwood, 1999) and in the bivalve, *Unio*

limidus exposed to copper (Cossu *et al.*, 2000). Decreased GSH and increased MDA were alsoreported in *Perna viridis* (Geret *et al.*, 2002) and *Ruditape sdecussatus* (Khebbeb *et al.*, 2010) exposed to cadmium and *Mytilus galloprovincialis* exposed tocopper (Canesi *et al.*, 1998).

Our study revealed decreased levels of AChE activity (Fig.1) in all treated brain snails collected after 15, 30 and 90 days.

Table 3. Glutathione content (µM/mg of protein) on control and treated snail livers (m±s, n=3; For each treatment period, means followed by the same letter are not significantly different from corresponding control at p<0.05).

Treatment	Treatment duration (days)		
	15	30	90
Control	0.84 ± 0.22a	0.84 ± 0.03a	0.83 ± 0.14a
Cd (0.8 µg)	0.55 ± 0.06b	0.65 ± 0.09b	0.62 ± 0.07b
Fungicide (2 mg)	0.55 ± 0.14b	0.61 ± 0.10b	0.64 ± 0.23b
Cd (0.8 µg) + Fungicide (2 mg)	0.57 ± 0.28b	0.73 ± 0.07c	0.47 ± 0.02c

This ACh E activity inhibition was due to the effect of heavy metalpollutant. As with MDA, decrease in ACh Eactivity was more important in snails treated with Cd + Fungicide. Moreover, AChE activity reached its highest inhibition after 90 days of treatment with Cd + Fungicide compared to that observed with Cd or fungicide alone.

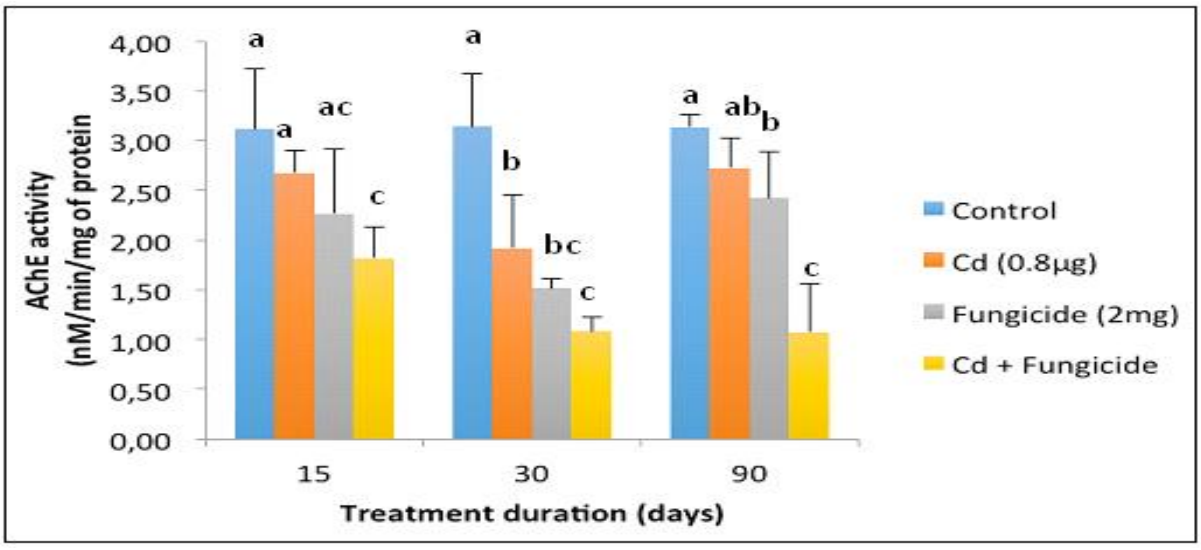

Fig. 1. Acetylcholine esterase activity (nM/min/mg of protein) on control and treated snail brains (m±s, n=3; For each treatment period means followed by the same letter are not significantly different from corresponding control at p<0.05).

Our results were consistent with studies on *Donax trunculus* from the coast of Annaba (Algeria) (Soltani *et al.*, 2012; Bensouda and Soltani-Mazouni, 2014; Mebarki *et al.*, 2015) and on *Lizaaurata* (Bouzenda *et al.*, 2017) who reported an inhibition in AChE and an induction in GST activities in the site of Sidi salem polluted by heavy metals compared to a less polluted site. Similarly, an inhibition of AChE was observed in clams *Ruditapes philippinarum* exposed to neurotoxic pollutants present in the water of agricultural land drainage (Matozzo *et al.*, 2012) and in *Donaxtrunculus* from a multi-contamination site

by the intensive agricultural and industrial activities in comparison with the reference site in the Gulf of Tunis (Tlili *et al.*, 2013).

In conclusion, this study focuses primarily on the "cocktail" effect of two or more pollutants simultaneously. In our work, this effect is real when cadmium is combined with a copper-based fungicide. Users of this fungicide should be vigilant about the rates used on soils that may be contaminated with various metals.

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