

TIME-DEPENDENT HISTOPATHOLOGICAL RESPONSES OF THE DIGESTIVE GLAND IN THE LAND SNAIL *CANTAREUS ASPERSUS* EXPOSED TO CADMIUM, A COPPER-BASED FUNGICIDE, AND THEIR MIXTURE

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Abstract: Agricultural intensification increases the accumulation of chemical mixtures in ecosystems, threatening non-target organisms. This study assessed the cocktail effect of cadmium and the copper-based fungicide Vacomil-Plus on the digestive gland of the land snail *Cantareus aspersus*. Snails were trophically exposed to cadmium (800 µg/L), fungicide (2 g/L), or their 1:1 mixture for 15, 30 and 90 days, and lesions were graded using a semi-quantitative histopathological score. Single contaminants induced relatively limited histopathological alterations at earlier exposure times, whereas severe and extensive tissue damage became evident at 90 days. In contrast, the mixture triggered hyperplasia and apoptosis as early as day 30, followed by marked fibrosis and necrosis at day 90; strongly suggesting synergistic toxicity. These results indicate that single-compound risk assessments underestimate mixture-related hazards in agrosystems.

Keywords: histopathology, heavy metals, fungicide, synergistic toxicity, digestive gland.

INTRODUCTION

The growing contamination of ecosystems by environmental pollutants represents a major threat to biodiversity. Many of these contaminants are able to penetrate exposed organisms at the cellular level, reaching intracellular organelles and altering their structure, abundance, and spatial organization (Fountoucidou *et al.*, 2019; Radwan *et al.*, 2020; Farfar *et al.* 2022). Such structural and ultrastructural disturbances are now widely recognized as sensitive and integrative biomarkers of environmental stress, particularly chemical pollution (Caricato *et al.*, 2018; Farfar *et al.* 2022). Histopathological approaches offer the advantage of providing an accurate assessment of tissue integrity at the individual level, thereby reflecting the overall physiological status of organisms (Snyman *et al.*, 2005; Hödl *et al.*, 2010).

In this context, the terrestrial snail *Cantareus aspersus* is extensively used as a bioindicator species in ecotoxicological studies (Louzon *et al.*, 2020; Colpaert *et al.*, 2021; Garcia *et al.*, 2021; Louzon *et al.*, 2021; Colpaert *et al.*, 2024; Zantis *et al.*, 2024; Colpaert *et al.*, 2025). Particular attention has been paid to its digestive gland, an organ central to metabolism and detoxification and representing a major entry point for xenobiotics (Dutta *et al.*, 2025; Onyena *et al.*, 2025; Noort *et al.*, 2025; Stoian *et al.*, 2025; Vazquez-Ortiz *et al.*, 2025). Physiologically, the digestive gland is responsible for enzyme secretion, nutrient absorption, intracellular digestion by endocytosis, and excretion (Rivera-Pérez *et al.*, 2011; Colpaert *et al.*, 2021; Farfar *et al.*, 2022). Owing to these functions, it is especially vulnerable to heavy metals such as cadmium (Ding, 2025; Madeddu, 2025) and to agricultural fungicides, which may induce

acute, sub-acute, and chronic toxicity (Snyman *et al.*, 2005; Louzon *et al.*, 2020).

The toxic mechanisms of copper-based fungicides and cadmium are largely mediated by oxidative stress (Elia *et al.*, 2021; Esposito *et al.*, 2022; Gnatyshyna *et al.*, 2023; Campoy-Diaz *et al.*, 2023; Ju *et al.*, 2024; Cheng *et al.*, 2025; Fu *et al.*, 2025; Ji *et al.*, 2025; Jing *et al.*, 2025; Majid *et al.*, 2025; Ren *et al.*, 2025). Excessive production of reactive oxygen species (ROS) results in lipid peroxidation, protein oxidation, and DNA damage (Fountoucidou *et al.*, 2019; Ali *et al.*, 2020; Onopiuk *et al.*, 2021; Yang *et al.*, 2022; Campoy-Diaz *et al.*, 2023; Li *et al.*, 2023; 2024; Sun *et al.*, 2024; Boukadida *et al.*, 2025; Fernández *et al.*, 2025), accompanied by enzymatic disturbances and metallothionein overexpression (Mleiki *et al.*, 2015; Colpaert *et al.*, 2021; Farfar *et al.*, 2022; Sun *et al.*, 2024; Boukadida *et al.*, 2025; Ren *et al.*, 2025). These enzymatic markers are widely recognized for assessing toxicity in terrestrial and aquatic fauna (Bebianno & Barreira, 2009; Banaee *et al.* 2019; Radwan *et al.*, 2020; Gazzaniga *et al.*, 2025).

These biochemical disruptions inevitably translate into *tissue-level alterations*, including vacuolization, lysosomal and mitochondrial damage, apoptosis, (Snyman *et al.*, 2005; Nica *et al.*, 2017; Caricato *et al.*, 2018) and impaired tissue regeneration following prolonged exposure (Snyman *et al.*, 2005; Ali *et al.*, 2020; Zheng *et al.*, 2022; Ju *et al.*, 2024; Fu *et al.*, 2025).

Despite extensive documentation of single-compound toxicity, the interactive effects of co-occurring xenobiotics remain insufficiently explored, particularly with respect to time-dependent responses (Fountoucidou *et al.*, 2019; Douafer *et al.*, 2020; Parra *et al.*, 2024; Tang *et al.*, 2024; Du *et al.*, 2025; Fogliano *et al.*, 2025). This

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knowledge gap is critical, as agricultural soils are typically contaminated by complex mixtures rather than isolated pollutants (Gimbert *et al.*, 2008; Zakari *et al.*, 2021; Saud *et al.*, 2023). In northeastern Algeria, including regions such as Annaba and Guelma, elevated concentrations of cadmium and pesticide residues have been reported in agricultural soils and sediments, often associated with industrial discharges, wastewater irrigation, and intensive use of phosphate fertilizers and copper-based fungicides (Louhi *et al.*, 2012; Mouassa *et al.*, 2018; Laribi *et al.*, 2019; Youbi *et al.* 2020; Boufekane *et al.*, 2021; Fekrache *et al.*, 2024). The frequent co-occurrence of these contaminants underscores the need to assess their combined toxicity.

In this context, the primary objective of the present study is to characterize the cocktail effect induced by the interaction between cadmium and the copper-based fungicide Vacomil-Plus in the terrestrial snail *Cantareus aspersus*. By assessing histopathological alterations of the digestive gland under acute, subacute, and chronic exposure scenarios, this study specifically aims to validate the relevance of these tissue injuries as sensitive biomarkers for the monitoring of synergistic pollution in Algerian agrosystems.

MATERIALS AND METHODS

Biological material and sampling site

Adult terrestrial snails (*Cantareus aspersus*; mean weight 8 ± 1 g) were collected from Seraïdi (Edough Massif, northeastern Algeria), a geographically isolated area considered a regional reference site due to its low anthropogenic pressure. Previous geochemical and ecotoxicological studies (Rabei *et al.*, 2018; Douafer *et al.*, 2020) have confirmed that heavy-metal concentrations in this area remain close to natural background levels (Maas *et al.*, 2010; Talbi & Kachi, 2019). Upon arrival at the laboratory, snails were acclimated for two weeks under controlled conditions as described by Vaufleury *et al.* (2006) (20 ± 2 °C; 18 h light/6 h dark photoperiod; 80–90% relative humidity).

Chemicals

Cadmium was administered as cadmium chloride (CdCl_2 , CAS No. 10108-64-2). The commercial fungicide Vacomil-Plus® (VAPCO) was selected for the pesticide treatment. It contains two active ingredients: metalaxyl (15%, CAS No. 57837-19-1) and copper oxychloride (35 %, CAS No. 1332-65-6). Stock solutions were prepared using distilled water.

Experimental design and exposure

Snails were randomly assigned to four experimental groups, each conducted in triplicate. Each replicate consisted of 12 individuals housed in ventilated polypropylene boxes ($40 \times 30 \times 15$ cm). Treatments included:

1. Control: Fed with fresh lettuce rinsed with distilled water.
2. Cadmium (Cd): Fed with lettuce treated with Cd ($800 \mu\text{g/L}$).
3. Fungicide (F): Fed with lettuce treated with Vacomil-Plus (2 g/L).
4. Mixture (Cd+F): Fed with lettuce treated with a mixture of Cd ($800 \mu\text{g/L}$) and Fungicide (2 g/L) (1:1, v/v).

Exposure was trophic, via lettuce leaves surface-treated with 1 mL of the corresponding solution. Uneaten food and waste were removed daily, and stock solutions were renewed weekly to maintain their chemical stability. The experiment lasted 90 days.

Sampling procedure

Sampling was performed after 15, 30, and 90 days. At each time point, three snails per group (one per replicate) were randomly selected. Sampled snails were placed in clean boxes, fasted for 48 h (to empty their digestive tract), then sacrificed by decapitation, and the digestive glands were excised for histological analysis.

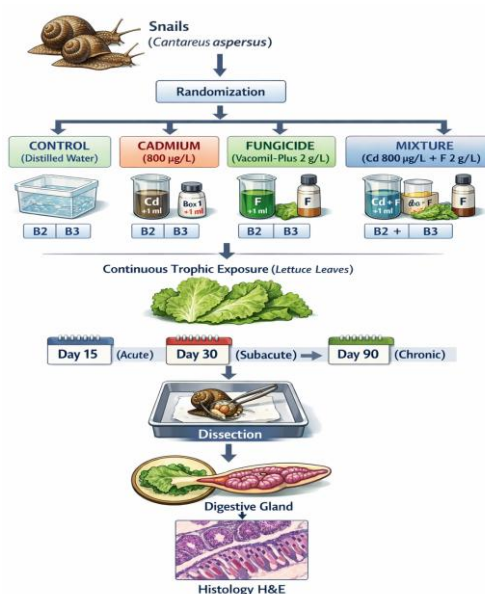


Fig. 1. Experimental protocol and method of exposing land snails *Cantareus aspersus* to cadmium, fungicide, and the cocktail (Cd+F).

Histological analysis

Tissue samples were fixed in 10% neutral buffered formalin. After fixation, samples were dehydrated through a graded ethanol series (70%, 95%, and 100%), then cleared in toluene to facilitate impregnation. Tissues were then embedded in paraffin.

Histological sections (2–3 µm thick) were cut using a microtome (Anglia Scientific), mounted on glass slides, and dried at 37 °C for 48 hours. Sections were deparaffinized and stained with Hematoxylin and Eosin (H&E) for structural analysis (Martoja & Martoja, 1967).

Microscopic observations and photographic documentation were performed using a Motic BA 400 light microscope equipped with a digital camera.

Semi-quantitative histopathological grading

To complement the qualitative description of tissue lesions, a semi-quantitative histopathological scoring system was applied to four lesion categories considered representative of the pathological cascade observed in the digestive gland: excretory/epithelial-cell hyperplasia, intertubular fibrosis, necrosis, and apoptosis. For each experimental group and exposure duration, lesion severity was independently graded by two observers on three non-consecutive sections per gland ($n = 3$ snails per condition) using a four-tier ordinal scale adapted from established histopathological indices (Bernet *et al.*, 1999): 0, lesion absent; 1, mild/focal lesion, affecting less than 25% of the tubulo-acinar units; 2, moderate/multifocal lesion, affecting 25-50% of the tubules; 3, severe/diffuse lesion, affecting more than 50% of the tubules. The modal score attributed by the two observers was retained for each lesion category and used to build the histopathological summary presented in section 3.5.

RESULTS AND DISCUSSION

Histological structure of the control digestive gland

Microscopic examination of the digestive gland in control snails revealed a normal tubulo-acinar architecture (Fig. 2 A, A1). The hepatopancreatic tubules were separated by a thin layer of intertubular connective tissue containing hemolymphatic vessels. The epithelium was simple and columnar, mainly composed of three cell types: Digestive Cells (DC), numerous and elongated; Excretory Cells (EC), characterized by large vacuoles; and Calcium Cells (CC), recognizable by their pyramidal shape and basal localization. The tubular lumen was clear, and nuclei exhibited regular contours with distinct chromatin organization (Fig. 2A1).

Histopathological effects of cadmium (Cd)

Acute exposure (15 days):

The glandular epithelium remained relatively preserved. However, early signs of stress were evident, including a reduction in the number of digestive cells and compensatory hyperplasia (proliferation) of excretory cells. The tubular lumen appeared slightly dilated (Fig. 3B).

Subacute exposure (30 days):

Tissue alterations became more pronounced. Cellular hypertrophy and disruption of the apical membrane were observed. Nuclear abnormalities were frequent, characterized by pyknosis (nuclear condensation) and the onset of karyolysis. Calcium cells appeared enlarged, with prominent spherules. Focal necrosis began to appear within the tubular epithelium (Fig. 3C).

Chronic exposure (90 days):

The tissue exhibited severe degeneration. Epithelial height was markedly reduced (atrophy), and cell walls were disrupted, leading to cell fusion. Notably, the cytoplasm contained large apoptotic bodies (condensed spherical structures), indicating advanced programmed cell death. Necrotic areas were extensive, with complete loss of cellular organization (Fig. 3D).

Histopathological effects of the fungicide (Vacomil-Plus)

Acute exposure (15 days):

Fungicide treatment induced a marked dilation of the tubulo-acinar units (Fig. 4B). The epithelium was generally preserved, although some digestive cells appeared vacuolated.

Subacute exposure (30 days):

Significant structural damage was recorded. Epithelial height decreased, accompanied by hydropic degeneration (cell swelling and cytoplasmic clarification). Marked fibrosis of the intertubular connective tissue was observed (eosinophilic thickening), suggesting an inflammatory response. Diffuse cytoplasmic aspects suggested intracellular accumulation of the xenobiotic (Fig. 4C).

Chronic exposure (90 days):

The degenerative process culminated in severe epithelial flattening, resulting in a quasi-squamous appearance. Fibrosis intensified, surrounding dilated hemolymphatic vessels. Cellular atrophy was associated with extensive nuclear pyknosis and necrosis. Distinct apoptotic figures were also identified (Fig. 4D).

Synergistic effects of the mixture (Cd + fungicide)

Acute exposure (15 days):

Exposure to the contaminant mixture induced more rapid degeneration compared with single-pollutant treatments. Partial epithelial disruption, acinar atrophy, and marked proliferation of excretory cells replacing digestive cells were observed (Fig. 5B).

Subacute exposure (30 days):

The mixture triggered severe alterations earlier than those observed in groups exposed to individual pollutants. Lesions included significant epithelial thinning, cellular hypertrophy, and widening of the tubular lumen. Notably, apoptotic bodies were frequently observed as early as day 30, whereas they appeared only at day 90 in single-contaminant treatments (Fig. 5C).

Chronic exposure (90 days):

The tissue exhibited a critical state of disintegration. The epithelium was completely flattened (severe atrophy), forming a thin continuous line. Intertubular connective tissue showed extensive fibrosis. Nuclear

alterations were extreme, ranging from karyolysis to severe karyorrhexis (nuclear fragmentation). The majority of cells were necrotic, strongly suggests synergistic potentiation toxicity of the mixture (Fig. 5D).

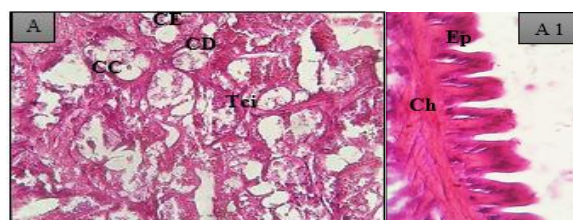


Fig. 2. Control group (A, A1) showing: an intact basement membrane and a regular lumen and a normal tubulo-acinar architecture with a columnar epithelium containing digestive cells (DC), excretory cells (EC) and calcium cells (CC). Digestive cells (DC); calcium cells (CC); excretory cells (EC); intertubular connective tissue (ICT); Epithelium (Ep). Staining: Hematoxylin–Eosin. Magnification : $\times 40$ and $\times 100$

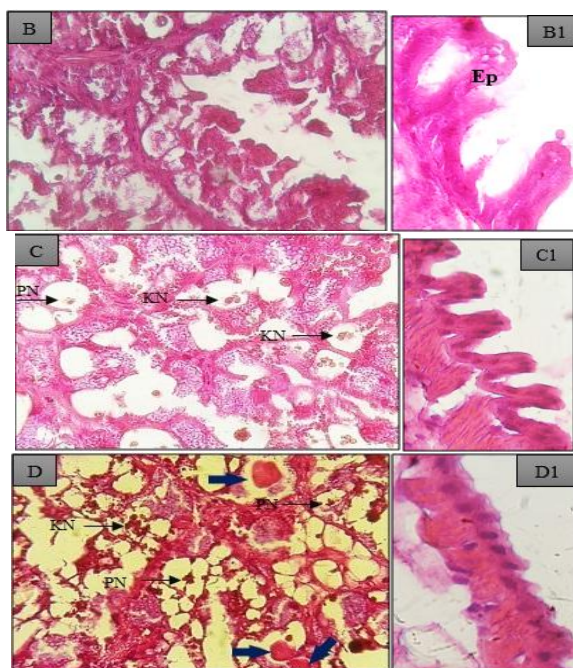


Fig. 3. Cadmium-induced time-dependent histopathological alterations in the digestive gland of *Cantareus aspersus* (800 $\mu\text{g/L}$). Digestive cells (DC); calcium cells (CC); excretory cells (EC); intertubular connective tissue (ICT); Epithelium (Ep) ; Apoptosis  ; Karyolytic nuclei (KN) ; Pyknotic nuclei (PN) ; Necrotic cell (NC).

(B, B1) Acute exposure (15 days) showing slight lumen (L) dilatation and compensatory hyperplasia of excretory cells (EC).

(C, C1) Subacute exposure (30 days) characterized by cellular hypertrophy, nuclear pyknosis (arrowheads), and focal necrosis.

(D, D1) Chronic exposure (90 days) revealing severe epithelial atrophy (flattening), cellular fusion, and the presence of large apoptotic bodies (thick arrows).

Staining: Hematoxylin–Eosin. Magnification : $\times 40$ and $\times 100$

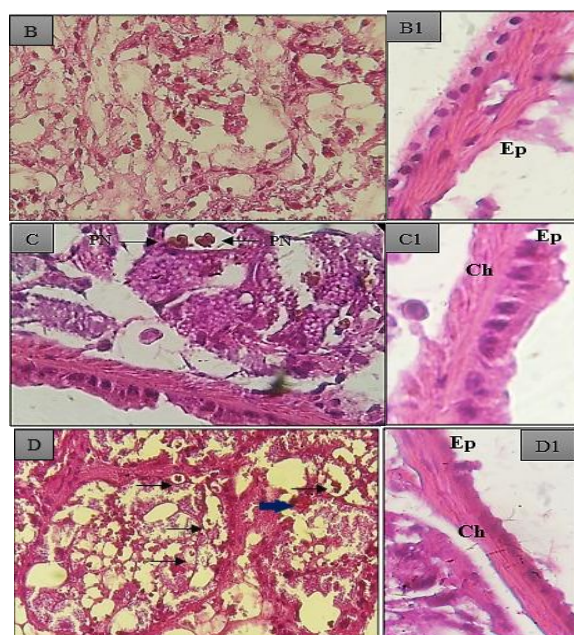


Fig. 4. Time-dependent effects of the copper-based fungicide Vacomil-Plus (2 g/L) on hepatopancreatic tubules of *Cantareus aspersus*. Digestive cells (DC); calcium cells (CC); excretory cells (EC); intertubular connective tissue (ICT); Epithelium (Ep); Apoptosis ➡; Karyolytic nuclei (KN); Pyknotic nuclei (PN); Necrotic cell (NC). (B, B1) Acute exposure (15 days) showing marked acinar dilatation and the onset of vacuolization. (C, C1) Subacute exposure (30 days) characterized by hydronic degeneration (HD) and distinct intertubular fibrosis (F, asterisk), indicative of inflammatory processes. (D, D1) Chronic exposure (90 days) illustrating severe squamous-type atrophy, extensive fibrosis surrounding dilated hemolymphatic vessels, and nuclear pyknosis (Pn).
Staining: Hematoxylin–Eosin. Magnification : $\times 40$ and $\times 100$

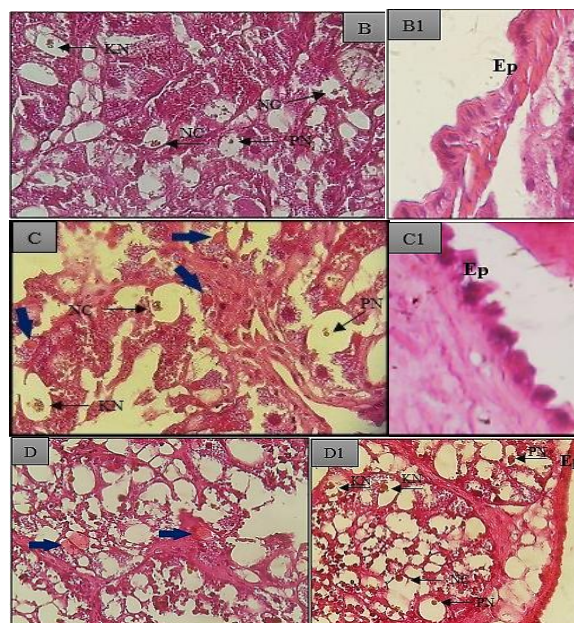


Fig. 5. Synergistic toxicity of the cadmium/fungicide mixture (1:1) in the digestive gland of *Cantareus aspersus*. Digestive cells (DC); calcium cells (CC); excretory cells (EC); intertubular connective tissue (ICT); Epithelium (Ep); Apoptosis ➡; Karyolytic nuclei (KN); Pyknotic nuclei (PN); Necrotic cell (NC). (B, B1) Acute exposure (15 days) showing rapid degeneration, acinar atrophy, and proliferation of excretory cells replacing digestive cells. (C, C1) Subacute exposure (30 days) highlighting the early appearance of apoptotic bodies (AB) and marked cellular hypertrophy compared with single-compound exposures. (D, D1) Chronic exposure (90 days) demonstrating complete tissue disorganization, severe atrophy (flattened epithelium), and extensive karyorrhexis (arrows). Note the accelerated degradation confirming the synergistic effect.
Staining: Hematoxylin–Eosin. Magnification : $\times 40$ and $\times 100$

Semi-quantitative histopathological scoring

Applying the four-tier ordinal scale defined in section 2.6 (0, absent; 1, mild/focal, <25% of tubulo-acinar units; 2, moderate/multifocal, 25–50%; 3, severe/diffuse, >50%) to the qualitative lesions described above yielded the histopathological severity profile summarized in Table 1.

The scores broadly reflected the time-dependent progression described above, with lesion severity generally increasing with prolonged exposure. The mixture showed earlier and/or more pronounced alterations, particularly for apoptosis at day 30 and fibrosis and necrosis at day 90, compared with the individual treatments.

Table 1.

Semi-quantitative histopathological severity scores (0–3) of the digestive gland of *Cantareus aspersus* exposed to cadmium (Cd), fungicide (F) and their mixture (Cd+F) for 15, 30 and 90 days

Exposure	Hyperplasia	Fibrosis	Necrosis	Apoptosis
Control	0	0	0	0
Cd– 15 d	1	0	0	0
Cd– 30 d	0	0	1	0
Cd– 90 d	0	0	3	2
F– 15 d	0	0	0	0
F– 30 d	0	2	0	0
F– 90 d	0	3	2	1
Cd+F – 15 d	2	0	0	0
Cd+F – 30 d	0	0	0	2
Cd+F – 90 d	0	3	3	2

Score: 0, absent; 1, mild/focal (<25% of tubulo-acinar units affected); 2, moderate/multifocal (25–50%); 3, severe/diffuse (>50%). Cd, cadmium; F, fungicide (Vacomil-Plus); Cd+F, binary mixture.

Discussion of the histopathological findings

The digestive gland (Hepatopancreas) of terrestrial gastropods, a true functional analogue of the vertebrate liver, represents the principal site of metabolism and xenobiotic detoxification (Vaufleury *et al.*, 2006; Drăghici *et al.*, 2023; Yu *et al.*, 2023; Ju *et al.*, 2024; Liu *et al.*, 2024; Zhang *et al.*, 2024; Benradia *et al.*, 2025). This biotransformation capacity relies on the activity of key enzymatic systems, notably cytochrome P450 monooxygenases and glutathione S-transferases (Nica *et al.*, 2019; Gnatyshyna *et al.*, 2020; Maroua *et al.*, 2021; Tian *et al.*, 2021; Yu *et al.*, 2023; Garbaly *et al.*, 2025; Stoian *et al.*, 2025; Xu *et al.*, 2025). Consequently, any alteration in its histological architecture reflects a disruption of organismal homeostasis, conferring on this organ a major diagnostic value for the assessment of environmental stressors (Nadji *et al.*, 2024; Baurand *et al.*, 2015; Abdel-Azeem *et al.*, 2023; Colpaert *et al.*, 2025).

The present study, conducted on specimens collected from the reference site of Edough (Seraïdi), confirms the relevance of *Cantareus aspersus* as a sentinel organism. Our results demonstrate that exposure to cadmium, the fungicide Vacomil-Plus, and their mixture induces progressive and severe *cytological alterations*. These findings validate the sensitivity of this biological model for monitoring mixed and complex pollution scenarios (Pagano *et al.*, 2017; Draghici *et al.*, 2019; Besnaci *et al.*, 2022; Yu *et al.*, 2023; Colpaert *et al.*, 2024; Zantis *et al.*, 2024; Colpaert *et al.*, 2025).

Acute phase: epithelial plasticity and adaptive strategy

During the initial exposure phase (15 days), histological examination revealed modifications in cellular dynamics characterized by hyperplasia of

excretory cells and a concomitant reduction in digestive cells, associated with acinar dilation. This response should not be interpreted solely as tissue damage, but rather as an *adaptive defensive strategy* (Brown *et al.*, 2025).

The proliferation of excretory cells suggests an active attempt by the organism to enhance its toxic sequestration capacity. These cells, functionally comparable to rhogocytes, are rich in cytoplasmic granules and respond early to detoxify hemocyanin and exogenous metals (El Mageed *et al.*, 2022; Fadlalla *et al.*, 2020; Colpaert *et al.*, 2024). A similar compensatory proliferation has been reported in other species exposed to metals (Gazzaniga *et al.*, 2025) as well as in *Cantareus aspersus* subjected to metal stress (Mleiki *et al.*, 2015).

Concurrently, the observed cytoplasmic vacuolization (hydropic degeneration) reflects lysosomal overload, as these compartments attempt to sequester xenobiotics and protect vital organelles (Caricato *et al.*, 2018; Brown *et al.*, 2024). The absence of massive necrosis at this stage is consistent with the findings of Mleiki *et al.* (2018), indicating that the epithelium retains functional plasticity and temporarily withstands cytotoxic stress.

Subacute/chronic phase: bioaccumulation and reactive fibrosis

Prolonged exposure (30 to 90 days) marked the exceedance of detoxification capacities. Recent studies show that prolonged exposure to cadmium and fungicides, or a combination of both, can exceed the detoxification capacities of the hepatopancreas (Motta *et al.*, 2024; Gazzaniga *et al.*, 2025). As early as the subacute phase (30 days), cellular hypertrophy and tubular disorganization were observed, corroborating the results of Chabicoovsky *et al.* (2003) and Zaldibar *et al.*

(2007). These changes in cellular proportions are reliable biomarkers of chronic environmental stress (Colpaert *et al.*, 2025).

At 90 days, the most striking alteration in the fungicide and mixture-exposed groups pronounced fibrosis of the intertubular connective tissue associated with severe epithelial atrophy. This interstitial fibrosis represents a chronic inflammatory response aimed at isolating areas damaged by persistent tissue necrosis (Hao *et al.*, 2020). Similar observations have been made in *Pomacea canaliculata*, where heavy metal accumulation leads to comparable structural destruction. (Wu *et al.*, 2015; Caricato *et al.*, 2018; Motta *et al.*, 2024).

Additionally, we observed the accumulation of distinct pigmented particles (yellow for the fungicide, blue for cadmium) as well as black granular deposits. According to Amaral *et al.* (2004) and Kruatrachue *et al.* (2011), these deposits correspond to metallic inclusions sequestered within connective tissue and lysosomes. These findings confirm the strong bioaccumulation capacity of the species, while also suggesting that heavy-metal homeostasis (initially modulated by intracellular granules) is overwhelmed during prolonged exposures (Hödl *et al.*, 2010; Baurand *et al.*, 2015; Motta *et al.*, 2024).

Mechanisms of cell death: apoptosis, necrosis, and ferroptosis

At the cytopathological level, detailed analysis of nuclear and cytoplasmic lesions reveals a complex dynamic of cell death evolving according to the intensity and duration of toxic stress. Initially, the marked presence of spherical condensed bodies and nuclear pyknosis indicates activation of apoptosis. As emphasized by Włostowski *et al.* (2014) and Thit *et al.* (2013, 2015), this programmed cell death mechanism represents a controlled attempt to eliminate cells with irreparable DNA damage caused by oxidative stress (ROS), thereby preventing excessive inflammatory responses.

However, the coexistence (particularly at 90 days) of areas of cell lysis, karyorrhexis, and complete epithelial destruction indicates an inevitable shift toward necrosis. This transition from “clean” to “dirty” cell death reflects the surpassing of cellular tolerance thresholds, triggering an uncontrolled response characterized by plasma membrane rupture, as described in contexts of acute toxicity (Brenner, 2002; Ali *et al.*, 2020).

From a more recent mechanistic perspective, the involvement of ferroptosis must also be considered. According to Zhang *et al.* (2025), this iron-dependent form of regulated cell death, characterized by lethal lipid peroxide accumulation, is highly relevant for explaining the severity of lesions observed in mollusks. Disruption of metal metabolism (cadmium and copper) induced by our treatments likely promotes a pro-oxidant environment conducive to ferroptosis, suggesting that the observed cellular mortality results from a synergistic interaction between antioxidant defence depletion and membrane lipid peroxidation.

The “cocktail” effect: synergy between cadmium and fungicide

The major contribution of this investigation lies in demonstrating the exacerbated toxicity induced by the “Cadmium + Fungicide” cocktail. The mixture-exposed group exhibited clinical signs of apoptosis as early as the subacute stage (30 days), preceding the necrosis and fibrosis that emerged by 90 days, in contrast to single-compound treatments where these lesions appeared later or not at all. This pathological acceleration cannot be attributed to simple additivity, but rather strongly suggests synergistic potentiation, in agreement with recent ecotoxicological data on agricultural co-exposures (Fountoucidou *et al.*, 2019; Gazzaniga *et al.*, 2025).

At the molecular and systemic levels, this deleterious synergy likely arises from the convergence of three major pathological processes. *First, metallothionein saturation* is probable: cadmium, a potent inducer of these detoxification proteins, monopolizes available binding sites. Once this chelation system is overwhelmed, copper from the fungicide Vacomil-Plus remains in a free, highly toxic form (Bebianno & Barreira, 2009). *Second, this chemical toxicity is amplified by deregulation of membrane transporters.* Simultaneous exposure appears to disrupt the expression of cationic metal transporters (notably ZIP and HMA families), facilitating massive and uncontrolled intracellular influx of pollutants (Moraes *et al.*, 2021; Liu *et al.*, 2024). *Finally, the gut–microbiota axis* must be considered as an amplifier of toxicity. As demonstrated by Ding (2025) and Luo *et al.* (2025), heavy metals disrupt microbial homeostasis, reduce the synthesis of protective short-chain fatty acids, and weaken the intestinal barrier, thereby exacerbating local inflammation and promoting systemic translocation of toxicants.

In summary, this study demonstrates that chronic exposure to cadmium and fungicide induces a cascade of irreversible histopathological damage (Besnaci *et al.*, 2022; Yu *et al.*, 2022; Yu *et al.*, 2023), beginning with adaptive hyperplasia and culminating in generalized necrotic collapse. The highlighted *cocktail effect* critically underscores that current risk-assessment frameworks based on single pollutants severely underestimate the real impact of co-exposures typical of modern agroecosystems (Gnatyshyna *et al.*, 2020; Cerezer *et al.*, 2020). These findings advocate for a revision of environmental biosurveillance strategies, integrating refined histological markers alongside advanced molecular indicators, including ferroptosis-related pathways, to achieve a more realistic assessment of ecosystem health.

Perspectives and future directions

Although the present study provides robust histological evidence of toxic synergy between cadmium and the fungicide Vacomil-Plus, it also opens several promising research avenues for a more refined elucidation of the underlying mechanisms.

First, a *molecular-level approach* is essential to substantiate the mechanistic hypotheses raised herein. Future investigations should focus on quantifying the

expression of genes encoding membrane metal transporters, particularly members of the ZIP and HMA families, to determine whether their dysregulation underlies the massive intracellular metal accumulation observed. In addition, the analysis of specific ferroptosis markers, such as glutathione peroxidase 4 (GPX4), would help to ascertain whether this iron-dependent form of regulated cell death represents a primary driver of the necrotic processes detected, in line with recent perspectives proposed by Zhang *et al.* (2025).

Second, *given the trophic exposure route employed*, the interaction between this pollutant cocktail and the gut microbiota of *Cantareus aspersus* warrants particular attention. Metagenomic analyses could elucidate whether metal-induced dysbiosis exacerbates tissue inflammation and intestinal permeability, thereby acting as an amplification factor of toxicity, as recently highlighted by Ding (2025).

Finally, from an applied perspective, *validating these histological biomarkers in natural populations* sampled in situ from Algerian agrosystems -particularly in the Seybouse plain- would represent a decisive step forward. Such an approach would allow calibration of biomarker sensitivity under realistic conditions of mixed environmental contamination and would strengthen national biomonitoring strategies aimed at assessing the ecological risks associated with complex pollutant mixtures.

CONCLUSIONS

This study provides clear histopathological evidence that cadmium and the copper-based fungicide Vacomil-Plus exert time-dependent cytotoxic effects on the digestive gland of *Cantareus aspersus*. Our results reveal a progressive disruption of tissue homeostasis, evolving from compensatory hyperplasia to reactive fibrosis and extensive necrosis.

The major contribution of this work lies in demonstrating a pronounced toxic synergy (cocktail effect): simultaneous exposure precipitates cellular damage (apoptosis/necrosis) as early as 30 days, compared with 90 days for individual pollutants, suggesting rapid saturation of detoxification systems.

These findings confirm that risk assessments based on single pollutants substantially underestimate the true ecological hazards. Accordingly, this study underscores the urgency of revising regulatory toxicity thresholds to account for multi-pollutant interactions and positions histological analysis as an essential early diagnostic tool for monitoring agricultural ecosystems in Algeria.

AUTHOR CONTRIBUTIONS

I, the corresponding author, Khadidja Farfar, confirm that the descriptions are accurate and agreed by all authors. In addition, I certify that we have participated sufficiently in the intellectual content, conception, and design and data collection/experimental work of this research or the analysis and interpretation of the data, as well as the writing of the manuscript: resources, A.Y., K.F. and F.I.; funding acquisition, M.R.D. and B.H.; writing - original draft, K.F.; writing - review & editing, A.Y.; visualization, A.Y.; formal analysis, A.Y.; conceptualization, K.F.; investigation, F.I.; supervision,

M.R.D.; validation, M.R.D. and B.H.; project administration, B.H.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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