# CELLULAR BIOMARKERS OF EXPOSURE TO THE FUNGICIDE COPPER OXYCHLORIDE, IN THE COMMON GARDEN SNAIL *HELIX ASPERSA*, IN WESTERN CAPE VINEYARDS

BY

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DISSERTATION
PRESENTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE
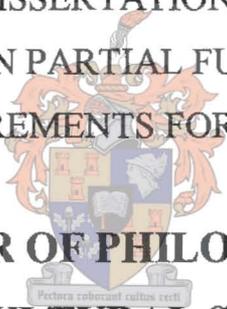
DOCTOR OF PHILOSOPHY
IN AGRICULTURAL SCIENCES
IN ZOOLOGY

FACULTY OF AGRICULTURAL SCIENCES
UNIVERSITY OF STELLENBOSCH

MARCH 2001

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: _____   Date: 23 - 01 - 2001

ABSTRACT

Copper oxychloride ($Cu_2Cl(OH)_3$) is a broad-spectrum fungicide, intensively sprayed in many South African vineyards and orchards. It is necessary to find accurate and effective methods of monitoring the effects of this fungicide on the biota of the agricultural environment. The use of biomarkers may be a possible method to employ for this purpose.

This study investigated the effects of copper, as a result of copper oxychloride exposure, on the biology of the common garden snail *Helix aspersa*, as well as a number of cellular responses to exposure to the fungicide. The possible use of these responses as biomarkers was also investigated.

Two groups of snails were exposed to 80 and 240 µg g^{-1} copper oxychloride respectively, for six weeks. A third group served as control. On a weekly basis, body mass, number of eggs produced, neutral red retention (NNR) times of haemocytic lysosomes, and body copper concentrations were determined for each individual. At the end of the experiment, the digestive glands, ovotestes and hermaphrodite ducts of a number of snails were prepared for histological analysis. The following parameters were investigated: tubule area, epithelium height and area in the digestive gland, spermatozoan area in the vesicula seminalis and ovotestis, as well as oocyte numbers in the ovotestis. To test the validity of the laboratory results, a field survey was conducted. Snails were collected from an uncontaminated vineyard and on two occasions from a contaminated vineyard in the Western Cape. The same cellular responses were investigated as in the laboratory study.

The results showed that growth, egg production and hatching success in *Helix aspersa* were affected by experimental exposure to copper oxychloride. In both the laboratory study and field survey, copper in the body of *H. aspersa* was shown to be compartmentalized and the digestive gland was the most important site of copper accumulation. NNR times of haemocytic lysosomes were shown to be affected by copper oxychloride exposure, already during the first week of exposure. A time evolution of copper accumulation and lysosomal damage existed. Epithelium height and area of digestive gland tubules, and spermatozoan and oocyte densities in the ovotestis, were also affected by copper oxychloride exposure and the concomitant copper burdens in the respective organs. Through the field survey it was ascertained that these histopathological changes were largely dependent on exposure time.

It was concluded that lysosomal response of *H. aspersa* haemocytes, as measured by the NNR time assay, could be considered a useful biomarker of copper oxychloride exposure, since it provides an early warning of stress induced by this fungicide. Changes in digestive gland epithelium cells, and gametes in the ovotestis, can also possibly serve as biomarkers of copper oxychloride exposure. However, these can not serve as an early warning. All of the cellular responses identified in the present study can be used in combination with other cellular and physiological parameters and toxicological endpoints in order to improve the reliability and accuracy of interpretations regarding cause and effect.

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UITTREKSEL

Koperoksichloried ($Cu_2Cl(OH)_3$) is 'n breë-spektrum fungisied wat intensief gespuit word in talle Suid-Afrikaanse wingerde en boorde. Dit is noodsaaklik om akkurate en effektiewe metodes te vind om die effekte van hierdie fungisied op die biota van die landbou omgewing te monitor. Biomerkers kan moontlik vir hierdie doel gebruik word.

Hierdie studie het die effek van koper, as gevolg van koperoksichloriedblootstelling, op die biologie van die tuinslak *Helix aspersa* ondersoek, sowel as 'n aantal sellulêre response op blootstelling aan die fungisied in hierdie dier. Die moontlike gebruik van hierdie response as biomerkers is ook ondersoek.

Twee groepe slakke is vir ses weke aan onderskeidelik 80 en 240 μg g^{-1} koperoksichloried blootgestel. 'n Derde groep het as kontrole gedien. Liggaamsmassa, aantal eiers gelê, neutraal-rooi retensietyd (NNR tyd) van hemositiese lisosome en liggaamskoperkonsentrasies is weekliks vir elke individu bepaal. Ná die eksperiment is die spysverteringskliere, ovotestes en hermafrodietbuise van 'n aantal slakke vir histologiese analise voorberei. Die volgende parameters is ondersoek: buisoppervlak, epiteelhoogte- en oppervlak in die spysverteringsklier, spermatosoönoppervlak in die vesicula seminalis en ovotestis, asook die aantal oösiete in die ovotestis. Om die laboratoriumresultate te staaf, is 'n veldstudie geloods. Slakke is versamel in 'n ongekontamineerde wingerd en tydens twee geleenthede in 'n gekontamineerde wingerd in die Wes-Kaap. Dieselfde sellulêre response as in die laboratoriumstudie is ondersoek.

Die resultate het getoon dat groei, eierproduksie en uitbroeisukses in *Helix aspersa* deur eksperimentele koperoksicloriedblootstelling geaffekteer is. In die laboratoriumstudie sowel as die veldstudie, het koper in die liggaam van *H. aspersa* gekompartementaliseer en was die spysverteringsklier die belangrikste plek vir koperakkumulasie. Daar is gevind dat NNR tye van hemositiese lisosome reeds tydens die eerste week van blootstelling geaffekteer is. 'n Tydevolusie van koperakkumulasie en lisosomale skade is gevind. Epiteelhoogte- en oppervlak in die spysverteringsklierbuise, sowel as spermatosoön- en oösietdigthede in die ovotestis, is ook geaffekteer deur koperoksichloriedblootstelling en die ooreenkomstige hoë kopervlakke in die betrokke organe. Deur

middel van die veldstudie is vasgestel dat hierdie histopatologiese veranderinge grootliks van die blootstellingsperiode afhanklik was.

Die gevolgtrekking was dat lisosomale respons van *H. aspersa* hemosiete, soos bepaal deur die NNR tyd tegniek, as 'n bruikbare biomerker van koperoksichloriedblootstellling beskou kan word, aangesien dit 'n vroeë waarskuwing van stres, geïndusseer deur die fungisied, kan bied. Veranderinge in spysverteringsklierepiteelselle en gamete in die ovotestis kan ook mootlik dien as biomerkers van koperoksichloriedblootstellling. Hierdie veranderinge kan egter nie as vroeë waarskuwing dien nie. Alle sellulêre response wat tydens die huidige studie geidentifiseer is kan in kombinasie met ander sellulêre en fisiologiese parameters en toksikologiese eindpunte gebruik word om die betroubaarheid en akkuraatheid van interpretasies aangaande oorsaak en gevolg te verhoog.

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DEDICATION

"I am the vine; you are the branches......apart from me you can do nothing. "

John 15:5

I dedicate this thesis to my Heavenly Father, without whom nothing is possible, and to the memory of Mr. Dion Sadie, whom I greatly admired for his integrity, dedication and enthusiasm.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following:

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INTRODUCTION

In agriculture throughout the world, great emphasis is placed on the protection of crops through the control of plant diseases. Over the past century, according to Vyas (1988), the development of fungicide protectants has progressed from simple fungicides acting as surface protectants (e.g. elemental sulfur, copper compounds and dithiocarbamates), to systemic fungicides (e.g. acetamides and organophosphates), to non-fungitoxic systemic compounds that suppress fungal pathogenicity or accentuate natural plant resistance systems (e.g. acylalanines and triazoles). Despite this, most of the older surface protectants (e.g. those containing heavy metals) are currently still being used extensively.

The copper-containing fungicides are amongst the most commonly used world-wide: e.g., McEwen & Stephenson (1979) stated that, by 1975, about 10 million lbs. of copper sulphate and related copper fungicides were still in use in the United States of America, whereas Teisseirre et al. (1998) and Lombardi et al. (2000) reported that copper-containing compounds (such as copper oxychloride), is still generously sprayed in French vineyards and on Brazilian crops respectively. Similarly, in Africa, the fungicide copper oxychloride, especially, is widely used in agriculture in countries such as Zambia (Javaid 1998), Kenya (Mwanthi 1998) and South Africa (Krause et al. 1996). Copper oxychloride ($Cu_2Cl(OH)_3$), also known as blue copper, Bordeaux A, Bordeaux Z and Miedzian, among others (Richardson & Gangolli 1993), is a broad-spectrum fungicide applied to the foliage of a wide variety of fruit and vegetable plants (Vyas 1988). In South African crop fields, orchards and vineyards, it is sprayed intensively at a rate of $1.25 - 7.5$ kg.ha^{-1} with up to nine applications per season, in the fight against numerous plant diseases such as downy and powdery mildew, anthracnose, leaf spot and early blight (Krause et al. 1996). In the Western and Eastern Cape regions of South Africa alone, the amount of copper-containing fungicides applied in vineyards during 1989 was in the order of 104 700 kg (London & Myers 1995).

It is well known that copper fungicides not only affect the target fungus but are also toxic to other non-target plants, e.g. copper oxychloride is toxic to the development of tomatoes and delays the ripening of coffee. Copper sprays also generally increase the susceptibility of plants to frost (Vyas 1988). In contrast, little is known about the effects of copper fungicides on animals. It is however clear from the literature that copper oxychloride may be either toxic or beneficial, depending on the dose and animal group involved. Richardson & Gangolli (1993) reported that this fungicide has been shown to cause gastroenteritis and to damage the capillaries and digestive tract mucous

membranes of mammals (unspecified) fed copper oxychloride. These animals also exhibited signs of heavy metal poisoning and loss of water and electrolytes. On the other hand, male commercial broiler strain chickens showed a significant increase in growth when fed copper oxychloride at 125 mg/kg diet and it is therefore considered as a valid option for copper supplementation in the USA (Ewing et al. 1998).

Copper is of course an essential trace element in animals, since it is contained in many proteins, such as oxygen-binding haemocyanin, cytochrome oxidase, tyrosinase and lactase (Piscator 1979; Moore & Ramamoorthy 1984 and Galvin 1996). The toxicity of copper to animals is also well documented. According to Moore & Ramamoorthy (1984), exposure to copper may cause loss of cellular adhesion in the gills of aquatic animals, cell necrosis, retarded growth and lowered rate of reproduction and egg survival. De Boeck et al. (1997) also demonstrated retarded growth in the common carp, *Cyprinus carpio*. Aloj Totaro et al. (1986) found that mitochondria degenerated in *Torpedo marmorata*, after exposure to copper. Aquatic as well as terrestrial invertebrates have been the subjects of numerous ecotoxicological studies on copper. For example, the damaging effects of this metal on fitness of the snail *Helix aspersa* (Laskowski & Hopkin 1996b), the hepatopancreas cells of the shrimp *Metapenaeus dobsoni* (Manisseri & Menon 1996), reproduction and sperm morphology of the earthworm *Eisenia fetida* (Reinecke & Reinecke 1996; 1997), reproduction of the terrestrial worm *Enchytraeus crypticus* (Posthuma et al. 1997), survival and reproduction of the springtail *Folsomia fimetaria* (Scott-Fordsmand et al. 1997), and fitness of the scallop *Argopecten purpuratus* (Troncoso et al. 2000) have been studied recently.

Despite the fact that copper oxychloride is so extensively used worldwide, it is clear that the specific effects thereof on the environment have not been carefully studied and documented. It has become necessary to find accurate and effective methods of monitoring the effects of this fungicide on the non-target biota of the agricultural environment. Currently, most ecological risk assessments are, to a great extent, based on chemical residue analysis of soil, sediments and surface waters (Dickerson et al. 1994). It is however well known that such information does not provide a good measure of the bioavailability of a chemical to animals (Arjonilla et al. 1994 and Dickerson et al. 1994). For example, in the case of copper in soils, part of the metal is insoluble and a portion of the soluble part is bound to ions such as Cl^-, SO_4^{-2} etc (Sauvé et al. 1997). The free metal activity therefore represents a very small fraction of the system's total metal burden (Sauvé et al. 1998). Also, the partitioning of copper, its solubility and its speciation in solution are dependent on several factors, including pH and organic matter. All these factors need to be taken into account when evaluating the bioavailability and toxicity of metals to animals (Sauvé et al. 1997; 1998 and Ge et

al. 2000). Therefore, the effects of metals and other chemicals on animals may be more accurately predicted by measuring chemical residues in animal tissues but, according to Dickerson et al. (1994), this type of assessment is more effective with metals and chemicals that bioaccumulate, rather than with those that are readily metabolized.

An alternative tool in environmental impact assessment is the biomarker (Peakall & Walker 1994; Weeks 1995 and Van Gestel & Van Brummelen 1996). According to the latter authors, a biomarker may be defined as a biological response to an environmental chemical at the sub-individual level, measured inside an animal or its products (e.g. hair or faeces). This should be an abnormal response that cannot be detected in the intact animal. Behavioural effects are therefore excluded and measurements are limited to biochemical, physiological and morphological responses. These authors also stated that a biomarker may serve as an early warning of pollutant-induced stress, i.e. it can be measured shortly after exposure, long before effects at the population and ecosystem levels can be detected. It should also be able to be measured at concentrations below those causing irreversible effects. However, the authors stressed that, although biomarkers can identify a deviation from health at the individual level, it is still doubtful whether they can be used to predict effects at higher levels of biological organization. This view is shared by authors such as Weeks (1995) and Fairbrother et al. (1998). Weeks (1995) also pointed out that, in biomarker studies, it is difficult to distinguish between measured alterations that are adaptive and reversible and those that are pathological and irreversible. This author suggested that the problem might be resolved by using a suite of different types of biomarkers in conjunction, since it is rare for one marker to be sufficiently reliable and specific.

Invertebrates, especially, have proved to be effective models in biomarker studies of exposure to pesticides and heavy metals. A wide range of biochemical, tissue and cellular biomarkers have been studied in groups such as annelids (Cikutovic et al. 1993; Davies et al. 1995; Reinecke et al. 1995; Reinecke & Reinecke 1996; 1997; Marcano et al. 1997 and Yongcan et al. 1998), echinoderms (Young & Nelson 1974; Castagna et al. 1981 and Au et al. 2000) and crustaceans (Manisseri & Menon 1995 and Rtal et al. 1996). In the case of molluscs, researchers have mainly concentrated on marine species (Viarengo et al. 1981a; 1985a; 1989; Lowe & Pipe 1986; Gould et al. 1988; Lowe 1988; Lowe & Clarke 1989; Vega et al. 1989; Marigomez et al. 1990; Cajaraville et al. 1991; Bebianno et al. 1992; Regoli 1992; Rubio et al. 1993; Etxeberria et al. 1994; 1995; Bauer et al. 1995; Cajaraville et al. 1996; Najimi et al. 1997; Soto & Marigomez 1997; Hamza-Chaffai et al. 1998; Blasco & Puppo 1999; Domouhtsidou & Dimitriadis 2000; Pavlica et al. 2000 and Wedderburn et al. 2000), and to a lesser extent on freshwater species (Bianchi et al. 1993; Rambabu

& Rao 1994; Baturo & Lagadic 1996; Jonnalagadda & Rao 1996; Doyotte et al. 1997 and Elangovan et al. 2000). Many of these authors used indices such as epithelial thickness, luminal radius, lysosomal density and size, cytoplasmic dense bodies, accumulations of lipids, and numbers and sizes of reproductive cells, to illustrate the negative effects of heavy metals and other xenobiotics on specifically, the reproductive organs and digestive gland of molluscs (Lowe & Pipe 1986; Gould et al. 1988; Lowe 1988; Lowe & Clarke 1989; Vega et al. 1989; Marigomez et al. 1990; Cajaraville et al. 1991; Regoli 1992; Rubio et al. 1993; Etxeberria et al. 1994; 1995; Bauer et al. 1995; Jonnalagadda & Rao 1996 and Wedderburn et al. 2000).

In general, very few biomarker studies, using terrestrial molluscs as models, have been conducted. The effects of pesticides and heavy metals on digestive tract and digestive gland cells of the slugs *Deroceras reticulatum* (Triebskorn 1989; Bourne et al. 1991 and Triebskorn & Köhler 1996), and *Arion ater* (Marigomez et al. 1998) have been studied to some extent, using indices such as digestive epithelial thickness, luminal radius and changes in cell organelle structure. Although Dallinger et al. (1993) stated that terrestrial snails are useful in ecotoxicological studies, since they represent biological sinks for various metals, these animals have been greatly neglected as models in biomarker studies. Russell et al. (1981) and Gomot-De Vaufleury & Kerhoas (2000) studied the effects of cadmium on the reproductive system of *Helix aspersa*, whilst Ireland & Marigomez (1992) studied histological changes in digestive tubule epithelium of *Achatina fulica*, after exposure to heavy metals. Hopkin (1989), Simkiss & Taylor (1981) and Berger et al. (1995) investigated the production of metallothioneins and other metal binding proteins in *Helix aspersa* and *H. pomatia*, as a result of heavy metal exposure.

On the subcellular level specifically, lysosomes have become increasingly popular in biomarker studies, since they have the ability to bioconcentrate a wide range of environmental contaminants, including lipophilic xenobiotics and metals (Moore 1990). George et al. (1978) and McIntosh & Robinson (1999) demonstrated the ability of molluscan haemocytes to sequester heavy metals such as copper and cadmium. Various authors have illustrated the effects of such bioconcentrated contaminants on the structure of molluscan lysosomes, notably Cajaraville et al. (1991; 1995), Regoli (1992), Etxeberria et al. (1994; 1995), Krishnakumar et al. (1994) Donval & Plana (1997) and Giamberini & Pihan (1997).

The use of lysosomal stability has been proposed by Allison & Young (1969) and Bayne et al. (1979) to provide an index of cellular condition that correlates significantly with physiological condition. According to Moore (1990) the lysosomal membrane permeability is increased as a result

of accumulated contaminants and this leads to a loss of the acid hydrolase content into the cytosol, eventually causing cellular damage. Ward (1990), Regoli (1992), Hole et al. (1993), Lin & Steichen (1994), Krishnakumar et al. (1994) and Regoli et al. (1998) have all demonstrated this membrane destabilization of molluscan lysosomes, resulting from environmental factors and exposure to xenobiotics, using various indices, e.g. the lysosome acid labilization period, the cytochemical ß-N-acetylhexosaminidase (NAH) latency period, and free and membrane-bound enzyme activity.

Lowe et al. (1995a) proposed that the efflux of lysosomal contents into the cytosol can be measured by a neutral red retention (NNR) time assay. According to Seglen (1983), the efficiency of neutral red retention in the lysosome is dependent on the efficiency of membrane bound proton pumps. Svendsen & Weeks (1995) stated that any event impairing this proton pump system will result in a lowered neutral red retention time. The NRR time assay reflects a normal physiological process that has become compromised following damage to the membranes (Lowe et al. 1995a) and can, according to Svendsen & Weeks (1995), serve as an early warning system, since it can indicate contamination even at low levels.

The neutral red retention time technique has been used for coelomocytic lysosomes of the earthworms *Eisenia andrei* and *Eisenia veneta*, exposed to organophosphates, polycyclic aromatic hydrocarbons (PAH's) and nickel (Eason et al. 1999 and Scott-Fordsmand et al. 1998). It has also been used extensively for haemocytic and digestive gland cell lysosomes of the mussels *Mytilis edulis* (Lowe & Pipe 1994; Lowe et al. 1995a; Grundy et al. 1996 and Wedderburn et al. 2000), and *M. galloprovincialis* (Lowe et al. 1995b), exposed to PAH's, organochlorines, mercury, cobalt and effluents, as well as of the oysters *Ostrea edulis* and *Crassostrea virginica*, exposed to various natural stressors such as water temperature and salinity (Hauton et al. 1998 and Ringwood et al. 1998). A number of researchers have used this assay as biomarker of, specifically, copper exposure: Weeks & Svendsen (1996), Svendsen & Weeks (1997), Harreus et al. (1997), Reinecke & Reinecke (1999) and Scott-Fordsmand et al. (2000b) used the technique on coelomocytic lysosomes of the oligochaetes *Lumbricus rubellus*, *Eisenia andrei*, *Aporrectodea rosea* and *Eisenia fetida* respectively. Svendsen & Weeks (1995) and Nicholson (1999) used haemocytic lysosomes of the freshwater snail *Viviparus contectus* and the mussel *Perna viridis* respectively, and Ringwood et al. (1998) digestive gland cell lysosomes of the oyster *Crassostrea virginica*, as models to test for copper exposure. Only one study has been done on the use of the neutral red retention assay as biomarker of exposure to copper oxychloride: Helling et al. (2000) used the assay on coelomocytic lysosomes of the earthworm *Eisenia fetida*.

Between 1997 and 2000, a study was undertaken in the Western Cape, South Africa, to determine the use of the garden snail *Helix aspersa* (Müller) as model in cellular biomarker studies of stress due to exposure to the fungicide copper oxychloride. This species was chosen for the study since it occurs commonly in vineyards and orchards in South Africa, where it is often considered to be a pest causing severe crop losses (general opinion among Western Cape farmers). Various *Helix* species have also been successfully reared in the laboratory by a number of researchers, e.g. Dan & Bailey 1982; Berger et al. 1993; Dallinger et al. 1993; Schuytema et al. 1994; Desbuquois & Madec 1998; Jess & Marks 1998 and Lazaridou-Dimitriadou et al. 1998.

Helix aspersa was also chosen for this study since it fulfills a number of requirements for qualification as bioindicator and/or biomonitor species. Firstly, its common occurrence may possibly render it a useful sentinel of prevailing environmental conditions resulting from the use of agrochemicals such as copper oxychloride. Secondly, much is known about the general biology and physiology of this species (Bailey 1975; 1983; Dan & Bailey 1982; Tompa 1984; Sanz Sampelayo et al. 1991; Almedros & Porcel 1992a; 1992b; Madec & Daguzan 1993; Iglesias et al. 1996; Desbuquois & Madec 1998; Elmslie 1998; Jess & Marks 1998; Lazaridou-Dimitriadou et al. 1998 and Iglesias & Castillejo 1999), as well as of other related species, namely *Helix lucorum* (Staikou et al. 1988 and Dimitriadis & Hondros 1992) and *H. pomatia* (Lind 1973, cited by Tompa 1984; Pollard 1975 and Trembacz & Jezewska 1993). Thirdly, the uptake and accumulation of various heavy metals (also copper) have been extensively studied in *Helix aspersa* (Coughtrey & Martin 1977; Simkiss & Taylor 1981; Berger et a. 1995; Beeby & Richmond 1987; 1991; Laskowski & Hopkin 1996a; 1996b; Gomot 1997; Gomot & Pihan 1997 and Gomot-De Vaufleury 2000) and in *Helix pomatia* (Moser & Wieser 1979; Berger et al. 1993; Dallinger et al. 1993 and Rabitsch 1996). The subject of possible tolerance and adaptation to heavy metals, specifically lead, in *H. aspersa*, have been touched on by Beeby & Richmond (1987; 1991). Finally, the histochemistry and cytology of the digestive glands of both *Helix aspersa* and *H. pomatia* have been described (Abolins-Krogis 1960 and Sumner 1965; 1966), which provides a basis for identification of cellular changes in this organ.

Very little is however known about the accumulation of pesticides in *Helix*. Schuytema et al. (1994) investigated the toxicity of 12 pesticides (e.g. paraquat, atrazine and aminocarb) to *H. aspersa*, but, to date, no information is available on the uptake of, and responses to the fungicide copper oxychloride, in this species.

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AIMS OF THE STUDY

General aim:

The aim of the study was to investigate the effects of copper, as a result of copper oxychloride exposure, on the biology of the common garden snail *Helix aspersa*, and to identify a number of cellular responses to experimental and field exposure to the fungicide, in this animal. Also, to evaluate the possible use of these responses as biomarkers of copper oxychloride exposure.

Specific aims:

1. To determine the LC_{50} for copper oxychloride for *Helix aspersa*.

2. To investigate the effects of experimental copper oxychloride exposure on food consumption, body mass, egg production and hatching success in this species.

3. To investigate the accumulation and distribution of copper in the body of *H. aspersa*.

4. To examine the following cellular responses of copper oxychloride exposure in this species:

   a) Changes in neutral red retention times of haemocytic lysosomes

   b) Changes in total tubule area, and epithelium area and height, in the digestive gland

   c) Changes in spermatozoan density in the vesicula seminalis and ovotestis

   d) Changes in oocyte numbers in the ovotestis

5. To correlate the above-mentioned responses with body copper concentrations.

6. To test the validity of the laboratory results through a field survey.

7. To discuss and compare the various cellular responses identified, and to evaluate their usefulness as possible biomarkers.

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MATERIALS AND METHODS

1. Study animal

The classification of the common garden snail *Helix aspersa* (Müller), according to Barnes (1987) and Godan (1983), is as follows:

Phylum:	Mollusca
Class:	Gastropoda
Subclass:	Pulmonata
Order:	Stylommatophora
Family:	Helicidae
Genus:	*Helix*
Species:	*aspersa*

The species is not endemic to South Africa, but originates from the western Meditteranean region (Madec & Daguzan 1993). It has been spread world wide and is considered a pest throughout its distribution (Godan 1983). The annual activity rhythm and life cycle of *Helix aspersa* present a high degree of flexibility, and have been shown to vary with seasons and geographic location (Madec & Daguzan 1993 and Iglesias et al. 1996). Although *H. aspersa* is considered a generalist herbivore, its diet has also been shown to vary seasonally. The age of the individual and nutritional value of the food also play important roles in the diet (Iglesias & Castillejo 1999). The species has a close relationship with soil, since its eggs are laid here, and since soil provides organic matter for normal snail growth (Elmslie 1998).

Despite the fact that *H. aspersa* is considered a pest in agriculture and horticulture, it plays an important role in the balance of nature: in the production of humus, the disposal of plant and animal waste products, and the control of fungi, algae and lichens. It is also an important prey item in the diets of many invertebrates and vertebrates, and considered a delicacy by humans in Westernized countries (Godan 1983).

2. Laboratory rearing and keeping of snails

Helix aspersa were originally collected from an uncontaminated vineyard on Helderberg Mountain, Western Cape, and were reared in aerated plastic containers on a clean soil substrate. Animals were kept in a temperature-controlled chamber, at 16 – 18 °C, 70 ± 2 % humidity and a constant photoperiod of 14:10 (light:dark). Fresh water was provided in the containers, and the animals, as well as the interiors of the containers, were moistened daily with a water bottle fitted with a spray nozzle. During the time of rearing of the snails, they were fed on fresh lettuce. The type of food given during the experiments was introduced two weeks before the experiments commenced. This was in the form of a mixture of Agar, distilled water and a commercial fruit and vegetable juice mixture, as described by Berger et al. (1993). A mixture of 10 g Agar and 250 ml distilled water was slowly brought to boil on a stirrer-hotplate. This mixture was then diluted with 250 ml commercial orange and carrot juice, after which the mixture was poured into plastic Petri dishes and allowed to cool and set at 5 °C for 30 minutes.

3. Range-finding tests and acute toxicity test (LC_{50} test)

3. 1 Range-finding tests

In order to determine the concentrations of copper oxychloride to be used in the LC_{50} test, *Helix aspersa* were exposed to a range of concentrations beforehand. Forty-eight individuals of equal size were starved for a period of two days, whereupon they were divided into two main groups of 24 animals each. These two groups were then exposed to high concentrations of 10 000 and 5000 $\mu g.g^{-1}$ copper oxychloride respectively, for a period of 72 hours. Animals were exposed through their food, in the form of contaminated Agar plates: the food was prepared according to the method described in section 2, and the fungicide was added before the solution was allowed to set. Snails were placed directly on the Agar plates, so that exposure also took place via the skin surface. The number of dead individuals were noted daily. After 72 hours, the process was repeated with a larger test population of uncontaminated animals, and for the following concentrations: 2500, 1250, 625 and 312.5 $\mu g.g^{-1}$ copper oxychloride.

The following concentrations of copper oxychloride were selected for the 14-day LC_{50} test: 2000, 1000, 500 and 250 $\mu g.g^{-1}$. Twenty-four animals, divided into six groups of four, were used per concentration. These were kept in aerated plastic containers, at constant conditions. Contaminated Agar plates were once again used, and exposure was therefore through the alimentary canal and skin surface. Animals were exposed for a period of 14 days, during which fresh water and food (Agar) were given every three days. Dead individuals were removed daily and the number of live animals noted. At the end of the experiment, the LC_{50} for copper oxychloride for *Helix aspersa* was determined with the Trimmed Spearman-Karber (TSK) Program Version 1.5.

4. Sublethal toxicity test

Healthy, uncontaminated snails of equal size and age were used as test animals. These were all starved for two days, and weighed on a Sartorius balance before the experiments commenced. Animals were kept under similar constant conditions as described in section 2. Two consecutive six-week exposure experiments were performed, using a total of 63 snails in the first experiment and 126 in the second. In both experiments the test animals were divided into three groups. In the first experiment three replicates per group were used, and in the second experiment six replicates per group. The same procedures were followed during both experiments. The first test group of *H. aspersa* served as control and received uncontaminated food. The other two test groups were exposed to 80 $\mu g.g^{-1}$ and 240 $\mu g.g^{-1}$ copper oxychloride respectively, which was mixed into the food (prepared as described in section 2), using 0.04 g and 0.12 g efekto Virikop (500 g copper per 850 g fungicide) respectively. The concentrations were chosen to represent concentrations below the previously determined LC_{50} for *Helix aspersa* for copper oxychloride. Before being given to the snails, each Agar plate was weighed. Agar plates were also renewed every three days, weighed directly after having been removed, and a subsample of each batch was frozen for future metal analysis.

4.1 Body mass, egg production and hatching success

Individual snails were weighed before the experiments started, once every week during the experiments, and at the end of the sixth week. Also, the soil substrates of the containers were

regularly searched for egg clutches. All eggs were removed from the soil, counted and placed in fresh soil in separate containers. These were regularly moistened and searched for hatchlings. Hatchlings were also removed from the soil, counted, weighed, killed by freezing at -10 °C, and stored at the same temperature for future copper analysis. At the end of the experiments, the total number of eggs produced, as well as the percentage of hatched eggs, were determined for each of the three test groups.

4.2 Neutral Red Retention Time Assay

4.2.1 Cell viability

The Eosin Y test, recommended by Svendsen & Weeks (1995) was used to determine whether the process of haemocyte extraction and subsequent handling did not seriously damage the cells. Healthy cells stained green and dead cells red. From this it was ascertained that, at the time of counting, 90 % of all haemocytes were alive, and that the extraction and handling of the haemocytes would therefore not influence the results of the NRR time assay to a great extent.

4.2.2 Experimental procedure

The neutral red retention (NRR) times of three individuals from each group in the first experiment, and six individuals from each group in the second experiment, were determined prior to the commencement of the experiments, as well as at the end of each of the following six weeks. According to the method described by Svendsen & Weeks (1995), a small hole was pierced in the shell of each animal, using a sterilized needle, and 20 μl haemolymph was then drawn from the visceral haemocoel, into an equal volume of temperature-adjusted snail Ringer, using a needle and syringe. The snail Ringer consisted of 5.0 g NaCl, 0.08 g KCl and 0.6 g $CaCl_2$, dissolved in 1 litre of distilled water. Two drops haemolymph/Ringer solution were placed on a microscope slide and 20 μl neutral red working solution was added to each drop. The working solution, which was renewed hourly, was prepared by mixing 2.5 ml snail Ringer and 10 μl neutral red stock solution. The latter consisted of 20 mg Sigma neutral red powder dissolved in 1 ml Sigma DMSO (dimethyl sulfoxide).

The slide was covered with a coverslip and was studied under a Nikon light microscope (40x magnification). The total number of haemocytes, as well as the number of stained (pink/red)

haemocytes in a haemolymph sample were counted at two-minute intervals. Only the most abundant cell type, namely the smaller, hyaline, agranular haemocytes with pseudopodia were counted. The point, expressed in minutes, at which 50 % of the total number of cells in a sample were stained, was taken as the neutral red retention time.

4.3 Copper Analysis

Subsequent to NRR time measurements animals were weighed, killed by freezing at -10 °C and stored at the same temperature. At the end of each experiment these snails, as well as the hatchlings and Agar samples were thawed, and the adult snails were dissected on a sterile surface, using stainless steel instruments. The digestive gland, ovotestis and hermaphrodite duct of each animal were removed. The latter two organs of each animal were digested as one sample. Apart from the Agar samples, all other samples were dried for 24 hours at 60 °C. Tissue, hatchling and Agar samples were then weighed and placed in metal free test tubes. The remainder of each adult's body (including the shell) was ground with a pestle and mortar, and a one gram homogenous subsample was used for metal analysis. Test tubes were placed in a Labcon dual digestor in a fume cabinet, and all samples were digested with 10 ml 55 % nitric acid at a temperature of 40 °C for a period of 2 hours, after which the temperature was increased to 120 °C for 1 hour. After cooling, samples were filtered through Whatman 6 (90 mm) filter paper, and then through Sartorius 0.45 μm cellulose nitrate filter paper, with a needle and syringe. Finally, the samples were diluted to 20 ml with distilled water and stored at 5 °C in sterilized plastic containers. Copper concentrations in the samples were determined with a Varian AA-1275 flame atomic absorption spectrophotometer and converted using the following formula:

$$\frac{\text{value obtained from AAS x dilution factor (20)}}{\text{mass of sample (g)}}$$

All copper concentrations were expressed as $\mu g.g^{-1}$ dry mass. Wet mass copper concentrations of the Agar samples were converted to $\mu g\ g^{-1}$ dry mass, using the known percentage water in these samples.

4.4 Histological Analysis

4.4.1 Fixation and histological technique

At the end of the six-week experimental periods, the remaining animals were all killed by decapitation and the digestive gland, ovotestis and hermaphrodite duct of each animal were rapidly excised and fixated in Bouin's fluid (Preece 1972) for 20-23 hours. The organs were then placed in stainless steel embedding cassettes and rinsed in 50% alcohol for 1 hour, after which the organs were taken through various steps of dehydration in alcohol and clearing in xylene (room temperature), as well as a number of impregnation steps with Paraplast wax, at 58 °C (Table 1). Finally, the organs were embedded in fresh Paraplast wax in non-stick stainless steel Tissue-Tek base moulds, covered with plastic Tissue-Tek embedding covers and cooled overnight at 5 °C.

Table 1: Duration of the various steps of dehydration, clearing and impregnation during histological preparation of the digestive gland, ovotestis and hermaphroditic (herm.) duct of *Helix aspersa*.

	Organs	
Chemicals used/Steps	**Ovotestis and Herm. duct**	**Digestive gland**
70% Alcohol	40 minutes	1 hour
90% Alcohol	30 minutes	1 hour
96% Alcohol I	30 minutes	1 hour
96% Alcohol II	30 minutes	1 hour
100% Alcohol I	30 minutes	1 hour
100% Alcohol II	30 minutes	1 hour
Xylene I	20 minutes	40 minutes
Xylene II	20 minutes	40 minutes
Xylene + Wax (1:1)	45 minutes	90 minutes
Wax II	4 hours	Overnight (± 12 hours)
Wax III	------	90 minutes in vacuum @ 300mmHg

4.4.2 Sectioning

Longitudinal and cross sections of the digestive glands, and cross sections of the ovotestes and hermaphrodite ducts were made with a Leica Rotary Microtome, at a thickness of 6-8 μm. Sections were placed on microscope slides, a few drops of Mayer's egg albumin and glycerol mixture (Preece 1972) were added to spread the wax, and the slides were dried on a slide-drying hotplate at 40 °C.

4.4.3 Staining and mounting

All slides were stained with Erlich Hematoxylin and alcohol dissolvable Eosin according to the recipes and method described by Presnell et al. (1997). The slides were taken through various steps of rinsing, dehydration, staining, differentiating and clearing, as shown in Table 2. Immediately after the final clearing step with xylene, a few drops of Entellan mounting fluid were placed on each slide and a coverslip was carefully added. The slides were then allowed to dry overnight.

Table 2: Duration of the various rinsing, dehydration, staining, differentiation and clearing steps during biological staining of sections of the digestive gland, ovotestis and hermaphrodite duct (vesicula seminalis region) of *Helix aspersa*.

Chemicals used/Steps	Duration	Function
Xylene I	3 minutes	Removes wax
Xylene II	3 minutes	Removes wax
100% Ethanol I	Dip	Removes xylene
100% Ethanol II	3 minutes	Dehydration
100% Ethanol III	3 minutes	Dehydration
96% Ethanol	2 minutes	Dehydration
70% Ethanol	2 minutes	Dehydration
50% Ethanol	2 minutes	Dehydration
Distilled H_2O	2 minutes	Removes ethanol
Hematoxylin	10-15 minutes	Staining for nuclei

Flowing tap water	3 minutes	Provides blue colour of nuclei
Scott's solution	3 minutes	Provides alkaline medium
1% HCl in 70% ethanol	2-3 dips	Differentiation
Flowing tap water	3 minutes	Rinsing
1% Eosin	30 seconds	Staining for cytoplasm
70% Ethanol	Dip	Differentiation
96% Ethanol	3 minutes	Dehydration
100% Ethanol I	3 minutes	Dehydration
100% Ethanol II	3 minutes	Dehydration
Xylene I	4 minutes	Clearing
Xylene II	5 minutes	Clearing

4.4.4.Processing of slides

Slides of the organs of at least four individuals from each experimental group were studied under a Nikon compound microscope at 10x and 40x magnification. Only slides made at approximately the middle of each organ were used. Measurements were made randomly across the slides, using the Leica QWin computer software package. Quantitative techniques were used, since differences between the three test groups could not be identified by mere visual observation. According to Lowe & Clarke (1989), some of the subjectivity associated with descriptive histology is certainly avoided with quantification. These authors also stated that quantitative techniques provide early indications of toxicity with greater sensitivity and precision than qualitative descriptions alone.

4.4.4.1. Digestive gland / hepatopancreas / midgut gland

The morphology and cytology of the digestive gland of *Helix* have been studied microscopically by authors such as Abolins-Krogis (1960) and Sumner (1965; 1966), using a variety of highly specialized staining techniques. They described the gland as a compound tubular gland with branched, blind-ending tubules separated by loose connective tissue (Figure 1). The secretory epithelium consists of one layer of differently modified glandular cells. According to Abolins-Krogis (1960) the digestive epithelium of *Helix pomatia* consists of two cell types, namely the long, narrow digestive cells and the shorter, pyramidal shaped calcium cells. Sumner (1966), however, found four different cell types in the digestive epithelium of *H. aspersa*: thin cells, calcium cells,

digestive cells and excretory cells. Abolins-Krogis (1960) argued that any other cells apart from the digestive and calcium cells are simply digestive cells in different functional stages of activity.

Such an in-depth investigation into the cytology of the digestive epithelium of *H. aspersa* did not fall within the scope of the present study. The aim was merely to identify a number of simple, quick and effective cellular responses to exposure to copper oxychloride, as potential biomarkers, using the routine H & E staining technique.

A set of parameters was used for the digestive gland, since Vega et al. (1989) stated that one parameter is not sufficient to indicate a pathological state in this organ. The following measurements were taken at random within each digestive gland: total areas of the individual digestive tubules, heights of the tubule epithelium cells, and total areas of the tubule epithelia. The latter was expressed as a percentage of the total area of the tubule. Percentages, rather than absolute values were used, since the digestive tubules of molluscs are known to vary in size between individuals of the same population (Robinson 1983) and during the digestion process (Vega et al. 1989). Molluscan digestive tubule diameter can also, according to Marigomez (1989, cited by Vega et al. 1989), be altered by trematode infestations.
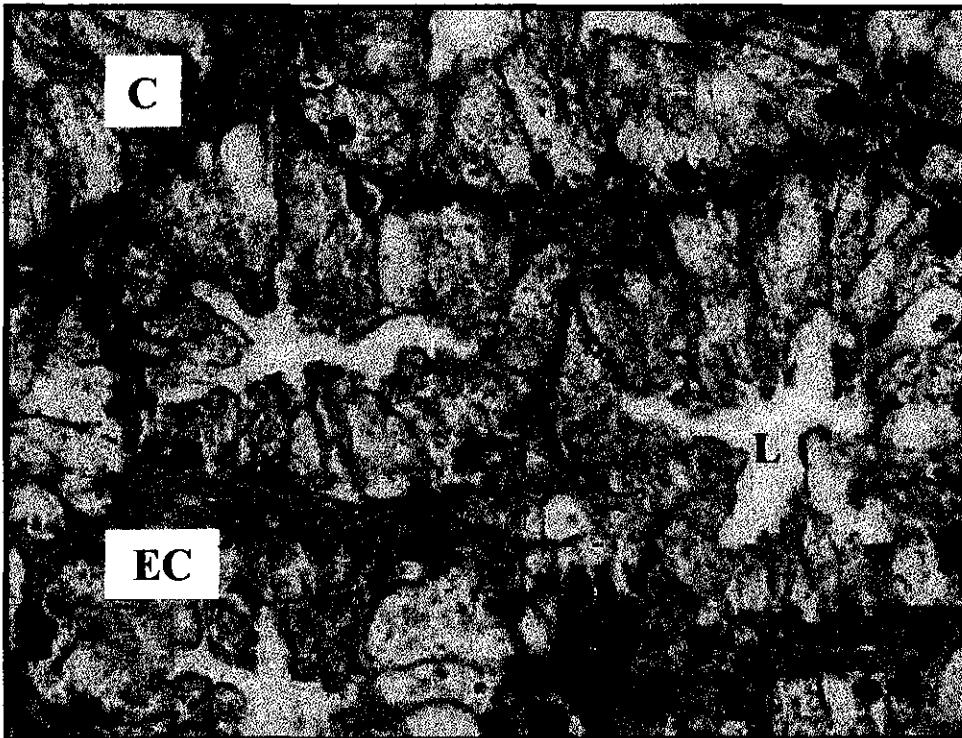
Figure 1: Cross section of the digestive tubules in the digestive gland of *Helix aspersa*, showing the lumen (L), digestive epithelium cells (EC) and connective tissue (C) (400x magnification).

4.4.4.2. Ovotestis and hermaphrodite duct

An overview of the morphology of the reproductive organs of terrestrial snails was given by Tompa (1984). This author described the ovotestis as a single organ, consisting of numerous acini, each with its own efferent ductule. This organ produces both sperm and ova (Figure 2) that originate and mature in every part of the ovotestis. The hermaphrodite duct is formed by the confluence of the efferent ductules of the ovotestis. This duct can be divided into three regions, of which the middle section, the vesicula seminalis, is the storage place for sperm for copulation. The seminal vesicle is fluid-filled and lined with ciliated epithelium. Spermatozoa lie immobile in this fluid (Figure 3). Lind (1973, cited by Tompa 1984) and Bailey (1983) found that the seminal vesicle contains sperm throughout the year, even during hibernation, after copulation and after oviposition.

The following measurements were taken at random within each ovotestis: total number of mature oocytes per 1 mm^2 ovotestis, as well as total area of spermatozoa per 1 mm^2 ovotestis. Within each hermaphrodite duct the total area of spermatozoa, expressed as a percentage of the total duct area was measured in the vesicula seminalis region (middle region) of the duct.
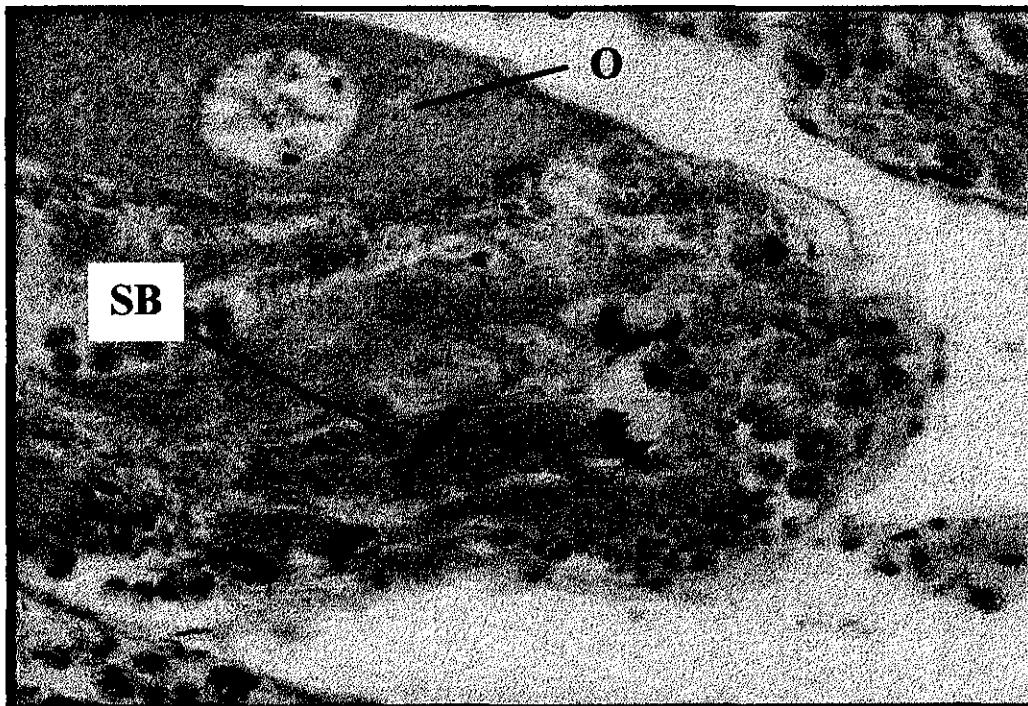
Figure 2: Cross section of an acinus in the ovotestis of *Helix aspersa*, showing a mature oocyte (O) and a bundle of spermatozoa (SB) (400x magnification).
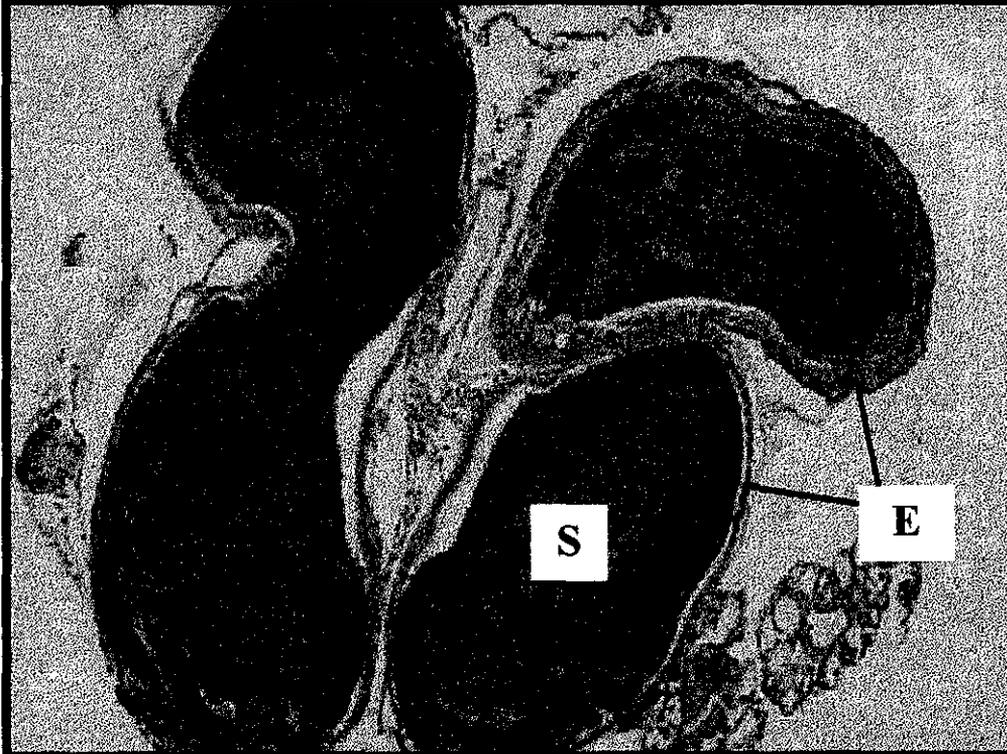
Figure 3: Cross and transverse sections of the convoluted vesicula seminalis region of the hermaphrodite duct of *Helix aspersa*, showing epithelium (E) and sperm (S) in the lumen (100x magnification).

5. Field survey

5.1 Sampling Sites and Method

In order to test the viability of the laboratory results, snails were collected from two vineyards in the Western Cape, South Africa. One of these sites represented a vineyard where copper oxychloride had been applied, and one site served as control, i.e. a vineyard with no history of contamination. The contaminated site (33°50'30"S; 18°51'35"E) was in a vineyard on the Delheim estate between the towns of Stellenbosch and Paarl, and the uncontaminated site (34°02'30"S; 18°51'35"E) in a vineyard on a small-holding on Helderberg Mountain, Somerset West. Animals from Delheim were collected in December 1999 and February 2000, i.e. one week after copper oxychloride application and two months after the last application respectively. Snails from the control site were collected once during December 1999. During each collection 10 – 13 individuals were taken, as well as a representative sample of soil and vine leaves.

The mean monthly rainfall and temperature for the months December 1999 to February 2000, as measured at Somerset West (representing the control site) and at Paarl (representing the contaminated site), are shown in Tables 3 and 4. All rainfall and temperature data were provided by the South African Weather Bureau, Department of Environmental Affairs and Tourism.

Table 3: Mean monthly temperature (°C) in Somerset West, representing the control field site, and in Paarl, representing the contaminated field site, for the period December 1999 to February 2000.

Month and Year	Somerset West		Paarl	
	Minimum	**Maximum**	**Minimum**	**Maximum**
December 1999	20.45 ± 3.56	28.87 ± 5.87	19.51 ± 3.63	33.25 ± 4.79
January 2000	17.17 ± 1.82	26.8 ± 4.23	18.01 ± 2.51	31.25 ± 5.02
February 2000	18.26 ± 1.73	27.08 ± 4.06	18.34 ± 1.97	31.81 ± 3.66

Table 4: Mean monthly rainfall (mm) in Somerset West, representing the control field site, and in Paarl, representing the contaminated field site, for the period December 1999 to February 2000.

Month and Year	Somerset West	Paarl
December 1999	0.19 ± 0.71	0.12 ± 0.44
January 2000	0.96 ± 3.32	1.61 ± 6.6
February 2000	1.0 ± 4.56	0.12 ± 0.63

5.2 Snail samples

The neutral red retention time technique was performed immediately on 6 – 10 snails from each site, after which they were killed by freezing and stored for future copper analysis. The methods described in sections 4.2 and 4.3 were followed. The remaining animals were kept in aerated plastic containers for 2 – 3 days and fed on leaves from the vineyards where they were collected. These animals were then used for histological analysis, according to the method described in section 4.4.

5.3 Leaf and soil samples

Three sieved soil samples and three unwashed leaf samples from each site were digested and analyzed for copper according to the method described in section 4.3. In the case of the soil samples, 5 ml of 70 % perchloric acid was added to each sample, and the solutions were boiled for an extra hour at 120 °C, after which they were cooled and filtered.

6. Statistical Analysis

The data were statistically analyzed and graphically portrayed with the Jandel Scientific Sigmastat 2.0, Microsoft Excel 97 and Microsoft PowerPoint 97 computer programs.

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RESULTS

1. Acute toxicity (LC$_{50}$ test) of copper oxychloride

Table 5 shows the number of *Helix aspersa* mortalities per exposure concentration of copper oxychloride, during the 14-day LC$_{50}$ test. The results of the Trimmed Spearman-Karber LC$_{50}$ test are presented in Table 6. The Spearman-Karber LC$_{50}$ estimate for *Helix aspersa* was 567.16 µg g^{-1} copper oxychloride.

Table 5: The total number and percentage of *Helix aspersa* mortalities per copper oxychloride exposure concentration (µg g^{-1}), after 14 days (n = number of snails).

Exposure concentration (µg g^{-1})	n	Number of mortalities	% mortalities
0	24	0	0
250	24	6	25
500	24	9	37.5
1000	24	20	83.33
2000	24	24	100

Table 6: Trimmed Spearman-Karber test to determine the 14-day LC$_{50}$ for *Helix aspersa* for the fungicide copper oxychloride.

Spearman-Karber trim	25%
LC$_{50}$ estimate	**567.16 µg g^{-1}**
95% lower confidence	418.35 µg g^{-1}
95% upper confidence	768.89 µg g^{-1}

2. Sublethal toxicity of copper oxychloride

2.1 Effects of copper oxychloride on food intake, body mass, egg production and hatching success, and accumulation of copper in hatchlings

2.1.1 Food intake

Table 7 and Figure 4 illustrate the mean individual food intake (grams) of *Helix aspersa*, per week per exposure concentration, over the six-week exposure period.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table I) revealed that there were no significant differences (p>0.05) between weekly individual food intake of the control and 80 μg g^{-1} copper oxychloride exposure groups over the six-week exposure period. In the 240 μg g^{-1} exposure group however, the individual food intake during week 6 of the experiment (0.17 $\pm$ 0.08 g) was significantly higher (p<0.05) than during week 1 (0.1 $\pm$ 0.08 g).

Comparisons between the three exposure groups (Appendix Table II) showed that, during the first week of exposure, the individual food intake of animals from the 80 and 240 μg g-1 groups (0.17 $\pm$ 0.14 and 0.1 $\pm$ 0.08 g respectively) were significantly lower (p<0.05) than that of the control group (0.3 $\pm$ 0.21 g). However, there were no significant differences in individual food intake between the three groups during the final week of exposure.
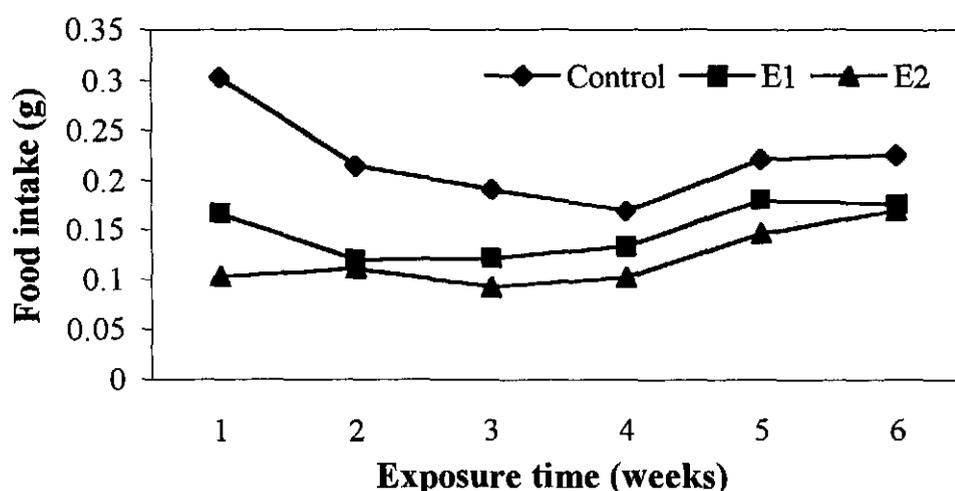
Figure 4: Mean individual food intake (grams) of *Helix aspersa* over a six-week period of exposure to three concentrations of copper oxychloride (Control = 0 μg g^{-1} copper oxychloride; E1 = 80 μg g^{-1} copper oxychloride; E2 = 240 μg g^{-1} copper oxychloride).

Table 7: Mean individual food intake (grams) of *Helix aspersa*, over six weeks in the control group, and two groups exposed to copper oxychloride (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; n = number of Agar blocks; SD = standard deviation).

Control				
Weeks	**n**	**Mean**	**SD**	**Range**
1	14	0.3	0.21	0.02-0.67
2	14	0.22	0.12	0.08-0.45
3	17	0.19	0.11	0.05-0.47
4	16	0.17	0.08	0.07-0.3
5	15	0.22	0.12	0.07-0.49
6	14	0.23	0.11	0.1-0.49
E1				
Weeks	**n**	**Mean**	**SD**	**Range**
1	14	0.17	0.14	0.01-0.38
2	14	0.12	0.08	0.003-0.24
3	17	0.12	0.06	0.04-0.27
4	16	0.13	0.08	0.03-0.27
5	14	0.18	0.09	0.04-0.31
6	16	0.18	0.08	0.0-0.33
E2				
Weeks	**n**	**Mean**	**SD**	**Range**
1	14	0.1	0.08	0.0-0.19
2	14	0.11	0.04	0.06-0.2
3	17	0.09	0.06	0.02-0.18
4	16	0.1	0.07	0.02-0.21
5	15	0.15	0.11	0.02-0.32
6	14	0.17	0.08	0.007-0.3

2.1.2 Changes in body mass

Table 8 and Figure 5 show the mean individual body mass (grams) of *Helix aspersa*, per week per exposure concentration, over the six-week exposure period.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table III) revealed that, within all three exposure groups, the individual body mass of snails were significantly ($p<0.05$) higher at the end of the first week of exposure, compared to the body mass prior to exposure. The control group also exhibited a significant ($p<0.05$) decrease in individual body mass from week 1 to week 2. However, this group, as well as the 80 µg g^{-1} exposure group exhibited significant ($p<0.05$) increases in individual body mass over the entire experimental period.

Comparisons between the three test groups (Appendix Table IV) showed that, neither prior to exposure, nor after six weeks of exposure, were there any significant differences ($p>0.05$) in individual body mass between the three groups. However, at the end of the first week of exposure, the individual body mass of animals from the 80 and 240 µg g^{-1} groups (6.45 ± 0.97 g and 6.26 ± 0.95 g respectively) were significantly lower ($p \leq 0.001$) than that of the control group (7.09 ± 1.3 g).
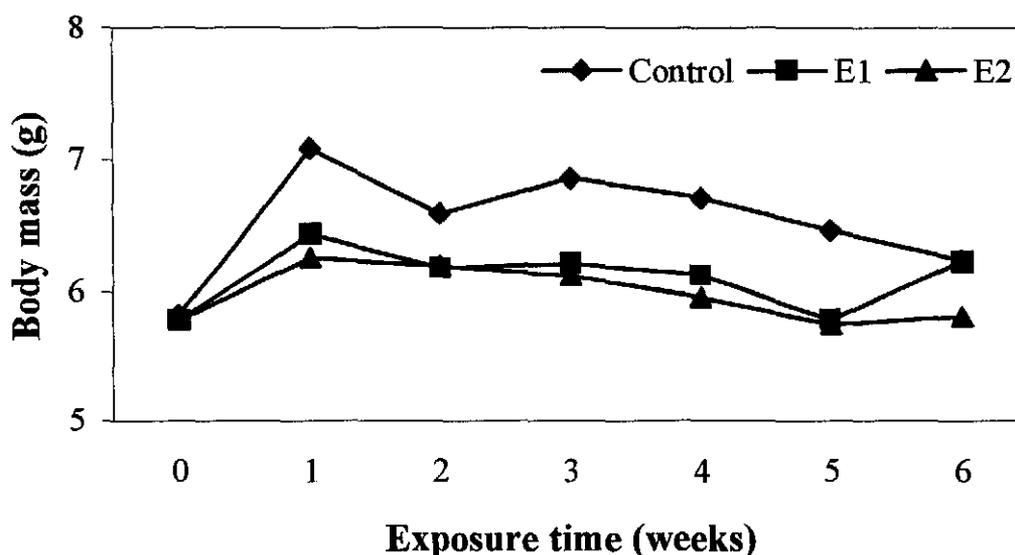
Figure 5: Mean individual body mass (grams) of *Helix aspersa* over a six-week period of exposure to copper oxychloride (Control = 0 µg g^{-1}; E1 = 80 µg g^{-1}; E2 = 240 µg g^{-1} copper oxychloride).

Table 8: Mean individual body mass (grams) of *Helix aspersa*, over six weeks of exposure to copper oxychloride (Control = 0 µg g^{-1}; E1 = 80 µg g^{-1}; E2 = 240 µg g^{-1} copper oxychloride; n = number of snails; SD = standard deviation).

Control				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	72	5.82	0.79	4.5-7.5
End of Week 1	72	7.09	1.3	4.1-10.5
End of Week 2	63	6.6	1.26	4.4-10.0
End of Week 3	54	6.86	1.37	4.9-10.6
End of Week 4	45	6.71	1.25	4.7-11.2
End of Week 5	36	6.47	0.9	4.7-9.2
End of Week 6	27	6.25	1.0	3.2-8.2
E1				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	72	5.77	0.79	4.5-8.3
End of Week 1	72	6.45	0.97	4.8-8.9
End of Week 2	63	6.18	1.03	4.1-8.25
End of Week 3	54	6.22	0.94	4.6-8.4
End of Week 4	45	6.13	0.82	4.6-7.8
End of Week 5	36	5.78	0.87	4.4-8.5
End of Week 6	27	6.24	1.0	4.5-8.6
E2				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	72	5.78	0.83	4.5-8.3
End of Week 1	72	6.26	0.95	4.2-8.7
End of Week 2	63	6.2	1.0	4.6-9.0
End of Week 3	54	6.12	0.97	4.1-8.7
End of Week 4	45	5.95	0.78	4.7-8.0
End of Week 5	36	5.75	0.93	4.3-8.4
End of Week 6	27	5.81	0.83	4.3-7.3

2.1.3 Egg production

2.1.3.1 Total egg production per exposure group

The mean number of eggs produced per exposure group over the six-week exposure period is shown in Figure 6. The Kruskal-Wallis One Way Analysis of Variance on Ranks test revealed that there were no significant differences ($p > 0.05$) in total egg production between the three test groups.

2.1.3.2 Individual egg production per week

The mean number of eggs produced per individual per week is shown in Figure 7 and Table 9 for each of the three test groups. Within-group comparisons, using the Kruskal-Wallis One Way Analysis of Variance on Ranks test revealed that the control group showed a significant ($p < 0.05$) increase in individual egg production over the six-week experimental period (Appendix Table V).

Comparisons between the three groups (Appendix Table VI) showed no significant differences ($p > 0.05$) in individual egg production during the first week of exposure but a significantly higher ($p < 0.05$) individual egg production in the control group during week 6 (18.82 ± 15.07 eggs per snail), compared to the 80 and 240 $\mu g\ g^{-1}$ exposure groups (both 0.0 ± 0.0 eggs per snail).

2.1.4 Hatching success

2.1.4.1 Mean hatching success per exposure group

Figure 8 illustrates the mean percentage of hatched *Helix aspersa* eggs per test group, over the six-week experimental period. No significant differences ($p > 0.05$) in hatching success were found between any of the groups, using the Kruskal-Wallis One Way Analysis of Variance on Ranks test.

2.1.4.2 Mean hatching success per egg clutch

Comparisons of the percentage of hatched eggs per egg clutch, between the three test groups are shown in Figure 9 and Table VII (Appendix). The control group had a significantly higher ($p < 0.05$) percentage of hatched eggs per egg clutch (33.41 ± 9.39 %) compared to the 240 $\mu g\ g^{-1}$ exposure group (9.64 ± 11.98 %).
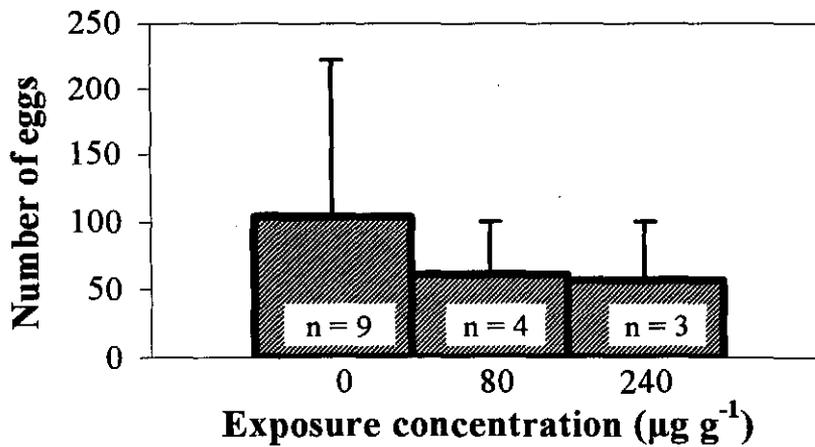
Figure 6: Mean number of eggs produced by the control group and two copper oxychloride exposure groups of *Helix aspersa*, over a period of six weeks (n = number of clutches).
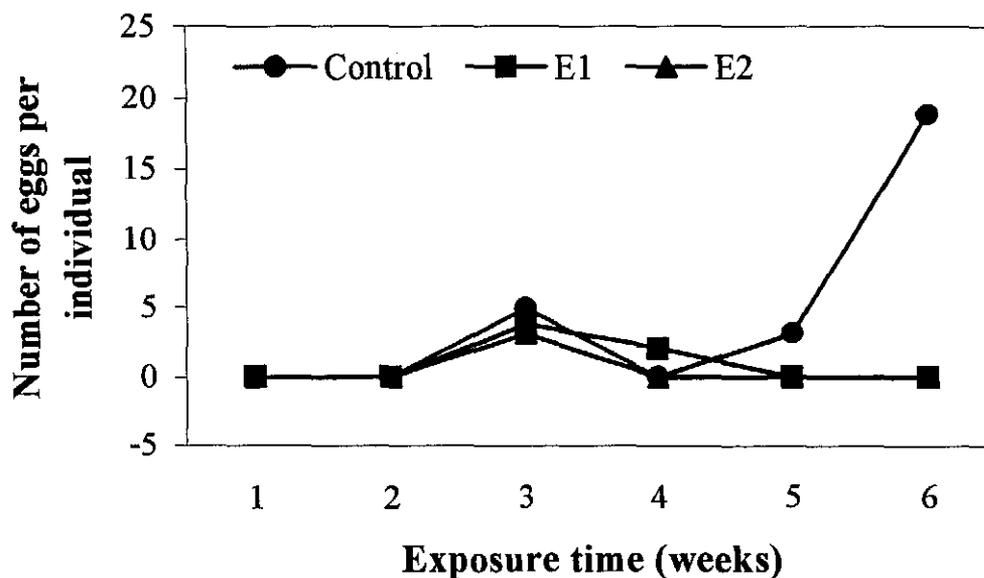
Figure 7: Mean number of eggs produced per individual per week within each of the three test groups of *Helix aspersa* (Control = 0 µg g^{-1}; E1 = 80 µg g^{-1}; E2 = 240 µg g^{-1} copper oxychloride).

Table 9: Mean number of eggs produced per individual per week within each of the three test groups of *Helix aspersa* (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; n = number of subgroups; SD = standard deviation).

Control				
Weeks	**n**	**Mean**	**SD**	**Range**
1	4	0.0	0.0	0.0-0.0
2	4	0.0	0.0	0.0-0.0
3	4	4.95	4.56	0.0-11.0
4	4	0.0	0.0	0.0-0.0
5	4	3.1	3.78	0.0-7.7
6	4	18.82	15.07	4.29-37.0
E1				
Weeks	**n**	**Mean**	**SD**	**Range**
1	4	0.0	0.0	0.0-0.0
2	4	0.0	0.0	0.0-0.0
3	4	3.74	3.6	0.0-8.57
4	4	2.04	4.08	0.0-8.17
5	4	0.0	0.0	0.0-0.0
6	4	0.0	0.0	0.0-0.0
E2				
Weeks	**n**	**Mean**	**SD**	**Range**
1	4	0.0	0.0	0.0-0.0
2	4	0.0	0.0	0.0-0.0
3	4	3.09	6.18	0.0-12.36
4	4	0.0	0.0	0.0-0.0
5	4	0.0	0.0	0.0-0.0
6	4	0.0	0.0	0.0-0.0
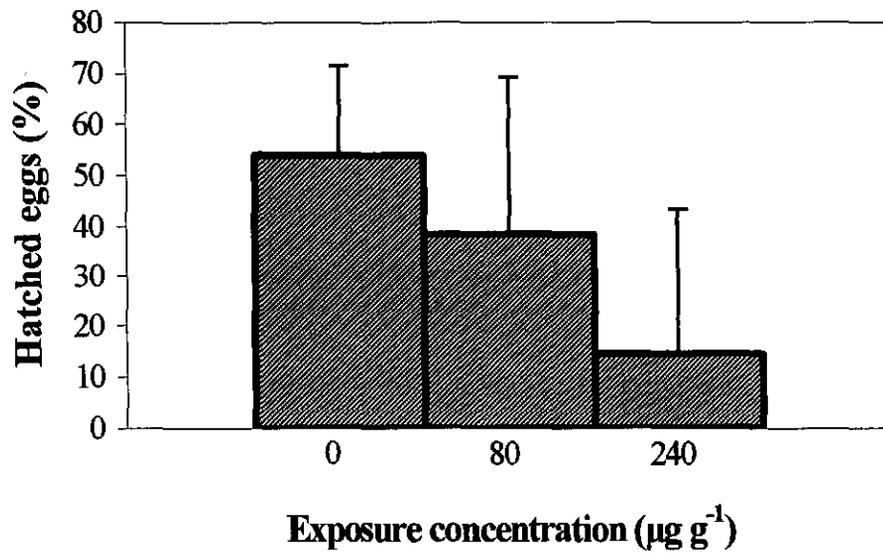
Figure 8: Mean percentage of hatched eggs over the six-week experimental period, in the control and two copper oxychloride exposure groups of *Helix aspersa* (n = 4, i.e. number of subgroups).
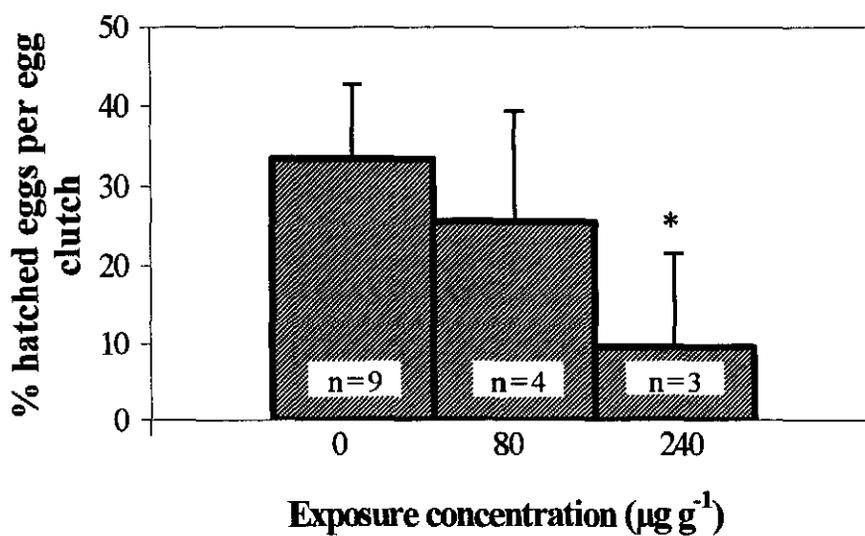
Figure 9: Mean percentage of hatched eggs per egg clutch over the six-week experimental period, in the control and two copper oxychloride exposure groups of *Helix aspersa* (n = number of egg clutches; * = significant difference from control).

2.1.5 Hatchlings

The mean copper concentration (μg g^{-1} dry mass) in hatchlings produced over the six-week experimental period within each test group is shown in Figure 10.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test revealed that there were no significant (p>0.05) differences in copper concentrations between hatchlings of the control and 80 μg g^{-1} copper oxychloride exposure groups, or between the 80 and 240 μg g^{-1} exposure groups. The copper concentrations in hatchlings from the 240 μg g^{-1} exposure group (74.18 ± 11.2 μg g^{-1} dry mass) were however significantly higher (p<0.05) than in hatchlings from the control group (44.08 ± 19.07 μg g^{-1} dry mass) (Appendix Table VIII).
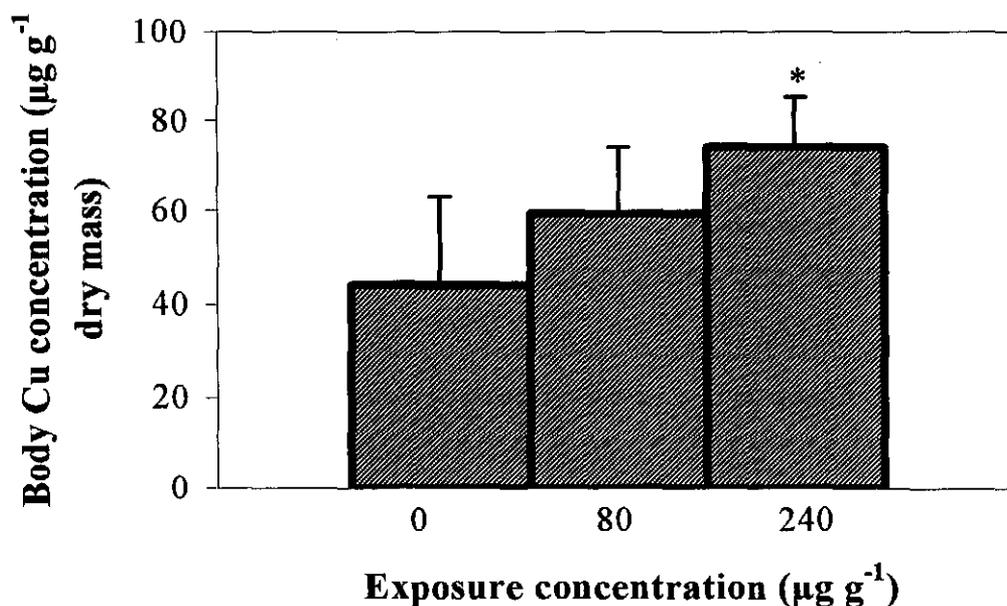
Figure 10: Mean copper concentrations (μg g^{-1} dry mass) in hatchlings from the control and two copper oxychloride exposure groups of *Helix aspersa* (n = 6, i.e. number of pooled samples; * = significant difference from control).

2.2 Copper exposure

The results of the copper analysis of the Agar blocks fed to the three test groups over the six-week experimental period are shown in Table 10.

Table 10: Mean wet mass and dry mass copper concentrations (μg g^{-1}) in the food of the control and two copper oxychloride exposure groups of *Helix aspersa* (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1} E2 = 240 μg g^{-1} copper oxychloride; SD = standard deviation).

Exposure group	μg g^{-1} wet mass		μg g^{-1} dry mass	
	Mean	SD	Mean	SD
Control	0.0	0.0	0.0	0.0
E1	40.11	4.03	200.57	20.13
E2	129.03	15.64	545.11	78.2

2.3 Copper uptake and distribution

2.3.1 Copper uptake

2.3.1.1 Copper uptake in the reproductive organs

The mean copper concentrations (μg g^{-1} dry mass) in the reproductive organs (ovotestis and hermaphrodite duct pooled) of the three test groups of *Helix aspersa* (weeks 1-6 pooled) are shown in Figure 11. There were no significant differences (p>0.05) in reproductive organ copper concentrations between the 80 μg g^{-1} and 240 μg g^{-1} copper oxychloride groups, but the copper concentrations in the reproductive organs of snails from these two groups (75.93 $\pm$ 164.1 and 158.03 $\pm$ 281.03 μg g^{-1} respectively) were significantly higher (p $\leq$ 0.001) than those of snails from the control group (2.15 $\pm$ 15.82 μg g^{-1}) (Appendix Table IX).

Table 11 and Figure 12 show the mean copper concentration (μg g^{-1} dry mass) in the reproductive organs of the control group and two copper oxychloride exposure groups of *Helix aspersa*, per week, over the six-week experimental period.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table X) revealed that, within the 80 and 240 μg g^{-1} exposure groups, the copper concentrations in the reproductive organs were significantly (p<0.05) higher at the end of the exposure period (week 6), compared to prior to exposure (week 0). The control group exhibited no significant (p>0.05) difference in reproductive organ copper concentrations between weeks 0 and 6 of the experiment.

Comparisons between the three test groups (Appendix Table XI) showed that prior to exposure there were no significant differences (p>0.05) in reproductive organ copper concentrations (μg g^{-1} dry mass) between any of the three groups. However, at the end of the final week of exposure, the reproductive organ copper concentrations in animals from the 80 and 240 μg g^{-1} groups (263.99 $\pm$ 260.47 and 328.09 $\pm$ 362.27 μg g^{-1} respectively) were significantly higher (p<0.05) than those of the control group (below detectable levels).
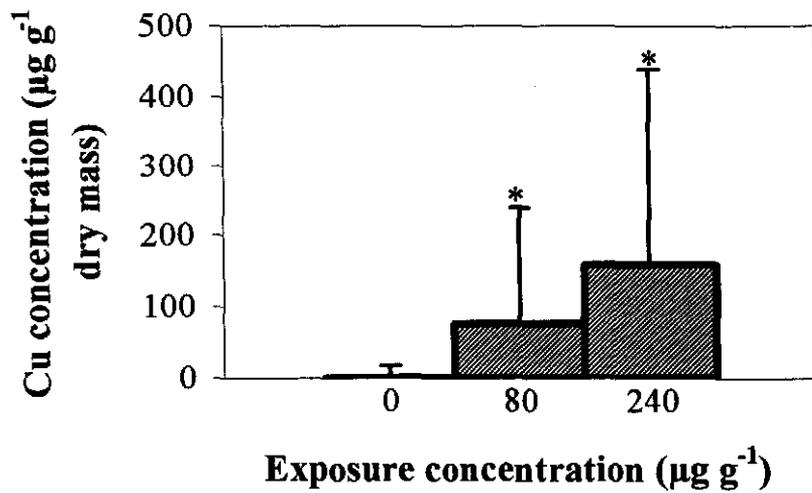
Figure 11: Mean copper concentration ($\mu g \ g^{-1}$ dry mass) in the reproductive organs of the control groups and two copper oxychloride exposure groups of *Helix aspersa* (n = 54 snails; * =significant difference from control).
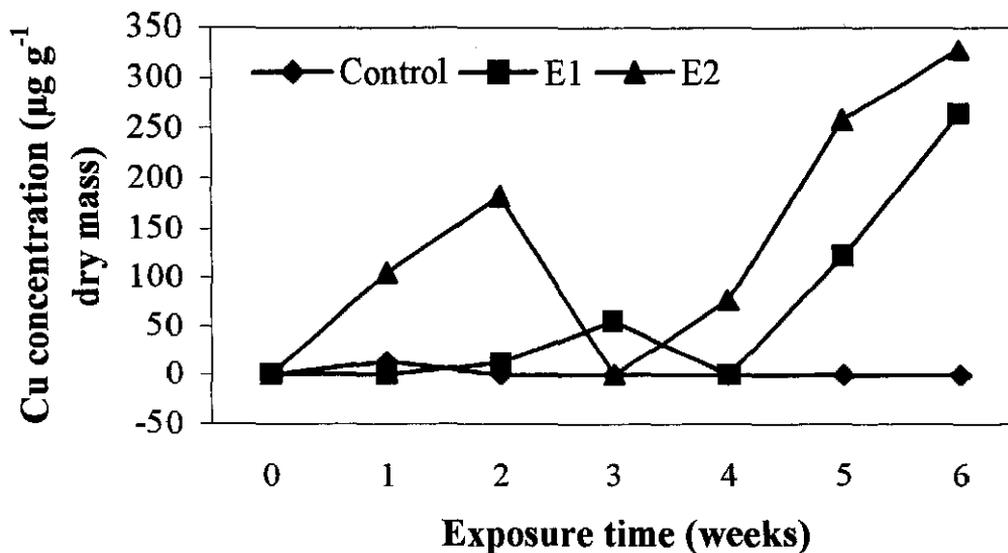
Figure 12: Mean copper concentrations ($\mu g \ g^{-1}$ dry mass) in the reproductive organs of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 $\mu g \ g^{-1}$; E1 = 80 $\mu g \ g^{-1}$; E2 = 240 $\mu g \ g^{-1}$ copper oxychloride).

Table 11: Mean copper concentrations ($\mu g \ g^{-1}$ dry mass) in the reproductive organs of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 $\mu g \ g^{-1}$; E1 = 80 $\mu g \ g^{-1}$; E2 = 240 $\mu g \ g^{-1}$ copper oxychloride; n = number of snails; SD = standard deviation).

Control				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	0.0	0.0	0.0-0.0
End of Week 1	9	12.92	38.76	0.0-116.28
End of Week 2	9	0.0	0.0	0.0-0.0
End of Week 3	9	0.0	0.0	0.0-0.0
End of Week 4	9	0.0	0.0	0.0-0.0
End of Week 5	9	0.0	0.0	0.0-0.0
End of Week 6	9	0.0	0.0	0.0-0.0
E1				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	0.0	0.0	0.0-0.0
End of Week 1	9	0.0	0.0	0.0-0.0
End of Week 2	9	12.43	37.29	0.0-118.86
End of Week 3	9	56.25	114.05	0.0-300.0
End of Week 4	9	0.0	0.0	0.0-0.0
End of Week 5	9	122.95	189.63	0.0-432.26
End of Week 6	9	263.99	260.47	0.0-800.0
E2				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	0.0	0.0	0.0-0.0
End of Week 1	9	104.94	69.56	0.0-500.00
End of Week 2	9	182.3	344.93	0.0-1000.0
End of Week 3	9	0.0	0.0	0.0-0.0
End of Week 4	9	76.74	130.51	0.0-869.57
End of Week 5	9	257.84	359.63	0.0-869.57
End of Week 6	9	328.09	362.27	0.0-930.23

2.3.1.2 Copper uptake in the digestive gland

The mean copper concentrations (μg g^{-1} dry mass) in the digestive glands of the three test groups of *Helix aspersa* (weeks 1-6 pooled) are shown in Figure 13. There were significant differences ($p<0.05$) in digestive gland copper concentrations between all three groups. The control group had the lowest mean copper concentration in the digestive gland (75.35 ± 64.04 μg g^{-1} dry mass) and the 240 μg g^{-1} exposure group the highest (468.81 ± 217.06 μg g^{-1} dry mass) (Appendix Table XII).

Table 12 and Figure 14 show the mean copper concentration (μg g^{-1} dry mass) in the digestive gland of the control group and two copper oxychloride exposure groups of *Helix aspersa*, per week, over the six-week experimental period.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XIII) revealed that, within the control group, there were no significant differences ($p>0.05$) in digestive gland copper concentrations between any of the consecutive weeks of observation. Within the 80 μg g^{-1} exposure group there were significant increases ($p<0.001$) in digestive gland copper concentrations from week 0 (i.e. prior to exposure) to week 1, as well as in total from week 0 to 6. The mean digestive gland copper concentration at the end of week 6 of exposure was the highest (567.82 ± 228.29 μg g^{-1} dry mass) and the mean digestive gland copper concentration prior to exposure the lowest (97.73 ± 74.35 μg g^{-1} dry mass). The 240 μg g^{-1} copper oxychloride exposure group showed significant increases ($p<0.05$) in digestive gland copper concentrations from week 0 (i.e. prior to exposure) to week 1, from week 3 to 4, from week 5 to 6, and in total from week 0 to 6. The mean digestive gland copper concentration prior to exposure was the lowest (97.73 ± 87.14 μg g^{-1} dry mass) and the mean digestive gland copper concentration at the end of the final week of exposure the highest (701.18 ± 99.51 μg g^{-1} dry mass).

Comparisons between the three test groups (Appendix Table XIV) showed that prior to exposure there were no significant differences ($p>0.05$) in digestive gland copper concentrations (μg g^{-1} dry mass) between any of the three exposure groups. However, at the end of the final week of exposure, the digestive gland copper concentrations of animals from the 80 and 240 μg g^{-1} groups were significantly higher ($p<0.001$) than that of the control group.
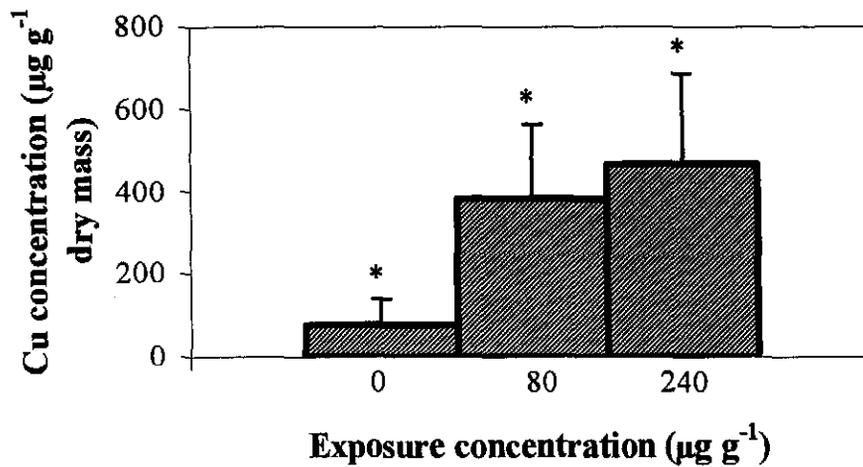
Figure 13: Mean copper concentrations (μg g^{-1} dry mass) in the digestive glands of the control group and two copper oxychloride exposure groups of *Helix aspersa* (n = 54 snails; * = significant difference).
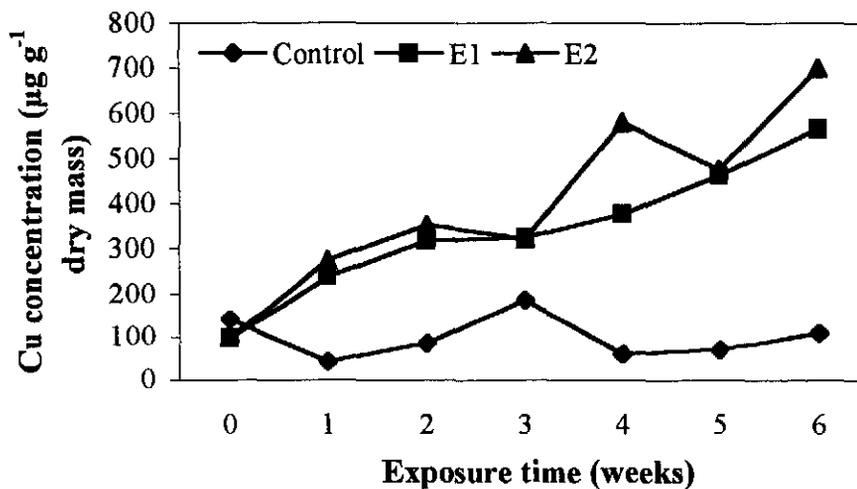
Figure 14: Mean copper concentrations (μg g^{-1} dry mass) in the digestive gland of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride).

Table 12: Mean copper concentrations ($\mu g \ g^{-1}$ dry mass) in the digestive gland of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 $\mu g \ g^{-1}$; E1 = 80 $\mu g \ g^{-1}$; E2 = 240 $\mu g \ g^{-1}$ copper oxychloride; n = number of snails; SD = standard deviation).

Control				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	139.18	306.18	0.0-951.68
End of Week 1	9	42.62	28.46	0.0-77.7
End of Week 2	9	85.27	76.59	0.0-250.05
End of Week 3	9	187.53	298.63	9.94-951.68
End of Week 4	9	60.22	32.82	0.0-99.36
End of Week 5	9	71.41	52.08	0.0-180.85
End of Week 6	9	109.68	76.46	8.43-252.38
E1				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	97.73	74.35	8.43-252.38
End of Week 1	9	238.97	73.88	98.68-340.14
End of Week 2	9	318.42	84.62	222.22-493.08
End of Week 3	9	324.63	96.05	230.7-531.91
End of Week 4	9	376.48	208.98	146.32-865.15
End of Week 5	9	462.8	167.23	88.65-634.24
End of Week 6	9	567.82	228.29	320.86-890.99
E2				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	97.1	87.14	9.94-300.0
End of Week 1	9	274.89	203.62	40.1-672.85
End of Week 2	9	351.19	185.16	89.16-718.23
End of Week 3	9	322.43	204.47	0.0-593.95
End of Week 4	9	580.16	103.86	389.0-754.24
End of Week 5	9	477.25	124.4	287.8-666.67
End of Week 6	9	701.18	99.51	552.32-873.27

2.3.1.3 Copper uptake in the rest of the snail body and shell (excluding reproductive organs and digestive gland)

The mean copper concentrations (μg g^{-1} dry mass) in the remainder of the snail body, including the shell, of the three test groups of *Helix aspersa* (weeks 1-6 pooled) are shown in Figure 15. There were significant differences (p<0.05) in "rest of the body" copper concentrations between all three test groups. The control group had the lowest mean "rest of the body" copper concentration (53.24 ± 25.54 μg g^{-1} dry mass) and the 240 μg g^{-1} exposure group the highest (159.98 ± 71.94 μg g^{-1} dry mass) (Appendix Table XV).

Table 13 and Figure 16 show the mean copper concentration (μg g^{-1} dry mass) in the "rest of the body" of the control group and two copper oxychloride exposure groups of *Helix aspersa*, per week, over the six-week experimental period.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XVI) revealed that, within the control group, there was a significant (p<0.05) decrease in "rest of the body" copper concentrations from week 3 to 4 of observation, but that none of the other concentrations in the consecutive weeks differed significantly from one another (p>0.05). Within the 80 μg g^{-1} exposure group there were significant increases (p<0.05) in "rest of the body" copper concentrations from week 0 (i.e. prior to exposure) to week 1, as well as from week 2 to 3, and in total from week 0 to 6. The mean copper concentration at the end of week 6 of exposure was the highest (160.28 ± 60.79 μg g^{-1} dry mass) and the mean copper concentration prior to exposure the lowest (53.9 ± 24.32 μg g^{-1} dry mass). The 240 μg g^{-1} copper oxychloride exposure group showed significant increases (p<0.05) in "rest of the body" copper concentrations from week 4 to 5 and in total from week 0 to 6. The mean "rest of the body" copper concentration prior to exposure was the lowest (81.43 ± 59.12 μg g^{-1} dry mass) and the mean "rest of the body" copper concentration at the end of the final week of exposure the highest (243.52 ± 69.42 μg g^{-1} dry mass).

Comparisons between the three exposure groups (Appendix Table XVII) showed that prior to exposure there were no significant differences (p>0.05) in "rest of the body" copper concentrations (μg g^{-1} dry mass) between any of the three test groups. However, at the end of the final week of the experiment, the "rest of the body" copper concentrations of animals from the 0 and 240 μg g^{-1} groups were, respectively, significantly lower and significantly higher (p<0.05) than that of the other two groups.
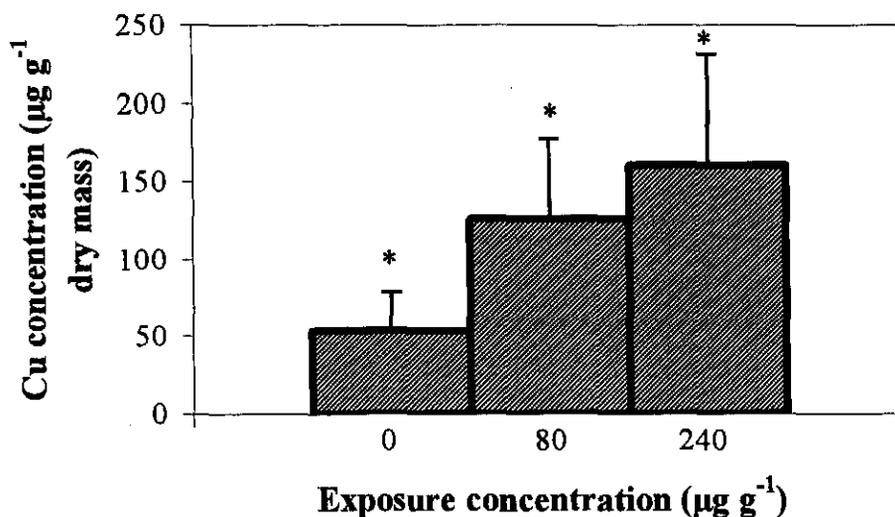
Figure 15: Mean copper concentration ($\mu g\ g^{-1}$ dry mass) in the body (excluding reproductive organs and digestive gland) of the control group and two copper oxychloride exposure groups of *Helix aspersa* (n = 54 snails; * = significant difference).
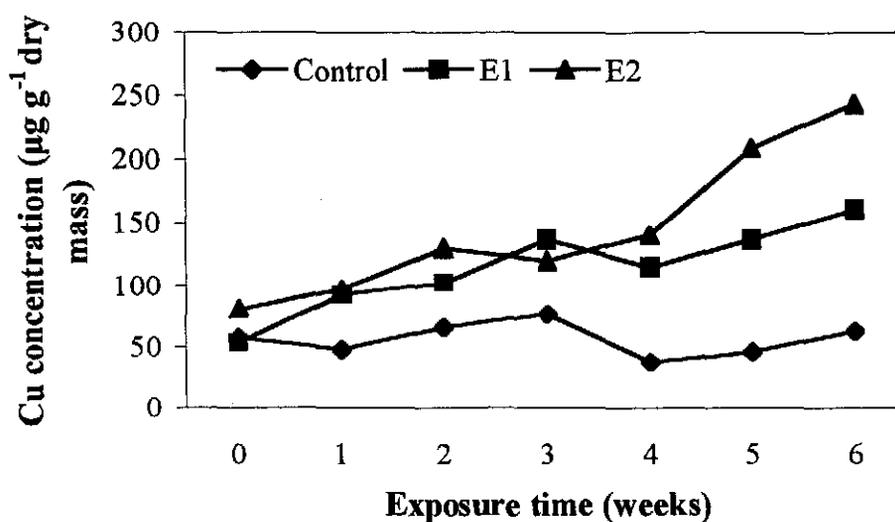
Figure 16: Mean copper concentrations ($\mu g\ g^{-1}$ dry mass) in the body (excluding reproductive organs and digestive gland) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 $\mu g\ g^{-1}$; E1 = 80 $\mu g\ g^{-1}$; E2 = 240 $\mu g\ g^{-1}$ copper oxychloride).

Table 13: Mean copper concentrations ($\mu g \ g^{-1}$ dry mass) in the body (excluding reproductive organs and digestive gland) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 $\mu g \ g^{-1}$; E1 = 80 $\mu g \ g^{-1}$; E2 = 240 $\mu g \ g^{-1}$ copper oxychloride; n = number of snails; SD = standard deviation).

Control				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	57.75	16.06	40.32-87.99
End of Week 1	9	47.57	11.46	33.22-66.84
End of Week 2	9	66.35	41.29	35.29-161.62
End of Week 3	9	77.52	49.5	36.15-196.49
End of Week 4	9	37.57	17.22	7.48-61.25
End of Week 5	9	45.56	11.32	34.3-69.99
End of Week 6	9	63.17	27.79	19.81-108.48
E1				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	53.9	24.32	30.45-108.48
End of Week 1	9	93.23	23.82	51.54-128.52
End of Week 2	9	102.61	24.69	61.27-140.15
End of Week 3	9	137.34	26.19	110.74-184.06
End of Week 4	9	115.43	44.53	65.93-190.18
End of Week 5	9	137.83	78.52	36.51-286.19
End of Week 6	9	160.28	60.79	70.37-237.06
E2				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	81.43	59.12	36.15-196.49
End of Week 1	9	97.56	47.24	11.09-171.73
End of Week 2	9	130.42	47.53	73.07-222.82
End of Week 3	9	120.67	52.66	31.67-188.28
End of Week 4	9	140.61	57.2	57.19-242.51
End of Week 5	9	208.79	52.58	150.45-318.23
End of Week 6	9	243.52	69.42	160.06-356.03

2.3.1.4 Whole body copper concentrations (reproductive organs, digestive gland, remainder and shell)

The mean whole body copper concentrations (μg g^{-1} dry mass) of the three test groups of *Helix aspersa* (weeks 1-6 pooled) are shown in Figure 17. There were significant differences (p<0.05) in whole body copper concentrations between all three groups. The control group had the lowest mean whole body copper concentration (55.28 ± 27.5 μg g^{-1} dry mass) and the 240 μg g^{-1} exposure group the highest (182.37 ± 76.15 μg g^{-1} dry mass) (Appendix Table XVIII).

Table 14 and Figure 18 show the mean whole body copper concentrations (μg g^{-1} dry mass) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, per week, over the six-week experimental period.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XIX) revealed that, within the control group, there was a significant (p<0.05) decrease in whole body copper concentrations from week 3 to 4 of observation, and a significant (p<0.05) increase from week 5 to 6. There was however no significant difference (p>0.05) in whole body copper concentrations between week 0 (i.e. prior to exposure) and week 6. Within the 80 μg g^{-1} exposure group, whole body copper concentrations increased significantly from week 0 to week 1 (p<0.001) and from week 2 to 3 (p<0.05). The whole body copper concentrations measured at the end of the sixth week of exposure (200.85 ± 53.5 μg g^{-1} dry mass) were also significantly higher (p<0.001) than those measured prior to exposure (49.87 ± 8.69 μg g^{-1} dry mass). Within the 240 μg g^{-1} exposure group significant increases (p<0.05) in whole body copper concentrations were found from week 0 to 1 and from week 4 to 5. Also, a significant (p<0.001) net increase in whole body copper concentrations was found over the six-week exposure period, i.e. from week 0 (56.41 ± 30.9 μg g^{-1} dry mass) to week 6 (272.24 ± 67.15 μg g^{-1} dry mass).

Comparisons between the three test groups (Appendix Table XX) showed that prior to exposure there were no significant differences (p>0.05) in whole body copper concentrations (μg g^{-1} dry mass) between any of the three groups. However, at the end of the final week of the experiment, the whole body copper concentrations of animals from all three groups differed significantly (p<0.05). The 240 μg g^{-1} exposure group had the highest mean whole body copper concentration (272.24 ± 67.15 μg g^{-1} dry mass) and the control group the lowest (67.84 ± 31.08 μg g^{-1} dry mass).
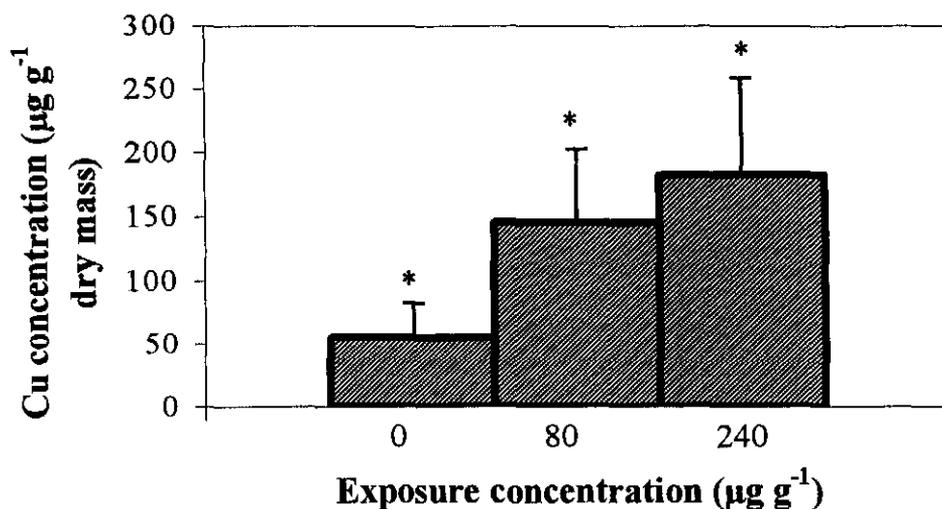
Figure 17: Mean whole body copper concentrations of the control group and two copper oxychloride exposure groups of *Helix aspersa* (n = 54 snails; * = significant difference).
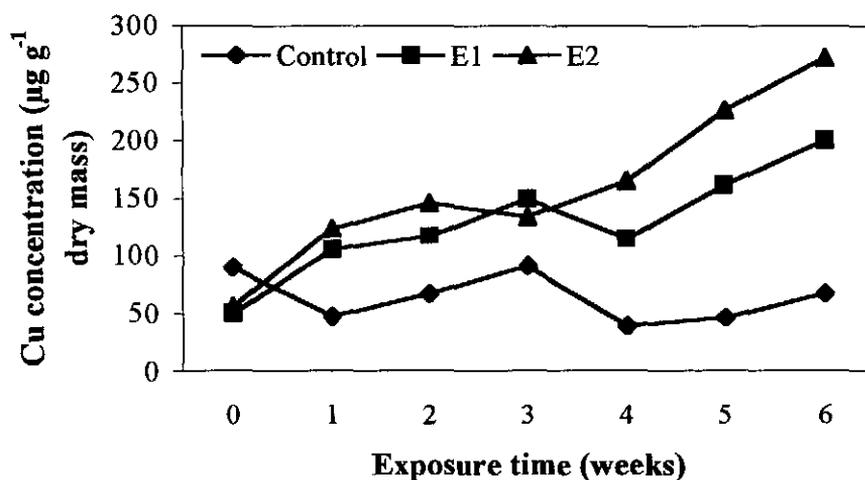
Figure 18: Mean whole body copper concentrations (μg g^{-1} dry mass) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride).

Table 14: Mean whole body copper concentrations ($\mu g \ g^{-1}$ dry mass) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 $\mu g \ g^{-1}$; E1 = 80 $\mu g \ g^{-1}$; E2 = 240 $\mu g \ g^{-1}$ copper oxychloride; n = number of snails; SD = standard deviation).

Control				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	89.62	77.38	34.19-267.41
End of Week 1	9	47.02	10.42	35.32-62.26
End of Week 2	9	66.74	40.84	34.19-160.4
End of Week 3	9	90.91	71.76	38.48-267.41
End of Week 4	9	38.92	17.45	6.8-56.14
End of Week 5	9	46.74	11.74	36.15-73.99
End of Week 6	9	67.84	31.08	25.01-123.67
E1				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	49.87	8.69	34.65-61.44
End of Week 1	9	105.21	26.88	53.02-151.62
End of Week 2	9	116.69	27.09	75.53-160.5
End of Week 3	9	150.14	27.16	109.62-190.11
End of Week 4	9	115.22	59.63	8.91-196.53
End of Week 5	9	162.36	85.53	37.57-309.88
End of Week 6	9	200.85	53.5	102.93-267.25
E2				
Time	**n**	**Mean**	**SD**	**Range**
Before exposure	9	56.41	30.9	35.09-113.43
End of Week 1	9	123.67	47.16	44.14-198.16
End of Week 2	9	145.98	59.68	81.36-268.21
End of Week 3	9	134.25	62.06	28.97-216.74
End of Week 4	9	165.35	62.08	79.91-283.03
End of Week 5	9	226.24	53.89	164.09-341.49
End of Week 6	9	272.24	67.15	185.01-387.64

2.3.2 Distribution of copper in the Helix aspersa *body*

Figure 19 shows the mean copper concentrations (weeks 1 to 6 pooled), in the reproductive organs, digestive gland and remainder of the body of animals from the three test groups of *Helix aspersa*.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXI) showed that, within the control group, the copper concentrations in the reproductive organs (2.15 ± 15.82 μg g^{-1} dry mass) were significantly ($p<0.001$) lower than those in the digestive gland and the rest of the body. The latter two did not differ significantly ($p>0.05$) from one another. In the 80 and 240 μg g^{-1} exposure groups the copper concentrations in the reproductive organs, digestive gland and rest of the body all differed significantly from one another ($p<0.001$). In both cases the mean copper concentration in the reproductive organs (75.93 ± 164.1 and 158.32 ± 281.03 μg g^{-1} dry mass respectively) was the lowest, and the mean copper concentration in the digestive gland (381.52 ± 183.5 and 468.81 ± 217.06 μg g^{-1} dry mass respectively) the highest.

Table XXII (Appendix) shows that, prior to exposure, the copper concentrations in the reproductive organs of animals from all three groups were significantly lower ($p<0.001$) than the copper concentrations in the digestive gland and rest of the body, whilst the latter two did not differ significantly ($p>0.05$) from each other. Similar results were also found for the control group at the end of the experimental period. Within the 80 and 240 μg g^{-1} exposure groups however, the copper concentrations in the digestive gland were significantly higher ($p<0.05$) at the end of week 6 than the copper concentrations in the reproductive organs and rest of the body, whilst the latter two did not differ significantly ($p>0.05$) from each other.
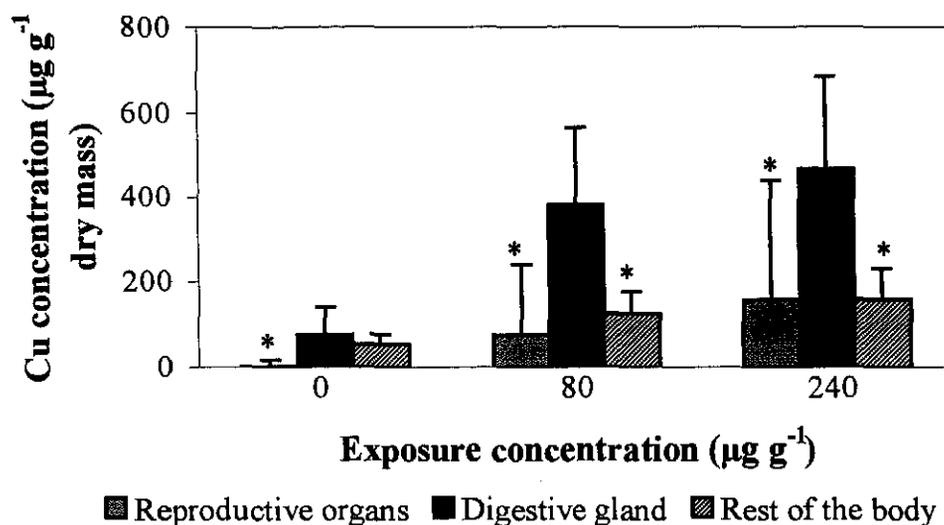
Figure 19: Mean copper concentrations (μg g^{-1} dry mass) in the reproductive organs, digestive gland and rest of the body, of animals from the control group and two copper oxychloride exposure groups of *Helix aspersa* (n = 54 in all cases; * = significantly different from digestive gland).

2.4 Helix aspersa *copper concentrations versus body mass and egg production*

2.4.1 *Whole body copper concentrations versus body mass changes over time*

The relationship between whole body copper concentrations and body mass changes over the six-week experimental period was tested for the three test groups using the Spearman Rank Order Correlation test. A strong significant (p<0.05) negative correlation (r = -0.886) was found for the 240 μg g^{-1} copper oxychloride exposure group (Appendix Table XXIII; Figure 20).

2.4.2 *Copper concentration in the reproductive organs versus egg production*

The relationship between reproductive organ copper concentrations and total egg production over the six-week experimental period was tested for the three test groups using the Spearman Rank Order Correlation test. A strong significant (p<0.05) negative correlation (r = -0.845) was found for the 240 μg g^{-1} copper oxychloride exposure group (Appendix Table XXIV; Figure 21).
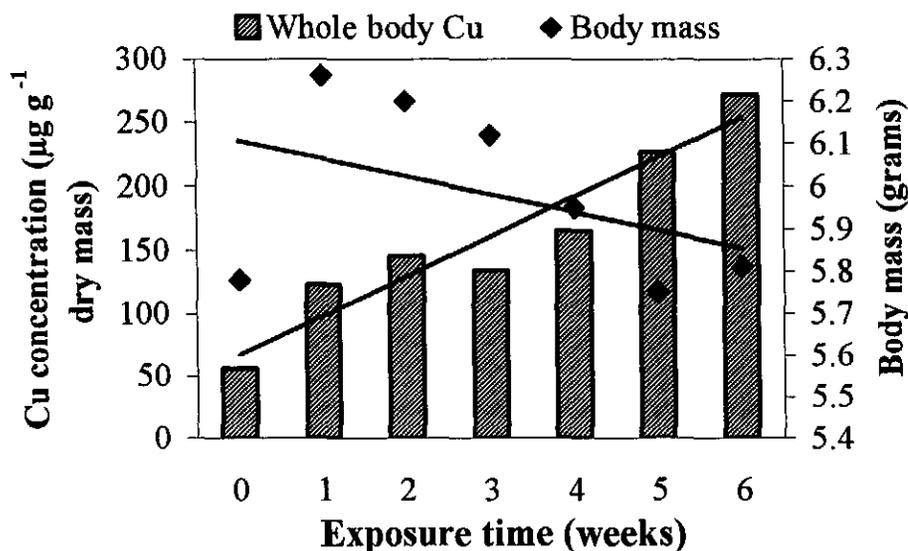
Figure 20: Relationship (r = -0.886) between whole body copper concentrations (μg g⁻¹ dry mass) and body mass (grams) in the 240 μg g⁻¹ copper oxychloride exposure group of *Helix aspersa*, over the six-week experimental period (lines = trendlines).
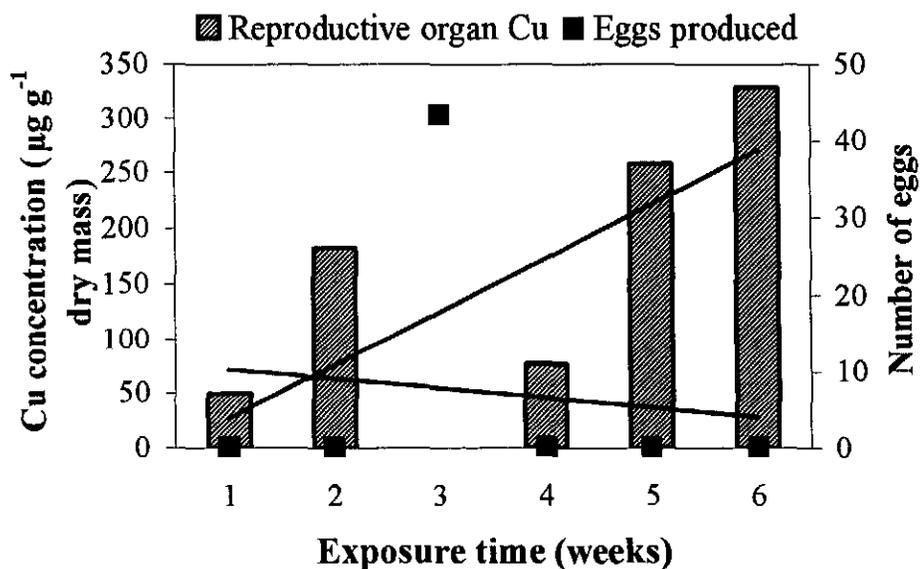
Figure 21: Relationship (r = -0.845) between reproductive organ copper concentrations (μg g⁻¹ dry mass) and total egg production in the 240 μg g⁻¹ copper oxychloride exposure group of *Helix aspersa*, over the six-week experimental period (lines = trendlines).

2.5 Neutral red retention time assay

Table 15 and Figure 22 show the mean neutral red retention times (minutes) of haemocytic lysosomes of the control group and two copper oxychloride exposure groups of *Helix aspersa*, per week, over the six-week experimental period. The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXV) revealed that, within the control group, there were no significant changes ($p>0.05$) in neutral red retention times between any of the consecutive weeks of observation. Within both the 80 $\mu g \; g^{-1}$ and 240 $\mu g \; g^{-1}$ copper oxychloride exposure groups there were significant decreases ($p<0.05$) in neutral red retention times from week 1 to 2, from week 2 to 3, and in total from week 0 (i.e. prior to exposure) to 6. In the cases of both exposure groups, the mean neutral red retention times measured at the end of week 6 of exposure were the shortest (10.22 ± 3.53 and 2.67 ± 2.83 minutes respectively) and the mean neutral red retention times measured prior to exposure the longest (28.22 ± 5.33 and 26.22 ± 6.04 minutes respectively).

Comparisons between the three test groups (Appendix Table XXVI) showed that prior to the exposure, there were no significant differences ($p>0.05$) in neutral red retention times between any of the three groups. However, at the end of the final week of exposure, the neutral red retention times of animals from all three test groups differed significantly ($p<0.05$) from one another. The mean neutral red retention time for the control group was the longest at the end of the experimental period, and the mean neutral red retention time for the 240 $\mu g \; g^{-1}$ group the shortest.
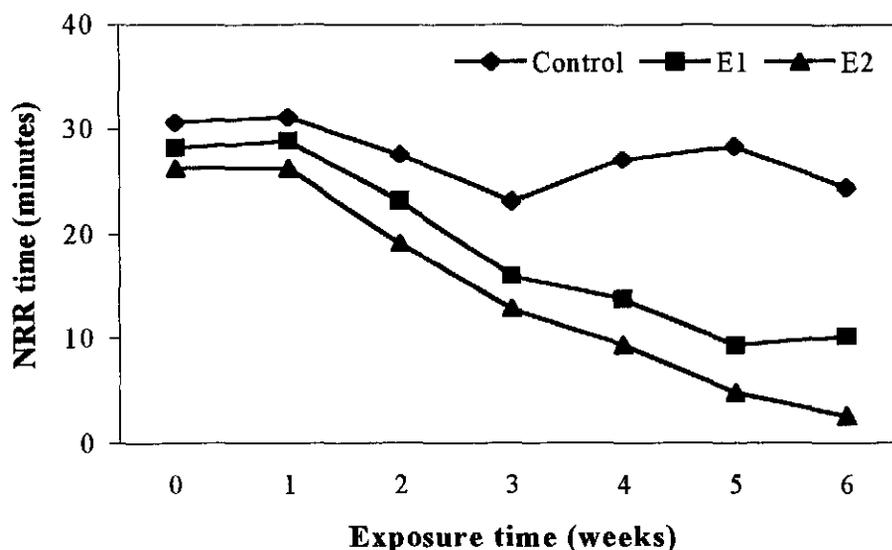
Figure 22: Mean neutral red retention (NRR) times (minutes) of haemocytic lysosomes of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 $\mu g \; g^{-1}$; E1 = 80 $\mu g \; g^{-1}$; E2 = 240 $\mu g \; g^{-1}$ copper oxychloride).

Table 15: Mean neutral red retention times (minutes) of haemocytic lysosomes of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; n = number of snails; SD = standard deviation).

Control				
Time	**N**	**Mean**	**SD**	**Range**
Before exposure	9	30.67	6.33	20.0-36.0
End of Week 1	9	31.11	4.81	24.0-36.0
End of Week 2	9	27.56	10.67	12.0-40.0
End of Week 3	9	23.11	5.58	16.0-32.0
End of Week 4	9	27.11	6.25	20.0-36.0
End of Week 5	9	28.44	5.81	20.0-36.0
End of Week 6	9	24.44	8.35	16.0-44.0
E1				
Time	**N**	**Mean**	**SD**	**Range**
Before exposure	9	28.22	5.33	20.0-36.0
End of Week 1	9	28.89	4.81	20.0-36.0
End of Week 2	9	23.11	5.93	16.0-32.0
End of Week 3	9	16.0	5.29	12.0-28.0
End of Week 4	9	13.78	4.52	8.0-24.0
End of Week 5	9	9.33	6.33	0.0-20.0
End of Week 6	9	10.22	3.53	4.0-16.0
E2				
Time	**N**	**Mean**	**SD**	**Range**
Before exposure	9	26.22	6.04	20.0-40.0
End of Week 1	9	26.22	6.04	20.0-36.0
End of Week 2	9	19.11	4.81	12.0-28.0
End of Week 3	9	12.89	2.67	8.0-16.0
End of Week 4	9	9.33	4.0	4.0-16.0
End of Week 5	9	4.89	5.21	0.0-16.0
End of Week 6	9	2.67	2.83	0.0-8.0

2.6 Neutral red retention times versus body copper concentrations over time

Figure 23 illustrates the relationship between mean NRR times and mean body copper concentrations in each of the three test groups over the six-week experimental period. The control clustered in the top left corner, whereas the two exposure groups both showed a marked change after one week of exposure and a strong negative correlation for the entire experimental period. Regression analysis showed a clear downward trend in the case of both exposure groups, with R^2 values ($p<0.05$) of 0.77 and 0.84 for the 80 μg g^{-1} and 240 μg g^{-1} copper oxychloride groups respectively.
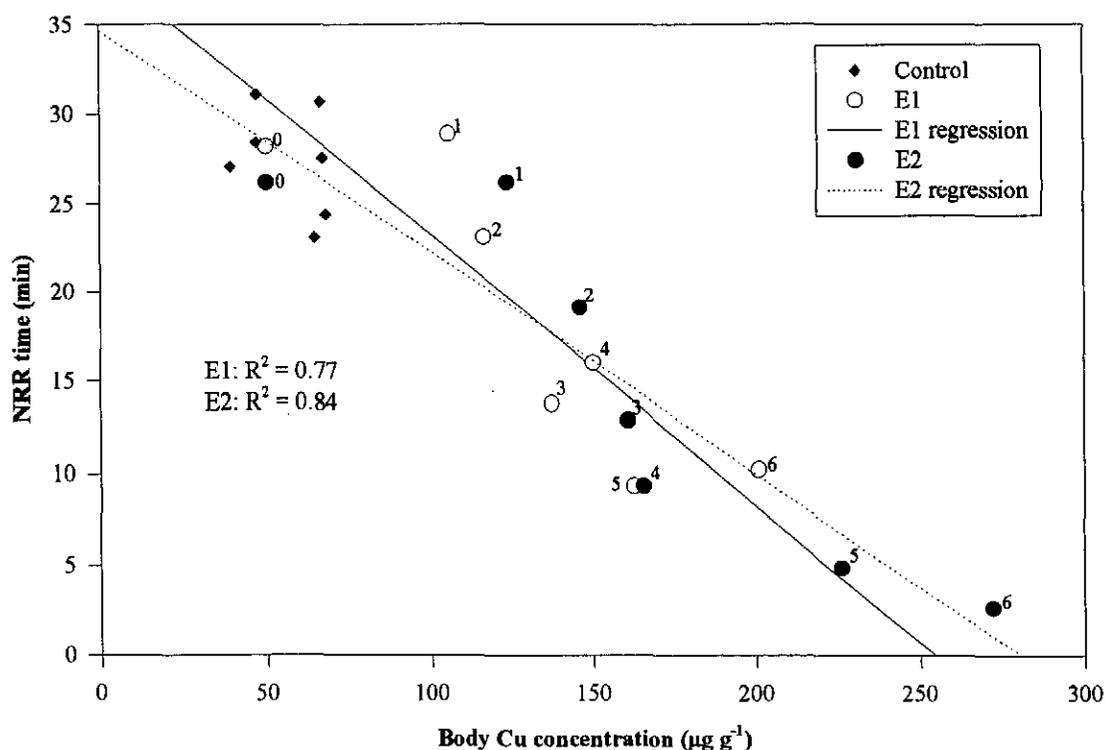
Figure 23: The relationship between neutral red retention (NRR) time (minutes) and whole body copper concentrations (μg g^{-1} dry mass) in the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; n = 9 individuals per week).

2.7 Histological analysis

2.7.1 Digestive gland

2.7.1.1. Digestive gland tubule area

Table 16 and Figure 24 show the mean area (μm^2) of digestive gland tubules of the control group and two copper oxychloride exposure groups of *Helix aspersa*, measured at the end of the six-week experimental period. The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXVII) revealed no significant differences ($p>0.05$) in digestive gland tubule area between any of the three test groups.
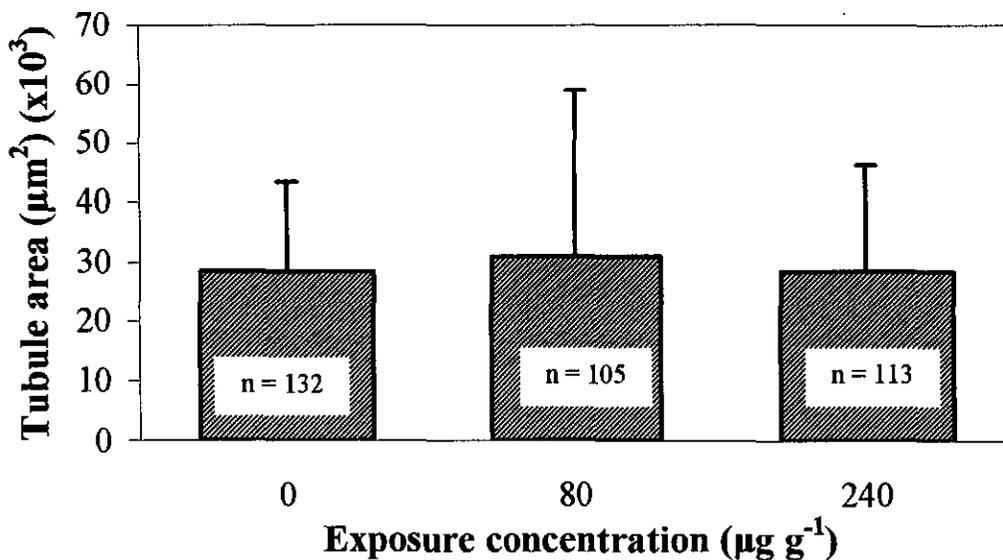
Figure 24: Mean ($\pm$SD) digestive gland tubule area (μm^2) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, measured at the end of a six-week experimental period.

Table 16: Mean tubule area (μm^2) of the digestive gland of the control group and two copper oxychloride exposure groups of *Helix aspersa*, measured at the end of the six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; n = number of digestive gland tubules; SD = standard deviation).

Groups	n	Mean ± SD	Range
Control	132	28388.4 ± 14962.79	7192.31-118810.2
E1	105	30811.67 ± 28103.22	7152.78-263233.31
E2	113	28347.86 ± 17865.09	8110.17-116101.5

2.7.1.2 Digestive gland epithelium height

Table 17 and Figure 25 show the mean height (μm) of the digestive gland epithelium of the control group and two copper oxychloride exposure groups of *Helix aspersa*, measured at the end of the six-week experimental period. The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXVIII) revealed that the epithelium height in the digestive glands of all three test groups differed significantly ($p<0.001$). The control exhibited the highest mean digestive gland epithelium height (32.88 ± 7.2 μm) and the 240 μg g^{-1} exposure group the lowest (28.71 ± 7.12 μm).
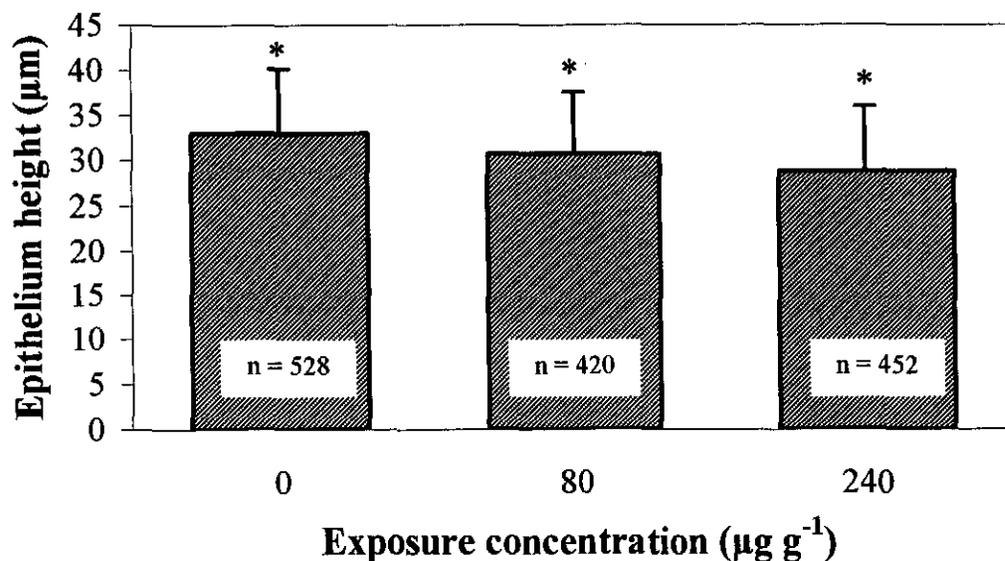
Figure 25: Mean (±SD) digestive gland epithelium height (µm) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (* = significant difference).

Table 17: Mean epithelium height (µm) in the digestive gland of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (Control = 0 µg g^{-1}; E1 = 80 µg g^{-1}; E2 = 240 µg g^{-1} copper oxychloride; n = number of cells; SD = standard deviation).

Groups	n	Mean ± SD	Range
Control	528	32.88 ± 7.2	17.22-55.4
E1	420	30.66 ± 6.89	12.02-59.11
E2	452	28.71 ± 7.12	5.27-55.38

2.7.1.3 Digestive gland epithelium area

Table 18 and Figure 26 show the mean digestive gland epithelium area of the control group and two copper oxychloride exposure groups of *Helix aspersa*, measured at the end of the six-week experimental period. The epithelium area is expressed as a percentage of the total area of the digestive tubule.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXIX) showed significant differences (p<0.001) in percentage epithelium area between the control group and the two exposure groups. The control had the highest mean epithelium area percentage (80.07 ± 7.59 %) and the 240 μg g^{-1} exposure group the lowest (69.65 ± 12.64 %).
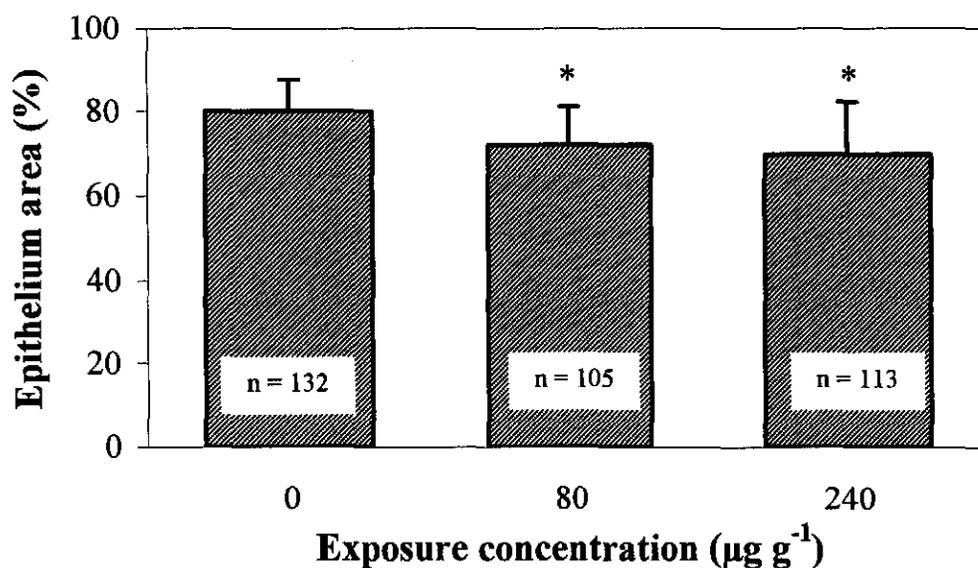
Figure 26: Mean (±SD) digestive gland epithelium area (%) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (* = significant difference from control).

Table 18: Mean digestive gland epithelium area (%) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; n = number of dugestive gland tubules; SD = standard deviation).

Groups	n	Mean ± SD	Range
Control	132	80.07 ± 7.59	52.88-94.44
E1	105	71.79 ± 9.42	34.15-87.05
E2	113	69.65 ± 12.63	34.17-91.66

2.7.2 Hermaphrodite duct: Spermatozoan area in the vesicula seminalis region

Table 19 and Figure 27 show the mean area of spermatozoa in the vesicula seminalis of the control group and two copper oxychloride exposure groups of *Helix aspersa*, as measured after the six-week experimental period. The spermatozoan area is expressed as a percentage of the total area of the vesicula seminalis in cross section.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXX) produced no significant differences (p>0.05) in percentage spermatozoan area between any of the three test groups.
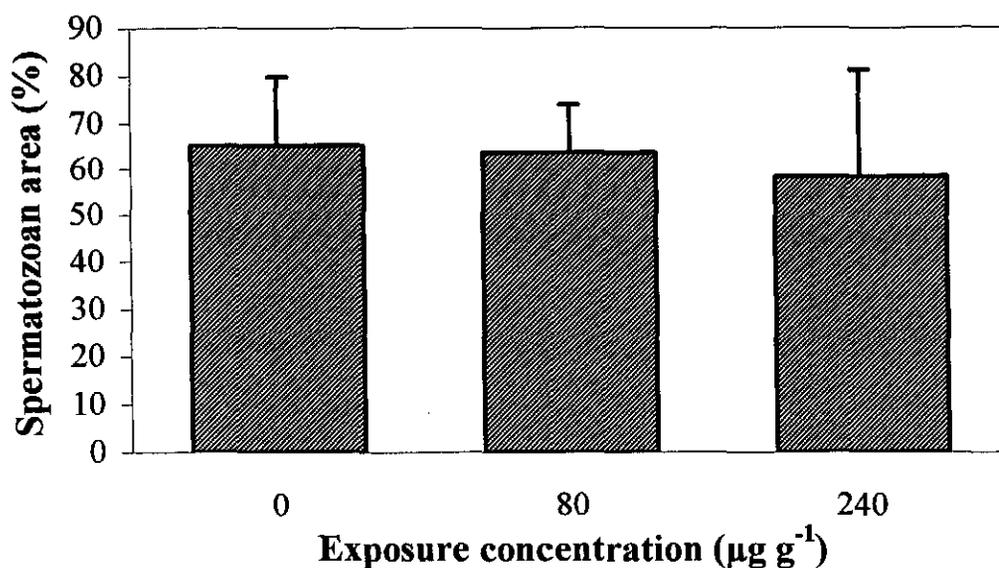
Figure 27: Mean (±SD) spermatozoan area (%) in the vesicula seminalis of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (n = 24 for all three groups).

Table 19: Mean spermatozoan area (%) in the vesicula seminalis of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (Control = 0 µg g^{-1}; E1 = 80 µg g^{-1}; E2 = 240 µg g^{-1} copper oxychloride; n = number of ducts; SD = standard deviation).

Groups	n	Mean ± SD	Range
Control	24	65.23 ± 14.44	28.76-83.26
E1	24	71.79 ± 9.42	41.85-78.09
E2	24	69.65 ± 12.63	12.11-87.23

2.7.3 Ovotestis

2.7.3.1 Spermatozoan area in the ovotestis

Table 20 and Figure 28 show the mean area (μm^2) covered by spermatozoa, per 1 mm^2 ovotestis, of the control group and two copper oxychloride exposure groups of *Helix aspersa*, measured after the six-week experimental period.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXXI) revealed significant differences (p<0.001) in spermatozoan area between the control group and the 240 μg g^{-1} copper oxychloride exposure group, as well as between the 80 and 240 μg g^{-1} exposure groups. The 80 μg g^{-1} exposure group had the highest mean spermatozoan area per 1 mm^2 of ovotestis (349 957.04 ± 128 837.62 μm^2), and the 240 μg g^{-1} exposure group the lowest (221 216.18 ± 193 448.17 μm^2).
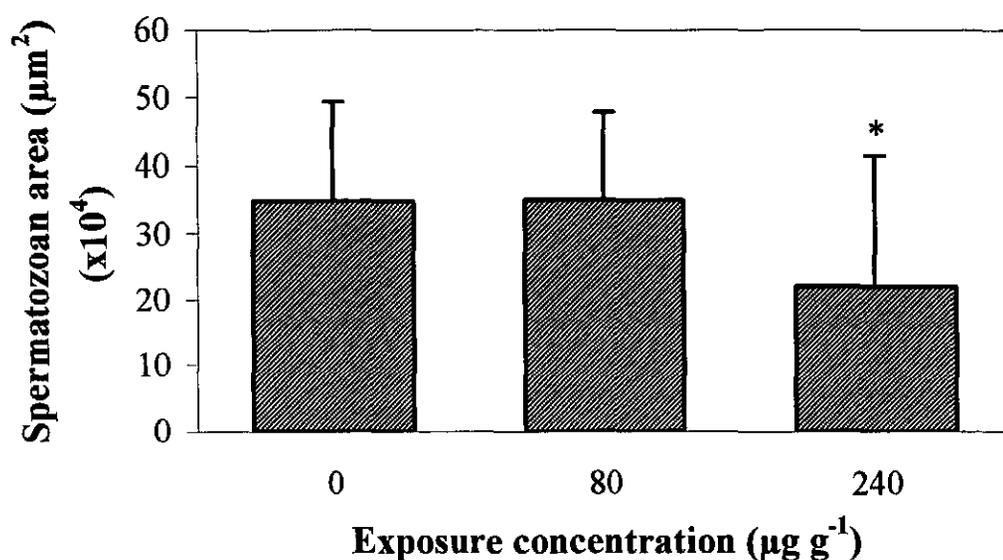
Figure 28: Mean (±SD) spermatozoan area per 1 mm^2 ovotestis of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (n = 80 for all three groups; * = significant difference from control).

Table 20: Mean spermatozoan area per 1 mm^2 ovotestis, of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (Control = 0 $\mu g\ g^{-1}$; E1 = 80 $\mu g\ g^{-1}$; E2 = 240 $\mu g\ g^{-1}$ copper oxychloride; n = number of measurements; SD = standard deviation).

Groups	n	Mean ± SD	Range
Control	80	346362.83 ± 147158.42	120312.29-681432.71
E1	80	349957.04 ± 128837.62	45918.57-620262.57
E2	80	221216.18 ± 193448.17	0.0-617362.0

2.7.3.2. Number of oocytes in the ovotestis

Table 21 and Figure 29 show the mean number of oocytes per 1 mm^2 ovotestis, of the control group and two copper oxychloride exposure groups of *Helix aspersa*, measured after the six-week experimental period.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXXII) produced significant differences (p<0.001) in oocyte numbers between all three test groups. The control group had the highest mean number of oocytes per 1 mm^2 ovotestis (5.09 ± 3.44) and the 240 $\mu g\ g^{-1}$ exposure group the lowest number (2.33 ± 1.64).
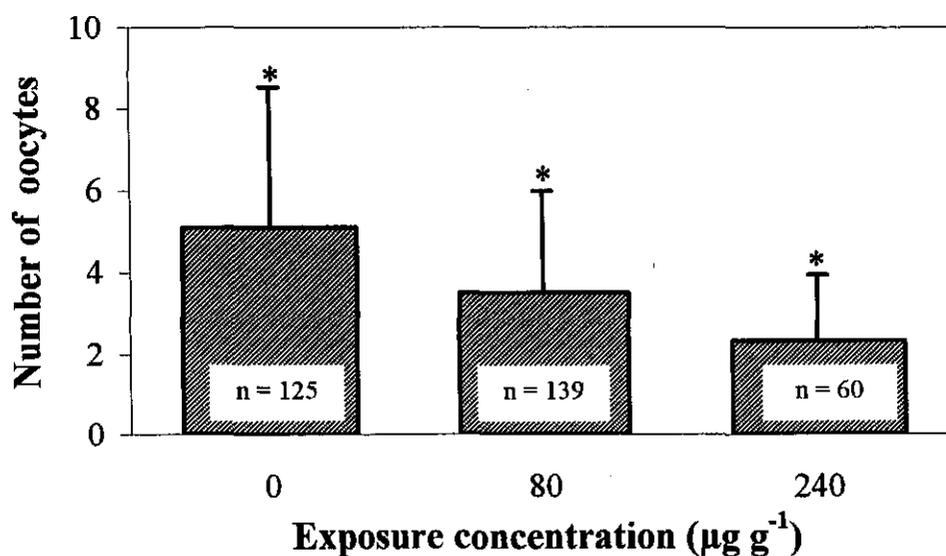
Figure 29: Mean (±SD) number of oocytes per 1 mm² ovotestis, of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (* = significant difference).

Table 21: Mean number of oocytes per 1 mm² ovotestis, of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (Control = 0 µg g⁻¹; E1 = 80 µg g⁻¹; E2 = 240 µg g⁻¹ copper oxychloride; n = number of measurements; SD = standard deviation).

Groups	n	Mean ± SD	Range
Control	125	5.09 ± 3.44	1.0-17.0
E1	139	3.52 ± 2.48	1.0-13.0
E2	60	2.33 ± 1.64	1.0-8.0

2.8 Digestive gland copper concentrations versus digestive epithelium height and area

2.8.1 Digestive gland copper concentrations versus digestive gland epithelium height

When the mean digestive gland epithelium heights measured for the three test groups were plotted, in decreasing order, against their corresponding mean digestive gland copper concentrations (μg g^{-1} dry mass), determined over the six-week experimental period, regression analysis revealed a strong significant (p<0.001) negative correlation (r = -0.963) (Figure 30).

2.8.2 Digestive gland copper concentrations versus digestive gland epithelium area

When the mean digestive gland epithelium area (percentage), measured for the three test groups were plotted, in decreasing order, against their corresponding mean digestive gland copper concentrations (μg g^{-1} dry mass), determined over the six-week experimental period, regression analysis revealed a strong significant (p<0.001) negative correlation (r = -0.999) (Figure 31).
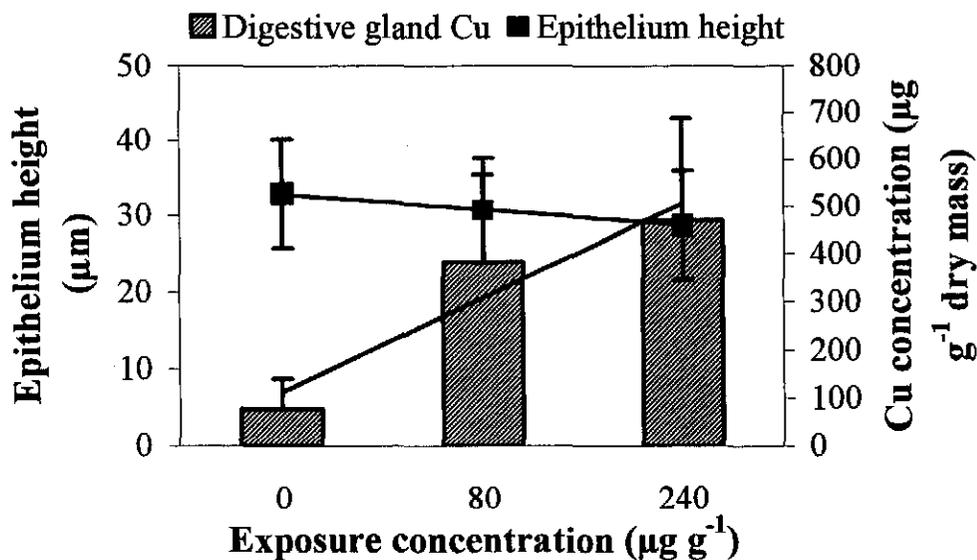
Figure 30: Relationship (r = -0.963) between digestive gland copper concentrations (μg g^{-1} dry mass) and digestive gland epithelium height (μm), in the control group and two copper oxychloride exposure groups of *Helix aspersa*, after the six-week experimental period (lines = trendlines).

■ Digestive gland Cu ■ Epithelium area
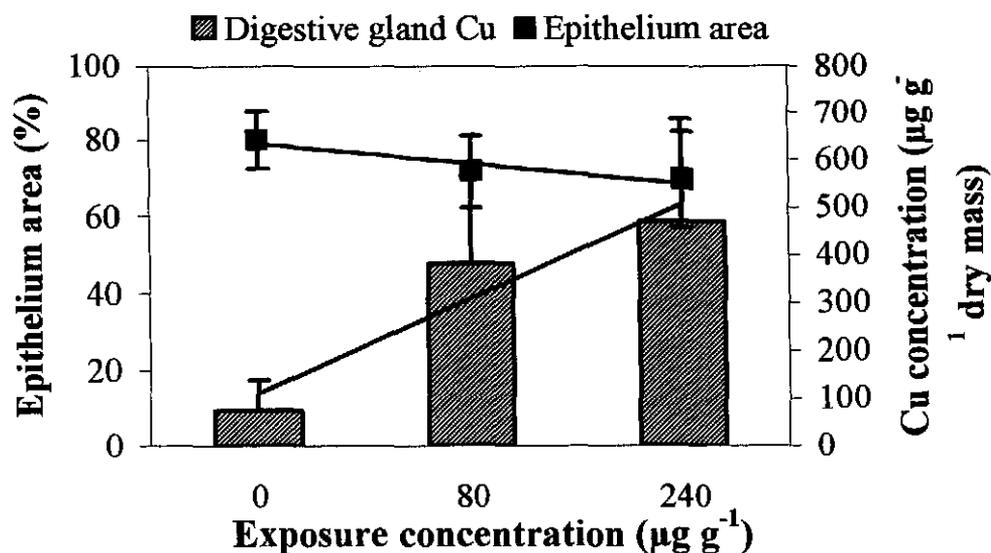
Figure 31: Relationship (r = -0.999) between digestive gland copper concentrations (μg g^{-1} dry mass) and digestive gland epithelium area (%), in the control group and two copper oxychloride exposure groups of *Helix aspersa*, after the six-week experimental period (lines = trendlines).

2.9 Reproductive organ copper concentrations versus spermatozoan area and oocyte numbers

2.9.1 Reproductive organ copper concentrations versus spermatozoan area

When the mean spermatozoan area (μm^2) per 1 mm^2 ovotestis, measured for the three test groups were plotted, in decreasing order, against their corresponding mean reproductive organ copper concentrations (μg g^{-1} dry mass), determined over the six-week experimental period, regression analysis revealed a strong significant ($p < 0.001$) negative correlation (r = -0.870) (Figure 32).

2.9.2 Reproductive organ copper concentrations versus oocyte numbers

When the mean oocyte numbers per 1 mm^2 ovotestis, measured for the three test groups were plotted, in decreasing order, against their corresponding mean reproductive organ copper concentrations (μg g^{-1} dry mass), determined over the six-week experimental period, regression analysis revealed a strong significant ($p < 0.001$) negative correlation (r = -0.994) (Figure 33).
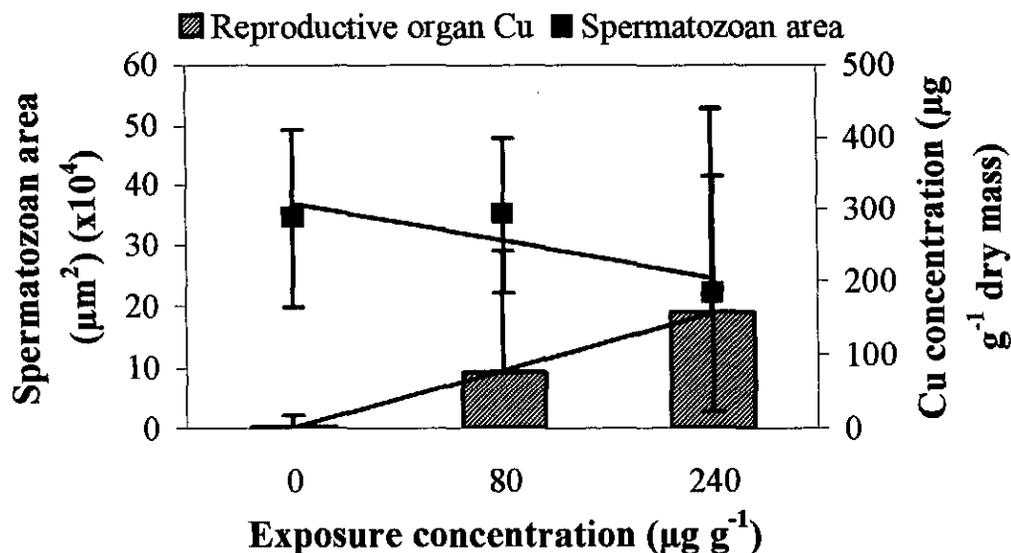
Figure 32: Relationship (r = -0.870) between reproductive organ copper concentrations ($\mu g\ g^{-1}$ dry mass) and spermatozoan area (μm^2) per 1 mm^2 ovotestis, in the control group and two copper oxychloride exposure groups of *Helix aspersa*, after the six-week experimental period (lines = trendlines).
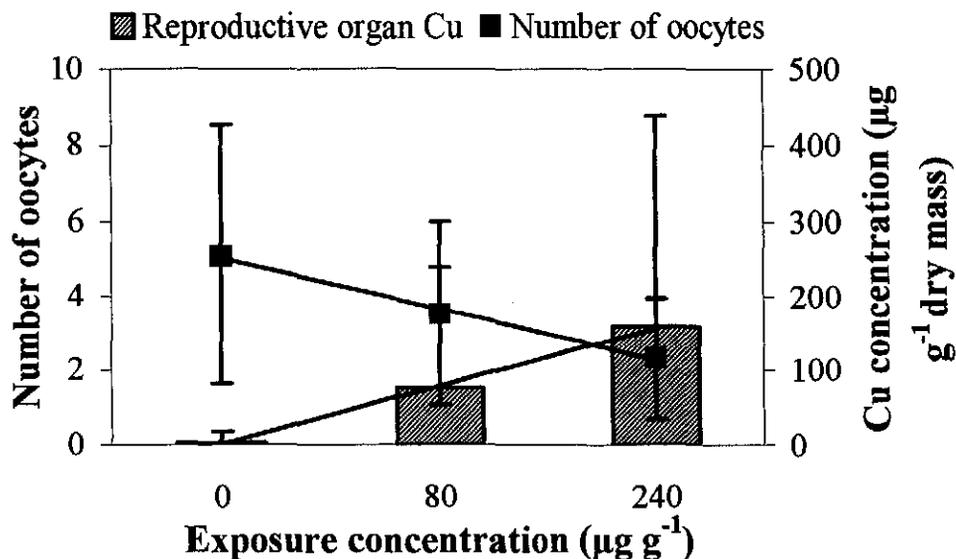
Figure 33: Relationship (r = -0.994) between reproductive organ copper concentrations ($\mu g\ g^{-1}$ dry mass) and oocyte numbers per 1 mm^2 ovotestis, in the control group and two copper oxychloride exposure groups of *Helix aspersa*, after the six-week experimental period (lines = trendlines).

3. Field survey

3.1 Environmental copper concentrations

Figure 34 and Table 22 show the mean copper concentrations (μg g^{-1} dry mass) in vine leaves and soil collected from the two field sites.

Vine leaves and soil from the Helderberg site had the lowest mean copper concentrations (8.77 ± 1.96 and 5.04 ± 0.14 μg g^{-1} dry mass respectively), whereas the highest mean copper concentrations were found in vine leaves from the Delheim site (first sampling) (1247.25 ± 342.12 μg g^{-1} dry mass) and in soil from the same site, second sampling (15.11 ± 0.6 μg g^{-1} dry mass). The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXXIII) produced no significant differences ($p > 0.05$) in vine leaf copper concentrations between the Helderberg (control) and Delheim sites, or between the two sampling dates at Delheim. There were however significant differences ($p < 0.05$) in soil copper concentrations between the Helderberg and Delheim (second sampling) sites, and between the first and second sampling from Delheim.
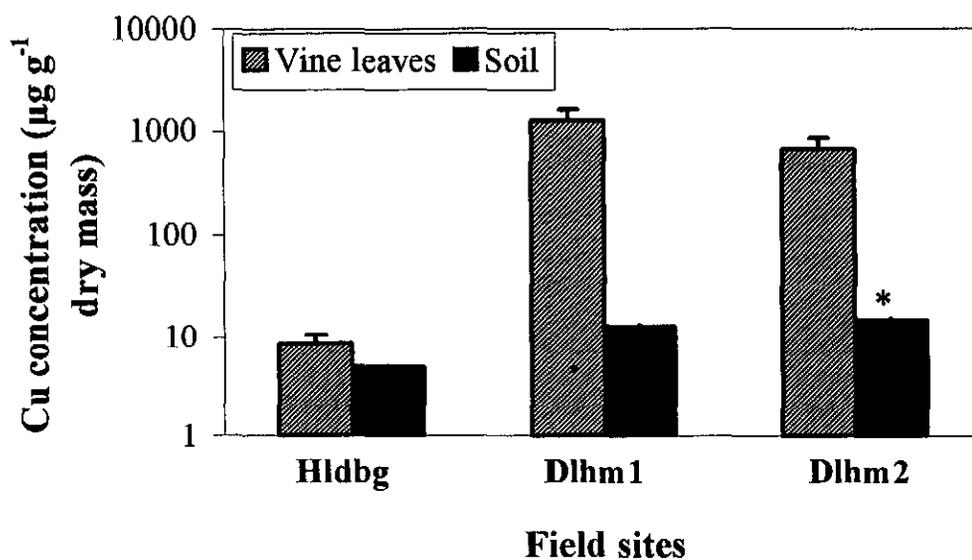
Figure 34: Mean (±SD) copper concentrations (μg g^{-1} dry mass) in vine leaves and soil collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (n = 3; * = significant difference from Helderberg site).

Table 22: Mean copper concentrations (μg g^{-1} dry mass) in vine leaves and soil collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (SD = standard deviation).

Site	Vine leaves			Soil		
	n	Mean ± SD	Range	n	Mean ± SD	Range
Helderberg	3	8.77±1.96	6.68-10.56	3	5.04±0.14	4.9-5.17
Delheim1	3	1247.25±342.12	907.86-1592.04	3	12.79±0.68	12.02-13.29
Delheim2	3	667.02±192.69	533.82-887.97	3	15.11±0.6	14.53-15.73

3.2 Copper concentrations and distribution in Helix aspersa

3.2.1 Copper concentrations

3.2.1.1 Copper concentrations in the reproductive organs

Figure 35 and Table 23 show the mean copper concentrations (μg g^{-1} dry mass) in the reproductive organs (ovotestis and hermaphrodite duct pooled) of *Helix aspersa*, collected from the two field sites.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXXIV) revealed no significant differences ($p>0.05$) in reproductive organ copper concentrations between the Helderberg (control) and Delheim field sites, or between the two sampling dates at Delheim.
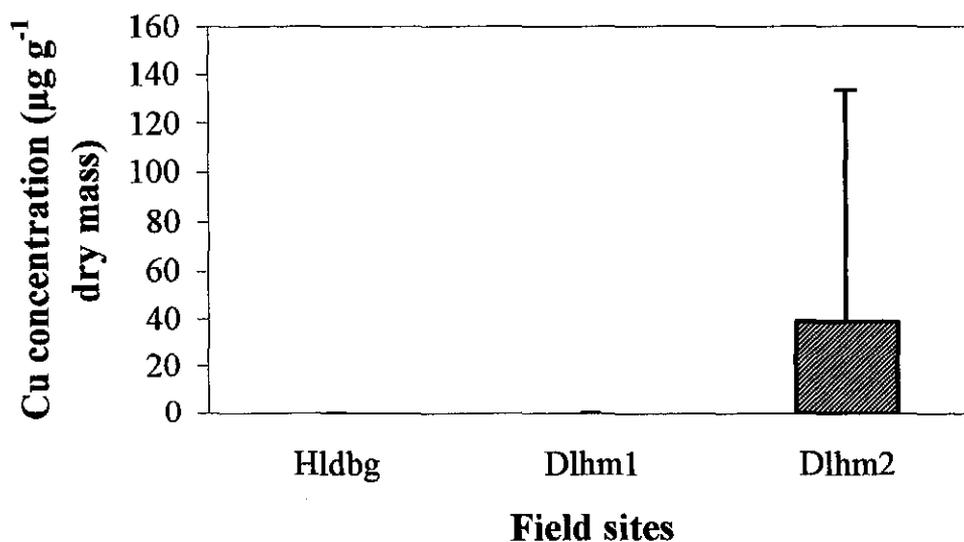
Figure 35: Mean ($\pm$SD) copper concentrations (μg g^{-1} dry mass) in the reproductive organs of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (n = 10 for Hldbg and Dlhm1, n = 6 for Dlhm2).

Table 23: Mean (±SD) reproductive organ copper concentrations (μg g^{-1} dry mass) of *Helix aspersa* collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (n = number of snails; SD = standard deviation).

Field sites	n	Mean ± SD	Range
Helderberg	10	0.0 ± 0.0	0.0-0.0
Delheim1	10	0.0 ± 0.0	0.0-0.0
Delheim2	6	38.76 ± 94.94	0.0-232.56

3.2.1.2 Copper concentrations in the digestive gland

Figure 36 and Table 24 show the mean copper concentrations (μg g^{-1} dry mass) in the digestive gland of *Helix aspersa*, collected from the two field sites.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXXV) produced significant differences (p<0.05) in digestive gland copper concentrations between the Helderberg (control) and Delheim (first and second sampling) sites, as well as between the first and second sampling dates at Delheim. Snails from the Helderberg site had the lowest mean digestive gland copper concentration (44.75 ± 19.43 μg g^{-1} dry mass), and snails from Delheim (second sampling) the highest (453.4 ± 123.68 μg g^{-1} dry mass).
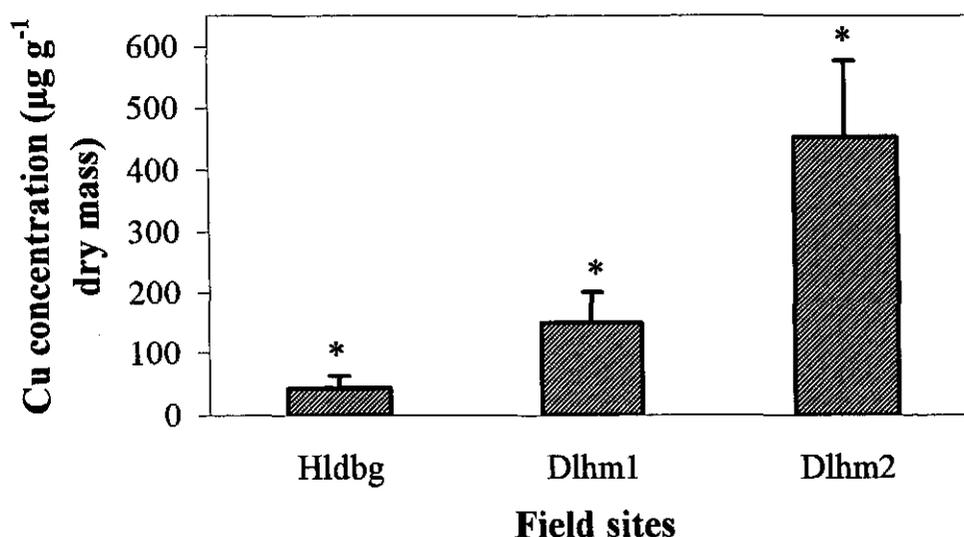
Figure 36: Mean (±SD) copper concentrations (μg g^{-1} dry mass) in the digestive gland of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (n = 10 for Hldbg and Dlhm1, n = 6 for Dlhm2; * = significant difference).

Table 24: Mean (±SD) digestive gland copper concentrations (μg g^{-1} dry mass) of *Helix aspersa* collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (n = number of snails; SD = standard deviation).

Field sites	n	Mean ± SD	Range
Helderberg	10	44.75 ± 19.43	20.43-83.43
Delheim1	10	151.55 ± 49.23	99.63-237.15
Delheim2	6	453.4 ± 123.68	306.24-651.16

3.2.1.3 Copper concentrations in the rest of the snail body and shell (excluding reproductive organs and digestive gland)

Figure 37 and Table 25 show the mean copper concentrations ($\mu g\ g^{-1}$ dry mass) in the "rest of the body" of *Helix aspersa*, collected from the two field sites.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXXVI) produced significant differences ($p<0.05$) in "rest of the body" copper concentrations between the Helderberg (control) and Delheim (first and second sampling) sites, as well as between the first and second sampling dates at Delheim. Snails from the Helderberg site had the lowest mean "rest of the body" copper concentration ($47.23 \pm 14.58\ \mu g\ g^{-1}$ dry mass), and snails from Delheim (second sampling) the highest ($254.33 \pm 112.76\ \mu g\ g^{-1}$ dry mass).
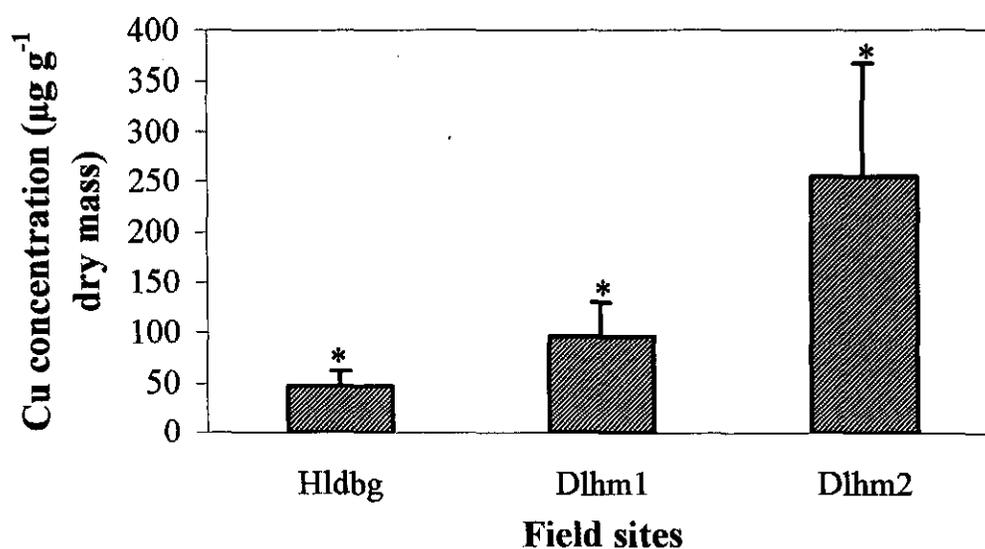
Figure 37: Mean ($\pm$SD) copper concentrations ($\mu g\ g^{-1}$ dry mass) in the body of *Helix aspersa* (excluding reproductive organs and digestive gland), collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (n = 10 for Hldbg and Dlhm1, n = 6 for Dlhm2; * = significant difference).

Table 25: Mean (±SD) copper concentrations ($\mu g\ g^{-1}$ dry mass) in the body of *Helix aspersa* (excluding reproductive organs and digestive gland), collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (n = number of snails; SD = standard deviation).

Field sites	n	Mean ± SD	Range
Helderberg	10	47.23 ± 14.58	22.38-78.88
Delheim1	10	95.88 ± 34.08	41.67-161.03
Delheim2	6	254.33 ± 112.76	139.68-397.25

3.2.1.4 Whole body copper concentrations (reproductive organs, digestive gland, remainder and shell)

Figure 38 and Table 26 show the mean whole body copper concentrations ($\mu g\ g^{-1}$ dry mass) in *Helix aspersa* collected from the two field sites.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXXVII) revealed significant differences ($p < 0.05$) in whole body copper concentrations between the Helderberg (control) and Delheim (first and second sampling) sites, as well as between the first and second sampling dates at Delheim. Snails from the Helderberg site had the lowest mean whole body copper concentration (47.03 ± 14.35 $\mu g\ g^{-1}$ dry mass) and those from Delheim (second sampling) the highest (274.63 ± 113.73 $\mu g\ g^{-1}$ dry mass).
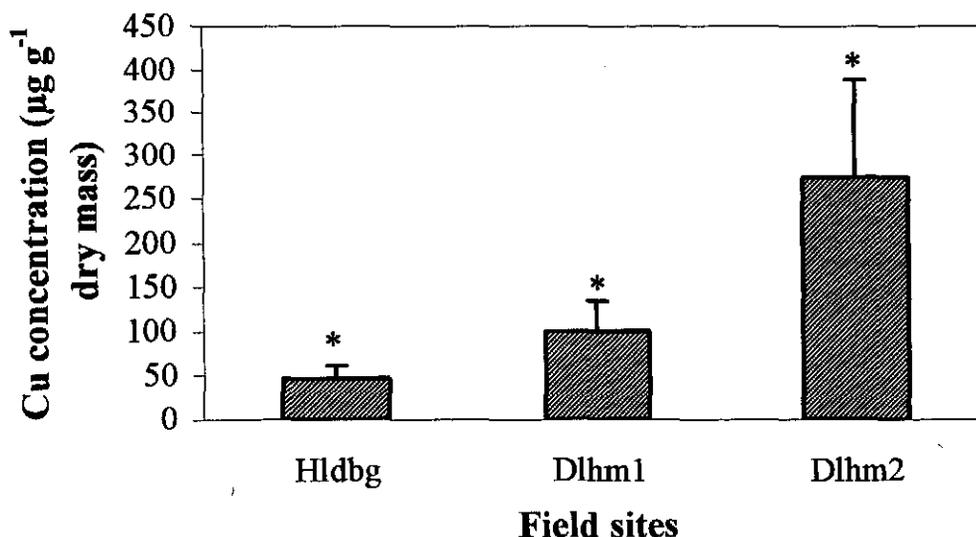
Figure 38: Mean (±SD) whole body copper concentrations (µg g^{-1} dry mass) in *Helix aspersa* collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (n = 10 for Hldbg and Dlhm1, n = 6 for Dlhm2; * = significant difference).

Table 26: Mean whole body copper concentrations (µg g^{-1} dry mass) of *Helix aspersa* collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (n = number of snails; SD = standard deviation).

Field sites	n	Mean ± SD	Range
Helderberg	10	47.03 ± 14.35	24.73-79.19
Delheim1	10	100.22 ± 33.62	46.87-161.14
Delheim2	6	274.63 ± 113.73	160.87-429.58

*3.2.2 Distribution of copper in the body of* Helix aspersa

Figure 39 shows the mean copper concentrations (μg g^{-1} dry mass), in the reproductive organs, digestive gland and remainder of the body of snails from the two field sites.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXXVIII) showed that copper concentrations in the digestive gland and "rest of the body" of animals from the Helderberg site did not differ significantly ($p > 0.05$). Copper concentrations in the reproductive organs of these snails were below detection limits. In the case of the animals collected from the Delheim site during both the first and second sampling, the copper concentrations in the reproductive organs, digestive gland and "rest of the body" all differed significantly from one another ($p < 0.05$). In both cases the mean copper concentration in the reproductive organs (below detection limits and 38.76 ± 94.94 μg g^{-1} dry mass respectively) was the lowest, and the mean copper concentration in the digestive gland (151.55 ± 49.23 and 453.4 ± 123.68 μg g^{-1} dry mass respectively) the highest.
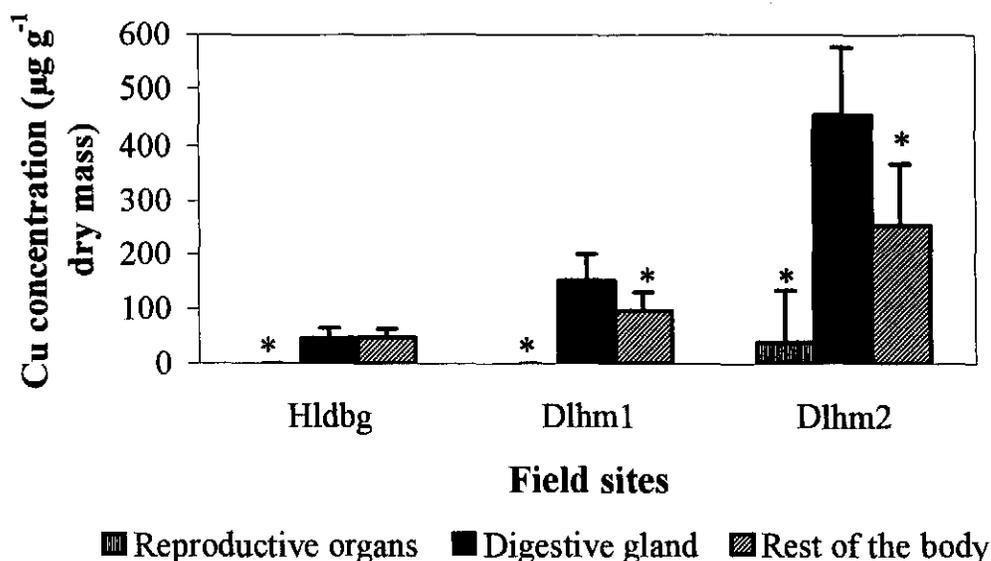
Figure 39: Mean ($\pm$SD) copper concentrations in the reproductive organs, digestive gland and rest of the body, of *Helix aspersa* collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (n = 10 for Hldbg and Dlhm1; n = 6 for Dlhm2; * = significantly different from digestive gland).

3.3 Neutral red retention time assay

Figure 40 and Table 27 show the mean neutral red retention times (minutes) of haemocytes of *Helix aspersa*, collected from the two field sites.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XXXIX) revealed significant differences (p<0.05) in neutral red retention times between the Helderberg and Delheim (first and second sampling) sites, as well as between the first and second sampling at Delheim. Snails from the Helderberg site had the longest mean neutral red retention time (27.6 ± 4.79 minutes), and snails from Delheim (second sampling) the shortest (12.0 ± 3.58 minutes).
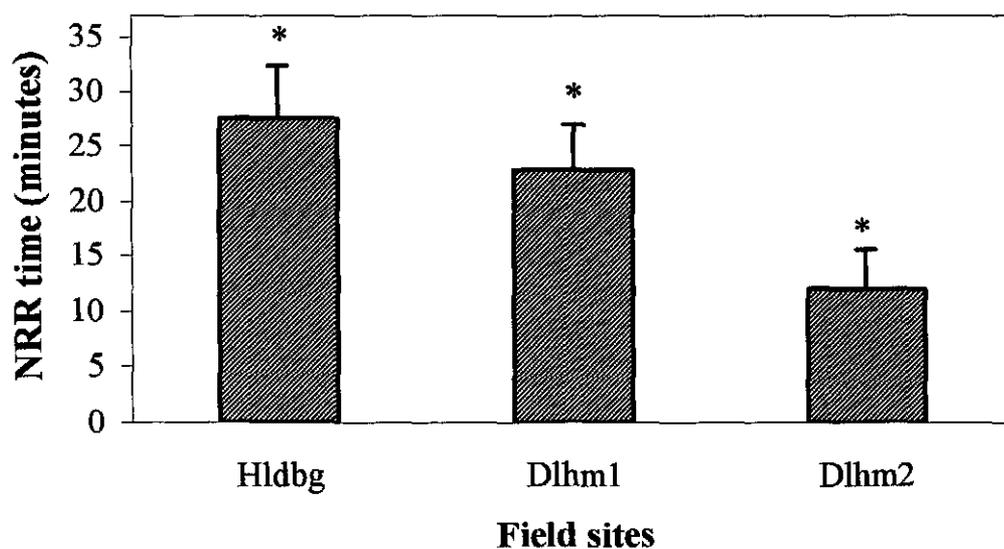
Figure 40: Mean (±SD) neutral red retention (NRR) times (minutes) of haemocytes of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (n = 10 for Hldbg and Dlhm1, n = 6 for Dlhm2; * = significant difference).

Table 27: Mean (±SD) neutral red retention times (minutes) of haemocytes of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (n = number of snails; SD = standard deviation).

Field sites	n	Mean ± SD	Range
Helderberg	10	27.6 ± 4.79	20.0-36.0
Delheim1	10	22.8 ± 4.24	16.0-32.0
Delheim2	6	12.0 ± 3.58	8.0-16.0

3.4 Neutral red retention times versus whole body copper concentrations

When the mean neutral red retention times (minutes) determined for the animals collected from each field site and on each sampling date were plotted, in decreasing order, against their corresponding mean whole body copper concentrations (μg g^{-1} dry mass), regression analysis revealed a strong significant (p<0.001) negative correlation (r = -0.997) (Figure 41).
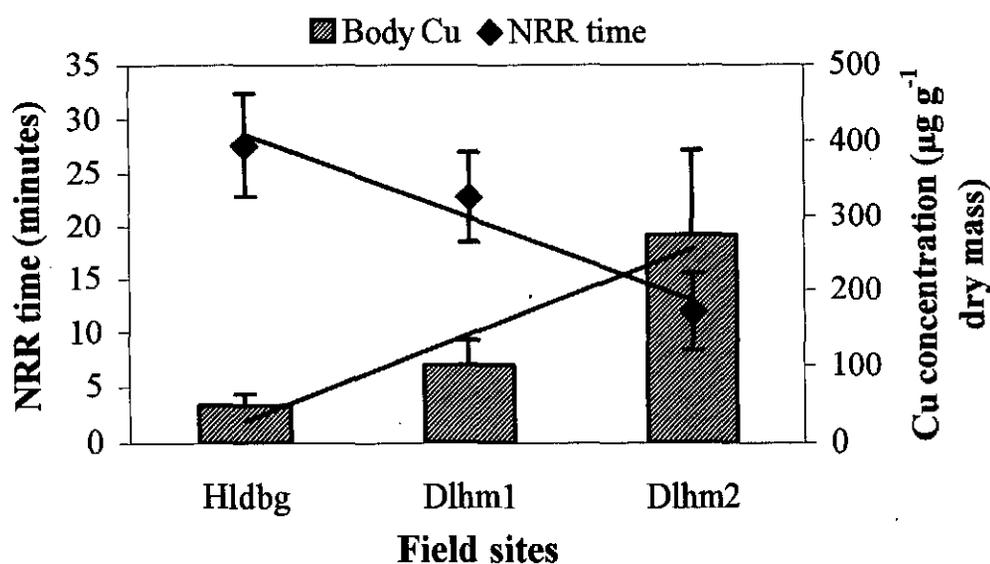
Figure 41: Relationship (r = -0.997) between mean ($\pm$SD) neutral red retention (NRR) times (minutes) and mean whole body copper concentrations (μg g^{-1} dry mass) of *Helix aspersa* collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (n = 10 for Hldbg and Dlhm1, n = 6 for Dlhm2; lines = trendlines).

3.5 *Histological analysis*

3.5.1 Digestive gland

3.5.1.1 Digestive gland tubule area

Table 28 and Figure 42 show the mean area (μm^2) of digestive gland tubules of *Helix aspersa*, collected from the two field sites. The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XL) revealed no significant differences ($p>0.05$) in digestive gland tubule area between snails from the Helderberg (control) and Delheim sites, or between snails collected during the first and second samplings at Delheim.
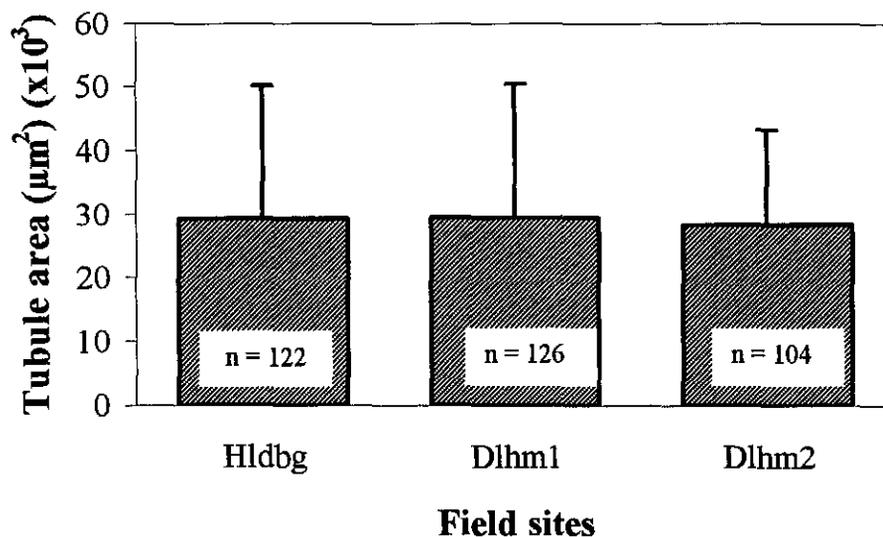
Figure 42: Mean (±SD) digestive gland tubule area (μm^2) of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride.

Table 28: Mean tubule area (μm^2) of the digestive gland of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (n = number of digestive gland tubules; SD = standard deviation).

Field sites	n	Mean ± SD	Range
Helderberg	122	29152.95 ± 21062.12	7192.31-118810.2
Delheim 1	126	29500.84 ± 20973.85	7004.56-118810.2
Delheim 2	104	28385.1 ± 15089.34	8709.69-78872.98

3.5.1.2 Digestive gland epithelium height

Table 29 and Figure 43 show the mean epithelium height (μm) in the digestive gland tubules of *Helix aspersa*, collected from the two field sites. The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XLI) produced significant differences ($p < 0.001$) in digestive gland epithelium height between the Helderberg site and second sampling date at Delheim, as well as between the two sampling dates at Delheim. Snails collected at Helderberg had the highest mean digestive gland epithelium height (33.99 ± 7.15 μm) and snails collected during the second sampling at Delheim the lowest (28.9 ± 7.29 μm).
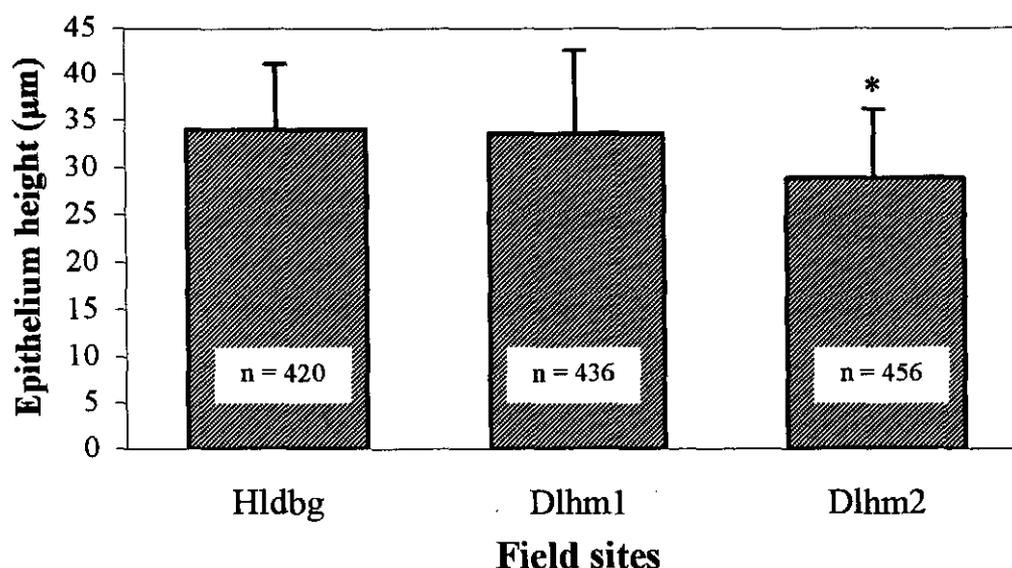
Figure 43: Mean (±SD) digestive gland epithelium height (μm) of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (* = significant difference from Helderberg site).

Table 29: Mean height (μm) of digestive gland epithelium of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (n = number of cells; SD = standard deviation).

Field sites	n	Mean ± SD	Range
Helderberg	420	33.99 ± 7.15	17.72-55.4
Delheim 1	436	33.67 ± 8.89	12.02-59.9
Delheim 2	456	28.9 ± 7.29	5.27-59.11

3.5.1.3 Digestive gland epithelium area

Table 30 and Figure 44 show the mean digestive gland epithelium area of *Helix aspersa*, collected from the two field sites. The area is expressed as a percentage of the total area of the digestive gland tubule.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XLII) produced significant differences (p<0.001) in percentage epithelium area between the Helderberg site and second sampling date at Delheim, as well as between the two sampling dates at Delheim. Snails collected at Helderberg had the highest mean epithelium area percentage (80.24 ± 9.19 %) and snails collected during the second sampling at Delheim the lowest (70.58 ± 10.5 %).
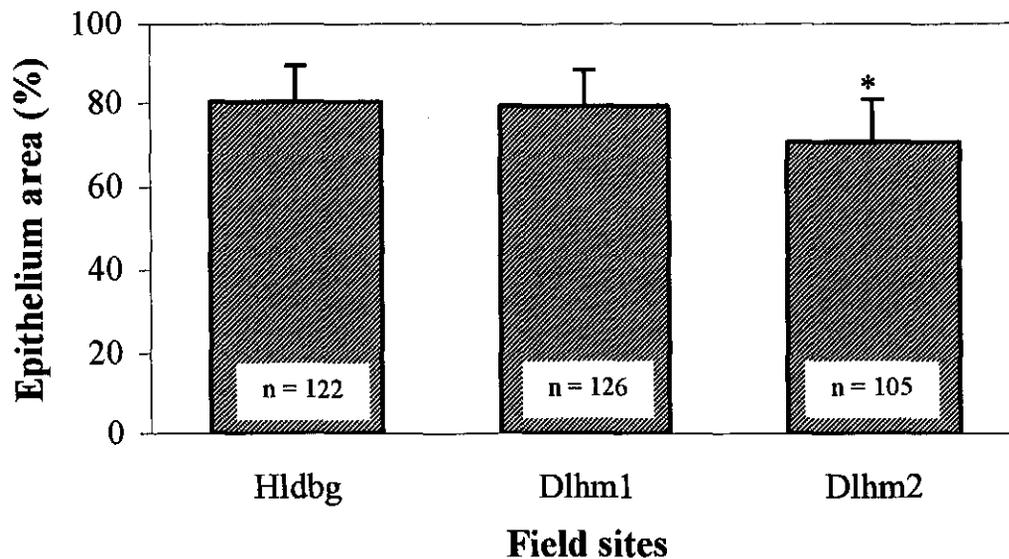
Figure 44: Mean (±SD) digestive gland epithelium area (%) of *Helix aspersa* collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (* = significant difference from Helderberg site).

Table 30: Mean digestive gland epithelium area (%) of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (n = number of digestive gland tubules; SD = standard deviation).

Field sites	n	Mean ± SD	Range
Helderberg	122	80.24 ± 9.19	34.15-95.44
Delheim 1	126	79.07 ± 9.24	34.15-92.27
Delheim 2	105	70.58 ± 10.5	46.03-94.44

3.5.2 Hermaphrodite duct: Spermatozoan area in the vesicula seminalis region

Table 31 and Figure 45 show the mean area of spermatozoa in the vesicula seminalis of *Helix aspersa*, collected from the two field sites. The area is expressed as a percentage of the total area of the vesicula seminalis in cross section.

The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XLIII) produced no significant differences ($p > 0.05$) in percentage spermatozoan area between snails from the Helderberg (control) and Delheim sites, or between snails collected during the first and second samplings at Delheim.
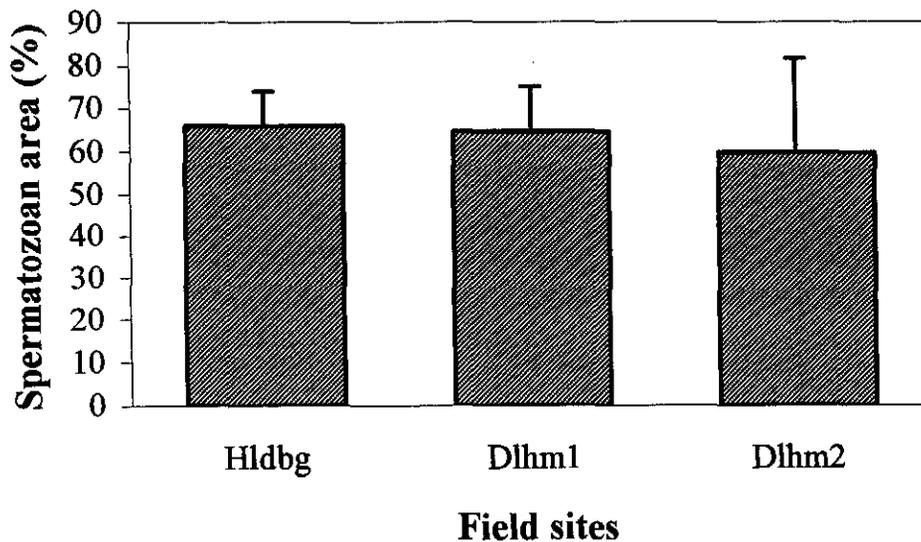
Figure 45: Mean (±SD) spermatozoan area (%) in the vesicula seminalis of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (n = 24).

Table 31: Mean spermatozoan area (%) in the vesicula seminalis of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (n = number of ducts; SD = standard deviation).

Field sites	n	Mean ± SD	Range
Helderberg	24	65.69 ± 8.26	52.06-82.36
Delheim 1	24	64.39 ± 10.76	41.85-78.24
Delheim 2	24	59.89 ± 21.96	12.12-87.23

3.5.3 Ovotestis

3.5.3.1 Spermatozoan area in the ovotestis

Table 32 and Figure 46 show the mean area (μm^2) covered by spermatozoa, per 1 mm^2 ovotestis of *Helix aspersa*, collected from the two field sites. The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XLIV) revealed significant differences ($p<0.05$) in spermatozoan area between the Helderberg site and second sampling date at Delheim, as well as between the two sampling dates at Delheim. Snails collected at Helderberg had the highest mean spermatozoan area per 1 mm^2 of ovotestis (360 065.14 ± 132 768.17 μm^2), and snails collected during the second sampling at Delheim the lowest (278 176.14 ± 190 357.09 μm^2).
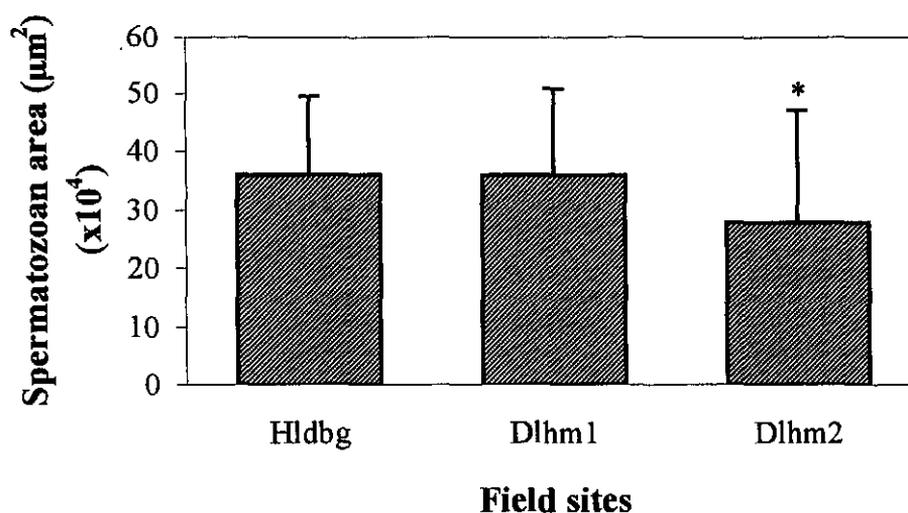
Figure 46: Mean (±SD) spermatozoan area per 1 mm^2 ovotestis of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (n = 80; * = significant difference from Helderberg site).

Table 32: Mean spermatozoan area per 1 mm^2 ovotestis of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (n = number of measurements; SD = standard deviation).

Field sites	n	Mean ± SD	Range
Helderberg	80	360065.14 ± 132768.17	100019.43-681432.71
Delheim 1	80	358545.2 ± 147785.0	45918.57-637557.86
Delheim 2	80	278176.14 ± 190357.09	0.0-617362.0

3.5.3.2 Number of oocytes in the ovotestis

Table 33 and Figure 47 show the mean number of oocytes per 1 mm^2 ovotestis of *Helix aspersa*, collected from the two field sites. The Kruskal-Wallis One Way Analysis of Variance on Ranks test (Appendix Table XLV) produced significant differences (p<0.05) in oocyte numbers between the Helderberg site and second sampling date at Delheim, as well as between the two sampling dates at Delheim. Snails collected at Helderberg had the highest mean number of oocytes per 1 mm^2 ovotestis (5.33 ± 3.58) and snails collected during the second sampling at Delheim the lowest number (3.87 ± 2.8).
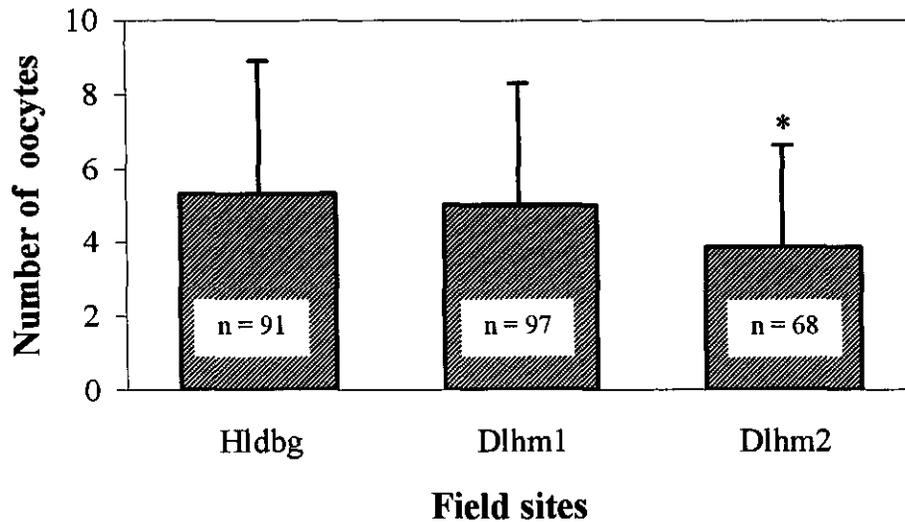
Figure 47: Mean (±SD) number of oocytes per 1 mm² ovotestis of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg), and from a contaminated vineyard (Dlhm = Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (* = significant difference from Helderberg site).

Table 33: Mean number of oocytes per 1 mm² ovotestis of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (n = number of measurements; SD = standard deviation).

Field sites	n	Mean ± SD	Range
Helderberg	91	5.33 ± 3.58	1.0-17.0
Delheim 1	97	5.02 ± 3.3	1.0-13.0
Delheim 2	68	3.87 ± 2.8	1.0-10.0

3.6 Digestive gland copper concentrations versus digestive epithelium height and area

3.6.1 Digestive gland copper concentrations versus digestive gland epithelium height

When the mean digestive gland epithelium heights measured for the snails from the Helderberg and Delheim sites were plotted, in decreasing order, against their corresponding mean digestive gland copper concentrations ($\mu g\ g^{-1}$ dry mass), regression analysis revealed a strong significant ($p<0.001$) negative correlation ($r = -0.980$) (Figure 48).

3.6.2 Digestive gland copper concentrations versus digestive gland epithelium area

When the mean digestive gland epithelium area (percentage), measured for the snails from the Helderberg and Delheim sites were plotted, in decreasing order, against their corresponding mean digestive gland copper concentrations ($\mu g\ g^{-1}$ dry mass), regression analysis revealed a strong significant ($p<0.001$) negative correlation ($r = -0.990$) (Figure 49).
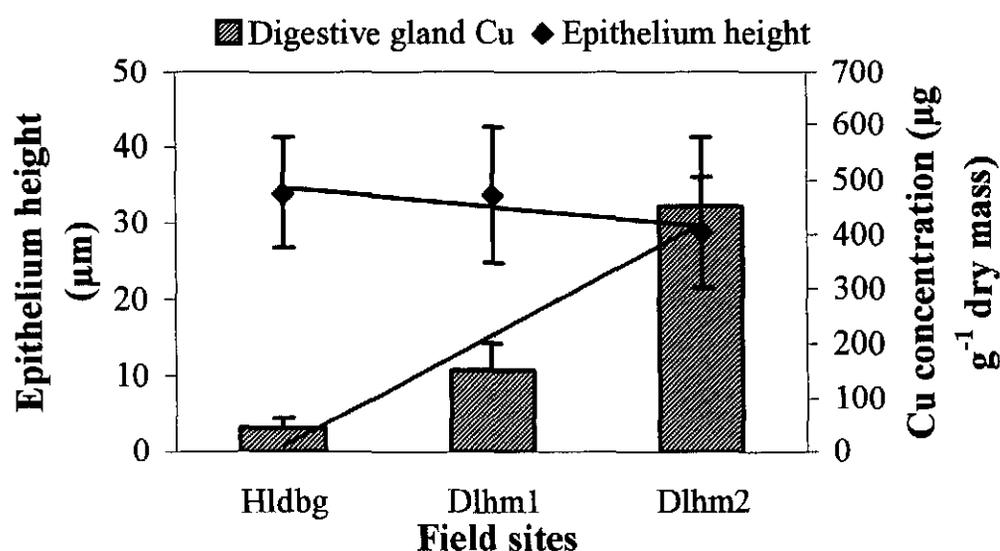

Figure 48: Relationship ($r = -0.980$) between digestive gland copper concentrations ($\mu g\ g^{-1}$ dry mass) and digestive gland epithelium height (μm), of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg) and a contaminated vineyard (Dlhm = Delheim), 1 week (Dlhm1 = Delheim 1) and 2 months (Dlhm2 = Delheim 2) after copper oxychloride application (lines = trendlines).
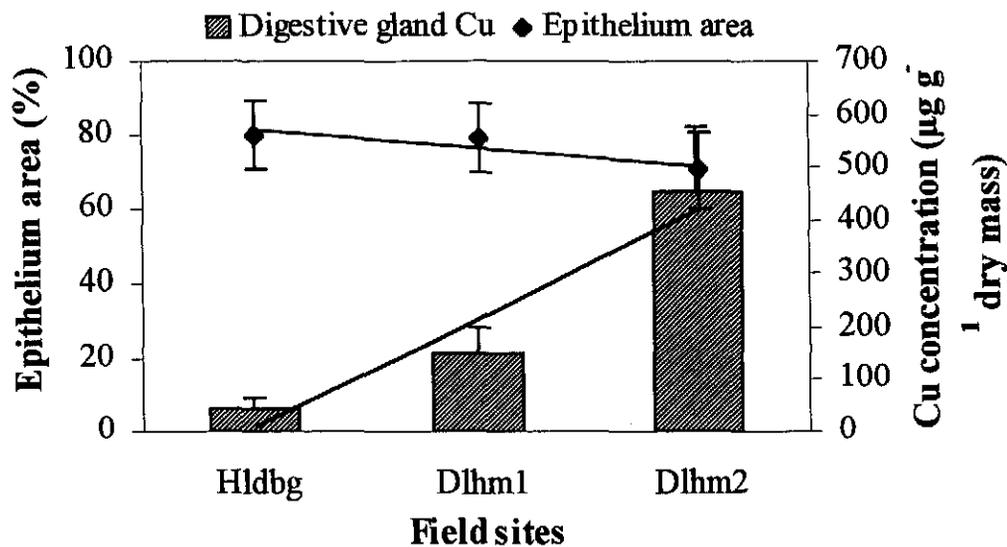
Figure 49: Relationship (r = -0.990) between digestive gland copper concentrations (μg g^{-1} dry mass) and digestive gland epithelium area (%), of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg) and a contaminated vineyard (Dlhm = Delheim), 1 week (Dlhm1 = Delheim 1) and 2 months (Dlhm2 = Delheim 2) after copper oxychloride application (lines = trendlines).

3.7 Reproductive organ copper concentrations versus spermatozoan area and oocyte numbers

3.7.1 Reproductive organ copper concentrations versus spermatozoan area

When the mean spermatozoan area (μm^2) per 1 mm^2 ovotestis, measured for snails from the Helderberg and Delheim sites were plotted, in decreasing order, against their corresponding mean reproductive organ copper concentrations (μg g^{-1} dry mass), regression analysis revealed a strong significant (p<0.001) negative correlation (r = -0.999) (Figure 50).

3.7.2 Reproductive organ copper concentrations versus oocyte numbers

When the mean oocyte numbers per 1 mm^2 ovotestis, measured for snails from the Helderberg and Delheim sites were plotted, in decreasing order, against their corresponding mean reproductive organ copper concentrations (μg g^{-1} dry mass), regression analysis revealed a strong significant (p<0.001) negative correlation (r = -0.980) (Figure 51).
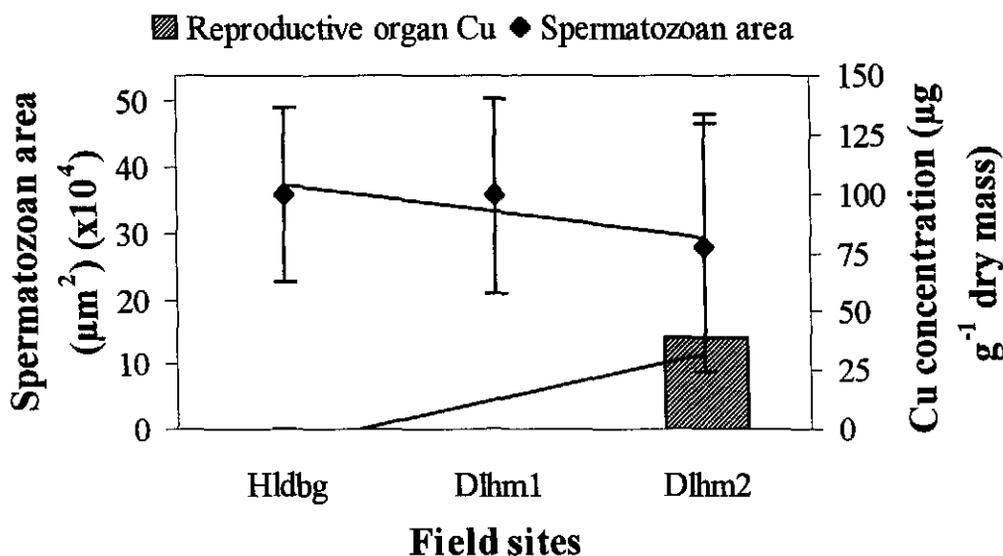
Figure 50: Relationship (r = -0.999) between reproductive organ copper concentrations (μg g^{-1} dry mass) and spermatozoan area (μm^2) per 1 mm^2 ovotestis, of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg) and a contaminated vineyard (Dlhm = Delheim), 1 week (Dlhm1 = Delheim 1) and 2 months (Dlhm2 = Delheim 2) after copper oxychloride application (lines = trendlines).
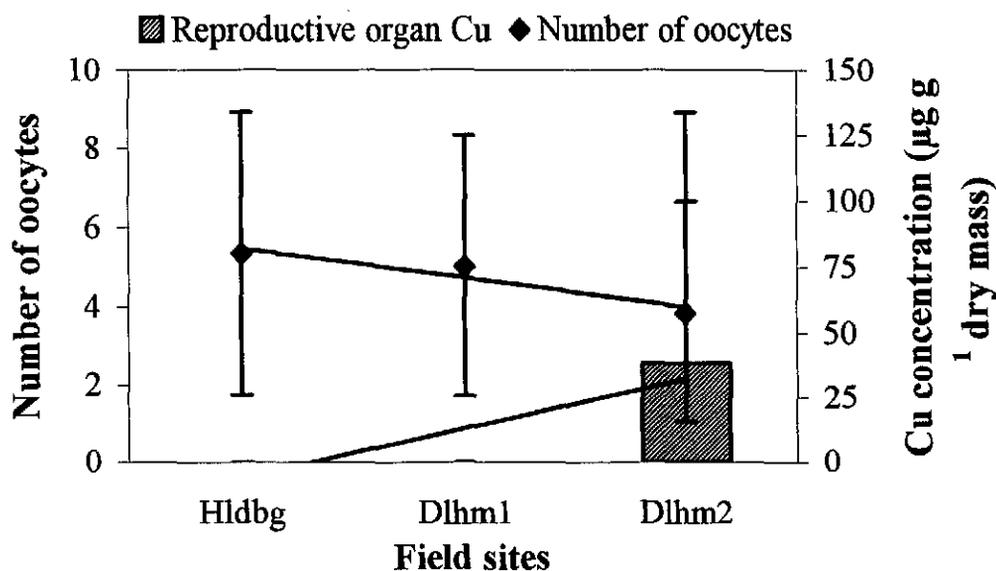
Figure 51: Relationship (r = -0.980) between reproductive organ copper concentrations (μg g^{-1} dry mass) and oocyte numbers per 1 mm^2 ovotestis, of *Helix aspersa*, collected from an uncontaminated vineyard (Hldbg = Helderberg) and a contaminated vineyard (Dlhm = Delheim), 1 week (Dlhm1 = Delheim 1) and 2 months (Dlhm2 = Delheim 2) after copper oxychloride application (lines = trendlines).

DISCUSSION

1. Acute toxicity (LC$_{50}$ test) of copper oxychloride

The 14-day LC$_{50}$ of copper oxychloride, determined for adult *Helix aspersa* during the present study (567.16 µg g^{-1}), was much higher than the LC$_{50}$'s of copper and copper oxychloride determined by various authors for other invertebrates. For example, the 96-hour LC$_{50}$ for copper oxychloride for the freshwater prawn *Macrobrachium rosenbergii*, determined by Lombardi et al. (2000), was in the order of 0.06 mg l^{-1}, and the estimated 96-hour LC$_{50}$ for copper for juvenile scallops, *Argopecten purpuratus*, 117 µg kg^{-1} (Troncoso et al. 2000). The estimated 15-day LC$_{50}$ for copper for mussels, *Mytilus edulis*, was 40-60 µg kg^{-1} for adults and 400 µg kg^{-1} for veligers (Beaumont et al. 1987, cited by Hoare & Davenport 1994). It therefore seems that copper oxychloride is less toxic for *Helix aspersa* than for the freshwater prawn, and also less toxic than copper for marine bivalves. One possible explanation for this is the bioavailability of copper, which is known to affect the metal's toxicity to animals (Sauvé et al. 1997 and Ge et al. 2000). It is possible that the copper contained in the fungicide copper oxychloride, given in the form of an Agar mixture, was less bioavailable to *H. aspersa*, than the copper in the freshwater and seawater used in the experiments of the above-mentioned authors.

2. Sublethal toxicity of copper oxychloride

2.1 Effects of copper oxychloride on food intake, body mass, egg production and hatching success, and accumulation of copper in hatchlings

2.1.1 Food intake

In the present study, the food intake per individual in the highest exposure group (240 µg g^{-1} copper oxychloride) of *Helix aspersa*, was significantly lower than that of the control group during the first week of exposure (Figure 4 (p 22); Tables 7 (p 23) & II (p 133)). The food intake of this exposure group increased gradually over the six week experimental period, resulting in a significantly higher food intake during the final week, compared to the first, and a mean individual food intake similar to

that of the other two test groups during week 6 (Figure 4 (p 22); Tables 7 (p 23), I & II (p 133)). These results are in agreement with the results of Marigomez et al. (1986), who found an exponential change in food intake of the slug *Arion ater*, at Cu concentrations between 100 and 1000 µg g^{-1}. They found that feeding rates at these concentrations decreased at first, then increased again after a period of acclimation, to rates similar to those observed in control animals. In contrast, Moser & Wieser (1979), Laskowski & Hopkin (1996a; 1996b) and Gomot-De Vaufleury (2000) all found no effects on food intake of *Helix* at low exposure concentrations, but marked drops in consumption at high Cu concentrations (>200 µg g^{-1} Cu in the case of the first two authors and >2000 µg g^{-1} Cu in the case of the latter author).

Marigomez et al. (1986) argued that the initial refusal of food that they observed in *Arion ater* was possibly due to disagreeable organoleptic characteristics and that animals started to increase their food intake again only because of hunger. The significantly lower food intake observed in the 240 µg g^{-1} exposure group during week 1 of exposure could have been due to unpalatability of the food, and the increase in feeding over the following weeks due to hunger. The animals could also possibly have become accustomed to the taste of the food.

2.1.2 Changes in body mass

The individual body mass of *Helix aspersa* increased significantly during the first week of exposure, in all three test groups (Figure 5 (p 24); Tables 8 (p 25) & III (p 134)). The phenomenon of hormesis was therefore observed in the two exposed groups. This increase in body mass was however more marked in the control group, as demonstrated by the significantly higher individual body mass at the end of week 1, compared to the two groups exposed to the fungicide (Table IV (p 134)). This may be ascribed to the higher food intake in this group during the first week. Marigomez et al. (1986) found that slugs exposed to low Cu concentrations (between 10 and 50 µg g^{-1} Cu) showed a benefit in growth until about day 18 of exposure. According to Simkiss (1979, cited by Marigomez et al. 1986), this apparent benefit may be due to Cu ions involved in normal metabolism. Therefore, the increases in body mass observed in the two exposed groups during week 1 could possibly have been due to beneficial effects of increased body copper concentrations (Table 14 (p 43)), as suggested by the above-mentioned author.

The control and 80 μ g g⁻¹ copper oxychloride exposure groups both exhibited a net increase in mean individual body mass over the six week experimental period, whilst no significant change in body mass was found in the 240 μg g⁻¹ exposure group (Table III (p 134)). Growth of snails in this group has thus, to some extent, been deterred. This can possibly be ascribed to increasing body copper concentrations, as a strong negative correlation between whole body copper concentrations and individual body mass was found for this exposure group (Figure 20 (p 46); Table XXIII (p 144)).

The negative effects of copper on the growth of invertebrates are well documented: Reinecke & Reinecke (1996) and Scott-Fordsmand et al. (1997; 2000a; 2000b) demonstrated negative effects of copper on the growth of the earthworm *Eisenia fetida* and the springtail *Folsomia fimetaria*. Helling et al. (2000) found that in earthworms (*Eisenia fetida*) exposed to copper oxychloride, growth was significantly reduced at exposure concentrations of 8.92 μg g⁻¹ and higher. In the case of *Helix aspersa*, growth only seems to be inhibited at copper exposure concentrations above 1000 μg g⁻¹ (Gomot-De Vaufleury 2000). At an exposure concentration of 250 μg g⁻¹ copper, which is comparable to the highest exposure concentration in the present study (240 μg g⁻¹ copper oxychloride), Laskowski & Hopkin (1996b) and Gomot & Pihan (1997) found no effects on the growth of *Helix aspersa*. The negative effect on growth of the 240 μg g⁻¹ exposure group, observed in the present study, could possibly have been due to a greater bioavailability of copper from copper oxychloride, and therefore higher body copper burdens, compared to the copper burdens in the snails used by the above-mentioned authors.

2.1.3 Egg production, hatching success and copper accumulation in hatchlings

The number of eggs produced and percentage of eggs hatched in the control group of *Helix aspersa* (Figures 6-9 (pp 27 & 29); Table 9 (p 28)), compared favourably with the results of Lazaridou-Dimitriadou et al. (1998) for the same species, and with the results of Pollard (1975) for *H. pomatia*. However, egg production and hatching success in the control group of *Helix aspersa* were much lower, compared to the results of Madec & Daguzan (1993), for *H. aspersa*. Various factors have been shown to influence egg production of *Helix aspersa* in the laboratory, such as temperature (Jess & Marks 1998), the number of clutches previously laid by an individual (Madec & Daguzan 1993), crowding, and the number of previous generations (Lazaridou-Dimitriadou et al. 1998). Staikou et al. (1988) also showed that only 75% of all adult *H. lucorum* lay eggs. In the case of hatching success of *Helix*, factors

such as parasitic infections (Madec & Daguzan 1993), within-clutch egg cannibalism (Desbuquois & Madec 1998), and desiccation (Staikou et al. 1988 and Desbuquois & Madec 1998) have been shown to play very important roles.

A number of the above-mentioned factors could have affected egg production and hatching success in the present study, but these fail to explain the significant differences in individual egg production and hatching success found between the three test groups (Tables VI & VII (pp 135 & 136)), since all animals were kept under similar conditions. The 80 and 240 μg g^{-1} copper oxychloride exposure groups produced significantly fewer eggs per individual than snails in the control group, during the final week of exposure. Only the control group showed a significant increase in individual egg production over the six-week experimental period (Table V (p 135)). A strong negative correlation between copper in the reproductive organs and egg production was found for the 240 μg g^{-1} exposure group (Figure 21 (p 46); Table XXIV (p 144)), indicating that, at least in this group, exposure to copper oxychloride and the concomitant increase in copper in the reproductive organs, affected egg production negatively, either by delaying or altogether preventing reproduction.

In the case of hatching success, the 240 μg g^{-1} exposure group exhibited a significantly lower percentage of hatched eggs per clutch compared to the control group (Figure 9 (p 29); Table VII (p 136)). The hatchlings of this group also exhibited significantly higher body copper concentrations, compared to hatchlings from the control group (Figure 10 (p 30); Table VIII (p 136)). It can therefore be argued that the transfer of high copper concentrations from the reproductive organs to the eggs was possibly the major factor that affected hatching success in this group.

The negative effects of copper exposure on invertebrate reproduction have been demonstrated by several authors, e.g. Laskowski & Hopkin (1996b), Reinecke & Reinecke (1996) and Scott-Fordsmand et al. (1997; 1998; 2000a; 2000b). Laskowski & Hopkin (1996b) found that the fecundity of *Helix aspersa* dropped sharply at high exposure concentrations of Cu (i.e. 250-1250 μg g^{-1}). The EC$_{20}$ (fecundity) and EC$_{50}$ (fecundity) for Cu for *H. aspersa* were estimated at 533 and 1050 μg g^{-1} respectively. In the case of copper oxychloride, Helling et al. (2000) demonstrated that the cocoon production and hatching success of *Eisenia fetida* was negatively affected at exposure concentrations of 8.92 and 15.92 μg g^{-1} respectively. Stringer & Morgan (1969; 1972, cited by Godan 1983) found that copper (II) oxychloride delayed and sometimes prevented egg hatching in *Biomphalaria*. Information on the accumulation of copper in the eggs and hatchlings of invertebrates is however lacking.

It is clear from the present results that the copper oxychloride exposure concentrations that affect the fecundity of *Helix aspersa* are well below the estimations of Laskowski & Hopkin (1996b) for copper, but much higher than the copper oxychloride concentrations given by Helling et al. (2000) for earthworms. Individual egg production and hatching success in *Helix aspersa* therefore seem to be sensitive parameters of copper oxychloride exposure. Compared to body mass of *H. aspersa* (section 2.1.2), egg production and hatching success seem to have been affected to a larger extent and are therefore possibly more sensitive parameters of copper oxychloride exposure. These findings are in agreement with those of Scott-Fordsmand et al. (1997; 2000a; 2000b), who also concluded that reproduction in the springtail *Folsomia fimetaria* and the earthworm *Eisenia fetida* is a more sensitive toxicological parameter of copper exposure than changes in body mass.

2.2 Copper uptake and distribution in adult Helix aspersa

2.2.1 Copper uptake

2.2.1.1 Reproductive organs (ovotestis and hermaphrodite duct)

The mean copper concentrations in the reproductive organs of snails from the control group were mostly below detectable levels and did not exceed a mean of 12.92 μg g^{-1} dry mass (Table 11 (p 34)). From Table 11 it can also be seen that copper concentrations in the reproductive organs of snails from the 80 and 240 μg g^{-1} copper oxychloride exposure groups already greatly exceeded these levels by the end of week 3 and the end of week 1 respectively. Pip (1995) also found low copper concentrations of between 1.6 and 7.6 μg g^{-1} dry mass in the gonads of various mussel species from a reasonably unpolluted river.

The copper concentrations in the reproductive organs of *H. aspersa* from the 80 and 240 μg g^{-1} exposure groups increased significantly over the six-week exposure period (Figure 12 (p 33); Tables 11 (p 34) & X (p 137)). The copper concentrations measured at the end of week 6 were significantly higher than those in the control group (Table XI (p 137)). Such an increase in reproductive organ copper content can easily be explained: it is known that molluscan plasma proteins and haemocytes are important in metal (also copper) transport (George et al. 1978; Robinson & Ryan 1988 and Robinson et

al. 1997). As the reproductive organs are bathed in haemolymph, it could be expected that metal concentrations within these organs would increase with increasing haemolymph metal concentrations.

It must however be stressed that only animals from the 80 µg g⁻¹ group exhibited increases in reproductive organ copper concentrations above the actual copper concentration in the food (Table 10 (p 31)). This can possibly be due to a different distribution pattern of copper in the bodies of these snails, compared to snails from the 240 µg g⁻¹ group: The 240 µg g⁻¹ exposure group had significantly higher copper concentrations in the "rest of the body" at the end of the experimental period (section 2.2.1.3), possibly indicating that copper in this group had been redistributed to other parts of the body by this time.

2.2.1.2 Digestive gland

Digestive gland copper concentrations were found to vary between 42.62 ± 28.46 and 187.53 ± 298.63 µg g⁻¹ dry mass for the control group, over the six-week experimental period (Table 12 (p 37)). The copper concentrations determined prior to the experiment (139.18 ± 306.18 µg g⁻¹ dry mass) and at the end of week three (187.53 ± 298.63 µg g⁻¹ dry mass) are high, compared to levels found in other unexposed molluscs. For example, Blasco & Puppo (1999) found Cu concentrations of 76.28 ± 13.09 µg g⁻¹ dry mass in the digestive glands of control marine bivalves, while Regoli (1998) found much lower concentrations in mussels from unpolluted sites, namely 14.9 ± 6.8 µg g⁻¹ dry mass. In the digestive glands of uncontaminated terrestrial snails and slugs, Ireland & Marigomez (1992) found copper levels of 21.9 ± 8.0 µg g⁻¹ dry mass for *Achatina fulica* and Marigomez et al. (1998) a mean of 84 µg g⁻¹ dry mass for slugs. Gomot & Pihan (1997) and Moser & Wieser (1979) found copper concentrations of 44.2 - 97.0 and 30 - 48 µg g⁻¹ dry mass in the digestive glands of *Helix aspersa* and *H. pomatia* respectively. The values found for control *Helix aspersa* in the present study were probably affected by the great individual variation, and therefore high standard deviations (Table 12 (p 37)). This wide range in intraspecific variation of copper levels in the mollusc body has been observed and commented on by various authors, such as Lobel et al. (1982, cited by Blasco & Puppo 1999), Greville & Morgan (1990), Ying et al. (1993) and Blasco & Puppo (1999).

For the 80 and 240 µg g⁻¹ copper oxychloride exposure groups, digestive gland copper concentrations of 567.82 ± 228.29 and 701.18 ± 99.51 µg g⁻¹ dry mass respectively, were measured at the end of the

six-week exposure period (Table 12 (p 37)). The concentrations measured for the 80 μg g^{-1} group fall within the range of concentrations given by Moser & Wieser 1979 for copper-exposed *H. pomatia* (192.0 – 638.0 μg g^{-1} dry mass), whilst the concentrations measured for the 240 μg g^{-1} group fall between the results of Moser & Wieser (1979), and the results of Gomot & Pihan (1997) for copper-exposed *H. aspersa* (915.0 ± 102.6 to 1304.6 ± 236.4 μg g^{-1} dry mass).

Snails from the 80 and 240 μg g^{-1} exposure groups accumulated significantly higher Cu concentrations already by the end of the first week of exposure (Figure 14 (p 36); Tables 12 (p 37) & XIII (p 138)). Also, the concentrations measured in these groups during the following weeks increased gradually and by the end of the final week of exposure, the digestive gland copper concentrations were higher than the copper concentrations in the food (Table 10 (p 31)) and significantly higher than in the control group (Table XIV (p 139)).

It is well known that heavy metals such as cadmium (Viarengo et al. 1985a; Bebianno et al. 1992 and Berger et al. 1995), aluminium (Elangovan et al. 2000), and copper (Viarengo et al. 1985a; 1989 and Hamza-Chaffai et al. 1998) are sequestered in the digestive glands of molluscs through the synthesis of specific metal-binding proteins called metallothioneins. Another type of Cu-binding protein has also been identified in the digestive gland of mussels (Viarengo et al. 1981a): these proteins are present in the lysosomes of digestive gland cells in an insoluble form (Viarengo et al. 1985b, cited by Viarengo et al. 1989) and, like the other metallothioneins, play an important role in metal detoxification (Viarengo et al. 1981b, cited by Viarengo et al. 1989). Various authors have also discussed the presence of insoluble intracellular granules in the calcium cells of molluscan digestive glands, into which copper is deposited (Simkiss & Taylor 1981; Hopkin 1989; Almedros & Porcel 1992b and Marigomez et al. 1998).

Although copper-binding proteins in the digestive gland of *Helix aspersa* have not specifically been identified and studied, the high concentrations of copper in this organ in exposed snails of the present study suggest that also in this species, the production of Cu-thioneins could be expected. Moser & Wieser (1979) also suggested the possibility for *Helix pomatia*.

Viarengo et al. (1981a) demonstrated that mussels are able to synthesize Cu-binding proteins within 48 hours of exposure to sublethal concentrations of copper. Moser & Wieser (1979) also suggested that, since it took only three days of exposure for the concentration of copper to increase dramatically in the

digestive gland of *H. pomatia*, the synthesis of complexing agents are probably induced during this period. The significant increases in digestive gland copper of the two copper oxychloride exposure groups, during the first week of exposure in the present study (Table XIII (p 138)), could possibly have been due to a rapid production of Cu-binding proteins in this gland, and sequestering of copper by these proteins immediately thereafter. The bioaccumulation of copper in the digestive glands of snails from these two exposure groups, during the following weeks of exposure, was probably due to an increased amount of the metal being sequestered by the Cu-thioneins.

2.2.1.3 Rest of the body (including shell)

The reproductive organs and digestive gland were analysed separately and the remainder of the soft tissues, together with the shell, were combined and referred to as "rest of the body". Copper concentrations in the "rest of the body" of control *H. aspersa* were between 37.57 ± 17.22 and $77.52 \pm 49.5 \ \mu g \ g^{-1}$ dry mass over the six-week experimental period (Table 13 (p 40)). These concentrations are lower than the soft body (minus shell) copper concentrations found by Laskowski & Hopkin (1996a) for control *H. aspersa* ($101 \pm 35 \ \mu g \ g^{-1}$ dry mass). This is however not surprising, since these authors included the digestive gland in their analyses. As mentioned in section 2.2.1.2, the digestive gland is an organ well known for accumulating high concentrations of copper. Snails from the 80 and 240 $\mu g \ g^{-1}$ exposure groups had "rest of the body" copper concentrations of 160.28 ± 60.79 and $243.52 \pm 69.42 \ \mu g \ g^{-1}$ dry mass respectively, after six weeks of exposure. These concentrations fall within the range given by Laskowski & Hopkin (1996a) for copper-exposed *H. aspersa* ($134 \pm 58 - 740 \pm 166 \ \mu g \ g^{-1}$ dry mass).

Copper concentrations in the "rest of the body" of animals from the two copper oxychloride exposure groups increased significantly over the six-week exposure period (Figure 16 (p 39); Table 13 (p 40); Table XVI (p 140)). Concentrations at the end of the final week were also significantly higher than in the control group, and differed significantly between these two exposure groups (Table XVII (p 140)). However, concentrations did not exceed the actual copper concentrations in the food (Table 10 (p 31)).

The question arises as to which of the tissues pooled in the "rest of the body" samples were primarily responsible for the observed increase in copper uptake in the present study. The foot of terrestrial snails seems to be an important route for the uptake of metals, since it comes into direct contact with

contaminated surfaces. This route of copper uptake has been investigated by authors such as Ryder & Bowen (1977), Moser & Wieser (1979) and Gomot & Pihan (1997). The former authors found copper concentrations between 133 ± 36 and 817 ± 55 µg g^{-1} dry mass in the foot of copper-exposed *Helix pomatia*, whereas the latter authors found very high copper concentrations of between 1261.9 and 2113 µg g^{-1} dry mass in the foot of exposed *H. aspersa*.

Authors seem to disagree on the importance of the snail shell in the storage of metals. Beeby & Richmond (1987) and Pyatt et al. (1997) found that most of the accumulated cadmium in *H. aspersa*, and most of the accumulated copper and manganese in *Lymnaea stagnalis*, is incorporated into the shells, whilst Laskowski & Hopkin (1996a) concluded that *H. aspersa* is not able to deposit significant quantities of metals in their shells. These authors found that even in copper-exposed snails, the concentration in the shells did not exceed 3.7 µg g^{-1} dry mass. They stated that during experiments lasting only a few months, shell growth and the opportunity to transport metals to the shell is negligible. However, they did concede that this scenario might change in animals exposed to copper for the duration of their lives.

Since the shell was not analysed separately in the present study, no conclusions can be drawn. However, since the exposure experiment was conducted over only six weeks, and since direct contact of the foot withh the food was possible, it is assumed that most of the copper found in the "rest of the body" samples of exposed *H. aspersa* originated from the foot, rather than the shell.

2.2.1.4 Whole body (reproductive organs, digestive gland, remainder and shell)

Whole body copper concentrations of control *Helix aspersa* varied between 38.92 ± 17.45 and 90.91 ± 71.76 µg g^{-1} dry mass over the six-week experimental period, whereas snails from the 80 and 240 µg g^{-1} exposure groups had whole body copper concentrations of 200.85 ± 85.53 and 272.24 ± 67.15 µg g^{-1} dry mass respectively, after six weeks of exposure to copper oxychloride (Table 14 (p 43)). Similarly, Marigomez et al. (1998) found that slugs (*Arion ater*) from unpolluted sites contained ±75 µg g^{-1} dry mass copper in their bodies, and those from a site polluted by run-off from an inactive Cu mine ±193 µg g^{-1} dry mass Cu. The whole body copper concentrations found for the control and two exposure groups of *Helix aspersa* in the present study also compare well with the soft body copper

concentrations found by Laskowski & Hopkin (1996a) for unexposed and exposed *H. aspersa* respectively (section 2.2.1.3).

Significant increases in whole body copper concentrations were already observed during the first week of exposure, in the two groups exposed to the fungicide (Figure 18 (p 42); Table XIX (p 141)). Body copper concentrations in these two groups also increased significantly over the six-week exposure period (Table 14 (p 43); Table XIX (p 141)) and were significantly higher than in the control group by the end of the exposure period (Table XX (p 142)). Whole body copper concentrations in the 240 µg g^{-1} exposure group were also significantly higher than in the 80 µg g^{-1} group at this time (Table XX (p 142)). These results can probably be explained by the digestive gland copper concentrations which followed a similar pattern in the two exposure groups (section 2.2.1.2) and would have greatly affected whole body copper concentrations.

2.2.2 Copper distribution in the body of Helix

Copper concentrations in the digestive gland of the control group did not differ significantly from the rest of the body (excluding reproductive organs) (Figure 19 (p 45); Table XXI (p 143)). This distribution pattern did not change over the six-week experimental period (Table XXII (p 143)). The reproductive organs also contained significantly lower amounts of copper than the remainder of the body throughout the experimental period (Table XXII (p 143)). Within the two groups of *H. aspersa* exposed to copper oxychloride, the importance of the digestive gland in copper accumulation was clearly demonstrated. When data obtained over the six weeks were pooled, the copper concentrations in the digestive glands of these two groups were shown to be significantly higher than in other parts of the body (Figure 19 (p 45); Table XXI (p 142)). Also, there was a clear difference in body copper distribution after six weeks of exposure, compared to prior to exposure (Table XXII (p 143)): the distribution of copper in the two exposure groups prior to exposure was similar to that of the control group. However, after exposure, the digestive gland had significantly higher copper concentrations than the other parts of the body. The accumulation of copper in this organ is discussed in section 2.2.1.2. A proportional increase in the copper concentrations of the reproductive organs was also observed in the two exposure groups over the experimental period (Table XXII (p 143)). Accumulation of copper in these organs is discussed in section 2.2.1.1.

Numerous authors have identified the digestive gland as the most important organ for heavy metal (e.g. copper) accumulation in molluscs, e.g. Ireland (1979), Simkiss & Taylor (1981), Viarengo et al. (1981a; 1985a; 1989), Bebianno et al. (1992), Berger et al. (1993; 1995), Hamza-Chaffai et al. (1998), Marigomez et al. (1998) and Blasco & Puppo (1999). In the case of *Helix*, varying results on the distribution of copper have been obtained: Simkiss & Taylor (1981) stated that metals, including copper, are largely concentrated in the digestive gland of *Helix aspersa*, whereas Moser & Wieser (1979) concluded that copper is equally distributed in the alimentary tract and digestive gland of *H. pomatia*. Gomot & Pihan (1997), on the other hand, found that most of the copper in *H. aspersa* is concentrated in the foot.

2.3 Neutral red retention time assay

Neutral red retention times for control *H. aspersa* were found to vary between 23.11 ± 5.58 and 31.11 ± 4.81 minutes over the six-week experimental period (Table 15 (p 48)). As no similar study on the NNR times of haemocytes of terrestrial molluscs has been done previously, no comparison with regard to control NRR times is possible. The NNR times measured for the control group during the present study were much lower than those obtained for marine Bivalvia, which were in excess of 80 minutes for all species studied (Lowe et al. 1995a; Grundy et al. 1996; Hauton et al. 1998) and as high as 160 minutes (Lowe & Pipe 1994) and 180 minutes (Lowe *et al.* 1995b) for *Mytilus edulis*. The NRR times determined for control *Perna viridis*, i.e. between 40 and 50 minutes (Nicholson 1999), were however closer to the results of the present study. Only one other gastropod has been used in a NRR time assay, namely the freshwater snail *Viviparus contectus* (Svendsen & Weeks 1995), that also had high NRR times for control animals (90 minutes).

Significant differences in NRR times were found between the three groups of *H. aspersa* at the end of the experimental period (Table XXVI (p 145)). This indicates that exposure concentration influences NRR times in *H. aspersa* haemocytes. Also, very strong negative correlations between whole body copper concentrations and NRR times, measured over the six week period, were found for the 80 $\mu g\ g^{-1}$ and 240 $\mu g\ g^{-1}$ exposure groups (Figure 23 (p 49)). These indicate that exposure time (with the concomitant increase in body copper burdens), is also an important factor influencing NRR times in *H. aspersa*.

Various anthropogenic stressors might possibly affect NNR times: Jussila et al. (1997) demonstrated how captivity and handling affected total and differential haemocyte count in the lobster *Panulirus cygnus*. It is possible that these factors may also influence membrane stability, and therefore neutral red retention of haemocytic lysosomes. In addition, the neutral red dye itself is known to be a cytotoxic chemical and to place stress on the lysosomal membrane (Lowe & Pipe 1994). However, in the present study, the concentration of neutral red used was very low and constant for all test groups. Also, since the control group showed no significant decrease in NRR times over the six week period (Table XXV (p 145)), it can be accepted that captivity and handling of the snails had minimal, if any effects on NRR times throughout the test groups, and that the results shown for the two exposure groups can indeed be attributed to stress resulting from exposure to copper oxychloride.

The results of the present study are in agreement with those of authors such as Svendsen & Weeks (1995; 1997), Weeks & Svendsen (1996), Harreus et al. (1997), Ringwood et al. (1998), Nicholson (1999) and Scott-Fordsmand et al. (2000b), who illustrated the negative effects of copper exposure, and copper oxychloride exposure in the case of Helling et al. (2000), on lysosomal membrane stability in their respective test animals, using the neutral red retention time assay. For molluscs specifically, Svendsen & Weeks (1995), Ringwood et al. (1998) and Nicholson (1999) found that NRR times decreased with increasing copper exposure concentration, in the freshwater snail *Viviparus contectus*, the oyster *Crassostrea virginica* and the mussel *Perna viridis* respectively. Additionally, Ringwood et al. (1998) demonstrated that not only exposure concentration, but also exposure time might be an influencing factor in NRR time assays.

The data for the snails exposed to 240 μg g^{-1} copper oxychloride show a typical dose response relationship (Figure 22 (p 47)). After one week of exposure the NRR times decreased rapidly over consecutive weeks of exposure, as the body copper concentrations increased. This indicates that copper regulation broke down in week 2 of exposure, and that increasing body copper concentrations in the following weeks were accompanied by severe lysosomal stress. The data fit for the snails exposed to 80 μg g^{-1} copper oxychloride (Figure 22 (p 47)) is not quite as clear but follows the same basic trend. When all the data plotted together in Figures 22 (p 47) and 23 (p 49) are considered, it is clear that a time evolution of copper accumulation and lysosomal damage exists. The latter has not previously been shown for this biomarker.

In conclusion, the results of the present study support the use of the lysosomal neutral red retention time assay for assessing the toxic effects of copper oxychloride. A clear relationship between body copper burdens, as a result of copper oxychloride exposure, and lysosomal responses of *Helix aspersa* haemocytes over time has been demonstrated. This also indicates that haemocytic lysosomes are targets for the toxicity of copper in *Helix aspersa*. In addition, the study also identified *Helix aspersa* as a terrestrial biomonitor species which is readily available and on which the NRR time technique can be used in a reasonably non-invasive manner.

2.4 Histological analysis

2.4.1 Digestive gland

2.4.1.1 Digestive gland tubule area

No significant differences in digestive gland tubule area were found between the three test groups of *Helix aspersa* (Figure 24 (p 50); Tables 16 (p 51) & XXVII (p 146)), indicating that exposure to copper oxychloride did not affect digestive gland tubule area to any extent. Since no similar study has been done with *Helix* as model, no comparisons can be drawn. Authors have found varying results for other molluscan species exposed to different chemicals: Vega et al. (1989) also found no significant changes in mean digestive tubule radius of the periwinkle *Littorina littorea*, after exposure to cadmium. In contrast, Marigomez et al. (1998) found that the mean radius of the digestive tubule of uncontaminated slugs was significantly higher than that of slugs exposed to copper, among other metals. Jonnalagadda & Rao (1996), on the other hand, observed total destruction of digestive tubules of the freshwater snail *Bellamya dissimilis* after exposure to the pesticide nuvan. It is therefore clear that there is great interspecific variation in the degree of change in digestive gland tubule area and radius, after exposure to different chemicals.

2.4.1.2 Digestive gland epithelium height and area

Within the two groups of *Helix aspersa* exposed to copper oxychloride, the height of the digestive gland epithelium was significantly shorter at the end of the exposure period, compared to that of

control animals (Figure 25 (p 52); Tables 17 (p 52) & XXVIII (p 146)). Digestive gland epithelium cells of snails from the 240 $\mu g \ g^{-1}$ exposure group were also significantly shorter than those of snails from the 80 $\mu g \ g^{-1}$ group. It was also shown that the areas of the digestive gland epithelia of snails from the two exposure groups, expressed as a percentage of the total tubule area, were significantly smaller, compared to the control group (Figure 26 (p 53); Tables 18 (p 54) & XXIX (p 146)).

It is known that the digestive stage of molluscan digestive gland tubules (Langton 1975, cited by Lowe & Clarke 1989), as well as various environmental stressors (Lowe & Clarke 1989; Vega et al. 1989; Jonnalagadda & Rao 1996; Marigomez et al. 1998 and Wedderburn et al. 2000) may affect the sizes of the tubules' lumina and the thickness of the epithelium. All animals in the present study were kept under similar conditions and starved for a period of two days at the end of the experiment. Therefore, the significantly shorter epithelium cells, and significantly smaller epithelium areas in the digestive glands of snails from the exposed groups, can be ascribed to exposure to copper oxychloride, and not differences in the digestive stages of the tubules. Similarly, Lowe & Clarke (1989), Vega et al. (1989) and Marigomez et al. (1998) also related the reduction in tubule epithelium thickness and concomitant increase in tubule lumen diameter that they observed in mussels, periwinkles and slugs respectively, to exposure to heavy metals (including copper).

In the present study, there was thus a marked dose-related reduction in the cell height of digestive gland epithelium of *H. aspersa*, and a strong negative correlation between digestive gland epithelium cell height and copper concentrations in the digestive gland (Figure 30 (p 59)). The differences in digestive gland epithelium area, on the other hand, were not dose-related, but also clearly related to copper concentrations in the digestive gland, as a strong negative correlation was found between digestive gland epithelium area and digestive gland copper concentrations (Figure 31 (p 59)).

The phenomenon of reduction in digestive gland epithelium thickness with the concomitant increase in lumen size is, according to Vega et al. (1989), due to losses of apical cytoplasm involved in the detoxification process. As discussed in section 2.2.1.2, heavy metals are sequestered in the lysosomes of molluscan digestive gland cells. It is also well known that the membrane stability of molluscan digestive cell lysosomes is greatly affected by the presence of xenobiotics, or abnormally high quantities of micronutrients such as copper, in these lysosomes (Lowe & Pipe 1994; Lowe et al. 1995; Ringwood et al. 1998 and Wedderburn et al. 2000). This results in a leakage of the lysosomal contents (digestive enzymes) into the cytoplasm of the digestive gland cell, and drastic changes in cell structure.

Under extreme conditions of metal exposure, this will lead to significant changes in the histological organization of the digestive gland and will eventually result in a reduction in the gland's accumulative capacity (Marigomez et al. 1990).

2.4.2 Vesicula seminalis and ovotestis

2.4.2.1 Vesicula seminalis: spermatozoan area

In the present study no significant differences in sperm area of the vesicula seminalis (expressed as a percentage of the total area of this duct) were found between the three test groups of *Helix aspersa* (Figure 27 (p 55); Tables 19 (p 55) & XXX (p 147)). A possible explanation for these results is discussed in section 2.4.2.2. No comparisons with the data of other researchers were possible, as sperm density in this duct has never been used as a parameter in histopathological studies.

2.4.2.2 Ovotestis: spermatozoan area

The area covered by spermatozoa, per 1 mm^2 of ovotestis, was shown to be significantly lower in *Helix aspersa* exposed to 240 $\mu g\ g^{-1}$ copper oxychloride, compared to the other two test groups (Figure 28 (p 56); Tables 20 (p 57) & XXXI (p 147)). Similarly, Russell et al. (1981) found fewer mature spermatozoa in ovotestis acini of *H. aspersa* exposed to cadmium. Authors such as Kanamadi & Saidapur (1992), Cikutovic et al. (1993), Davies et al. (1995) and Sinha et al. (1997) also found similar results for frogs, earthworms, leeches and rats respectively, exposed to cadmium and various pesticides.

In the present study, the copper concentrations in the reproductive organs of *Helix* exposed to 240 $\mu g\ g^{-1}$ copper oxychloride were, in turn, significantly higher than in the control group, and higher than in the 80 $\mu g\ g^{-1}$ exposure group (although not significantly) (Table XI (p 137)). Therefore, the differences in sperm densities could possibly be ascribed to these increased copper burdens. This is supported by the strong negative correlation found between copper concentrations in the reproductive organs and spermatozoan area in the ovotestis (Figure 32 (p 61)).

The question arises as to the possible reason for the fact that sperm densities in the 240 μg g^{-1} exposure group were only lower in the ovotestis, compared to the control group, and not lower in the vesicula seminalis. It is possible that sperm present in the vesicula seminalis of these snails had been produced much earlier during the exposure period, and had been stored in this duct for some time. Also, that exposure to copper oxychloride had only started to affect spermatogenesis in the ovotestis towards the end of the exposure period, either by blocking maturation or retarding it. An intensive histological study, on a weekly basis, of spermatogenesis in the ovotestis is of course necessary to test these hypotheses.

In conclusion, it is clear that these observed changes in sperm densities in the ovotestes of snails from the 240 μg g^{-1} group can be related to copper oxychloride exposure, and that this parameter has the potential to be used as biomarker of exposure to this fungicide.

2.4.2.3 Ovotestis: oocyte numbers

The numbers of oocytes per 1 mm^2 ovotestis were significantly different in all three test groups of *Helix aspersa*. The lowest mean was recorded for the 240 μg g^{-1} exposure group (Figure 29 (p 58); Tables 21 (p 58) & XXXII (p 147)). Davies et al. (1995) also found fewer ova in the ovisacs of leeches exposed to cadmium, but Russell et al. (1981) concluded that the ova of *Helix aspersa* did not seem to be affected by sublethal cadmium concentrations. It must however be stressed that the latter authors made visual observations and that, in the present study, the differences in oocyte numbers could only be identified with computer-assisted image analysis.

As in the case of the sperm density in the ovotestis, oocyte numbers also correlate well with the copper concentrations in the reproductive organs (Table XI (p 137) and Figure 33 (p 61)). In fact, this seems to be a more sensitive response to experimental copper oxychloride exposure than sperm density, since the response was clearly dose dependent. Davies et al. (1995) also found that ova of the leech *Nephelopsis obscura* were more sensitive to cadmium exposure than spermatozoa.

The mechanisms leading to gamete damage are not clear. It seems that the lysosomal compartment of oocytes could be an important target of the toxic action of environmental pollutants (Cajaraville et al. 1991). De Jong-Brink & Geraerts (1982) suggested that the final oocyte maturation involves a massive

increase in the number of yolk granules, and Lowe & Pipe (1986) speculated that gamete atresia is brought about by the cytotoxic effects of xenobiotics (specifically hydrocarbons) on the yolk granule lysosomes. Alternatively, Cajaraville et al. (1991) suggested that hydrocarbons could exert a direct toxic action on the cytoskeleton of oocytes. Any of the above-mentioned mechanisms could possibly also apply to heavy metals.

Finally, Davies et al. (1995) pointed out the short- and long-term implications of a reduction in gametes, especially ova: lowered fecundity and fitness, and ultimately lowered population abundance. The lowered egg production in the 240 µg g^{-1} group after six weeks of exposure to copper oxychloride (section 2.1.3) might have been a direct result of a reduction in oocyte numbers. However, more information is necessary to test this hypothesis. An ultrastructural study and motility test of the sperm in the vesicula seminalis also need to be done, since the sperm stored here could also have affected egg production: it is known that sperm ultrastructure and motility, and therefore sperm viability are affected by heavy metals (Young & Nelson 1974; Castagna et al. 1981; Reinecke & Reinecke 1997 and Au et al. 2000). Furthermore, an organ crucial in egg production has been ignored in histopathological studies, i.e. the albumin gland. This gland contributes all of the egg albumin fluid (Tompa 1984) and has also been shown to greatly reduce in weight as a result of cadmium exposure (Gomot-De Vaufleury & Kerhoas 2000).

3. Field survey

3.1 Environmental copper concentrations

In the present study, vine leaf copper concentrations decreased over the two-month period after copper oxychloride application at the Delheim site, while soil copper concentrations increased (Figure 34 (p 62)). This was probably due to the fungicide being washed from the leaves, as a result of rainfall during January and February 2000 (Table 4 (p 20)), as well as due to decaying leaf matter on the soil surface. Published data on the copper levels in contaminated soils in African orchards and vineyards are scarce. More information is available on copper levels in contaminated soils from other continents such as North America: Sauvé et al. (1997) found copper concentrations of between 26 ± 4 and 50 ± 10 µg g^{-1} dry mass in Canadian orchards contaminated with copper-based pesticides. The same authors also found copper concentrations of $62 - 3083$ µg g^{-1} dry mass in contaminated agricultural soils in New York State, USA. Compared to these results, the "contaminated" vineyard site in the present study seems reasonably unpolluted in terms of copper pollution (Table 22 (p 63)). However, it is well known that chemical residue analysis of soils does not provide a good measure of the bioavailability of a chemical (Arjonilla et al. 1994 and Dickerson et al. 1994), and that the bioavailability and toxicity of copper to animals also depends on its speciation, which is influenced by several factors, such as organic matter and pH (Sauvé et al. 1997; 1998 and Ge et al. 2000).

3.2 Copper concentrations and distribution in adult Helix aspersa

3.2.1 Copper concentrations

3.2.1.1 Reproductive organs (ovotestis and hermaphrodite duct)

In the field survey, the copper concentrations in the reproductive organs of uncontaminated snails, and of those collected one week after copper oxychloride application, were below detectable levels (Figure 35 (p 64); Table 23 (p 65)). These concentrations are therefore expected to be similar to the copper concentrations found in the reproductive organs of control snails of the laboratory experiment (Figures 11 & 12 (p 33); Table 11 (p 34)). Despite the fact that snails collected from the field one week after copper oxychloride application had been exposed to very high copper concentrations (Figure 34 (p 62);

Table 22 (p 63)), the concentrations in the reproductive organs were still below detectable levels (Table 23 (p 65)). These results differ from the laboratory results: the 240 µg g^{-1} copper oxychloride exposure group exhibited an increase (not statistically significant) in reproductive organ copper concentrations after only one week of exposure (Table 11 (p 34)). In snails collected two months after copper oxychloride application, copper concentrations in the reproductive organs increased to detectable levels, but these were still not significantly higher than the concentrations in snails from the control site (Table XXXIV (p 148)).

3.2.1.2 Digestive gland

The results of the laboratory study showed that the digestive gland is an important storage site for copper in *Helix aspersa*. Results also showed that the copper concentrations in this organ increase over time, with a significant increase after only one week of exposure to copper oxychloride (section 2.2.1.2).

In the field survey, a significant increase in digestive gland copper concentrations was found in field snails exposed to copper oxychloride for one week, compared to snails from the control site (Figure 36 (p 66); Table XXXV (p 149)). Furthermore, a significant increase in digestive gland copper was also found in snails exposed to the fungicide for two months, compared to those exposed for only one week (Figure 36 (p 66); Table XXXV (p 149)). The results of the field survey therefore validate the above-mentioned laboratory results.

The range of copper concentrations in the digestive glands of snails from the control site (Figure 36 (p 66); Table 24 (p 66)), compare favourably with the results found for the laboratory control group (Figures 13 & 14 (p 36); Table 12 (p 37)). In snails collected one week after copper oxychloride application, the mean digestive gland copper concentration falls between the concentrations recorded for the laboratory control group over six weeks (Table 12 (p 37)), and the concentrations recorded for the two laboratory exposure groups after one week of exposure to the fungicide (Table 12 (p 37)). Finally, snails collected two months after copper oxychloride application had a mean digestive gland copper concentration (Table 24 (p 66)) approaching the concentrations in the vine leaves (Table 22 (p 63)), and which compares well with the digestive gland copper concentrations of snails experimentally exposed to 240 µg g^{-1} copper oxychloride for five weeks (Table 12 (p 37)).

3.2.1.3 Rest of the body (including shell)

There were significant differences in "rest of the body" copper concentrations between all three groups of field snails (Table XXXVI (p 149)). "Rest of the body" copper concentrations of snails from the control (Helderberg) site (Figure 37 (p 67); Table 25 (p 68)), compared well with the "rest of the body" copper concentrations of snails from the laboratory control group (Figures 16 & 17 (p 39); Table 13 (p 40)). Also, snails collected in the field one week after copper oxychloride application (Figure 37 (p 67); Table 25 (p 68)), had similar "rest of the body" copper concentrations to snails from the 80 and 240 µg g^{-1} exposure groups, at the end of the first week of exposure (Table 13 (p 40)). Similarly, the "rest of the body" copper concentrations of snails collected from Delheim, 2 months after application of the fungicide (Figure 37 (p 67); Table 25 (p 68)), also compared very favourably with the "rest of the body" concentrations of snails from the two laboratory exposure groups, at the end of the experimental period (Table 13 (p 40)). Although the "rest of the body" copper concentrations of snails from the contaminated vineyard greatly exceeded concentrations in the soil, they did not however exceed concentrations in the vine leaves (Table 22 (p 63)).

3.2.1.4 Whole body (including reproductive organs, digestive gland, remainder and shell)

As in the case of the "rest of the body" copper concentrations, whole body copper concentrations differed significantly between all three groups of field snails (Table XXXVII (p 150)). Whole body copper concentrations determined during the laboratory experiment and field survey also compared very well. Whole body copper concentrations of snails from the control (Helderberg) site (Figure 38 (p 69); Table 26 (p 69)), were similar to whole body copper concentrations of snails from the laboratory control group (Table 14 (p 43)). Also, snails collected in the field one week after copper oxychloride application (Figure 38 (p 69); Table 26 (p 69)), had similar whole body copper concentrations to snails from the 80 and 240 µg g^{-1} exposure groups, at the end of the first week of exposure (Table 14 (p 43)). Similarly, the whole body copper concentrations of snails collected from Delheim, 2 months after application of the fungicide (Figure 38 (p 69); Table 26 (p 69)), also compared very favourably with the whole body copper concentrations of snails from the two laboratory exposure groups, at the end of the experimental period (Table 14 (p 43)). Whole body copper concentrations of snails from the contaminated vineyard did not exceed vine leaf copper concentrations, but greatly exceeded the concentrations in the soil (Table 22 (p 63)).

3.2.2 Distribution of copper in the body of Helix aspersa

As in the case of snails from the laboratory control group (Figure 19 (p 45); Table XXII (p 143)), snails from the control field site also had lower copper concentrations in the reproductive organs, compared to other parts of the body (Figure 39 (p 70); Table XXXVIII (p 150)). The role of the digestive gland as the most important copper storage site in the *H. aspersa* body is evident in snails from the contaminated vineyard, since these animals, collected 1 week and 2 months after copper oxychloride application, all exhibited significantly higher digestive gland copper concentrations compared to other parts of the body (Figure 39 (p 70); Table XXXVIII (p 150)). Similar results were found in snails experimentally exposed to copper oxychloride for six weeks (Figure 19 (p 45); Table XXII (p 143)). The only difference between the laboratory and field survey results is in the proportional increase in reproductive organ copper concentrations, observed in the 80 and 240 µg g^{-1} groups after six weeks of exposure (Table XXII (p 143)). Such an increase was not observed in the field snails collected two months after application of the fungicide (Table XXXVIII (p 150)).

In conclusion, it is clear that the uptake and distribution of copper in *Helix aspersa* exposed to copper oxychloride in the field followed a similar pattern to that of experimentally exposed snails. However, the uptake and distribution of copper in field snails seemingly occurred at a slower rate, even though these animals had been exposed to higher copper concentrations. This could have been due to various environmental factors, such as pH and organic matter, which are known to lower the bioavailability of copper to animals (Sauvé et al. 1997 and Ge et al. 2000). Factors such as temperature and rainfall can also affect the activity patterns and feeding behaviour of *Helix* (Pollard 1975; Iglesias et al. 1996 and Iglesias & Castillejo 1999), which in turn affect uptake rates of heavy metals. During the field sampling months in the present study, rainfall was very low and temperatures high (Tables 3 & 4 (pp 19 & 20). These could have resulted in significantly lower activity and feeding in *Helix*. Snails in the field can also feed more selectively, since the fungicide is not evenly distributed in the snails' food source (e.g. leaves are not always totally covered with the fungicide, and smaller leaves are often covered by larger ones and thus "protected" from the fungicide). It is also possible that the field snails had, by the time that they were sampled (two months after fungicide application), already excreted some of the excess copper in their bodies through regulatory mechanisms.

Irrespective of the rate of copper uptake and distribution in snails in contaminated vineyards, concentrations of copper in the *Helix* body are still high. Snails are predated on by many animals,

including rodents and birds (Pollard 1975) and are also considered a delicacy by humans. The importance of the apparent role of contaminated snails in Cu transfer along the food chain, and the threat they pose to these higher trophic levels, need to be thoroughly investigated (Laskowski & Hopkin 1996a).

3.3 Neutral red retention time assay

The neutral red retention time results of the field survey seem to confirm the results of the laboratory experiment (section 2.3), since, in the field survey (Figure 40 (p 71); Tables 27 (p 72) & XXXIX (p 151)), exposure time was also shown to be an important factor affecting lysosomal response and therefore NRR times: Field snails collected two months after copper oxychloride application had significantly shorter NRR times than snails collected only one week after application of the fungicide. Snails from the control site also exhibited significantly longer NRR times than those from the contaminated vineyard.

As in the laboratory study, a clear relationship between increasing whole body copper concentrations and decreasing NRR times was also found in the field survey (Figure 41 (p 73)). Since field snails collected one week after copper oxychloride application already exhibited significantly shorter NRR times than snails from the control site, Cu regulation probably broke down in the first week of exposure.

Unlike in the laboratory study, exposure concentration did not seem to be a major factor affecting NRR times of snails in the field. Although snails collected one week after application of the fungicide were exposed to very high copper concentrations (Table 22 (p 63)), these animals exhibited longer NRR times than snails exposed to lower copper concentrations but for a longer period (2 months). It therefore seems that actual body copper burdens and exposure time are the crucial factors which determine lysosomal membrane response and therefore NRR times of *Helix aspersa* haemocytes in the field.

In conclusion, the results from both the laboratory study and field survey support the findings of Svendsen & Weeks (1995; 1997), Weeks & Svendsen (1996), Harreus et al. (1997), Ringwood et al. (1998), Nicholson (1999), Helling et al. (2000) and Scott-Fordsmand et al. (2000b), who all found the

NNR time assay to be a reliable biomarker of exposure to copper, and copper oxychloride in the case of Helling et al. (2000). From the present results it is evident that the lysosomal responses in haemocytes of *Helix aspersa*, and the measuring thereof by the neutral red retention time assay, can be considered a useful biomarker of stress resulting from exposure to copper oxychloride. Used in conjunction with other cellular and physiological parameters and toxicological endpoints this biomarker could improve the reliability and accuracy of interpretations regarding cause and effect.

3.4 Histological analysis

3.4.1 Digestive gland

3.4.1.1 Digestive gland tubule area

The results of the field survey (Figure 42 (p 74); Table XL (p 151)) are in agreement with the results of the laboratory study (Figure 24 (p 50); Table 16 (p 51)): no significant differences in digestive gland tubule area were found between the two field sites and different sampling dates. Digestive gland tubule area can therefore not be considered a useful parameter in histopathological studies of the effects of copper oxychloride on the digestive gland of *Helix aspersa*.

3.4.1.2 Digestive gland epithelium height and area

In the laboratory study changes in digestive gland epithelium height were shown to be dose-dependent (section 2.4.1.2). This was not the case in the field survey (Figure 43 (p 76); Tables 29 (p 76) & XLI (p 152)): snails collected one week after copper oxychloride application did not have significantly shorter digestive epithelium cells, compared to snails from the control site, even though they had been exposed to very high copper concentrations (Table 22 (p 63)). Also, the snails collected two months after the application of the fungicide, had significantly shorter digestive gland epithelium cells than snails collected only one week after application (Figure 43 (p 76); Tables 29 (p 76) & XLI (p 152)), even though they had seemingly been exposed to lower environmental copper concentrations (Table 22 (p 63)). It therefore seems that exposure time is a far more important factor affecting digestive gland epithelium height of *H. aspersa* than exposure concentration.

In the case of digestive gland epithelium area, exposure concentration was also shown to be an important influencing factor: the digestive gland epithelium of snails collected two months after application of the fungicide, covered a significantly smaller area of the total tubule area, compared to snails collected one week after fungicide application (Figure 44 (p 77); Tables 30 (p 78) & XLII (p 152)). Here too, in the laboratory study as well as the field survey, exposure concentration did not seem to be an important factor affecting digestive gland epithelium area.

In both the laboratory study and field survey, decreases in epithelium height and area were well correlated with increases in digestive gland copper concentrations (Figures 30, 31, 48 & 49 (pp 59, 60, 83 & 84)). Therefore, actual digestive gland copper concentration is clearly also an important factor associated with these changes seen in the digestive epithelium.

It is therefore concluded that changes in the height and area of *H. aspersa* digestive gland tubules can be considered clearly measurable responses to copper oxychloride exposure. Used in conjunction with other cellular and physiological parameters and toxicological endpoints they too could improve the reliability and accuracy of interpretations regarding cause and effect. However, it has become evident through the field survey that these responses do not provide an early warning of contaminant-induced stress, since they can only be measured after several weeks of exposure, when irreparable damage to the digestive gland and other organs may already have been done. Also, since the length of the period after copper oxychloride application is clearly such an important factor affecting these parameters, knowledge of the specific spray programs used in the vineyards is crucial, in order to improve the usefulness of these measurements as possible biomarkers.

3.4.2 Vesicula seminalis and ovotestis

3.4.2.1 Vesicula seminalis: spermatozoan area

The results of the field survey (Figure 45 (p 79); Table XLIII (p 153)) are in agreement with the results of the laboratory study (Figure 27 (p 55); Table XXX (p 147)): no significant differences in spermatozoan area, expressed as a percentage of the total area of the vesicula seminalis, were found between the two field sites and different sampling dates. The area covered by spermatozoa in the

vesicula seminalis is therefore not considered a useful parameter in histopathological studies of the effects of copper oxychloride on the digestive gland of *Helix aspersa*.

3.4.2.2 Ovotestis: spermatozoan area and oocyte numbers

In the laboratory study, spermatozoan area and oocyte numbers per 1 mm^2 ovotestis correlated well with reproductive organ copper concentrations (Figures 32 & 33 (p 61)). Such relationships between increasing reproductive organ copper concentrations and decreasing spermatozoan area and oocyte numbers in the ovotestis were also found in the field survey (Figures 50 & 51 (p 85)).

From the field survey it is clear that changes in sperm density and oocyte numbers are also largely dependent on exposure time, rather than exposure concentration: spermatozoa of snails collected two months after copper oxychloride application covered a significantly smaller area per 1 mm^2 ovotestis, and oocyte numbers per 1 mm^2 ovotestis of this group were significantly lower than in snails collected only one week after fungicide application (Figures 45 & 46 (pp 79 & 80); Tables 31, 32 (pp 79 & 81), XLIV & XLV (pp 153 & 154). Snails from the former group had in turn been exposed to lower environmental copper concentrations than snails from the latter group (Table 22 (p 63)).

In conclusion, sperm density and oocyte numbers in the ovotestis of *Helix aspersa* can be considered useful measurable responses to copper oxychloride exposure, which could be used in conjunction with other cellular and physiological parameters and toxicological endpoints. However, as in the case of the digestive gland parameters, these responses do not provide an early warning of contaminant-induced stress, since they have been shown to be largely time-dependent. By the time that these responses can be measured, reproduction in the population may already have been seriously affected. Knowledge of the specific spray programs used in the vineyards is also crucially important, in order to improve the usefulness of these measurements as possible biomarkers.

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CONCLUSIONS

Results of the acute toxicity test (LC$_{50}$ test) and comparisons with the results of other authors, revealed that copper oxychloride was less toxic for *Helix aspersa* than for many other invertebrates, exposed to either copper oxychloride or copper. This was possibly due to a lower bioavailability of copper from the fungicide, given to the snails in the form of an Agar mixture.

From the sublethal toxicity test, feeding in the 240 µg g^{-1} exposure group of *H. aspersa* was shown to be affected initially, probably due to the unpalatability of the food. Feeding was gradually resumed after one week of exposure, probably due to hunger. Over the six-week exposure period, growth in this exposure group was also deterred to some extent. This was shown to be due to stress, resulting from increased body copper concentrations. Both egg production and hatching success were strongly affected in the highest copper oxychloride exposure group. These effects were related to increased copper concentrations in the reproductive organs. The transfer of high copper concentrations from the eggs to the hatchlings was also shown. It is concluded that egg production and hatching success in *H. aspersa* are more sensitive parameters of copper oxychloride exposure than changes in body mass.

An investigation into the uptake and distribution of copper in the snail body, as a result of experimental and field exposure to copper oxychloride, revealed that copper was compartmentalized in the body. The digestive gland was the most important site of copper accumulation in snails exposed to the fungicide. Copper concentrations in the digestive gland of these snails approached or exceeded the copper concentrations in the food/environment. Results from the field survey indicated that copper accumulation in the various organs and tissues of these snails probably occurred at a slower rate, compared to the accumulation rate in snails from the laboratory study. From the literature it is suggested that several factors could possibly have played a role in uptake rates in the field snails: copper speciation and bioavailability, temperature and rainfall (affecting animal behaviour), and copper regulation mechanisms in the snail body.

Despite the slower rate of uptake, it was found that body copper concentrations of field snails exposed to the fungicide, were high. Since *Helix* is predated on by various animals, it is necessary that the threat posed to higher trophic levels be thoroughly investigated.

Lysosomal membrane response of *Helix aspersa* haemocytes, as possible biomarker of copper oxychloride exposure, was investigated. In both the laboratory study and field survey strong negative correlations between neutral red retention times and body copper concentrations were found. It was also evident that regulation mechanisms broke down in the first week of exposure to copper oxychloride. The results of the laboratory study showed a clear time evolution of copper accumulation and lysosomal damage. This has not previously been shown for this biomarker. It is concluded that lysosomal response of *Helix aspersa* haemocytes, and the measuring thereof by the NNR time assay, can be considered a useful biomarker of stress resulting from exposure to copper oxychloride.

The responses of digestive gland cells and gametes of *H. aspersa* to copper oxychloride exposure were investigated. Neither digestive gland tubule area, nor spermatozoan area in the vesicula seminalis was affected by experimental or field exposure to the fungicide. These can therefore not be considered useful parameters in histopathological studies of the effects of copper oxychloride on the digestive gland and vesicula seminalis of *Helix aspersa*. In both the laboratory study and field survey, strong negative correlations were found between digestive gland epithelium height and area, spermatozoan area and oocyte numbers in the ovotestis, and copper concentrations in these respective organs. In the laboratory study, changes in digestive gland epithelium height and oocyte numbers in the ovotestis were also shown to be related to exposure concentration. In the field survey, however, changes in these parameters, as well as changes in digestive gland tubule area, epithelium area, and spermatozoan area in the ovotestis, were all shown to be strongly dependent on exposure time. It is concluded that these changes could all be considered measurable responses of copper oxychloride exposure and copper accumulation, and can therefore possibly be used as biomarkers.

In conclusion, changes in NNR times of haemocytic lysosomes of *H. aspersa* can serve as an early warning of stress induced by copper oxychloride exposure. The NNR time assay is also a reasonably quick, simple and inexpensive technique. It is however only a general biomarker of stress, induced by natural and anthropogenic stressors. The changes in digestive epithelium height and area, and in spermatozoan and oocyte densities, as a result of copper oxychloride exposure, can, unlike the NRR time assay, not serve as a very early warning of exposure to this fungicide, since they were shown to be dependent on exposure time. These responses might however have greater predictive value of higher-level effects than the NRR time assay, since changes in gamete numbers, especially, could possibly seriously affect reproduction in the population (as shown by the lowered egg production and hatching success in the laboratory study).

Finally, all the cellular responses to copper oxychloride identified during the present study certainly have value in terms of improving the reliability and accuracy of interpretations regarding cause and effect, if used in conjunction with other cellular and physiological parameters and toxicological endpoints.

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APPENDIX

Table I: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in individual food intake (grams) of *Helix aspersa*, over the six-week experimental period, within each of the three test groups (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value; W1-W6 = Week 1-Week 6).

Control		E1		E2	
Weeks	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**
W1 vs W2	0.185; >0.05	W1 vs W2	0.491; >0.05	W1 vs W2	0.89; >0.05
W2 vs W3	0.576; >0.05	W2 vs W3	0.93; >0.05	W2 vs W3	0.382; >0.05
W3 vs W4	0.526; >0.05	W3 vs W4	0.663; >0.05	W3 vs W4	0.589; >0.05
W4 vs W5	0.166; >0.05	W4 vs W5	0.125; >0.05	W4 vs W5	0.304; >0.05
W5 vs W6	0.895; >0.05	W5 vs W6	0.873; >0.05	W5 vs W6	0.544; >0.05
W6 vs W1	0.491; >0.05	W6 vs W1	0.708; >0.05	W6 vs W1	0.039; <0.05

Table II: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences between the three groups of *Helix aspersa*, in individual food intake (grams) during weeks 1 and 6 of exposure to copper oxychloride (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	**p; >/< 0.05**
Control week 1 vs E1 week 1	0.053; >0.05
Control week 1 vs E2 week 1	0.009; <0.05
E1 week 1 vs E2 week 1	0.154; >0.05
Control week 6 vs E1 week 6	0.228; >0.05
Control week 6 vs E2 week 6	0.122; >0.05
E1 week 6 vs E2 week 6	0.802; >0.05

Table III: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in individual body mass (grams) of *Helix aspersa*, before, during and after the six-week experimental period, within each of the three test groups (Control = 0 µg g^{-1}; E1 = 80 µg g^{-1}; E2 = 240 µg g^{-1} copper oxychloride; p = p-value; W0 = prior to exposure; W1-W6 = Week 1-Week 6).

Control		E1		E2	
Weeks	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**
W0 vs W1	<0.001	W0 vs W1	<0.001	W0 vs W1	0.002; <0.05
W1 vs W2	0.028; <0.05	W1 vs W2	0.198; >0.05	W1 vs W2	0.457; >0.05
W2 vs W3	0.38; >0.05	W2 vs W3	0.852; >0.05	W2 vs W3	0.852; >0.05
W3 vs W4	0.579; >0.05	W3 vs W4	0.696; >0.05	W3 vs W4	0.423; >0.05
W4 vs W5	0.328; >0.05	W4 vs W5	0.066; >0.05	W4 vs W5	0.148; >0.05
W5 vs W6	0.368; >0.05	W5 vs W6	0.053; >0.05	W5 vs W6	0.776; >0.05
W6 vs W0	0.025; <0.05	W6 vs W0	0.02; <0.05	W6 vs W0	0.848; >0.05

Table IV: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in individual body mass (grams) between the three test groups of *Helix aspersa*, measured before exposure, and at the end of weeks 1 and 6 of exposure (Control = 0 µg g^{-1}; E1 = 80 µg g^{-1}; E2 = 240 µg g^{-1} copper oxychloride; p = p-value).

Exposure groups	**p; >/<0.05**
Control week 0 vs E1 week 0	0.654; >0.05
Control week 0 vs E2 week 0	0.553; >0.05
E1 week 0 vs E2 week 0	0.957; >0.05
Control week 1 vs E1 week 1	0.001; <0.05
Control week 1 vs E2 week 1	<0.001
E1 week 1 vs E2 week 1	0.316; >0.05
Control week 6 vs E1 week 6	0.978; >0.05
Control week 6 vs E2 week 6	0.088; >0.05
E1 week 6 vs E2 week 6	0.094; >0.05

Table V: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in individual egg production of *Helix aspersa* over the six-week experimental period, within each of the three test groups (Control = 0 μg g⁻¹; E1 = 80 μg g⁻¹; E2 = 240 μg g⁻¹ copper oxychloride; p = p-value; W1-W6 = Week 1-Week 6).

Control		E1		E2	
Weeks	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**
W1 vs W2	1.0; >0.05	W1 vs W2	1.0; >0.05	W1 vs W2	1.0; >0.05
W2 vs W3	0.114; >0.05	W2 vs W3	0.114; >0.05	W2 vs W3	0.686; >0.05
W3 vs W4	0.114; >0.05	W3 vs W4	0.556; >0.05	W3 vs W4	1.0; >0.05
W4 vs W5	0.152; >0.05	W4 vs W5	0.686; >0.05	W4 vs W5	1.0; >0.05
W5 vs W6	0.114; >0.05	W5 vs W6	1.0; >0.05	W5 vs W6	1.0; >0.05
W6 vs W1	0.047; <0.05	W6 vs W1	1.0; >0.05	W6 vs W1	1.0; >0.05

Table VI: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in individual egg production between the three test groups of *Helix aspersa*, during the first and sixth week of exposure to copper oxychloride (Control = 0 μg g⁻¹; E1 = 80 μg g⁻¹; E2 = 240 μg g⁻¹ copper oxychloride; p = p-value).

Exposure groups	**p; >/<0.05**
Control week 1 vs E1 week 1	1.0; >0.05
Control week 1 vs E2 week 1	1.0; >0.05
E1 week 1 vs E2 week 1	1.0; >0.05
Control week 6 vs E1 week 6	0.047; <0.05
Control week 6 vs E2 week 6	0.047; <0.05
E1 week 6 vs E2 week 6	1.0; >0.05

Table VII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in percentage hatched eggs per egg clutch between the three test groups of *Helix aspersa* (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Groups	p; >/<0.05
Control vs E1	0.251; >0.05
Control vs E2	0.002; <0.05
E1 vs E2	0.06; >0.05

Table VIII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in hatchling copper concentrations (μg g^{-1} dry mass) between the control and two copper oxychloride exposure groups of *Helix aspersa* (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control vs E1	0.152; >0.05
Control vs E2	0.026; <0.05
E1 vs E2	0.08; >0.05

Table IX: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in copper concentrations (μg g^{-1} dry mass) in the reproductive organs of *Helix aspersa*, between the three test groups (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control vs E1	0.001; <0.05
Control vs E2	<0.001
E1 vs E2	0.176; >0.05

Table X: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in copper concentrations (μg g^{-1} dry mass) in the reproductive organs of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value; W0 = prior to exposure; W1-W6 = Week 1-Week 6).

Control		E1		E2	
Weeks	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**
W0 vs W1	0.317; >0.05	W0 vs W1	1.0; >0.05	W0 vs W1	0.146; >0.05
W1 vs W2	0.317; >0.05	W1 vs W2	0.317; >0.05	W1 vs W2	0.615; >0.05
W2 vs W3	1.0; >0.05	W2 vs W3	0.455; >0.05	W2 vs W3	0.067; >0.05
W3 vs W4	1.0; >0.05	W3 vs W4	0.146; >0.05	W3 vs W4	0.067; >0.05
W4 vs W5	1.0; >0.05	W4 vs W5	0.067; >0.05	W4 vs W5	0.34; >0.05
W5 vs W6	1.0; >0.05	W5 vs W6	0.278; >0.05	W5 vs W6	0.671; >0.05
W6 vs W0	1.0; >0.05	W6 vs W0	0.004; <0.05	W6 vs W0	0.012; <0.05

Table XI: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in copper concentrations (μg g^{-1} dry mass) in the reproductive organs, between the three test groups of *Helix aspersa*, measured before the experiment commenced, and at the end of week 6 of the experiment (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	**p; >/<0.05**
Control week 0 vs E1 week 0	1.0; >0.05
Control week 0 vs E2 week 0	1.0; >0.05
E1 week 0 vs E2 week 0	1.0; >0.05
Control week 6 vs E1 week 6	0.004; <0.05
Control week 6 vs E2 week 6	0.012; <0.05
E1 week 6 vs E2 week 6	0.672; >0.05

Table XII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in digestive gland copper concentrations (μg g^{-1} dry mass), between the control and two copper oxychloride exposure groups of *Helix aspersa* (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control vs E1	<0.001
Control vs E2	<0.001
E1 vs E2	0.026; <0.05

Table XIII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in copper concentrations (μg g^{-1} dry mass) in the digestive gland of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value; W0 = prior to exposure; W1-W6 = Week 1-Week 6).

Control		E1		E2	
Weeks	p; >/< 0.05	Weeks	p; >/< 0.05	Weeks	p; >/< 0.05
W0 vs W1	0.86; >0.05	W0 vs W1	<0.001	W0 vs W1	0.024; <0.05
W1 vs W2	0.137; >0.05	W1 vs W2	0.05	W1 vs W2	0.31; >0.05
W2 vs W3	0.566; >0.05	W2 vs W3	0.886; >0.05	W2 vs W3	0.758; >0.05
W3 vs W4	0.402; >0.05	W3 vs W4	0.509; >0.05	W3 vs W4	0.004; <0.05
W4 vs W5	0.593; >0.05	W4 vs W5	0.348; >0.05	W4 vs W5	0.075; >0.05
W5 vs W6	0.233; >0.05	W5 vs W6	0.282; >0.05	W5 vs W6	<0.001
W6 vs W0	0.085; >0.05	W6 vs W0	<0.001	W6 vs W0	<0.001

Table XIV: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in copper concentrations (μg g^{-1} dry mass) in the digestive gland, between the three test groups of *Helix aspersa*, measured before and after the experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control week 0 vs E1 week 0	0.145; >0.05
Control week 0 vs E2 week 0	0.2; >0.05
E1 week 0 vs E2 week 0	0.987; >0.05
Control week 6 vs E1 week 6	<0.001
Control week 6 vs E2 week 6	<0.001
E1 week 6 vs E2 week 6	0.233; >0.05

Table XV: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in "rest of the body" copper concentrations (μg g^{-1} dry mass), between the three test groups of *Helix aspersa* (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control vs E1	<0.001
Control vs E2	<0.001
E1 vs E2	0.009; <0.05

Table XVI: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in copper concentrations (μg g^{-1} dry mass) in the body (excluding reproductive organs and digestive gland) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value; W0 = prior to exposure; W1-W6 = Week 1-Week 6).

Control		E1		E2	
Weeks	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**
W0 vs W1	0.141; >0.05	W0 vs W1	0.003; <0.05	W0 vs W1	0531; >0.05
W1 vs W2	0.207; >0.05	W1 vs W2	0.425; >0.0.5	W1 vs W2	0.161; >0.05
W2 vs W3	0.508; >0.05	W2 vs W3	0.011; <0.05	W2 vs W3	0.686; >0.05
W3 vs W4	0.031; <0.05	W3 vs W4	0.221; >0.05	W3 vs W4	0.453; >0.05
W4 vs W5	0.262; >0.05	W4 vs W5	0.467; >0.05	W4 vs W5	0.018; <0.05
W5 vs W6	0.097; >0.05	W5 vs W6	0.507; >0.05	W5 vs W6	0.249; >0.05
W6 vs W0	0.619; >0.05	W6 vs W0	<0.001	W6 vs W0	<0.001

Table XVII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in copper concentrations (μg g^{-1} dry mass) in the snail body (excluding reproductive organs and digestive gland), between the three test groups of *Helix aspersa*, measured before and after the experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	**p; >/<0.05**
Control week 0 vs E1 week 0	0.697; >0.05
Control week 0 vs E2 week 0	0.263; >0.05
E1 week 0 vs E2 week 0	0.354; >0.05
Control week 6 vs E1 week 6	0.002; <0.05
Control week 6 vs E2 week 6	<0.001
E1 week 6 vs E2 week 6	0.016; <0.05

Table XVIII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in whole body copper concentrations ($\mu g \ g^{-1}$ dry mass) between the control group and two copper oxychloride exposure groups of *Helix aspersa* (Control = 0 $\mu g \ g^{-1}$; E1 = 80 $\mu g \ g^{-1}$; E2 = 240 $\mu g \ g^{-1}$ copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control vs E1	<0.001
Control vs E2	<0.001
E1 vs E2	0.01; <0.05

Table XIX: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in whole body copper concentrations ($\mu g \ g^{-1}$ dry mass) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 $\mu g \ g^{-1}$; E1 = 80 $\mu g \ g^{-1}$; E2 = 240 $\mu g \ g^{-1}$ copper oxychloride; p = p-value; W0 = prior to exposure; W1-W6 = Week 1-Week 6).

Control		E1		E2	
Weeks	**p; >/< 0.05**	**Weeks**	**P; >/< 0.05**	**Weeks**	**p; >/< 0.05**
W0 vs W1	0.25; >0.05	W0 vs W1	<0.001	W0 vs W1	0.009; <0.05
W1 vs W2	0.18; >0.05	W1 vs W2	0.38; >0.05	W1 vs W2	0.392; >0.05
W2 vs W3	0.31; >0.05	W2 vs W3	0.019; <0.05	W2 vs W3	0.688; >0.05
W3 vs W4	0.031; <0.05	W3 vs W4	0.129; >0.05	W3 vs W4	0.304; >0.05
W4 vs W5	0.281; >0.05	W4 vs W5	0.194; >0.05	W4 vs W5	0.041; <0.05
W5 vs W6	0.047; <0.05	W5 vs W6	0.269; >0.05	W5 vs W6	0.129; >0.05
W6 vs W0	0.965; >0.05	W6 vs W0	<0.001	W6 vs W0	<0.001

Table XX: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in whole body copper concentrations (μg g^{-1} dry mass), between the three test groups of *Helix aspersa*, measured before and after the experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control week 0 vs E1 week 0	0.354; >0.05
Control week 0 vs E2 week 0	0.27; >0.05
E1 week 0 vs E2 week 0	0.549; >0.05
Control week 6 vs E1 week 6	<0.001
Control week 6 vs E2 week 6	<0.001
E1 week 6 vs E2 week 6	0.024; <0.05

Table XXI: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences between copper concentrations (μg g^{-1} dry mass) in the reproductive organs, digestive gland and rest of the body, within each group of *Helix aspersa* (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Control	p; >/< 0.05
Reproductive organs vs Digestive gland	<0.001
Reproductive organs vs Rest of the body	<0.001
Digestive gland vs Rest of the body	0.052; >0.05
E1	**p; >/< 0.05**
Reproductive organs vs Digestive gland	<0.001
Reproductive organs vs Rest of the body	<0.001
Digestive gland vs Rest of the body	<0.001
E2	**p; >/< 0.05**
Reproductive organs vs Digestive gland	<0.001
Reproductive organs vs Rest of the body	<0.001
Digestive gland vs Rest of the body	<0.001

Table XXII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences between copper concentrations ($\mu g\ g^{-1}$ dry mass) in the reproductive organs, digestive gland and rest of the body, within each group of *Helix aspersa*, measured before (W0) and after (W6) the experimental period (Control = 0 $\mu g\ g^{-1}$; E1 = 80 $\mu g\ g^{-1}$; E2 = 240 $\mu g\ g^{-1}$ copper oxychloride; p = p-value).

Control	
Week 0	**p; >/< 0.05**
Reproductive organs vs Digestive gland	<0.001
Reproductive organs vs Rest of the body	<0.001
Digestive gland vs Rest of the body	0.2; >0.05
Week 6	**p; >/< 0.05**
Reproductive organs vs Digestive gland	<0.001
Reproductive organs vs Rest of the body	<0.001
Digestive gland vs Rest of the body	0.171; >0.05
E1	
Week 0	**p; >/< 0.05**
Reproductive organs vs Digestive gland	<0.001
Reproductive organs vs Rest of the body	<0.001
Digestive gland vs Rest of the body	0.122; >0.05
Week 6	**p; >/< 0.05**
Reproductive organs vs Digestive gland	0.018; <0.05
Reproductive organs vs Rest of the body	0.4; >0.05
Digestive gland vs Rest of the body	<0.001
E2	
Week 0	**p; >/< 0.05**
Reproductive organs vs Digestive gland	<0.001
Reproductive organs vs Rest of the body	<0.001
Digestive gland vs Rest of the body	0.691; >0.05
Week 6	**p; >/< 0.05**
Reproductive organs vs Digestive gland	0.03; <0.05
Reproductive organs vs Rest of the body	0.965; >0.05
Digestive gland vs Rest of the body	<0.001

Table XXIII: Spearman Rank Order Correlation test for the relationship between whole body copper concentrations (μg g^{-1} dry mass) and body mass (grams) in the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure group	r	p; >/< 0.05
Control	-0.029	1.0; >0.05
E1	-0.2	0.714; >0.05
E2	-0.886	0.03; <0.05

Table XXIV: Spearman Rank Order Correlation test for the relationship between reproductive organ copper concentrations (μg g^{-1} dry mass) and total egg production in the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure group	r	p; >/< 0.05
Control	-0.417	0.419; >0.05
E1	-0.223	0.658; >0.05
E2	-0.845	0.03; <0.05

Table XXV: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in neutral red retention times (minutes) of haemocytic lysosomes of the control group and two copper oxychloride exposure groups of *Helix aspersa*, over the six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value; W0 = prior to exposure; W1-W6 = Week 1-Week 6).

Control		E1		E2	
Weeks	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**	**Weeks**	**p; >/< 0.05**
W0 vs W1	0.963; >0.05	W0 vs W1	0.784; >0.05	W0 vs W1	1.0; >0.05
W1 vs W2	0.622; >0.05	W1 vs W2	0.037; <0.05	W1 vs W2	0.014; <0.05
W2 vs W3	0.325; >0.05	W2 vs W3	0.013; <0.05	W2 vs W3	0.004; <0.05
W3 vs W4	0.171; >0.05	W3 vs W4	0.272; >0.05	W3 vs W4	0.05
W4 vs W5	0.646; >0.05	W4 vs W5	0.11; >0.05	W4 vs W5	0.059; >0.05
W5 vs W6	0.256; >0.05	W5 vs W6	0.718; >0.05	W5 vs W6	0.277; >0.05
W6 vs W0	0.094; >0.05	W6 vs W0	<0.001	W6 vs W0	<0.001

Table XXVI: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in neutral red retention times (minutes) of haemocytic lysosomes, between the three test groups of *Helix aspersa*, measured before and after the experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	**p; >/<0.05**
Control week 0 vs E1 week 0	0.389; >0.05
Control week 0 vs E2 week 0	0.147; >0.05
E1 week 0 vs E2 week 0	0.467; >0.05
Control week 6 vs E1 week 6	<0.001
Control week 6 vs E2 week 6	<0.001
E1 week 6 vs E2 week 6	0.001; <0.05

Table XXVII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in tubule area (μm^2) of the digestive gland of the control group and two copper oxychloride exposure groups of *Helix aspersa* at the end of the six-week experimental period (Control = 0 $\mu g\ g^{-1}$; E1 = 80 $\mu g\ g^{-1}$; E2 = 240 $\mu g\ g^{-1}$ copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control vs E1	0.816; >0.05
Control vs E2	0.284; >0.05
E1 vs E2	0.386; >0.05

Table XXVIII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in epithelium height (μm) in the digestive gland of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (Control = 0 $\mu g\ g^{-1}$; E1 = 80 $\mu g\ g^{-1}$; E2 = 240 $\mu g\ g^{-1}$ copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control vs E1	<0.001
Control vs E2	<0.001
E1 vs E2	<0.001

Table XXIX: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in digestive epithelium area (%) of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (Control = 0 $\mu g\ g^{-1}$; E1 = 80 $\mu g\ g^{-1}$; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control vs E1	<0.001
Control vs E2	<0.001
E1 vs E2	0.54; >0.05

Table XXX: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in spermatozoan area (%) in the vesicula seminalis of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control vs E1	0.675; >0.05
Control vs E2	0.523; >0.05
E1 vs E2	0.853; >0.05

Table XXXI: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in spermatozoan area per 1 mm^2 ovotestis, of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control vs E1	0.455; >0.05
Control vs E2	<0.001
E1 vs E2	<0.001

Table XXXII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in oocyte numbers per 1 mm^2 ovotestis, of the control group and two copper oxychloride exposure groups of *Helix aspersa*, after a six-week experimental period (Control = 0 μg g^{-1}; E1 = 80 μg g^{-1}; E2 = 240 μg g^{-1} copper oxychloride; p = p-value).

Exposure groups	p; >/<0.05
Control vs E1	<0.001
Control vs E2	<0.001
E1 vs E2	<0.001

Table XXXIII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in copper concentrations (μg g^{-1} dry mass) of vine leaves and soil collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (p = p-value).

Field Sites	Vine leaves	Soil
	p; >/<0.05	p; >/<0.05
Helderberg vs Delheim1	0.1; >0.05	0.1; >0.05
Helderberg vs Delheim2	0.005; <0.05	<0.001
Delheim1 vs Delheim2	0.063; >0.05	0.011; <0.05

Table XXXIV: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in reproductive organ copper concentrations (μg g^{-1} dry mass) of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (p = p-value).

Field sites	p; >/<0.05
Helderberg vs Delheim1	1.0; >0.05
Helderberg vs Delheim2	0.197; >0.05
Delheim1 vs Delheim2	0.197; >0.05

Table XXXV: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in digestive gland copper concentrations ($\mu g \ g^{-1}$ dry mass) of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (p = p-value).

Field sites	p; >/<0.05
Helderberg vs Delheim1	<0.001
Helderberg vs Delheim2	0.001; <0.05
Delheim1 vs Delheim2	0.001; <0.05

Table XXXVI: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in body copper concentrations ($\mu g \ g^{-1}$ dry mass) of *Helix aspersa* (excluding reproductive organs and digestive gland), collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (p = p-value).

Field sites	p; >/<0.05
Helderberg vs Delheim1	0.002; <0.05
Helderberg vs Delheim2	0.001; <0.05
Delheim1 vs Delheim2	0.002; <0.05

Table XXXVII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in whole body copper concentrations (μg g^{-1} dry mass) of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (p = p-value).

Field sites	p; >/<0.05
Helderberg vs Delheim1	<0.001
Helderberg vs Delheim2	0.001; <0.05
Delheim1 vs Delheim2	0.002; <0.05

Table XXXVIII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences between copper concentrations (μg g^{-1} dry mass) in the reproductive organs, digestive gland and rest of the body of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim 1) and 2 months after application (Delheim 2) of copper oxychloride (p = p-value).

Helderberg site	p; >/< 0.05
Reproductive organs vs Digestive gland	<0.001
Reproductive organs vs Rest of the body	<0.001
Digestive gland vs Rest of the body	0.75; >0.05
Delheim 1	**p; >/< 0.05**
Reproductive organs vs Digestive gland	<0.001
Reproductive organs vs Rest of the body	<0.001
Digestive gland vs Rest of the body	0.009; <0.05
Delheim 2	**p; >/< 0.05**
Reproductive organs vs Digestive gland	0.002; <0.05
Reproductive organs vs Rest of the body	0.015; <0.05
Digestive gland vs Rest of the body	0.015; <0.05

Table XXXIX: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in neutral red retention times (minutes) of haemocytic lysosomes of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (p = p-value).

Field sites	p; >/<0.05
Helderberg vs Delheim1	0.028; <0.05
Helderberg vs Delheim2	<0.001
Delheim1 vs Delheim2	<0.001

Table XL: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in tubule area (μm^2) of the digestive gland of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (p = p-value).

Field sites	p; >/<0.05
Helderberg vs Delheim 1	0.839; >0.05
Helderberg vs Delheim 2	0.557; >0.05
Delheim 1 vs Delheim 2	0.703; >0.05

Table XLI: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in digestive gland epithelium height (µm) of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (p = p-value).

Field sites	p; >/<0.05
Helderberg vs Delheim 1	0.129; >0.05
Helderberg vs Delheim 2	<0.001
Delheim 1 vs Delheim 2	<0.001

Table XLII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in digestive gland epithelium area (%) of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (p = p-value).

Field sites	p; >/<0.05
Helderberg vs Delheim 1	0.343; >0.05
Helderberg vs Delheim 2	<0.001
Delheim 1 vs Delheim 2	<0.001

Table XLIII: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in spermatozoan area (%) in the vesicula seminalis of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (p = p-value).

Field sites	p; >/<0.05
Helderberg vs Delheim 1	0.642; >0.05
Helderberg vs Delheim 2	0.665; >0.05
Delheim 1 vs Delheim 2	0.918; >0.05

Table XLIV: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in spermatozoan area per 1 mm^2 ovotestis of *Helix aspersa*, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Dlhm1) and 2 months after application (Dlhm2) of copper oxychloride (p = p-value).

Field sites	p; >/<0.05
Helderberg vs Delheim 1	0.981; >0.05
Helderberg vs Delheim 2	0.016; <0.05
Delheim 1 vs Delheim 2	0.011; <0.05

Table XLV: Kruskal-Wallis One Way Analysis of Variance on Ranks test for differences in oocyte numbers per 1 mm^2 of *Helix aspersa* ovotestis, collected from an uncontaminated vineyard (Helderberg), and from a contaminated vineyard (Delheim), 1 week after application (Delheim1) and 2 months after application (Delheim2) of copper oxychloride (p = p-value).

Field sites	p; >/<0.05
Helderberg vs Delheim 1	0.642; >0.05
Helderberg vs Delheim 2	0.007; <0.05
Delheim 1 vs Delheim 2	0.015; <0.05

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