

---

**ECOLOGICAL RELEVANCE OF SUBORGANISMAL AND POPULATION  
RESPONSES OF TERRESTRIAL OLIGOCHAETA TO THE FUNGICIDE  
COPPER OXYCHLORIDE**

BY

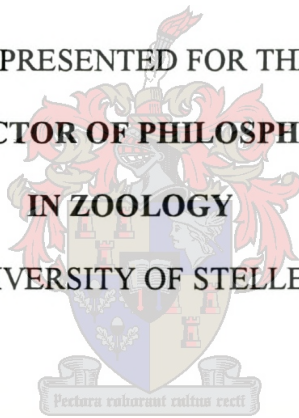
**MARK STEVE MABOETA**

DISSERTATION PRESENTED FOR THE DEGREE OF

**DOCTOR OF PHILOSOPHY**

**IN ZOOLOGY**

AT THE UNIVERSITY OF STELLENBOSCH



PROMOTOR: PROF. A.J. REINECKE

CO-PROMOTOR: DR. S.A. REINECKE

OCTOBER 2000

---

---

***DECLARATION...***

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

**Signature**

**Date**

---

## ***ABSTRACT...***

Copper oxychloride is a fungicide that is extensively used in vineyards in the Western Cape to treat and prevent fungal diseases. It is however not clear what the effects are on soil organisms, which play an important role in soil fertility, in South African soils. There is paucity of data linking results obtained in the laboratory to effects observed in the field, which will only become useful if a clear relation can be demonstrated.

The aims of this study were to:

- Determine the effects of copper oxychloride on field populations of earthworms and simultaneously monitor lysosomal membrane stability, measured as neutral red retention time (NRRT).
- Validate experimental field studies by doing inventories of earthworm populations in long-term sprayed vineyards.
- Determine the LC<sub>50</sub> of copper oxychloride and simultaneously measuring NRRT, and linking them to the experimental field studies.
- Conduct bioassays, burrowing activity- and soil-avoidance experiments to investigate their relations to earthworm population responses in the experimental field studies.

Earthworms were sampled by hand-sorting in the field tests on treated and untreated field plots in the Western- (October 1998 - July 1999) and Northern Cape (April 1998 - October 1999). Soil samples and worms were analysed for copper contents and coelomocytes of live earthworms were used to conduct the neutral red retention assays.

Acute toxicity tests were conducted over a period of 28 days during which the earthworms (*Eisenia fetida*) were exposed to different concentrations of copper oxychloride. Change in biomass and mortality were measured as endpoints, as well as NRRT.

Bioassays, burrowing activity and soil-avoidance were conducted by exposing *Aporrectodea caliginosa* to grassland- and vineyard soil as well as grassland soil spiked with 60 µg.g<sup>-1</sup> copper in the form of copper oxychloride. Growth and mortality were recorded in the bioassays as well as copper concentrations in earthworm body tissues and substrates used over a period of 28 days.

Burrowing activity and soil-avoidance were determined by measuring the length of tunnels burrowed by *A. caliginosa* in soil profiles over a period of 4 days under different exposure regimes.

Results from the field tests showed that spraying of copper oxychloride had a negative effect on earthworm populations at the prescribed application rates. NRRT in earthworms from the exposure plots was significantly ( $p < 0.05$ ) lower after just one spraying application. It was concluded that spraying copper oxychloride at prescribed application rates caused a decrease in field populations of earthworms and that NRRT was an early and reliable biomarker since it was indicative of later effects observed at the population level. Results obtained from the field inventory of earthworms in vineyards at Nietvoorbij, Robertson and Worcester confirmed data from the two field studies.

The calculated  $LC_{50}$  of  $882.78 \mu\text{g}\cdot\text{g}^{-1}$  for copper oxychloride and  $519.40 \mu\text{g}\cdot\text{g}^{-1}$  for copper was ecologically relevant if a safety factor of 10 was applied. NRRT which manifested earlier than effects on biomass change in the acute toxicity tests, were significant when viewed against the background of responses of field populations of earthworms.

From the bioassay experiments it was found that *A. caliginosa* exposed to copper oxychloride spiked soil had significantly ( $p < 0.05$ ) higher weight loss and mortality than those in grassland- and vineyard soil. This indicated that changes in biomass and mortality were indicative of population responses in the field and can be considered as ecologically relevant.

Burrowing activity of *A. caliginosa* was significantly ( $p < 0.05$ ) lower in vineyard and copper oxychloride spiked soil than in grassland soil. Similarly in the soil avoidance experiments it was found that *A. caliginosa* avoided vineyard- and copper oxychloride contaminated soil. It is therefore concluded that burrowing activity and soil avoidance were ecologically relevant endpoints since they corresponded with population responses in the field.

The study thus revealed that the long-term usage of copper oxychloride could have negative effects on earthworm populations. The spraying of copper oxychloride can have important implications on the sustainable use of agricultural soils since earthworms and other soil organisms play such an important role in soil fertility. The use of biomarkers and other ecotoxicological indicators can provide an early warning that soil organisms are under environmental stress.

---

## OPSOMMING...

Die fungisied koperoksichloried word wyd gebruik in die Wes-Kaap om swamsiektes in wingerde te beheer en te voorkom. Dit is egter nie bekend wat die effek daarvan op Suid Afrikaanse grondbiota, wat 'n belangrike rol speel in grondvrugbaarheid, is nie. Daar is ook 'n tekort aan inligting wat die resultate van laboratoriumondersoeke in verband bring met veldstudies.

Die doelstellings van die studie was om:

- Die effek van koperoksichloried op erdwurmpopulasies in die veld te ondersoek en terselfdertyd membraanstabieliteit, as moontlike biomerker, gemeet as neutraal rooi retensietye (NRRT), te monitor.
- Die geldigheid van eksperimentele veldstudies te toets deur ook grondanalises te doen in wingerde wat oor langtermyn met koperoksichloried bespuit is.
- Die  $LC_{50}$  van koperoksichloried vir erdwurms te bepaal en terselfdertyd NRRT te meet asook om dié gegewens in verband te bring met die resultate van seisoenale veldstudies oor die uitwerking op erdwurmpopulasies.
- Bio-evaluerings ("bioassays"), tonnelaktiwiteit- en vermydingseksperimente te onderneem en die verband tussen die toksiteitstoetse en populasieresponse, soos waargeneem in die veld, te ondersoek.

Erdwurms is versamel deur handsortering tydens die veldtoetse in die Wes- (Oktober 1998 - Julie 1999) en Noord-Kaap (April 1998 - Oktober 1999) op kontrole en bespuite persele. Grondmonsters en erdwurms is spektrofotometries geanaliseer om koperinhoud te bepaal. Die selomosiete van lewende wurms is gebruik om NRRT te bepaal. Akute toksisiteitstoetse is uitgevoer oor 'n tydperk van 28 dae waartydens *Eisenia fetida* blootgestel is aan verskillende koperoksichloried konsentrasies. Veranderinge in biomassa en mortaliteit is bepaal asook NRRT.

Bioevaluerings ("bioassays"), tonnelaktiwiteit- en vermydingseksperimente is uitgevoer deur *Aporrectodea caliginosa* bloot te stel aan grasveld- en wingerdgrond asook grasveldgrond wat met koperoksichloried gekontamineer is. Groei en mortaliteit is bepaal in die "bioassays" asook koperkonsentrasies in die grond en erdwurm liggaamswaarsels oor 'n tydperk van 28 dae. Tonnelaktiwiteit en grondvermyding is bepaal deur die lengte van tonnels wat deur *A. caliginosa* gegrawe is te meet oor 'n tydperk van vier dae vir die verskillende blootgestelde groepe.

Die resultate het aangedui dat koperoksichloriedbespuiting 'n negatiewe invloed het op erdwurmpopulasies teen die voorgeskrewe toedieningsprogram. NRRT in erdwurms van die blootstellingperseel, was beduidend ( $p < 0.05$ ) laer na 'n enkele bespuiting. Daar is verder bevind dat NRRT 'n betroubare en vroeë biomerker is, aangesien dit 'n aanduiding gee van latere effekte wat op populasievlak na vore getree het. Veldopnames in Nietvoorbij, Robertson en Worcester het die geldigheid van data verkry uit die veldstudies ondersteun.

Die berekende  $LC_{50}$  van  $882.78 \mu\text{g.g}^{-1}$  vir koperoksichloried en  $519.40 \mu\text{g.g}^{-1}$  vir koper was ekologies relevant indien 'n veiligheidsfaktor van 10 toegepas is. NRRT se ekologiese relevansie is bevestig deur dit te vergelyk met response wat in die veldtoetse waargeneem is.

Deur bioëvalueringseksperimente is bevind dat gewigsverlies en mortaliteit van *A. caliginosa* beduidend hoër was in koperoksichloried gekontameneerde grond as in die grasveld- (kontrole) en wingerdgronde. Veranderinge in biomassa en mortaliteit was aanduidend van populasieresponse soos waargeneem in die veldstudies en kan dus as ekologies relevante eindpunte beskou word.

Tonnelaktiwiteit van *A. caliginosa* was beduidend ( $p < 0.05$ ) laer in wingerd- en koperoksichloried gekontameneerde grond as in grasveldgrond. Dieselfde is gevind in die grondvermydingstoetse waar *A. caliginosa* wingerd- en koperoksichloried gekontameneerde grond vermy het. Dit kan dus afgelei dat tonnelaktiwiteit en grondvermyding ook ekologies bruikbare eindpunte is aangesien dit verband hou met populasieresponse soos waargeneem in die veldstudies.

Hierdie studie het getoon dat die herhaalde gebruik van koperoksichloried 'n nadelige invloed kan hê op erdwurmbevolking. In die lig van die belangrike rol wat erdwurms en ander grondorganismes speel in grondvrugbaarheid kan die oormatige gebruik van hierdie fungisied ernstige implikasies inhou vir volhoubare benutting van landbougronde. Die gebruik van biomerkers en ander ekotoksikologiese eindpunte kan egter as vroeë waarskuwingsmetode dien dat die grondorganismes onder omgewingstres verkeer.

## **TABLE OF CONTENTS...**

<b>Declaration</b>	i
<b>Abstract</b>	ii
<b>Opsomming</b>	iv
<b>List of figures</b>	x
<b>List of tables</b>	xiv
<b>Acknowledgements</b>	xviii
<b>1. Introduction</b>	
1.1. General	1
1.2. Copper oxychloride	1
1.3. Environmental relevance of pesticides	2
1.4. Earthworms in ecotoxicology	3
1.5. Ecotoxicological test methods	4
1.5.1. Acute-, sublethal-, bioassay- and field toxicity tests	4
1.5.2. Avoidance-behaviour tests	5
1.5.3. Biomarkers	6
1.6. Aims	8
<b>2. Material and Methods</b>	
2.1. Field studies	
2.1.1. Field studies at Nieuwoudtville and Vergenoegd (Stellenbosch)	10
2.1.2. Field studies at Nietvoorbij (Stellenbosch), Robertson and Worcester	13
2.2. Acute toxicity tests with copper oxychloride	
2.2.1. Species used	14
2.2.2. Substrates utilised	14
2.2.3. Experimental design of acute toxicity tests	15
2.3. Lysosomal membrane stability assays with copper oxychloride	
2.3.1. Stock- and working solutions	16
2.3.2. Collection of coelomic fluid and coelomocytes	17
2.3.3. Staining of coelomocytes	17

2.3.4. Measurement of neutral red times	17
2.4. Sublethal toxicity tests (bioassay) with copper oxychloride	
2.4.1. Species used, substrates and experimental design	17
2.5. Burrow activity and avoidance response	
2.5.1. Species used, substrates and experimental design	19
2.5.2. Burrow activity and avoidance-behaviour	19
2.6. Acid digestion of earthworms	20
2.7. Acid digestion of soil samples and substrate	20
2.8. Copper analysis	21
2.9. Statistical analysis of data	21

### 3. Results

3.1. Field tests with copper oxychloride conducted in the Nieuwoudtville area	
3.1.1. Physical parameters of the soil	22
3.1.2. Changes in mean biomass of collected earthworms and rainfall	23
3.1.3. Changes in mean number of earthworms collected and rainfall	24
3.1.4. Changes in lysosomal membrane stability of coelomocytes: Neutral red retention times	25
3.1.5. Changes in copper content of soils	26
3.1.6. Change in copper content of earthworm body tissues	28
3.2. Field-tests with copper oxychloride conducted in the Vergenoegd area	
3.2.1. Physical parameters of the soil	30
3.2.2. Changes in mean biomass of collected earthworms and mean rainfall	30
3.2.3. Changes in mean number of collected earthworms and mean rainfall	32
3.2.4. Changes in lysosomal membrane stability of coelomocytes: Neutral red retention times	34
3.2.5. Changes in copper content of soils	35
3.2.6. Change in copper content of earthworm body tissues	37
3.3. Field Studies conducted at Nietvoorbij, Robertson and Worcester	
3.3.1. Physical parameters of the soils at Nietvoorbij	39



3.3.2. Mean biomass of collected earthworms	39
3.3.3. Mean number of collected earthworms	40
3.3.4. Mean copper content in Nietvoorbij soils	41
3.3.5. Mean copper content of earthworm body tissues	43
3.3.6. Mean copper content in the Robertson and Worcester soils	44
3.4. Acute toxicity tests	
3.4.1. Change in mean biomass	45
3.4.2. Changes in lysosomal membrane stability of coelomocytes: Neutral red retention times	46
3.4.3. Mortality (LC <sub>50</sub> )	48
3.4.4. Copper content of substrate	49
3.4.5. Copper content of earthworm body tissues	50
3.5. Bioassay	
3.5.1. Change in biomass	53
3.5.2. Mortality	55
3.5.3. Mean copper content of substrates	55
3.5.4. Mean copper content of earthworm body tissues	56
3.6. Burrow activity and avoidance response of <i>Aporrectodea caliginosa</i>	
3.6.1. Burrow rate of earthworms	58
3.6.2. Soil avoidance by earthworms	
3.6.2.1. Grassland- vs. Vineyard soil	58
3.6.2.2. Grassland- vs. Copper contaminated soil	58

#### 4. Discussion

4.1. Field experiments at Nieuwoudtville and Vergenoegd	
4.1.1. Changes in biomass and numbers in relation to copper content of soils and earthworms	60
4.1.2. Changes in neutral red retention times in relation to copper content of soils and earthworms, and the link of these changes to population changes	62
4.2. A comparison between cellular and population responses at both localities	63
4.3. Field experiments at Nietvoorbij (Stellenbosch), Robertson and Worcester	66

4.4. Acute toxicity tests ( <i>Eisenia fetida</i> )	67
4.5. Bioassays of soils with <i>Aporrectodea caliginosa</i>	71
4.7. Burrow activity and avoidance response	72
<b>5. Conclusions</b>	<b>75</b>
<b>6. References</b>	<b>77</b>

---

## *List of figures...*

**Figure 1** Map of study areas where field studies where the effects of copper oxychloride on field populations of earthworm were conducted

**Figure 2** Layout of plots used at Nieuwoudtville and Stellenbosch for the duration of the study.

**Figure 3** Layout of plots and sampling used at Nietvoorbij during the study to determine earthworm biomass and numbers (x=vine; \*=sampling spot)

**Figure 4** Mean biomass (grams)  $\pm$ SD of earthworms (*Microchaetus* sp.) per m<sup>2</sup> in the control and copper oxychloride treated plots and mean rainfall (mm) for the duration of the study in the Nieuwoudtville area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

**Figure 5** Mean number  $\pm$ SD of earthworms (*Microchaetus* sp.) per m<sup>2</sup> in the control and copper oxychloride treated plots and mean rainfall (mm) for the duration of the study in the Nieuwoudtville area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

**Figure 6** Mean neutral red retention times (mins)  $\pm$ SD of earthworms (*Microchaetus* sp.) collected from the Nieuwoudtville area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 6$ ).

**Figure 7** Change over time in the mean copper concentration ( $\mu\text{g}\cdot\text{g}^{-1}$ )  $\pm$ SD in soils from the control and copper oxychloride exposed plots in the Nieuwoudtville area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 10$ ).

**Figure 8** Change over time in the mean copper concentration ( $\mu\text{g}\cdot\text{g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*Microchaetus* sp.) from the control and copper oxychloride exposed plots in the Nieuwoudtville area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 10$ ).

**Figure 9** Mean biomass (grams)  $\pm$ SD of earthworms (*Aporrectodea caliginosa*) per m<sup>2</sup> in the control and copper oxychloride sprayed plots and mean rainfall (mm) for the duration of the study in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control, p<0.05; n=5).

**Figure 10** Mean number  $\pm$ SD of earthworms (*Aporrectodea caliginosa*) per m<sup>2</sup> in the control and copper oxychloride sprayed plots and mean rainfall (mm) for the duration of the study in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control, p<0.05; n=5).

**Figure 11** Mean neutral red retention times (mins.)  $\pm$ SD of earthworms (*Aporrectodea caliginosa*) collected from the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control, p<0.05; n=6).

**Figure 12** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in the soils in the soils from the control and copper oxychloride sprayed plots in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control, p<0.05; n=10).

**Figure 13** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*Aporrectodea caliginosa*) from the control and copper oxychloride sprayed plots in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control, p<0.05; n=10).

**Figure 14** Mean biomass (grams)  $\pm$ SD of earthworms (*Aporrectodea caliginosa*) per m<sup>2</sup> in grassland-, interrow- and vineyard soils in the Nietvoorbij area (\*significantly different from grassland, p<0.05).

**Figure 15** Mean number  $\pm$ SD of earthworms (*Aporrectodea caliginosa*) per m<sup>2</sup> in grassland-, interrow- and vineyard soils in the Nietvoorbij area (\*significantly different from grassland, p<0.05).

**Figure 16** Mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in grassland-, interrow- and vineyard soils in the Nietvoorbij area (\*significantly different from grassland,  $p < 0.05$ ;  $n = 10$ ).

**Figure 17** Mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*Aporrectodea caliginosa*) collected from grassland-, interrow- and vineyard soils in the Nietvoorbij area (\*significantly different from grassland,  $p < 0.05$ ;  $n = 6$ ).

**Figure 18** Mean bodyweight (gram)  $\pm$ SD of earthworms (*Eisenia fetida*) over 28 days in groups exposed to different copper oxychloride concentrations ( $n = 60$ ).

**Figure 19** Mean neutral red retention times (mins.)  $\pm$ SD of earthworms (*Eisenia fetida*) over 28 days in groups exposed to different copper oxychloride concentrations ( $n = 6$ ).

**Figure 20.** Mean percentage of mortality in earthworms (*Eisenia fetida*) exposed to different copper oxychloride concentrations ( $\mu\text{g.g}^{-1}$ ) after 28 days ( $n = 60$  per group).

**Figure 21** Mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in the substrates from the control and copper oxychloride exposed groups in acute toxicity tests (\*significantly different from control,  $p < 0.05$ ;  $n = 10$ ).

**Figure 22** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*Eisenia fetida*) from control and copper oxychloride exposed groups in acute toxicity tests (\*significantly different from control,  $p < 0.05$ ;  $n = 6$ ).

**Figure 23** Mean weight loss (%) over time of earthworms (*Aporrectodea caliginosa*) in the grassland-, vineyard- and copper oxychloride ( $60\mu\text{g.g}^{-1}$ ) treated soil for the duration of the study (\*significantly different from initial bodyweight,  $p < 0.05$ ;  $n = 30$ ).

**Figure 24** Mean mortality (%) of earthworms (*Aporrectodea caliginosa*) in the grassland-, vineyard- and copper oxychloride ( $60\mu\text{g.g}^{-1}$ ) treated soil at the end of the study (\*significantly different from grassland soil,  $p < 0.05$ ;  $n = 30$ ).

**Figure 25** Mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in substrates from the grassland, vineyard and copper oxychloride exposed groups in the bioassay tests (\*significantly different from control,  $p < 0.05$ ,  $n=6$ )

**Figure 26** Mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*Aporrectodea caliginosa*) from the grassland, vineyard and copper oxychloride exposed groups in the bioassay tests (\*significantly different from control,  $p < 0.05$ ,  $n=6$ ).

---

## *List of tables...*

**Table 1** Comparative concentrations of Effekto Virikop<sup>®</sup>, copper oxychloride and copper

**Table 2** Mean pH  $\pm$ SD of soils in the control and copper oxychloride treated plots for the duration of the study in the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

**Table 3** Mean biomass (grams)  $\pm$ SD of earthworms (*Microchaetus* sp.) per  $m^2$  in the control and copper oxychloride treated plots for the duration of the study in the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

**Table 4** Mean number  $\pm$ SD of earthworms (*Microchaetus* sp.) per  $m^2$  in the control and copper oxychloride treated plots for the duration of the study in the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

**Table 5** Mean neutral red retention times (mins)  $\pm$ SD of earthworms (*Microchaetus* sp.) collected from the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ).

**Table 6** Change over time in the mean copper concentration ( $\mu\text{g}\cdot\text{g}^{-1}$ )  $\pm$ SD in soils from the control and copper oxychloride exposed plots in the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 10$ ).

**Table 7** Change over time in the mean copper concentration ( $\mu\text{g}\cdot\text{g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*Microchaetus* sp.) from the control and copper oxychloride exposed plots in the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 10$ )

**Table 8** Mean pH  $\pm$  SD of soils in the control and copper oxychloride sprayed plots for the duration of the study in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

**Table 9** Mean biomass (grams)  $\pm$ SD of earthworms (*Aporrectodea caliginosa*) per  $m^2$  in control and copper oxychloride sprayed plots for the duration of the study in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

**Table 10** Mean number  $\pm$ SD of earthworms (*Aporrectodea caliginosa*) per  $m^2$  in the control and copper oxychloride sprayed plots for the duration of the study in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

**Table 11** Mean neutral red retention times (mins.)  $\pm$ SD of earthworms (*Aporrectodea caliginosa*) collected from the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 6$ ).

**Table 12** Change over time in the mean copper concentration ( $\mu\text{g}\cdot\text{g}^{-1}$ )  $\pm$ SD in the soils from the control and copper oxychloride sprayed plots in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 10$ ).

**Table 13** Change over time in the mean copper concentration ( $\mu\text{g}\cdot\text{g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*Aporrectodea caliginosa*) from the control and copper oxychloride sprayed plots in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 10$ ).

**Table 14** Mean biomass (grams)  $\pm$ SD and number  $\pm$ SD of earthworms per  $m^2$  in comparison to the copper concentrations ( $\mu\text{g}\cdot\text{g}^{-1}$ )  $\pm$ SD found in soils ( $n = 10$ ) and earthworm body tissues ( $n = 10$ ) from the grassland- ( $n = 18$ ), interrow- ( $n = 18$ ) and vineyard soils ( $n = 17$ ) in the Nietvoorbij sampling area (\*significantly different from grassland soils,  $p < 0.05$ ).



**Table 15** Mean growth (gram)  $\pm$ SD of earthworms (*Eisenia fetida*) over 28 days in groups exposed to different copper oxychloride concentrations ( $\mu\text{g}\cdot\text{g}^{-1}$ ) and % change in bodyweight ("-" = decrease) after 28 days; (\*significantly different from control,  $p<0.05$ ).

**Table 16** Mean neutral red retention times (mins.)  $\pm$ SD of earthworms over 28 days in groups exposed to different copper oxychloride concentrations ( $\mu\text{g}\cdot\text{g}^{-1}$ ); (\*significantly different from control,  $p<0.05$ ;  $n=6$ )

**Table 17** The mean percentage of mortality of earthworms (*Eisenia fetida*) exposed to different concentrations for the duration of the experiment.

**Table 18** Change over time in the mean copper concentration ( $\mu\text{g}\cdot\text{g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*Eisenia fetida*) from control and copper oxychloride exposed groups in acute toxicity tests (\*significantly different from control,  $p<0.05$ ;  $n=6$ ; nd=not detected).

**Table 19** Comparative concentrations ( $\mu\text{g}\cdot\text{g}^{-1}$ ) of Effekto Virikop<sup>®</sup>, a.i. copper oxychloride, effective copper, measured copper content of substrates and earthworm body tissues and the bioconcentration factors (BCF) for copper after 28 days. [nd = not detected].

**Table 20** Mean percentage change in biomass (weight loss/gain) over time of earthworms (*Aporrectodea caliginosa*) in the grassland-, vineyard- and copper oxychloride ( $60\mu\text{g}\cdot\text{g}^{-1}$ ) treated soil for the duration of the study (\*significantly different from initial bodyweight,  $p<0.05$ ).

**Table 21** Mean percentage mortality  $\pm$ SD of earthworms (*Aporrectodea caliginosa*) in comparison to copper concentrations ( $\mu\text{g}\cdot\text{g}^{-1}$ )  $\pm$ SD in soils and earthworm body tissues exposed to grassland-, vineyard- and copper oxychloride contaminated soil (effective [Cu] of  $60\mu\text{g}\cdot\text{g}^{-1}$ ) over 28 days and the bioconcentration factors (BCF) for copper in earthworm body tissues ( $n=10$ ; \*significantly different from grassland soils,  $p<0.05$ ).

**Table 22** Mean tunnelling activity (cm)  $\pm$ SD of earthworms in the burrow activity experiments (n=18) in comparison to the mean copper concentrations ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD (n=6) in grassland-, vineyard- and copper oxychloride contaminated soils (effective [Cu] of  $60\mu\text{g.g}^{-1}$ ) over a period of 4 days (\*significantly different from grassland soils,  $p < 0.05$ ).

**Table 23** Mean tunnelling activity (cm)  $\pm$ SD of earthworms in the soil avoidance experiments (n=12) in comparison to the mean copper concentrations ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD (n=6) in grassland-, vineyard- and copper oxychloride contaminated soils (effective [Cu] of  $60\mu\text{g.g}^{-1}$ ) over a period of 4 days (\*significantly different from grassland soils,  $p < 0.05$ ).

**Table 24** Comparative table of measured parameters and % change from the control in the copper oxychloride sprayed plots ( $4250 \mu\text{g.g}^{-1}$  per application) at the two different study areas (Nieuwoudtville and Vergenoegd) for *Microchaetus sp.* and *A. caliginosa* for the duration of the study [arrows = significant ( $p < 0.05$ ) increase ( $\uparrow$ ) or decrease ( $\downarrow$ ) in comparison to values from the control plots]; (OM = Organic Matter). X = Long-term assessment of earthworm populations 12 months (Nieuwoudtville) and 6 months (Vergenoegd) after spraying was stopped.  $p > 0.05$  = no statistically significant difference between control and exposure plots.

---

## ***ACKNOWLEDGEMENTS...***

Hereby I wish to thank the following people and institutions that made this study possible:

- My promotors, Prof. A.J. Reinecke & Dr. S.A. Reinecke for their guidance and support.
- Prof. H. Eijsackers for guidance and support.
- Mr. Neil McGregor for allowing me to conduct part of my study on his farm, Glen Lyon, Nieuwoudtville area.
- The Stellenbosch Sports Bureau for allowing me to conduct part of my study on their premises at Vergenoegd, Stellenbosch.
- Nietvoorbij Institute for wine and vine allowing the conducting of a part of my study in their vineyards in Stellenbosch.
- Mr. Ulrich Deutschländer for his help with the atomic absorption spectrophotometer.
- The staff and postgraduate students at the Department of Zoology, University of Stellenbosch.
- J. Odendaal, C. Philander and M. Timmey for assistance in field work.
- The NRF for financial assistance.
- My parents for their encouragement and support.

# CHAPTER 1

## *INTRODUCTION...*

### **1.1. General**

In the nineteenth century, the need to relieve the threat of famine, the potential loss of a wine industry and the question of rotting railroad beams started and inspired investigations into controlling the detrimental actions of fungi (Lukens, 1971). In 1876 the antifungal properties of copper was accidentally discovered in France. This was brought about when a brew of copper sulphate and lime that was applied to roadside rows of grapes to initially discourage theft, had surprisingly protected the plants against disease (Lukens, 1971). Since then, pesticides, a generic term used for a group of compounds that include herbicides, fungicides, insecticides and nematicides to kill target organisms (Edwards *et al.*, 1995; Malkomes, 1997), have become an important component of the agricultural industry. Because of their use, developed countries have become self-sufficient in terms of their total food need (Somerville, 1990).

Some of the most effective pesticides are broad-spectrum biocides which by killing pests have extensive benefits such as higher crop yields (Lee, 1985; Somerville, 1990). Before the development of synthetic organic chemicals, metal containing pesticides were widely used (Walker *et al.*, 1997). The greater quantity of these pesticides applied to crops ends up in the soil, either by aerial drift, runoff from plants, or eventual death of plants (Edwards, 1993; Pugh *et al.*, 1975). Since contact with beneficial, non-target species cannot be avoided, pesticides have the potential of destroying these species (Edwards, 1992; Lee, 1985; Nimmo and McEwen, 1994; Somerville, 1990). These non-target species include terrestrial wildlife, aquatic organisms, beneficial insects, soil organisms, etc. (Edwards, 1993; Somerville, 1990).

### **1.2. Copper oxychloride**

Fungicides and other pesticides are considered to be indispensable aids in agriculture (Pugh *et al.*, 1975). Fungicides are currently extensively used on vines, comprising 88% by weight of the agrochemicals used in the wine/grape sector in the southern region of South Africa the bulk of which is applied as a preventative measure after the rainy season (London

and Meyers, 1995). After sulphur, copper containing products contribute the second highest category of chemicals used for this purpose in South Africa (London and Meyers, 1995). Many successful chemical control agents contain copper (Lukens, 1971) and, as effective fungicide, it has been applied to a wide range of crops at high dosage rates (Hopkin, 1989). One such substance is copper oxychloride which is widely used for the prevention of a wide spectrum of fungal diseases and, which is considered as one of the least expensive (Van der Merwe, 1991; Walker *et al.*, 1997).

Copper oxychloride ( $3\text{Cu}[\text{OH}]_2 \text{CuCl}_2$ ), (IUPAC name: dicopper chloride trihydroxide) is a bluish-green powder which is insoluble in water but is soluble on decomposition in dilute acids and ammonium hydroxide solutions. It is manufactured by the action of air on scrap copper in a solution of copper dichloride and sodium chloride. It is applied on crops as a wettable powder by mixing it with water in which the microgranules (500 g/kg copper) are dispersed (Martin, 1973). Per definition, wettable powders are mixed with water and the powder is dispersed in tiny flakes throughout the water (Arendse *et al.*, 1989). Copper oxychloride is applied after the rainy season at a rate of 1.25-7.5 kg.ha<sup>-1</sup> in vineyards in Southern Africa (Krause *et al.*, 1996) with several applications per season (De Klerk, 1988).

### 1.3. Environmental relevance of pesticides

Copper oxychloride is sprayed directly onto crops and may directly or indirectly contaminate soils (Malkomes, 1997). Many pesticides are harmless or only slightly toxic to earthworms at normal application rates (Edwards *et al.*, 1995). They do however have an impact on soil organisms, since all pesticides designed to control pests are biocides (Edwards, 1993). The diversity of known sublethal effects of pesticides on organisms range from slowing down of activity to hyperactivity as well as many other complex behavioural and physiological changes that result from low dose and long term usage (Edwards, 1993).

There are insufficient data on many pesticides from field and laboratory assays to make accurate assessments of their relative toxicity (Edwards *et al.*, 1995), especially since the use of fungicides has increased rapidly in the last decades (Edwards and Bohlen, 1992). Copper can have toxic effects on earthworm populations. In pastures with high copper levels worm numbers are, on average, the lowest and it is known that soils with a copper level of 85 µg.g<sup>-1</sup> are not tolerated by earthworms (Van Rhee, 1975). Lee (1985) concluded that copper oxychloride can be very toxic with long and continued use resulting in complete extermination

of earthworms in orchard soils. The general toxicity of copper oxychloride is however considered low (Thompson, 1978; Edwards and Bohlen, 1992) but only a few studies have been undertaken to determine the effects of this substance on soil fauna (Kula, 1994). Helling *et al.* (2000) have shown that copper oxychloride can affect growth and reproduction in *Eisenia fetida* at relatively low concentrations. There is no clarity on the effects of copper oxychloride on earthworm populations both in the field and in laboratory studies.

#### 1.4. Earthworms in ecotoxicology

At the time when fungicides are made available commercially to farmers, many physical, chemical and biological properties are already known (Malkomes, 1997). It is, however, important to know what effects these substances will have on non-target organisms. Although it may seem logical to physically measure the amounts of contaminants in soils, this may result in misleading indications of the bioavailability of the contaminants (Morgan *et al.*, 1992; Cook and Hendershot, 1996). Earthworms live in soil and are near the bottom of the terrestrial trophic level (Reinecke, 1992) and are a major component of the primary animal biomass (Bouché, 1992; Edwards and Bohlen, 1992). They also play several biological roles (Ash and Lee, 1980; Bengtsson *et al.*, 1988; Edwards and Bäter, 1992; Edwards and Bohlen, 1996) and are vulnerable to impacts on soils (Reinecke, 1992). It is therefore important that chemicals should be tested for toxicity towards earthworms (Stenersen *et al.*, 1992) and to know how earthworms are affected by different agricultural practices (Edwards *et al.*, 1995). Pesticides can kill earthworms and can also accumulate in their body tissues (Edwards, 1980). Earthworms were selected as one of five important indicator organisms in ecotoxicological tests by the European Economic Community (EEC), Organisation for Economic Co-operation and Development (OECD), Food and Agriculture Organisation of the United Nations (FAO), pesticide registration authorities and environmental pollution authorities (Edwards and Bäter, 1992). They are readily available, easy to handle (Reinecke, 1992) and make non-controversial research subjects as there are few ethical objections to their use. Metal concentrations found in their body tissues reflect the bioavailability of these metals at specific sampling areas since earthworms are usually restricted to small localities (Depledge *et al.*, 1994). Earthworms could be seen as a monitoring species in a prolonged sense of ecological thinking: “what is bad for earthworms might be bad for humans” (Morgan *et al.*, 1992).

Soil contamination is heavily emphasised in the environmental literature underscoring the importance of ecotoxicological assays to determine the side effects of these chemicals in order to protect our environment (Tarradellas and Biton, 1997). Many pesticides which are toxic to terrestrial organisms can retard the breakdown of organic matter in the soil which, in turn, could have major effects on the primary productivity of the ecosystem (Edwards, 1993). Nevertheless, there is a relative paucity of data on the effects of copper oxychloride on earthworms in ecotoxicological laboratory and field tests.

## **1.5. Ecotoxicological test methods**

### *1.5.1. Acute- sublethal-, bioassay- and field toxicity tests*

Evaluation of the ecotoxicological effects of chemicals is generally achieved by undertaking laboratory or field tests. When comparing pollution induced stress on soil organisms under both field and laboratory conditions, the effects in the field often seems less serious since many ecological factors can mask ecotoxicity (Malkomes, 1997). Acute and sublethal toxicity tests are useful in determining the relative toxicity of chemicals to earthworms but have little ecological significance (Reinecke, 1992). In fields tests however, the exposure of earthworms to contaminants are identical to that experienced in the environment under natural climatic and environmental conditions (Edwards, 1992; Edwards and Bohlen, 1992). According to Somerville (1990), the ultimate assessment on the effects of a pesticide resides in field-testing.

Laboratory tests play an important role in the risk assessment of chemicals and pesticides towards earthworms (Kula, 1998) and can be considered valuable if they predict the effects of earthworms under field conditions (Heimbach, 1998). Of these, acute toxicity tests are the most relevant (Kula, 1998). The advantages of laboratory experiments are reproducibility, short duration and low cost (Edwards and Bohlen, 1992). The effects are reported as an  $LC_{50}$ . Change in the biomass of the earthworms, another parameter measured in the present study, can provide additional information about the sublethal sensitivities of the worms (Reinecke, 1992). These tests are important in order to gain an understanding of the toxicity of environmental contaminants, although effects on reproduction and behaviour are not revealed (Reinecke, 1992). Heimbach (1992) found a reasonable correlation between the results of acute toxicity tests and effects observed in the field. The relevance of acute toxicity

tests is considered to be low since actual exposure in the field is not taken into account (Kula, 1998).

Contaminated soils from the field contain a range of contaminants with bioavailabilities that may be different from that of substrates used in laboratory tests. Bioassays, using soil from contaminated field sites, are therefore recommended for determining the actual toxicity and risk of contaminated soil (Van Gestel, 1997).

### 1.5.2. Avoidance-behaviour tests

Behaviour may be defined as anything organisms do to adjust to, or interact with their environment (Tomlin, 1992), and it may influence the exposure of organisms to contaminants (Edwards and Coulson, 1992). Studying this could provide rapid and sensitive bioindication of poor environmental quality (Pascoe *et al.* 1991). If avoidance tests could be used to provide results predicative of chronic responses of earthworms to contaminants, a useful tool will have been added to the existing battery of tests available to risk assessors (Stephenson, 1998). For most species behaviour with respect to preference for contaminated and uncontaminated food is unknown (Everts, 1990). Avoidance tests in aquatic toxicity testing have been utilised for some time (De Peyster and Long, 1993) but little has been done to develop a soil avoidance test using earthworms. Exceptions to this are the contributions by Slimak (1997), Yeardley *et al.* (1996) and Eijssackers (1987). Avoidance-behaviour tests have a number of potential advantages over acute and sublethal toxicity tests. These include increased sensitivity, ability to assess sublethal stress quickly and the relative ease of the method (Yeardley *et al.*, 1996). Toxicant-induced behavioural changes can alter the exposure of organisms to toxicants which could be important in environmental risk assessment. Earthworms play an important role in soil fertility (Ash and Lee, 1980; Bengtsson *et al.*, 1988; Edwards and Bater, 1992; Edwards and Bohlen, 1996) and behavioural responses such as their absence from soil due to pollution (Wentzel and Guelta, 1988) may result in decreased soil fertility (Bengtsson *et al.*, 1988). This, in turn, may affect the numbers of earthworm predators (Yeardley *et al.*, 1996). Avoidance-behaviour is more directly related to survival and distribution, but this relationship has not yet been quantified (Peakall, 1994), although it could be a sensitive measure of the sublethal effects of chemicals applied to soils (Wentzel and Guelta, 1988).



### 1.5.3. Biomarkers

The use of biomarkers<sup>1</sup> in modern ecotoxicology is well established (Van Gestel and Van Brummellen, 1996), because it is indicative of exposure to contaminants (Shane, 1994) and certain pollutant induced biochemical responses (Stürzenbaum, *et al.*, 1998). Actions of chemicals that produce disease and death in animals are ultimately exerted at the cellular level (Lowe *et al.*, 1992). Biomarkers can therefore offer rapid and sensitive indications of adaptive responses to environmental contamination before pollutant-induced injury manifests at the level of the whole organism (Giamberini and Pihan, 1997; Moore, 1985). Biomarker techniques also give additional information on possible harmful effects that cannot be obtained from the chemical residue levels in environmental and biological media (Depledge and Fossi, 1994) in laboratory and field tests. These techniques integrate exposure in time and space, and may avoid difficulties in the interpretation of chemical concentration measurements due to differences in bioavailability (Van Gestel and Van Brummellen, 1996). In recent years there has been an increasing interest in the use of biomarkers for the assessment of the potential adverse effects of chemicals on the environment (Svendsen and Weeks, 1997a; 1997b). The major strength of biomarkers lies in the fact that they have the potential to circumvent the serious limitations of the classical approach to toxicity testing, i.e. measuring the residue of a chemical in either the organism or the environment (Peakall, 1994).

The earliest changes in an organism following exposure to a pollutant occurs at the cellular level (Shane, 1994). In many cases the earliest detectable changes are associated with subcellular organelles such as lysosomes (Moore, 1985; 1990) because they are a target for the toxic action of xenobiotics (Lowe *et al.*, 1992). Lysosomes are a morphological heterogeneous group of membrane-bound subcellular organelles which contain acid hydrolase; its function is to stabilise organelles and macromolecules (Scott-Fordsmand and Weeks, 1998). They are also involved in various aspects of the metabolism of metals and act as protectors of cellular homeostasis. Excessive concentrations of metals in or around the lysosomes lead to alterations of their structure or their enzymatic content which may impair their functioning (Sternlieb and Goldfisher, 1976). Experimental studies have demonstrated that lysosomal alterations can be induced by single toxicants such as copper and polycyclic aromatic

---

<sup>1</sup> Any biological response to an environmental chemical at the below -individual level, measured inside an organism or in its products (Van Gestel and Van Brummellen, 1996).

hydrocarbons (Moore *et al.*, 1982). The non-specificity of the lysosomal reactions is therefore of value as a general indicator of the deterioration of the health of the animal (Moore, 1990).

The most widely used test for lysosomal membrane fragility, neutral red retention times (NRRT) of lysosomes, has been applied successfully to molluscan and fish species (Moore, 1990). These tests have predicative potential and can be used in a suite of tests applicable for environmental monitoring (Moore, 1990). The neutral red ecotoxicity assay is based on the ability of viable cells to incorporate and bind neutral red, a supravital dye, within their lysosomes (Babich and Borenfreund, 1990). It relies on the fact that only lysosomes in healthy cells take up and retain supravital dye. Membrane stability decreases in response to stress as membrane permeability increases (Scott-Fordsmand and Weeks, 1998).

Biomarkers are applicable under both laboratory and field conditions, and may act as an early warning signal of effects at the population level (Scott-Fordsmand and Weeks, 1998). They are intended for use as instruments in the field to tell if there is a deviation from health at the individual level (Van Gestel and Van Brummellen, 1996). Further they should be responsive to either general toxicity (e.g. heavy metals or pesticides) a specific group of toxicants (e.g. fungicides) or even a single pollutant (Stürzenbaum, *et al.*, 1998). However, further research is needed to develop a better understanding of the ecological relevance of biomarkers (Bembridge 1998) and more effort is needed in the area of linking biomarker responses with effects at the population level, especially under natural conditions (Scott-Fordsmand and Weeks, 1998).

## 1.6. Aims

In so far as the exposure of individual earthworms could assist us in measuring or predicting ecological change, laboratory assays will be useful, but only if a clear relation can be demonstrated. There is a continuing need to ascertain the effects of chemicals relevant to agriculture, on earthworm abundance, diversity and distribution (Hendrix, 1998). The next step in developing suitable ecotoxicological tests would be to show quantitative and valid relationships between responses obtained in laboratory tests and those observed in the field (Reinecke, 1992; Abdul Rida and Bouché, 1997). For terrestrial field trials with pesticides it is important to ascertain if the use of a pesticide had some effect on the environment, with the intent of predicting future events (Turner, 1990). There is an urgent need for laboratory and field results to be correlated with the ultimate aim of using the laboratory tests for hazard prediction without the need for expensive field tests (Edwards, 1992; Van Gestel, 1997). The extrapolation of data obtained in the laboratory to field situations is a fundamental problem, which have not been examined in detail (Walker, 1992; Spurgeon and Hopkin, 1995). Everts (1990) suggested that field trials should provide confirmation that predictions made on the basis of laboratory tests are valid, because the ecological relevance of laboratory measured parameters are not generally known.

The specific aims of the study were:

- ❶ To determine the effects of copper oxychloride on earthworm populations of *Microchaetus sp.* and *Aporrectodea caliginosa* in the field over a set period of time. Simultaneously, changes in the lysosomal membrane stability were monitored to establish whether there was any relationship between results found at the population and suborganismal level.
- ❷ Compare field populations of earthworms present in long-term copper oxychloride sprayed vineyards with earthworm populations from non-sprayed fields to investigate the effect of long-term copper oxychloride use on earthworm populations and to establish whether these findings could be utilised to validate results obtained from the field studies conducted.
- ❸ Determine the acute toxicity of the fungicide copper oxychloride for the earthworm species, *Eisenia fetida* by using a standardised acute toxicity test and to simultaneously monitor changes in biomass and lysosomal membrane stability responses. These were then related to body concentrations of copper in earthworm body tissues. Moreover, there was

sought to establish whether results from acute toxicity tests could be related to findings from previously performed field studies on the effects of copper oxychloride on two field populations of earthworms. The premise being that this would further clarify the relevance (if any) of the acute toxicity test as well as the neutral red retention assay for predicting expected changes in field populations.

- ④ Conduct bioassay experiments on soils obtained from vineyards to determine if there was any relationship between the various test parameters and population responses in the field.
- ⑤ Investigate the effects of copper oxychloride on burrow activity and avoidance response of *A. caliginosa* to see if there was any relation with population responses found in the field.

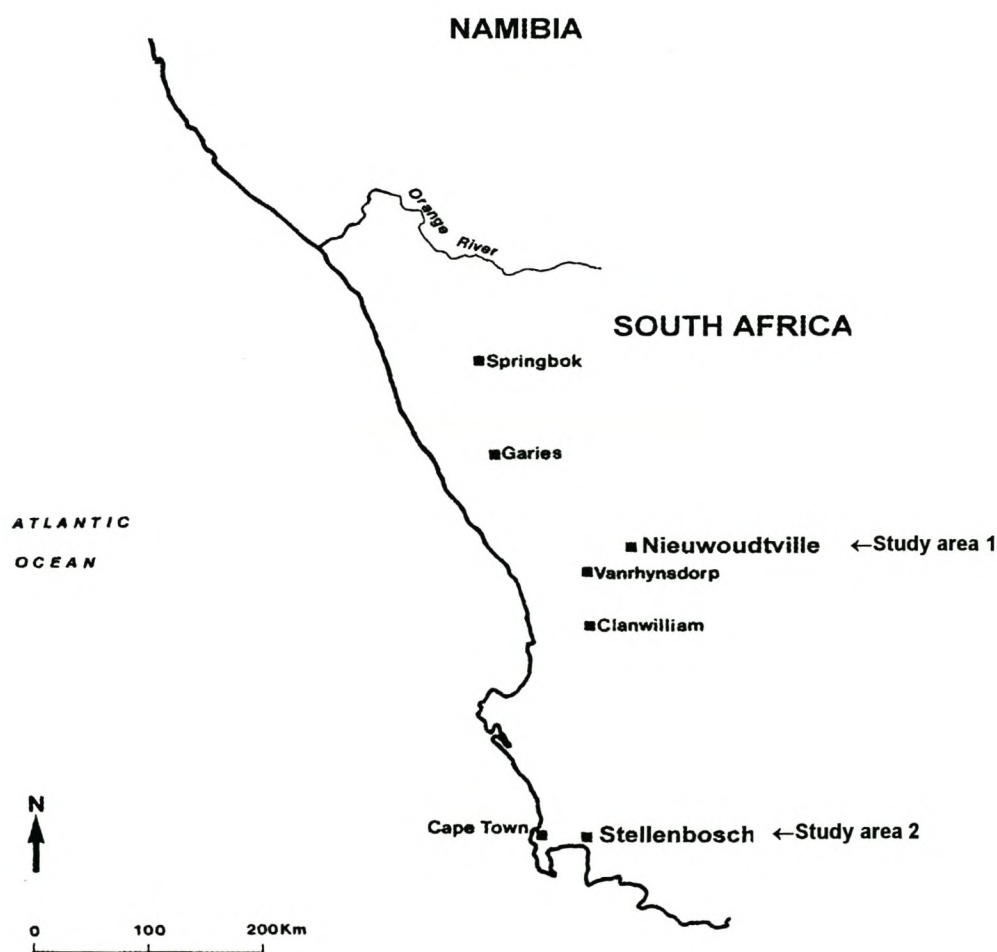
## CHAPTER 2

### *MATERIALS & METHODS...*

#### 2.1. Field studies

##### 2.1.1. Field Studies at Nieuwoudtville and Vergenoegd (Stellenbosch)

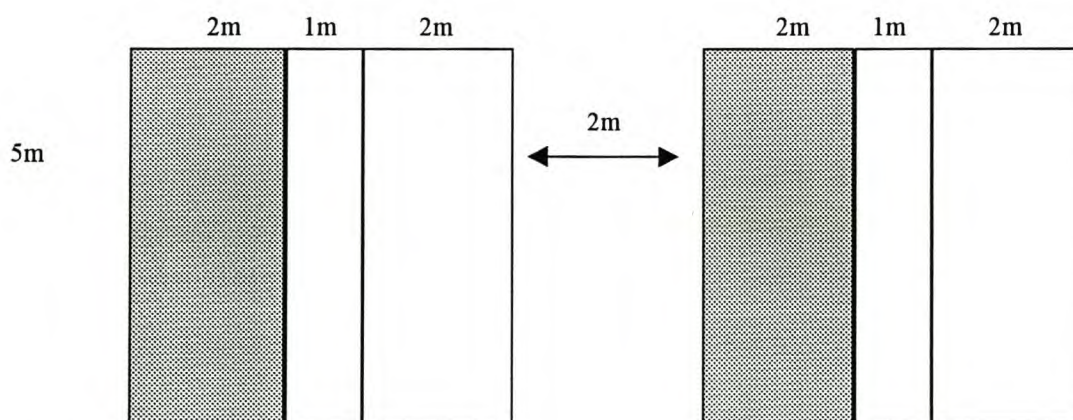
Field studies were conducted at two localities. The first was done on a farm, Glen Lyon near Nieuwoudtville in the Northern Cape (Figure 1). The second was done at Vergenoegd Sports grounds next to the Eersteriver in Stellenbosch, in the Western Cape (Figure 1).



**Figure 1** Map of study areas where field studies of the effects of copper oxychloride on field populations of earthworm were conducted

Two different locations were chosen to compare the effects of differences in soil qualities and climate towards the effects of copper oxychloride. In both these localities high earthworm populations existed. In Nieuwoudtville it was due to the fact that for the past 30 years conservation farming was practised and no agrochemicals were used (N. McGregor, pers. comm.). At Vergenoegd it is due to the fact that the grounds are situated next to a river and is an undisturbed grassland. The earthworms predominantly found in the Nieuwoudtville area belong to an indigenous earthworm species, viz. *Microchaetus*. No previous studies have been undertaken on the ecotoxicology of this indigenous earthworm species in the field in South Africa. The species found predominantly in the Stellenbosch area are exotic, mainly *A. caliginosa*.

Two plots, 5m x 5m were demarcated and cordoned off with hazard tape attached to metal poles at each site. Each plot was subdivided into three strips, two of 2m x 5m on the sides with a 1m x 5m safety strip in between to get a relatively realistic comparison of the effects of copper oxychloride on the earthworm populations present. The two plots were at least two metres apart.



**Figure 2** Layout of plots used at Nieuwoudtville and Stellenbosch for the duration of the study.

On each of the plots one 2m x 5m strip was utilised as an exposure strip (shaded on sketch) and the other as a control strip (Figure 2).

Sampling was done by extracting five random subsamples of 25cm x 25cm x 25cm from the strips on each plot, at least one metre apart, which resulted in a total of five control and five exposure subsamples. Samples were dug and handsorted to collect the

earthworms. Handsorting is laborious but gives the most accurate estimates of earthworm populations (Kula, 1992). Collected earthworms were counted and weighed to give an indication of the biomass and size of the earthworm population in the specific plots. According to Lofs (1992) the most commonly measured parameter in field studies is abundance, second is the concentration of the chemical in the soil and earthworms, and the third is biomass. All these parameters were taken into account for the purposes of this study. Soil samples from each plot were also collected for determination of the copper content and pH, which was determined with a Crison pH meter, by making a suspension of one gram of the substrate in distilled water.

Each of the exposure strips was sprayed with Effekto Virikop copper oxychloride, as is prescribed by the manufacturers. This gave an effective copper oxychloride concentration of  $4250 \mu\text{g}\cdot\text{g}^{-1}$ . To achieve this, five grams of copper oxychloride was dissolved in one litre of distilled water and applied with a Matabe compress spraypack. Sampling in Nieuwoudtville was done in April, June, July, September and October 1998. After sampling in April the exposure plots were sprayed with copper oxychloride and again during June, July and September 1998. During October 1999 the plots were sampled to determine if delayed (long-term) effects occurred due to spraying with copper oxychloride.

Sampling in the Stellenbosch area was conducted in October, November and December 1998, as well as January 1999. After sampling in October 1998 the exposure plots were sprayed with copper oxychloride and again during November and December 1998 as well as January 1999. The site was reinvestigated during July 1999 to determine the delayed (long-term) effects spraying may have had on earthworm populations. These specific months were chosen after the completion of the rainy season in Nieuwoudtville and in Stellenbosch since this is when copper oxychloride is sprayed (De Klerk, 1988). During these periods the exposure plots were sprayed to the equivalent of nine times with copper oxychloride as was specified by De Klerk (1988). The soil texture and organic matter content were determined for both of the areas and the mean rainfall in the respective areas were also recorded.

### 2.1.2. Field studies at Nietvoorbij (Stellenbosch), Robertson and Worcester

Field investigations on the effects of long-term use of copper oxychloride were investigated at Nietvoorbij in Stellenbosch in the Western Cape (Figure 1) during September and October 1999. An assessment of the copper concentrations found in vineyard soils in Robertson and Worcester was done during August 2000. Sampling was done the same as in Nieuwoudtville and Vergenoegd (refer to 2.1.1) except that the sampling plots differed. Samples were collected from grassland, interrow and vineyard soils. The samples taken from the grassland plots were  $\pm 2\text{m}$  from the vineyard and interrow sampling areas. Sampling was done from the bare border into the vineyard between the 1st and 2nd, the 3rd and 4th and between the 5th and 6th vine plants. Adjacently, samples were taken in the interrow between the vine plants. Similar samples were taken at equal distances into the grassland (Figure 3).

#### Vineyard

x		x		x	
*	*			*	*
x		x		x	
x		x		x	
*	*			*	*
x		x		x	
*	*			*	*
x		x		x	

#### Bare strip

*	*			*	*
---	---	--	--	---	---

#### Grassland

*	*			*	*
---	---	--	--	---	---

*	*			*	*
---	---	--	--	---	---

**Figure 3** Layout of plots and sampling used at Nietvoorbij, Robertson and Worcester during the study to determine earthworm biomass and numbers (x=vine; \*=sampling spot)



## 2.2. Acute toxicity tests with copper oxychloride

### 2.2.1. Species used

The earthworm species used in the acute toxicity tests was *E. fetida* ("tiger worm") which is epigeic and is a potential waste composting worm (Edwards & Bohlen, 1996). This species was used because its life cycle is well documented (Venter and Reinecke, 1988) and it has been prescribed by the OECD and EPA as test organism in ecotoxicological tests (Edwards and Bater, 1992). Likewise, Edwards and Coulson (1992) have also recommended *E. fetida* as a standard test species in ecotoxicological studies. The breeding stock of *E. fetida* used in this study was obtained from cultures maintained at the Department of Zoology at Stellenbosch University over several years. They were incubated in a Labcon® growth chamber at a constant temperature of  $\pm 25^{\circ}\text{C}$ . Only mature clitellate worms were used for the purposes of this investigation.

The classification of Simms & Gerard (1985) for the species is followed:

Phylum	: Annelida
Subphylum	: Clitellata
Class	: Oligochaeta
Order	: Haplotaxida
Suborder	: Lumbricina
Superfamily	: Lumbricoidea
Family	: Lumbricidae (Rafinesque-Schmaltz, 1815)
Subfamily	: Lumbricinae (Rafinesque-Schmaltz, 1815)
Genus	: <i>Eisenia</i>
Species	: <i>Eisenia fetida</i> (Savigny, 1826)

### 2.2.2. Substrates utilised

An artificial soil substrate was used in this study (OECD, 1984). It consisted of a dry weight mixture of 68% silica, 20% Kaolin clay and 10% sphagnum peat moss. 2% calcium carbonate ( $\text{CaCO}_3$ ) was added to adjust the pH to  $\pm 7$ . The ingredients were weighed and mixed in these proportions and moistened with distilled water to a 35% (by weight) moisture content. It was prepared in large batches to yield enough substrate for three replicates for each of the control and exposure groups. The substrate was placed into plastic containers (15 x 10 x 5cm), covered with a piece of plastic before sealing with a

perforated lid and kept in an environmental chamber ( $\pm 25^{\circ}\text{C}$ ) for 24 hours to stabilise prior to introducing the worms.

### 2.2.3. Experimental design of acute toxicity tests

Tests were performed according to guidelines formulated by the Office of Prevention, Pesticides and Toxic Substances (OPPTS) a subdirectorate of the United States Environmental Protection Agency (EPA). Twenty earthworms were placed in each of the three replicate containers filled with 540g of the prepared substrate. At no stage during the experiment were the worms fed. For the determination of the  $\text{LC}_{50}$  a control and seven copper oxychloride concentrations (200, 300, 450, 675, 1000, 1500 and  $2250\mu\text{g.g}^{-1}$  Effekto Virikop<sup>®</sup>) were used. The five lowest exposure concentrations (200 - 1000  $\mu\text{g.g}^{-1}$  Effekto Virikop<sup>®</sup>) were examined to determine the relationship between changes in biomass, NRRT and copper oxychloride. Table 1 presents a comparison of the effective exposure concentrations of copper oxychloride and copper in the substrates of each of these groups exposed to the fungicide formulation.

**Table 1** Comparative concentrations of Effekto Virikop<sup>®</sup>, copper oxychloride and copper.

Effekto Virikop $\mu\text{g.g}^{-1}$	Copper oxychloride $\mu\text{g.g}^{-1}$ (effective concentration)	Copper $\mu\text{g.g}^{-1}$ (effective concentration)
200	170	100
300	255	150
450	383	225
675	574	338
1000	850	500
1500	1275	750
2250	1913	1125

Copper concentrations in the control and exposure group substrates were determined prior to the start of the experiment (refer to 2.7). Every seven days, for a period of 28 days, the biomass and the mortality of the earthworms were determined and the pH and the moisture content of the substrates monitored for stability (pH  $\pm 7$  and moisture content of 25-30%). Moisture content was determined by analysing one gram of

the substrate with a Sartorius infrared moisture detector. pH was determined as described in the field tests (refer to 2.1.1). Samples of the substrate were also placed in polytop vials and frozen for copper analysis.

The biomass was determined by removing the worms from the substrate, washing them in distilled water and drying them on paper towels. They were then weighed in a waterfilled weighing boat using a Sartorius balance. This was done to prevent the worms desiccating and hence affect the weight of the earthworms.

Mortality rate was determined by counting the number of dead worms every seven days. Worms were considered as dead if they showed no movement and did not respond to a definite tactile stimulus to the anterior end. Because earthworms tend to disintegrate quickly after death, the earthworms absent from the containers examined were considered to have died.

Two worms were removed from each container every seven days to determine the NRRT of the coelomocytes (refer to 2.3). Afterwards these worms were placed on wet filter paper in Petri dishes for a period of 24-48 hours to allow the depuration of their gut contents. This was done to prevent misleading results concerning the actual copper content in the body tissues as a result of copper present in the gut contents. After this 24-48 hour period the worms were washed in distilled water, dried on paper towels and killed by placing them in a test tube which were placed in a beaker filled with hot water ( $\pm 70^{\circ}\text{C}$ ). They were individually weighed and frozen ( $\pm 0^{\circ}\text{C}$ ) in polytop vials for copper analysis at a later stage.

### **2.3. Lysosomal membrane stability assays with copper oxychloride**

NRRT (neutral red retention time) were determined according to a method described by Weeks and Svendsen, (1996).

#### *2.3.1. Stock- and Working Solutions*

A neutral red stock solution was prepared by dissolving 20mg of neutral red (Toluene red,  $\text{C}_{15}\text{H}_{17}\text{N}_4\text{Cl}$ ) in 1ml of dimethyl sulfoxide (DMSO,  $\text{C}_2\text{H}_6\text{OS}$ ). 10 $\mu\text{l}$  of the stock solution was added to 2.5ml of physiological earthworm Ringer and mixed to give a working solution with a concentration of 80  $\mu\text{l}.\text{ml}^{-1}$ .

### 2.3.2. Collection of coelomic fluid and coelomocytes

Coelomocytes to be examined for their NRRT were harvested by collecting coelomic fluid from clitellate earthworms used in the subchronic toxicity tests. This was done by extracting  $\pm 0.5$  ml of temperature adjusted physiological earthworm Ringer (25°C) into a syringe. An equal amount of coelomic fluid was extracted from earthworms. This was done by puncturing the body wall posterior to the clitellum with a syringe and extracting  $\pm 0.5$  ml of coelomic fluid from the coelomic cavity. This method differs from the one described by Weeks and Svendsen, (1996) in the sense that the authors first extracted coelomic fluid before adding the earthworm Ringer.

### 2.3.3. Staining of coelomocytes

20  $\mu$ l of the suspension (coelomocytes plus Ringer) was placed on a microscope slide. The cells were left for 30 seconds to adhere to the slide surface. 20  $\mu$ l of the neutral red working solution was added to this suspension and a cover slip placed over it. Thereafter the coelomocytes were counted.

### 2.3.4. Measurement of neutral red retention times

Each slide was examined continuously under a light microscope (x400 magnification) for two minutes with an interval of two minutes between the counting periods. During this period the number of observed live basophilic coelomocytes were recorded as well as those with fully stained cytosols (pinkish-red colour) by using a mechanical counter. The stained cytosols indicated the coelomocytes in which the dye had leaked from the lysosomes. The slides were kept in a humidity chamber to prevent desiccation during the two-minute intervals. Counting was continued until >50% of the counted coelomocytes had fully stained cytosols. This interval was recorded as the NRRT of the lysosomes in the specific cells.

## 2.4. Sublethal toxicity tests (Bioassay) with copper oxychloride

### 2.4.1. Species used, substrates and experimental design

*A. caliginosa* is a common and widespread endogeic species of grassland and agro-ecosystems can be used as an alternative test species with more relevance to field conditions. This species is more difficult to handle in the lab than *E. fetida* and cannot be

recommended for regular testing because of slow reproduction cycle and difficulties maintaining it in the laboratory for long periods (Kula & Larink, 1998).

*A. caliginosa* was collected from the field and exposed to grassland, vineyard and grassland soil spiked with 60  $\mu\text{g.g}^{-1}$  copper in the form of copper oxychloride (effective concentration, 102  $\mu\text{g.g}^{-1}$ ). The substrate consisted of 360g of soil and 40g peat moss, which was wetted with distilled water to a moisture content of 20%. The pH of the substrate was adjusted to  $\pm 7$  by adding calcium carbonate ( $\text{CaCO}_3$ ). It was prepared in big batches to yield enough for the three replicate groups. The substrate was placed in plastic containers, covered with a piece of plastic before replacing the perforated lid, and kept in an environmental chamber ( $\pm 15^\circ\text{C}$ ) for 24 hours to stabilise. Thereafter the earthworms were introduced into the test substrate.

Three plastic containers, with a volume of one litre each were used for each experimental group. Ten earthworms were placed in each of the three replicate containers, filled with 400g of prepared substrate. At no stage during the experiment were the earthworms fed.

Copper concentrations in the substrates were determined prior to the start of the experiment (refer to 2.7). The growth and mortality of the earthworms was recorded every seven days over a period of 28 days (refer to 2.2.3). The moisture content and pH were monitored for stability (pH  $\pm 7$  and moisture content  $\pm 20\%$ ), (refer to 2.2.3). After termination of the experiment the copper content of the earthworm body tissues and substrates was analysed spectrophotometrically (refer to 2.6).

The classification of Simms & Gerard (1985) is followed for the species:

Phylum	: Annelida
Subphylum	: Clitellata
Class	: Oligochaeta
Order	: Haplotaxida
Suborder	: Lumbricina
Superfamily	: Lumbricoidea
Family	: Lumbricidae (Rafinesque-Schmaltz, 1815)
Subfamily	: Lumbricinae (Rafinesque-Schmaltz, 1815)
Genus	: <i>Aporrectodea</i>
Species	: <i>Aporrectodea caliginosa</i> (Savigny, 1826)

## 2.5. Burrow activity and avoidance response

### 2.5.1. Species used, substrates and experimental design

The species used in this study was *A. caliginosa* (refer to 2.3.1). Grassland soil was collected, dried, ground and sieved to a particle size of <5mm. The profiles utilised were assembled from Perspex sheets, 1 mm thick. In the burrowing activity experiments two sheets (each 8 cm wide and 40 cm high) were placed next to each other, on a Perspex base, with a space of 1 cm between the two sheets. A second batch of profiles for burrowing activity experiments were assembled, which had the same dimensions as the latter, but with a space of 0.8 cm between the Perspex sheets. For the avoidance-behaviour experiments a similar design was followed and profiles had a width of 16 cm, a height of 40 cm with a space of 1 cm between the sheets.

The profiles used for burrow activity was filled with respectively 240g (8 x 0.8 x 40cm) and 300g (8 x 1 x 40cm) of soil. The profiles used to determine avoidance response were divided into two halves (each was 8 x 1 x 40cm) with a plastic partitioning. They were then filled with 300g of the respective treatment (grass-, vineyard- or copper contaminated) soils on each side of the plastic partitioning. The soil was carefully homogenised to completely fill the profiles, closed and carefully placed upright. After filling the profiles, they were gently tapped to compact the soil. The profiles were placed in a waterfilled container to completely soak the soil and left for an additional hour after soaking was completed. They were removed from the water and placed on a dry surface for one hour to stabilise the soil. The experiment was performed in an environmental chamber at a temperature of  $\pm 15^{\circ}\text{C}$  as recommended by Kula and Larink (1998) when using *A. caliginosa* as test species.

### 2.5.2. Burrow activity and avoidance-behaviour

In both the burrow activity and avoidance-behaviour experiments, one earthworm was placed in the middle of each of the profiles, on top of the substrate, and its activity was tracked every hour for the three first hours and daily for the next three days. The tunnelling tracks of the earthworms were copied on transparencies and measured by using an opisometer.

In the burrow activity experiments nine replicates were done for each of the profile sizes and treatment groups, viz. Grassland, vineyard and grassland soil contaminated with copper oxychloride to a copper content of  $60\mu\text{g}\cdot\text{g}^{-1}$ . In the avoidance behaviour experiment

12 replicates were done for each of the treatment groups, grassland vs. vineyard soil and grassland vs. copper contaminated ( $60\mu\text{g}\cdot\text{g}^{-1}$ ) soil.

## **2.6. Acid digestion of the earthworms**

Worms were digested as described by Katz & Jennis (1983). The polytop vials containing the frozen worms were taken out of the freezer and left at room temperature to thaw. After thawing the worms were placed individually in test tubes and 10 ml of 55% nitric acid ( $\text{HNO}_3$ ) was added. It was left overnight at room temperature to start the digestion process. The following day the samples were heated to  $40\text{-}60^\circ\text{C}$  for two hours and then  $120\text{-}130^\circ\text{C}$  for an hour, after which it was left to cool. 1 ml of 70% perchloric acid ( $\text{HClO}_4$ ) was added and this mixture was reheated to  $120\text{-}130^\circ\text{C}$  for an hour. The samples were allowed to cool before 5 ml of distilled water was added. Samples were then reheated to  $120\text{-}130^\circ\text{C}$  until white fumes were emitted. The samples were allowed to cool finally before they were microfiltered. For every batch of worms that was digested a blank was made to eliminate possible contamination during the digestion process.

The solutions were filtered through Whatman no. 6 filter paper into  $20\text{ cm}^3$  volumetric flasks using Sartorius microfilter-holders and plastic syringes. Distilled water was used to make up a  $20\text{ cm}^3$  filtered solution. These  $20\text{ cm}^3$  solutions were microfiltered through  $0.45\text{ }\mu\text{m}$  Sartorius Cellulose Nitrate filter paper into polyvinyl containers. Polyvinyl containers were used because metals do not adsorb to their surface. Sample solutions were stored in a refrigerator until they were spectrophotometrically analysed (refer to 2.8).

## **2.7. Acid digestion of soil samples and substrate**

The polytop vials containing the frozen samples were taken out of the freezer and left to thaw at room temperature. After thawing, one gram of soil was dried for  $\pm 48$  hours at  $106^\circ\text{C}$  in a Memmert drying oven. These dried samples were ground and sieved to a particle size of  $500\text{-}1000\text{ }\mu\text{m}$  to assist in the digestion process. These samples were digested and filtered in the same way as with the worm samples, except that 5 ml of perchloric acid was used instead of 1 ml. Before being filtered the samples were centrifuged using a DAMON/IEC centrifuge for five minutes at 3000 rpm (revolutions per minute).

The solutions were then decanted from the sludge and the supernatant was microfiltered and stored in plastic containers in the refrigerator until spectrophotometric analyses.

## **2.8. Copper analysis**

The copper content of the samples was determined by using a Varian AA-1275 Atomic Absorption Spectrophotometer. Freshly made 2, 5, 10 and 50  $\mu\text{g}\cdot\text{g}^{-1}$  copper standards were used to calibrate the spectrophotometer. The mean recovery percentage of this method is  $\pm 75\%$ . The concentrations are expressed as wet weight for the earthworms and dry weight for the soil samples analysed.

## **2.9. Statistical analysis of data**

The data in this study was analysed by using the SigmaStat<sup>®</sup> computer software package and all values presented as the mean  $\pm$  SD (standard deviation). The probability levels used for statistical significance were  $p < 0.05$  for the field and subchronic toxicity tests as well as NRRT. Parametric or non-parametric tests were used to compare the control and exposure groups. The  $\text{LC}_{50}$  values for copper oxychloride and copper in the acute toxicity tests were determined by using the Trimmed Spearman-Kärber (TSK) Program Version 1.5.



## CHAPTER 3

### RESULTS...

#### 3.1. Field tests with copper oxychloride conducted in the Nieuwoudtville area

##### 3.1.1. Soil physical parameters

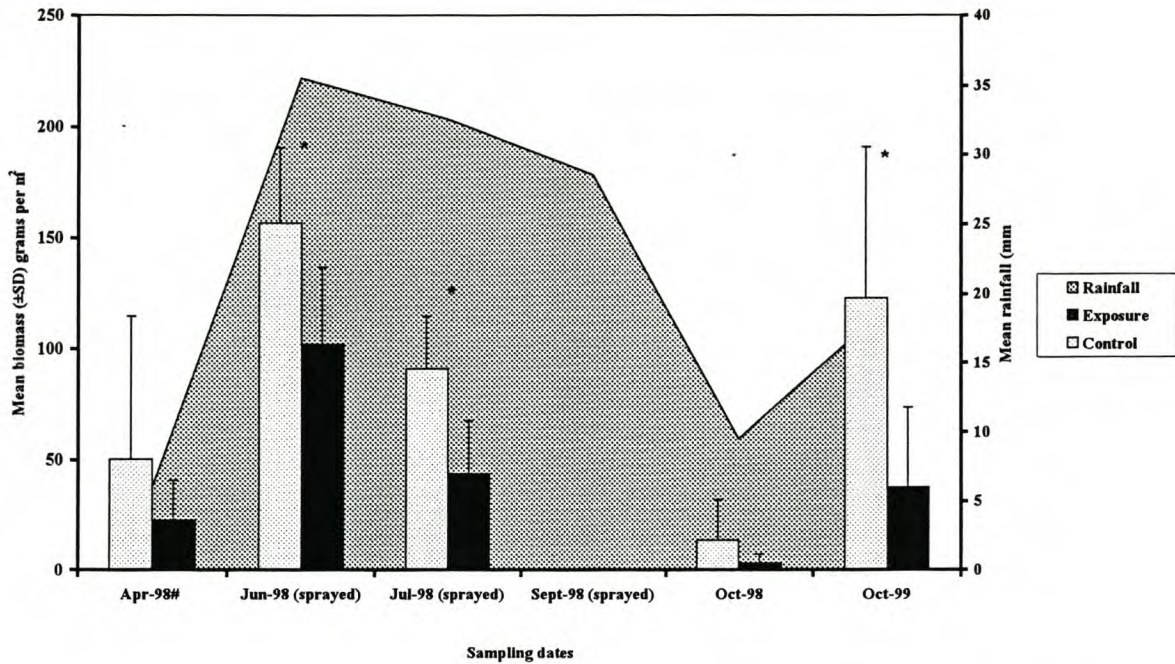
Soils consisted of 6.4% organic matter, 6.7% clay, 8.8% silt, 36.3% fine sand, 31.9% medium sand, 16.3% coarse sand and 21.2% stone. The pH was  $5.38 \pm 0.10$  in the control plots and  $5.43 \pm 0.06$  in the exposure plots (Table 2) at the beginning of the experiment prior to spraying. From June 1998 until October 1998 the pH of soils from the exposure plots was significantly higher ( $p < 0.05$ ) than in the control plots due to spraying with copper oxychloride (Table 1). The rainfall data for the duration of the study are depicted graphically in Figures 4 and 5.

**Table 2** Mean pH  $\pm$ SD of soils in the control and copper oxychloride treated plots for the duration of the study in the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n=5$ ).

Sampling date	n	Mean pH	
		Control plots	Exposure plots
Apr. 1998 <sup>#</sup>	5	$5.38 \pm 0.10$	$5.43 \pm 0.06$
June 1998 (sprayed)	5	$5.32 \pm 0.08$	$4.94 \pm 0.11^*$
July 1998 (sprayed)	5	$5.48 \pm 0.08$	$4.82 \pm 0.13^*$
Sept. 1998 (sprayed)	5	$5.44 \pm 0.11$	$4.68 \pm 0.15^*$
Oct. 1998	5	$5.46 \pm 0.05$	$4.46 \pm 0.11^*$
Oct. 1999	5	$5.44 \pm 0.11$	$5.46 \pm 0.09$

### 3.1.2. Changes in mean biomass of collected earthworms and rainfall

At the start of the study in April 1998 the mean biomass per m<sup>2</sup> of collected earthworms in the control plots was 50.15±64.29 g and 22.38±18.35 g in the exposure plots prior to spraying (Figure 4, Table 3). There was no statistical significant difference ( $p > 0.05$ ) between the mean earthworm biomasses.



**Figure 4** Mean biomass (grams) ±SD of earthworms (*Microchaetus* sp.) per m<sup>2</sup> in the control and copper oxychloride treated plots and mean rainfall (mm) for the duration of the study in the Nieuwoudtville area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n=5$ ).

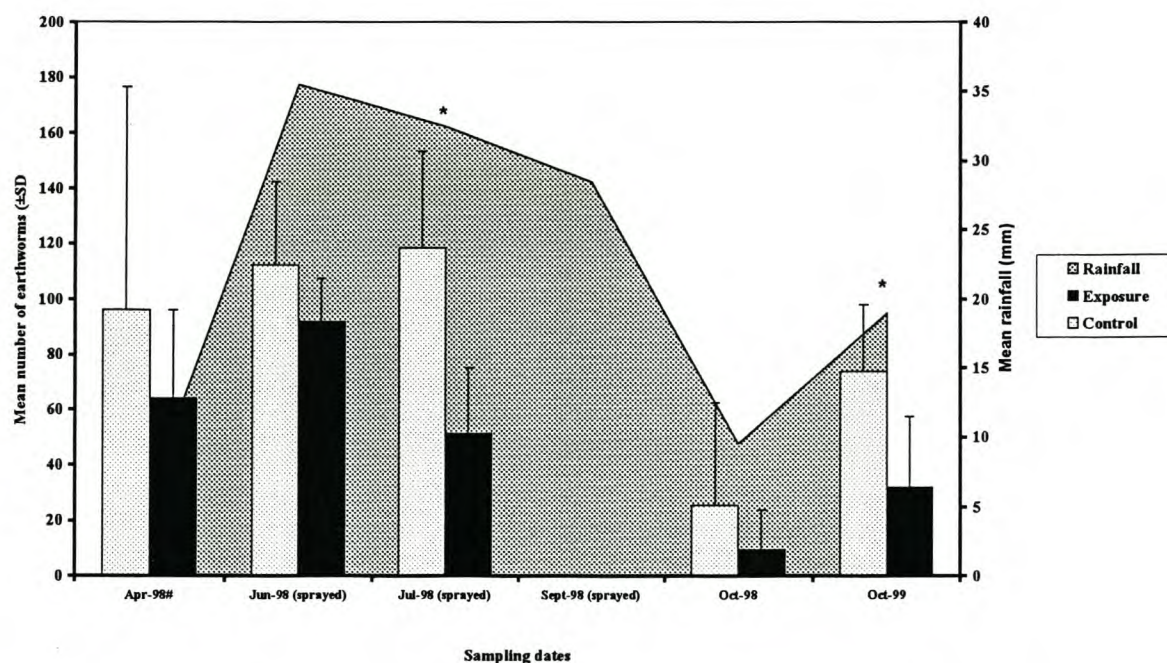
There was a significant difference ( $p < 0.05$ ) in the mean biomasses of earthworms from the control and copper oxychloride sprayed plots, except during October 1998 (Figure 4, Table 3). During October 1999 (after the termination of the experiment) the mean biomass of earthworms in the control plots was 122.86±67.74 g and 37.47±35.93 g in the sprayed plots (Figure 4, Table 3). The mean biomass of earthworms per m<sup>2</sup> in the control plots was significantly higher ( $p < 0.05$ ) than in the sprayed plots (Figure 4, Table 3).

**Table 3** Mean biomass (grams)  $\pm$ SD of earthworms (*Microchaetus* sp.) per m<sup>2</sup> in the control and copper oxychloride treated plots for the duration of the study in the Nieuwoudtville area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

Sampling date	n	Mean biomass	
		Control plots	Exposure plots
Apr. 1998 <sup>#</sup>	5	50.15 $\pm$ 64.29	22.38 $\pm$ 18.35
June 1998 (sprayed)	5	156.45 $\pm$ 33.93	101.90 $\pm$ 34.76*
July 1998 (sprayed)	5	90.82 $\pm$ 23.61	43.59 $\pm$ 23.70*
Sept. 1998 (sprayed)	5	-	-
Oct. 1998	5	13.37 $\pm$ 18.42	2.95 $\pm$ 4.05
Oct. 1999	5	122.86 $\pm$ 67.74	37.47 $\pm$ 35.94*

### 3.1.3. Changes in mean number of earthworms collected and mean rainfall

During April 1998, at the start of the experiment the mean number of earthworms per m<sup>2</sup> was  $96.00 \pm 80.53$  and  $64.00 \pm 32.00$  in the control and exposure plots respectively, prior to spraying (Figure 5, Table 4).



**Figure 5** Mean number  $\pm$ SD of earthworms (*Microchaetus* sp.) per m<sup>2</sup> in the control and copper oxychloride treated plots and mean rainfall (mm) for the duration of the study in the Nieuwoudtville area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

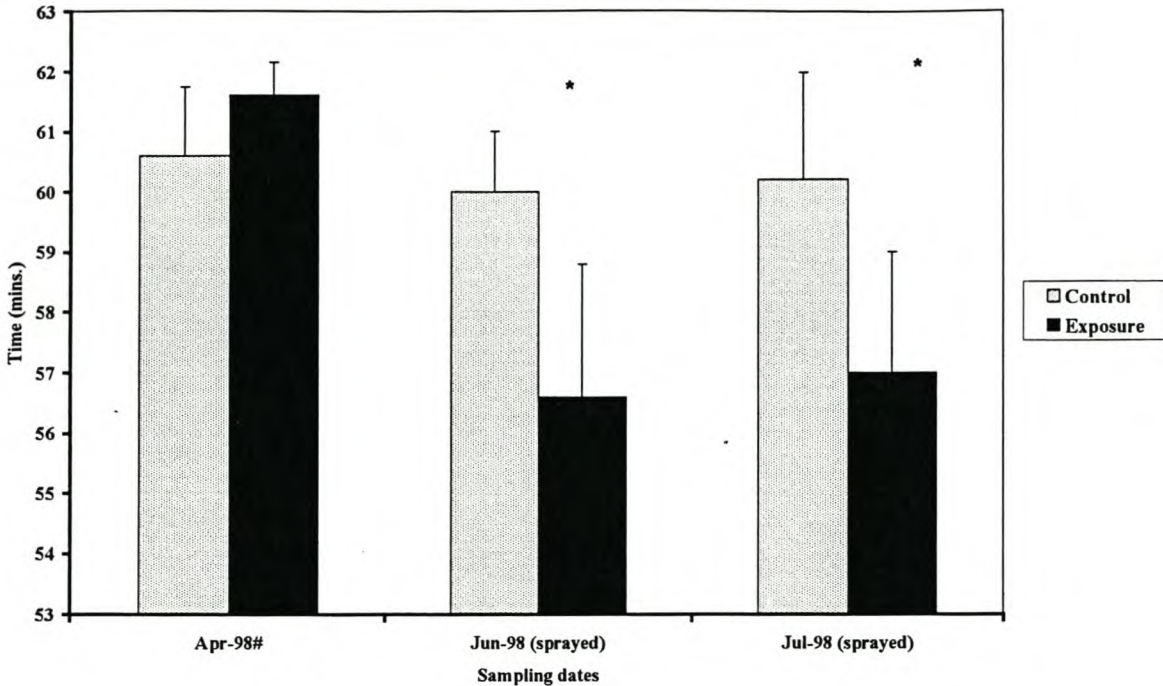
There was no significant difference ( $p > 0.05$ ) between the mean number of earthworms per  $m^2$  in the two plots. At the end of the experiment (October 1999) the mean number of earthworms per  $m^2$  was  $73.60 \pm 24.26$  and  $32.00 \pm 25.29$  in the control and exposure plots respectively (Figure 5, Table 4). The mean number of earthworms in the control plots was significantly higher ( $p < 0.05$ ) than those in the copper oxychloride sprayed plots.

**Table 4** Mean number  $\pm$ SD of earthworms (*Microchaetus* sp.) per  $m^2$  in the control and copper oxychloride treated plots for the duration of the study in the Nieuwoudtville area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

Sampling date	n	Mean biomass	
		Control plots	Exposure plots
Apr. 1998 <sup>#</sup>	5	$96.00 \pm 80.53$	$64.00 \pm 32.00$
June 1998 (sprayed)	5	$112.00 \pm 30.36$	$92.00 \pm 15.32$
July 1998 (sprayed)	5	$118.40 \pm 35.05$	$51.20 \pm 23.73^*$
Sept. 1998 (sprayed)	5	-	-
Oct. 1998	5	$25.60 \pm 36.83$	$9.60 \pm 14.31$
Oct. 1999	5	$73.60 \pm 24.26$	$32.00 \pm 25.29^*$

#### 3.1.4. Changes in lysosomal membrane stability of coelomocytes: Neutral red retention times

At the start of the experiment prior to spraying with copper oxychloride (April 1998) the NRRT of the coelomocytes in earthworms obtained from the control plots and exposure plots were  $60.60 \pm 1.14$  min. and  $61.60 \pm 0.55$  min. respectively (Figure 6, Table 5). There was no significant difference ( $p > 0.05$ ) in these NRRT values. The NRRT of coelomocytes from earthworms in the control plots during June 1998 and July 1998 were respectively  $60.00 \pm 1.00$  min. and  $60.20 \pm 1.79$  min. The NRRT of coelomocytes from earthworms in the exposure plots during June 1998 and July 1998 were respectively  $56.60 \pm 2.19$  min. and  $57.00 \pm 2.00$  min. (Figure 6, Table 5). For both these months the NRRT of earthworms from the control plots were significantly higher ( $p < 0.05$ ) than in the exposure plots.



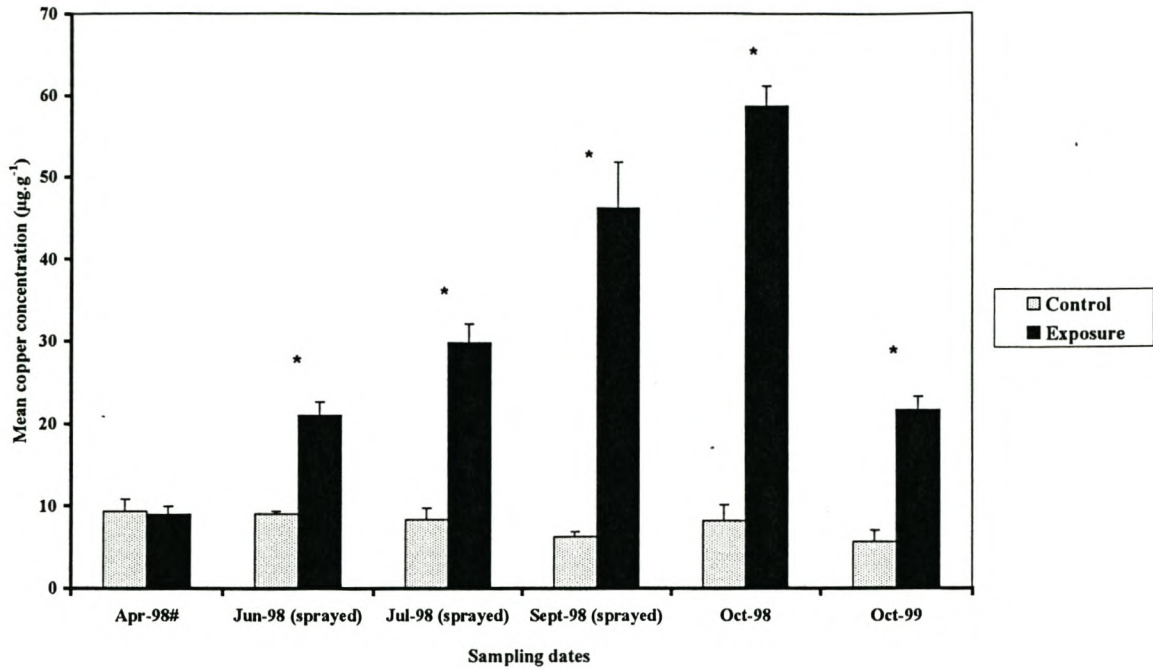
**Figure 6** Mean NRRT (min)  $\pm$ SD of earthworms (*Microchaetus* sp.) collected from the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n=6$ ).

**Table 5** Mean NRRT (min)  $\pm$ SD of earthworms (*Microchaetus* sp.) collected from the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ).

Sampling date	n	Mean NRRT (mins.)	
		Control plots	Exposure plots
Apr. 1998 <sup>#</sup>	6	60.60 $\pm$ 1.14	61.60 $\pm$ 0.55
June 1998 (sprayed)	6	60.00 $\pm$ 1.00	56.60 $\pm$ 2.19*
July 1998 (sprayed)	6	60.20 $\pm$ 1.79	57.00 $\pm$ 2.00*

### 3.1.5. Changes in copper content of soils

The copper content of soils prior to spraying (April 1998) was  $9.31 \pm 1.43 \mu\text{g.g}^{-1}$  in the control plots and  $8.92 \pm 1.04 \mu\text{g.g}^{-1}$  in the exposure plots (Figure 7, Table 6). There was no significant difference ( $p > 0.05$ ) between these values. The copper concentration in sprayed plots increased over the duration of the study period (Figure 7, Table 6). A year after the end of the experiment, in October 1999, the copper content in soils from the control plots was  $5.62 \pm 1.33 \mu\text{g.g}^{-1}$  and  $21.64 \pm 1.60 \mu\text{g.g}^{-1}$  in the exposure plots (Figure 7, Table 6).



**Figure 7** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in soils from the control and copper oxychloride exposed plots in the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n=10$ ).

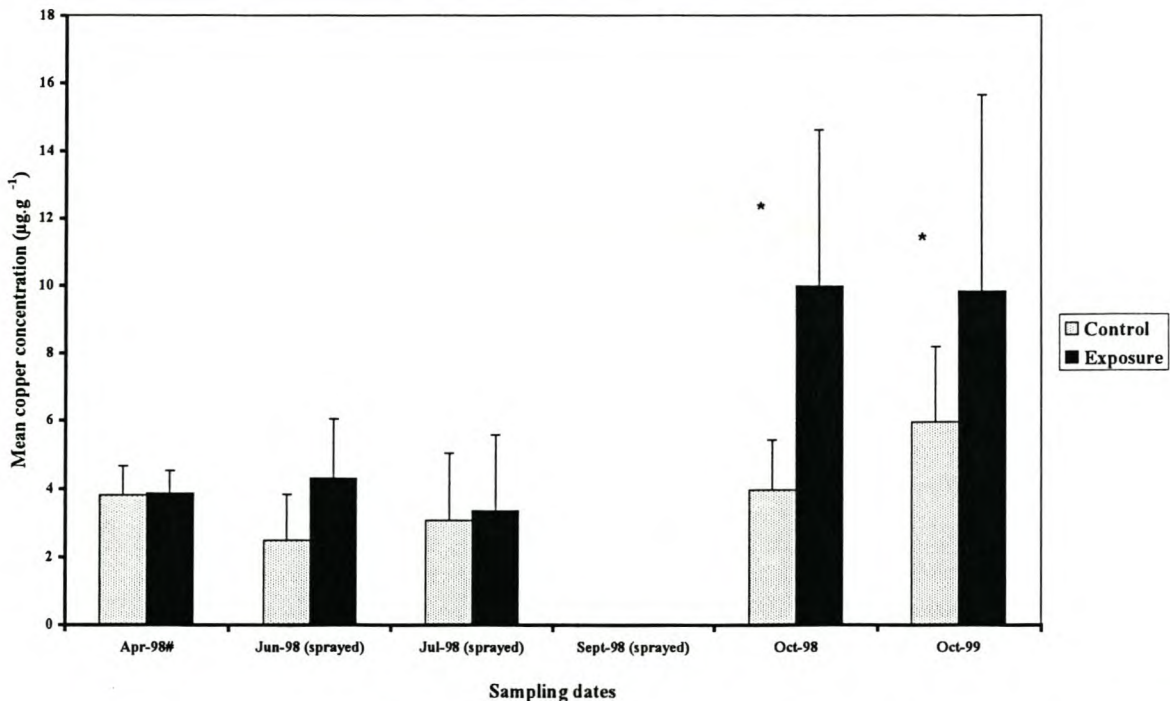
The copper content in soils from the control plots was significantly lower ( $p < 0.05$ ) than in the exposure plots. The copper concentration in the exposure plots in October 1999 was also significantly lower ( $p < 0.05$ ) than in October 1998.

**Table 6** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in soils from the control and copper oxychloride exposed plots in the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 10$ ).

Sampling date	n	Mean copper concentration ( $\mu\text{g.g}^{-1}$ )	
		Control plots	Exposure plots
Apr. 1998 <sup>#</sup>	10	9.31 $\pm$ 1.43	8.92 $\pm$ 1.04
June 1998 (sprayed)	10	9.00 $\pm$ 0.34	21.01 $\pm$ 1.59*
July 1998 (sprayed)	10	8.30 $\pm$ 1.41	29.76 $\pm$ 2.28*
Sept. 1998 (sprayed)	10	6.22 $\pm$ 0.64	46.19 $\pm$ 5.57*
Oct. 1998	10	8.11 $\pm$ 1.99	58.67 $\pm$ 2.49*
Oct. 1999	10	5.62 $\pm$ 1.33	21.64 $\pm$ 1.60*

### 3.1.6. Change in copper content of earthworm body tissues

At the beginning of the experiment prior to spraying (April 1998) the mean copper concentration in the body tissues of earthworms was  $3.79 \pm 0.85 \mu\text{g.g}^{-1}$  and  $3.85 \pm 0.67 \mu\text{g.g}^{-1}$  in earthworms from the control and exposure plots respectively (Figure 8, Table 7).



**Figure 8** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*Microchaetus* sp.) from the control and copper oxychloride exposed plots in the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 10$ ).

There was no significant difference ( $p > 0.05$ ) between these mean copper concentrations. The mean copper concentrations in earthworm body tissues at the end of the study (October 1999) were  $5.96 \pm 2.24 \mu\text{g.g}^{-1}$  and  $9.83 \pm 5.80 \mu\text{g.g}^{-1}$  in earthworms from the control and exposure plots respectively (Figure 8, Table 7). A year after spraying had stopped in October 1998 the mean copper concentration in earthworm body tissues from the control plots was significantly lower ( $p < 0.05$ ) than in the exposed earthworms.

**Table 7** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*Microchaetus* sp.) from the control and copper oxychloride exposed plots in the Nieuwoudtville area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 10$ )

Sampling date	n	Mean copper concentration ( $\mu\text{g.g}^{-1}$ )	
		Control plots	Exposure plots
Apr. 1998 <sup>#</sup>	10	$3.79 \pm 0.85$	$3.85 \pm 0.67$
June 1998 (sprayed)	10	$2.48 \pm 1.35$	$4.29 \pm 1.76$
July 1998 (sprayed)	10	$3.08 \pm 1.94$	$3.35 \pm 2.22$
Sept. 1998 (sprayed)	10	-	-
Oct. 1998	10	$3.96 \pm 1.45$	$9.98 \pm 4.64^*$
Oct. 1999	10	$5.96 \pm 2.24$	$9.83 \pm 5.80^*$



### 3.2 Field-tests with copper oxychloride conducted in the Vergenoegd area

#### 3.2.1. Soil physical parameters

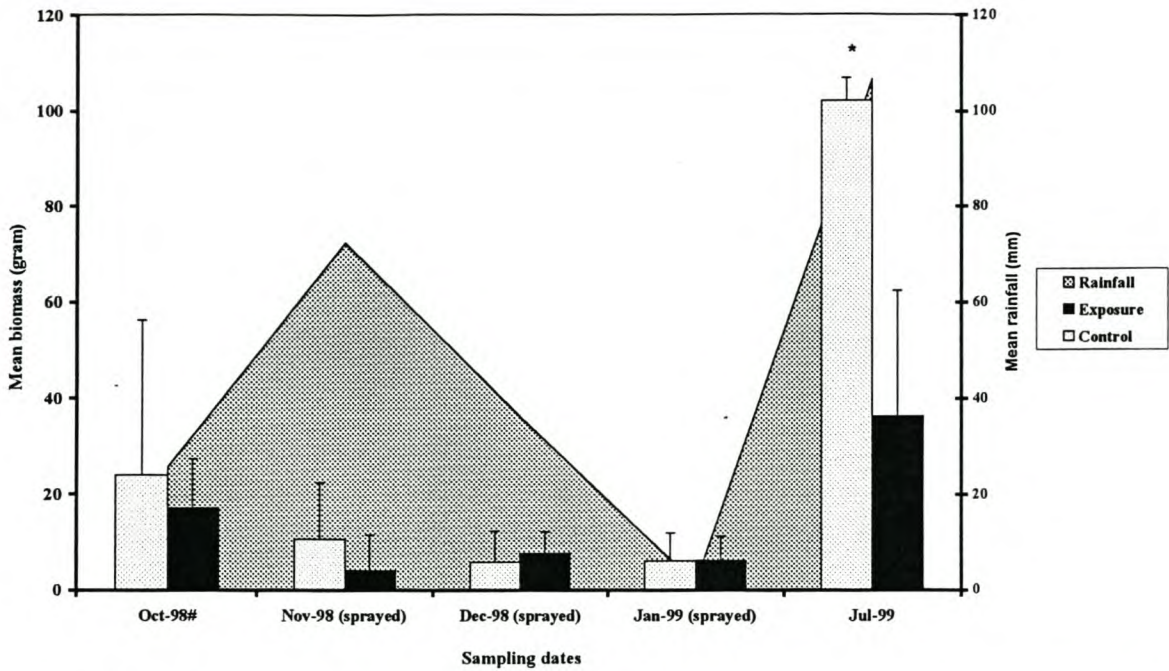
Soils consisted of 6.2% organic matter, 5.4% clay, 15.3% silt, 34.8% fine sand, 25.8% medium sand, 18.7% coarse sand and 23.6% stone. The pH was  $6.44 \pm 0.11$  in the control plots and  $6.40 \pm 0.12$  in the exposure plots at the beginning of the experiment before spraying with copper oxychloride was started (Table 8). From November 1998 until January 1999 the pH in the exposure plots was significantly higher ( $p < 0.05$ ) than in the control plots as a result of spraying with copper oxychloride (Table 8). The rainfall data for the duration of the study are graphically shown in Figures 9 and 10.

**Table 8** Mean pH  $\pm$ SD of soils in the control and copper oxychloride sprayed plots for the duration of the study in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n=5$ ).

Sampling date	n	Mean pH	
		Control plots	Exposure plots
Oct. 1998 <sup>#</sup>	5	$6.44 \pm 0.11$	$6.40 \pm 0.12$
Nov. 1998 (sprayed)	5	$6.40 \pm 0.14$	$5.96 \pm 0.10^*$
Dec. 1998 (sprayed)	5	$6.42 \pm 0.04$	$5.84 \pm 0.05^*$
Jan. 1999 (sprayed)	5	$6.42 \pm 0.13$	$5.82 \pm 0.08^*$
July 1999	5	$6.38 \pm 0.16$	$6.36 \pm 0.17$

#### 3.2.2. Changes in mean biomass of collected earthworms and mean rainfall

Prior to spraying (October 1998) the mean biomass of earthworms per  $m^2$  in the control plots was  $23.92 \pm 32.36$  g and  $17.16 \pm 10.15$  g in the exposure plots (Figure 9, Table 9). There was no statistical difference ( $p > 0.05$ ) between the mean earthworm biomass per  $m^2$ .



**Figure 9** Mean biomass (grams)  $\pm$ SD of earthworms (*A. caliginosa*) per  $m^2$  in the control and copper oxychloride sprayed plots and mean rainfall (mm) for the duration of the study in the Vergenoegd area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

For the duration of the study, until January 1999, there was no statistical difference ( $p > 0.05$ ) between the mean biomass of earthworms per  $m^2$  from the control and exposure plots (Figure 9, Table 9).

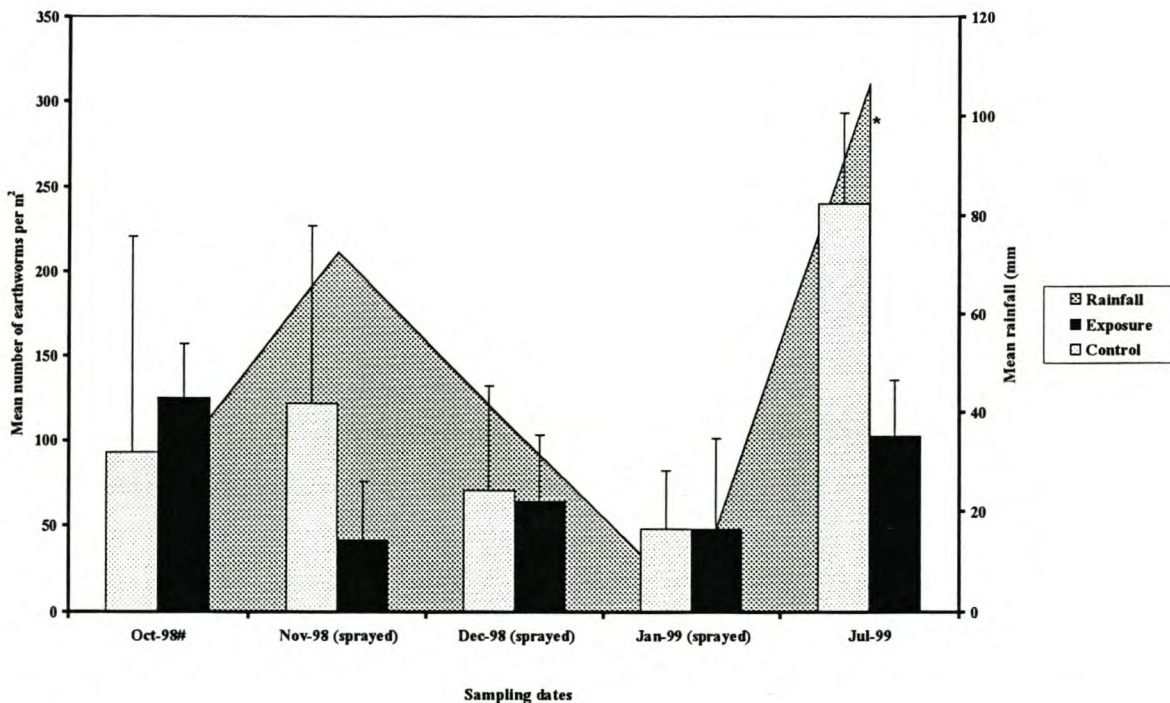
**Table 9** Mean biomass (grams)  $\pm$ SD of earthworms (*A. caliginosa*) per  $m^2$  in control and copper oxychloride sprayed plots for the duration of the study in the Vergenoegd area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

Sampling date	n	Mean biomass	
		Control plots	Exposure plots
Oct. 1998#	5	23.92 $\pm$ 32.36	17.16 $\pm$ 10.15
Nov. 1998 (sprayed)	5	10.62 $\pm$ 11.65	4.04 $\pm$ 7.50
Dec. 1998 (sprayed)	5	5.75 $\pm$ 6.54	7.66 $\pm$ 4.51
Jan. 1999 (sprayed)	5	6.04 $\pm$ 5.81	6.29 $\pm$ 5.04
July 1999	5	102.16 $\pm$ 4.48	36.23 $\pm$ 26.18*

Six months after termination of the experiment, in July 1999, the mean biomass of earthworms per m<sup>2</sup> in the control plots was 102.16±4.48 g and 36.23±26.18 g in the exposure plots. The mean biomass of earthworms per m<sup>2</sup> in the control plots was significantly higher ( $p < 0.05$ ) than in the exposure plots (Figure 9, Table 9).

### 3.2.3. Changes in mean number of collected earthworms and mean rainfall

At the start of the experiment, prior to spraying (October 1998), the mean numbers of earthworms per m<sup>2</sup> were 92.80±27.69 and 124.80±32.00 in the control and exposure plots respectively (Figure 10, Table 10). There was no significant difference ( $p > 0.05$ ) between the mean number of collected earthworms. The same was found during November 1998 until January 1999 (Figure 10, Table 10).



**Figure 10** Mean number ±SD of earthworms (*A. caliginosa*) per m<sup>2</sup> in the control and copper oxychloride sprayed plots and mean rainfall (mm) for the duration of the study in the Vergenoegd area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 5$ ).

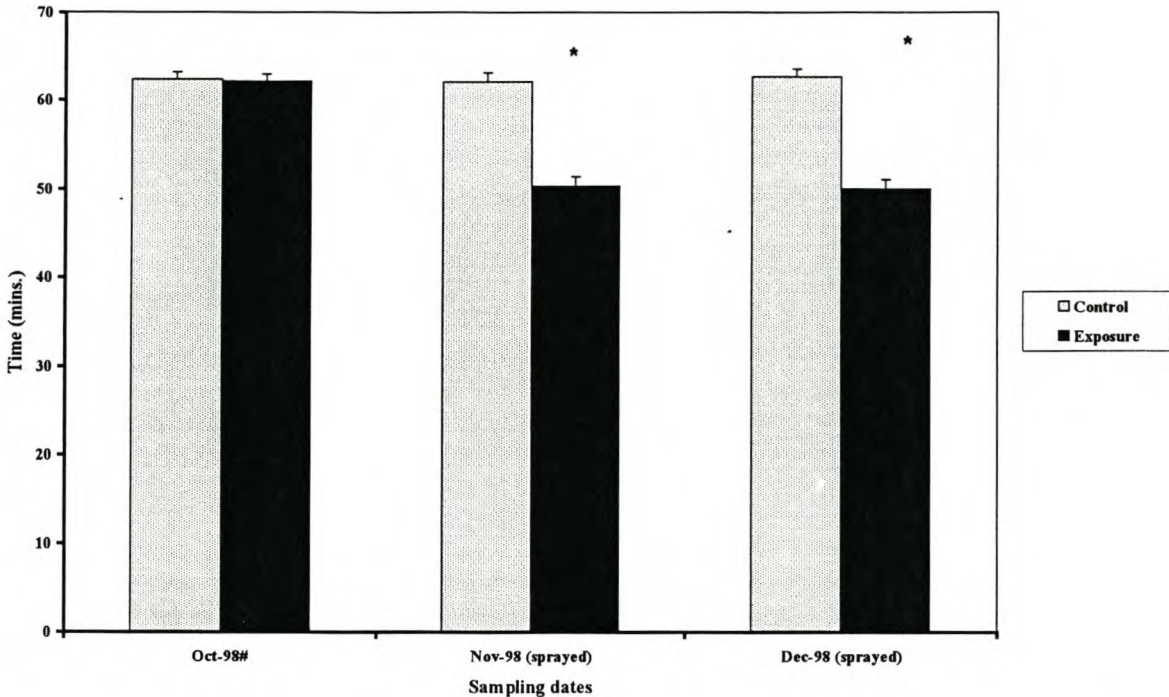
At the end of the experiment (July 1999) the mean numbers of earthworms per m<sup>2</sup> in the control plots were 240±53.06 and 102.40±33.18 in the exposure plots (Figure 10, Table 10). The mean number of earthworms per m<sup>2</sup> in the control plots was significantly higher (p<0.05) than in the exposure plots.

**Table 10** Mean number ±SD of earthworms (*A. caliginosa*) per m<sup>2</sup> in the control and copper oxychloride sprayed plots for the duration of the study in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control, p<0.05; n=5).

Sampling date	n	Mean biomass	
		Control plots	Exposure plots
Oct. 1998 <sup>#</sup>	5	92.80 ± 127.69	124.80 ± 32.00
Nov. 1998 (sprayed)	5	121.60 ± 105.28	41.60 ± 33.94
Dec. 1998 (sprayed)	5	70.40 ± 61.55	64.00 ± 39.19
Jan. 1999 (sprayed)	5	48.00 ± 33.94	48.00 ± 53.06
July 1999	5	240.00 ± 53.06	102.40 ± 33.18*

### 3.2.4. Changes in lysosomal membrane stability of coelomocytes: Neutral red retention times

During October 1998, prior to spraying with copper oxychloride, the NRRT of coelomocytes in earthworms from the control plots were  $62.33 \pm 0.81$  min. and  $62.17 \pm 0.75$  min. in earthworms from the exposure plots (Figure 11, Table 11).



**Figure 11** Mean NRRT (mins.)  $\pm$ SD of earthworms (*A. caliginosa*) collected from the Vergenoegd area (# sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n = 6$ ).

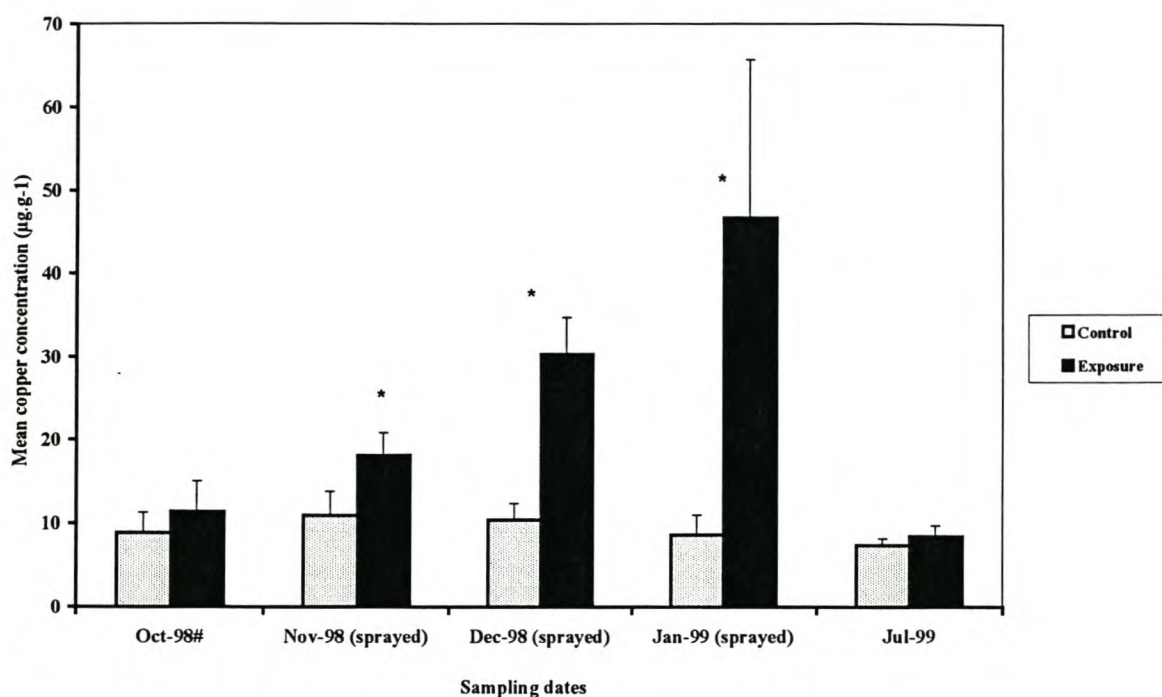
There was no significant difference ( $p > 0.05$ ) between these values. The NRRT of earthworms collected from the control plots during November 1998 and December 1998 were  $62.00 \pm 1.10$  min. and  $62.66 \pm 0.82$  min. respectively (Figure 11, Table 11). The NRRT of earthworms from the exposure plots for November 1998 and December 1998 were respectively  $50.33 \pm 1.03$  min. and  $50.00 \pm 1.10$  min. (Figure 11, Table 11). The NRRT of earthworms from the control plots were significantly higher ( $p < 0.05$ ) than in the exposure plots for both these months.

**Table 11** Mean NRRT (mins.)  $\pm$ SD of earthworms (*A. caliginosa*) collected from the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n=6$ ).

Sampling date	n	Mean NRRT (mins.)	
		Control plots	Exposure plots
Oct. 1998 <sup>#</sup>	6	62.33 $\pm$ 0.81	62.17 $\pm$ 0.75
Nov. 1998 (sprayed)	6	62.00 $\pm$ 1.10	50.33 $\pm$ 1.03*
Dec. 1998 (sprayed)	6	62.66 $\pm$ 0.82	50.00 $\pm$ 1.10*

### 3.2.5. Changes in copper content of soils

The mean copper concentration in soils prior to spraying (October 1998) was  $8.87 \pm 2.36 \mu\text{g.g}^{-1}$  and  $11.38 \pm 3.58 \mu\text{g.g}^{-1}$  in the control and exposure plots respectively (Figure 12, Table 12). There was no statistical difference ( $p > 0.05$ ) between these copper concentrations. During November 1998, December 1998 and January 1999 the copper concentration in soils from the control plots was significantly lower ( $p < 0.05$ ) than in the exposure plots (Figure 12, Table 12). At the end of the study in July 1999 the copper concentration in soils from the control plots was  $7.41 \pm 0.75 \mu\text{g.g}^{-1}$  and  $8.48 \pm 1.26 \mu\text{g.g}^{-1}$  in the exposure plots (Figure 12, Table 12).



**Figure 12** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in the soils in the soils from the control and copper oxychloride sprayed plots in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n=10$ ).

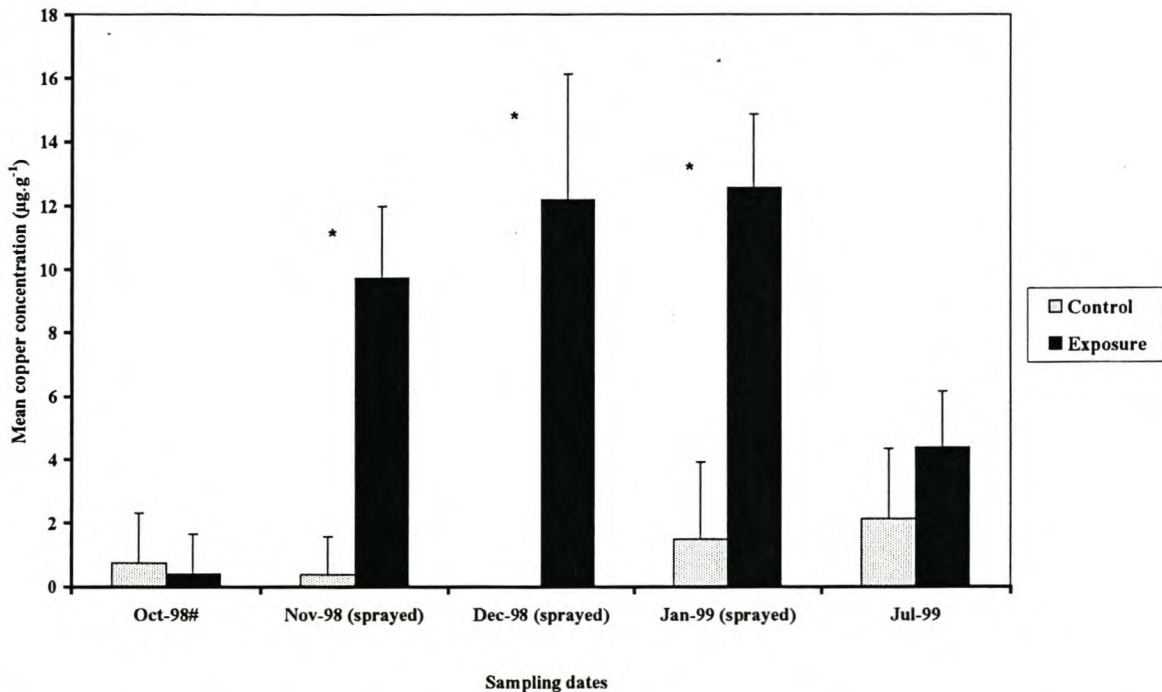
There was no significant difference ( $p > 0.05$ ) between these copper concentrations. The mean copper concentration in soils from the exposure plots in July 1999 was significantly lower ( $p < 0.05$ ) than in January 1999.

**Table 12** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in the soils from the control and copper oxychloride sprayed plots in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n=10$ ).

Sampling date	n	Mean copper concentration ( $\mu\text{g.g}^{-1}$ )	
		Control plots	Exposure plots
Oct. 1998 <sup>#</sup>	10	8.87 $\pm$ 2.36	11.38 $\pm$ 3.58
Nov. 1998 (sprayed)	10	10.96 $\pm$ 2.78	18.16 $\pm$ 2.68*
Dec. 1998 (sprayed)	10	10.41 $\pm$ 1.92	30.30 $\pm$ 4.35*
Jan. 1999 (sprayed)	10	8.65 $\pm$ 2.26	46.74 $\pm$ 18.89*
July 1999	10	7.41 $\pm$ 0.75	8.48 $\pm$ 1.26

### 3.2.6. Copper concentrations in earthworm body tissues

During October 1998 the mean copper concentration in the body tissues of earthworms was  $0.74 \pm 1.57 \mu\text{g.g}^{-1}$  and  $0.39 \pm 1.24 \mu\text{g.g}^{-1}$  in earthworms from the control and exposure plots respectively (Figure 13, Table 13). There was no significant difference ( $p > 0.05$ ) between these copper concentrations. During November 1998, December 1998 and January 1999 the copper concentration in earthworm body tissues from the control plots was significantly lower ( $p < 0.05$ ) than that in the exposure plots (Figure 13, Table 13).



**Figure 13** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*A. caliginosa*) from the control and copper oxychloride sprayed plots in the Vergenoegd area (#sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n=10$ ).

During July 1999, after the end of the exposure experiment, the mean copper concentrations in earthworm body tissues were  $2.12 \pm 2.22 \mu\text{g.g}^{-1}$  and  $4.36 \pm 1.80 \mu\text{g.g}^{-1}$  in earthworm body tissues from control and the exposure plots respectively (Figure 13, Table 13). There was no significant difference ( $p > 0.05$ ) between the copper concentrations. The mean copper concentration in earthworm body tissues from the exposure plots in July 1999 was significantly lower ( $p < 0.05$ ) than in January 1999.



**Table 13** Change over time in the mean copper concentration ( $\mu\text{g}\cdot\text{g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*A. caliginosa*) from the control and copper oxychloride sprayed plots in the Vergenoegd area (<sup>#</sup>sprayed after sampling, \*significantly different from control,  $p < 0.05$ ;  $n=10$ ).

Sampling date	n	Mean copper concentration ( $\mu\text{g}\cdot\text{g}^{-1}$ )	
		Control plots	Exposure plots
Oct. 1998 <sup>#</sup>	10	0.74 $\pm$ 1.57	0.39 $\pm$ 1.24
Nov. 1998 (sprayed)	10	0.37 $\pm$ 1.17	9.71 $\pm$ 2.27*
Dec. 1998 (sprayed)	10	not detected	12.18 $\pm$ 3.93*
Jan. 1999 (sprayed)	10	1.48 $\pm$ 2.43	12.54 $\pm$ 2.30*
July 1999	10	2.12 $\pm$ 2.22	4.36 $\pm$ 1.80

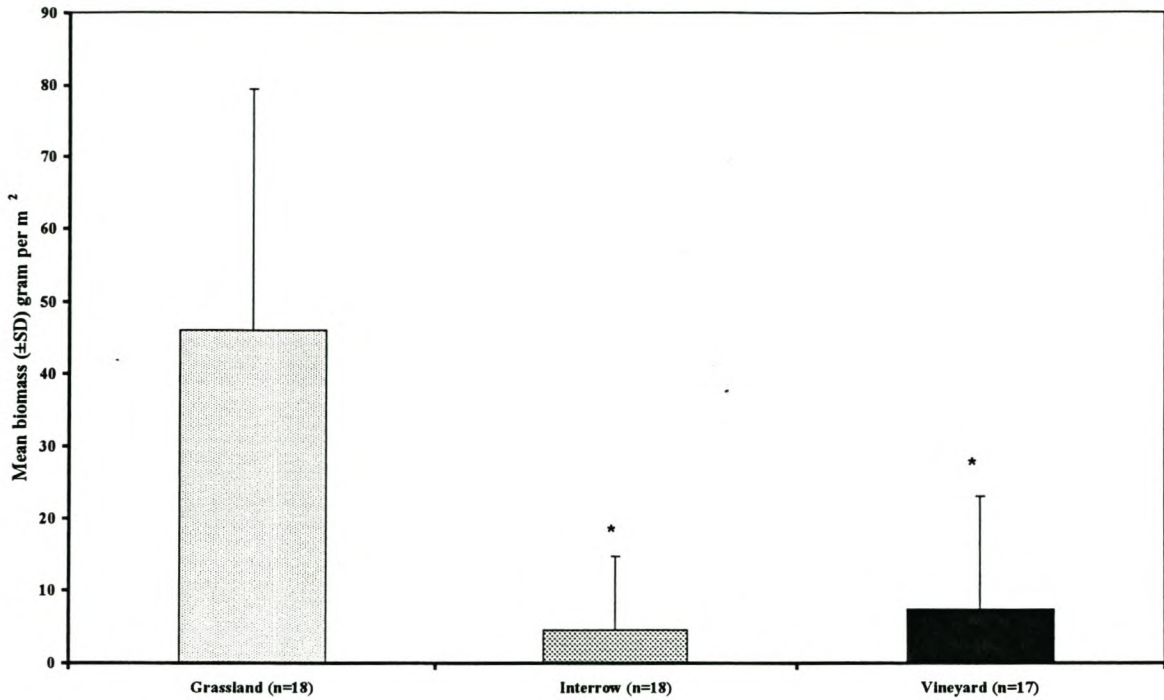
### **3.3. Field Studies conducted at Nietvoorbij, Robertson and Worcester**

#### *3.3.1. Soil physical parameters of soils at Nietvoorbij*

The pH of soils in the grassland soil was  $\pm 6.76$  and that in the vineyard- and interrow soils respectively  $\pm 6.96$  and  $\pm 6.95$ . There was no statistically significant difference ( $p > 0.05$ ) between the pH values of the different soil samples. The organic matter content of the grassland soil was  $\pm 0.83\%$  and that in the vineyard- and interrow soils respectively  $\pm 1.23\%$  and  $\pm 1.20\%$ . There was no statistically significant difference ( $p > 0.05$ ) between the organic matter contents of the different soil samples.

#### *3.3.2. Mean biomass of collected earthworms*

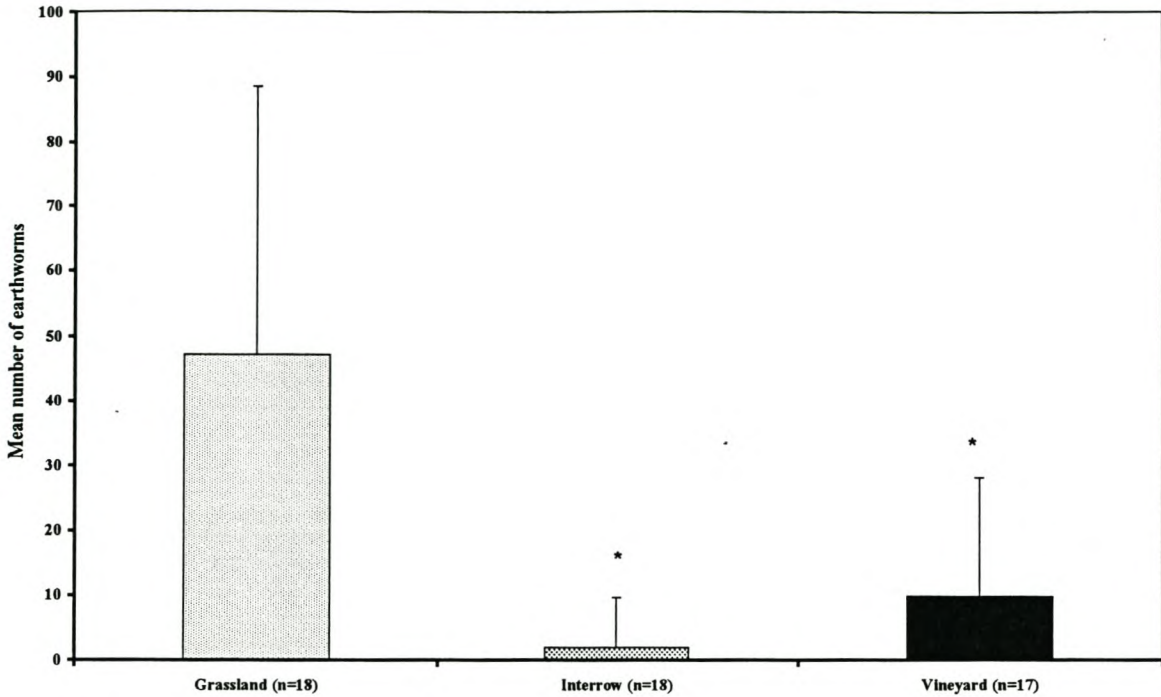
The mean biomass of earthworms per  $m^2$  at the Nietvoorbij area was  $46.00 \pm 33.45$  g in the grassland soils ( $n=18$ ),  $4.53 \pm 10.13$  g in the interrow soils ( $n=18$ ) and  $7.31 \pm 15.82$  g in the vineyard soils ( $n=17$ ), (Figure 14; Table 14). There was no significant difference ( $p > 0.05$ ) between the different mean biomasses. The mean biomass of earthworms per  $m^2$  in the grassland soils was significantly higher ( $p < 0.05$ ) than in the interrow- and vineyard soils. There was no significant difference ( $p > 0.05$ ) between the mean biomass of earthworms per  $m^2$  in the interrow- and vineyard soils.



**Figure 14** Mean biomass (grams)  $\pm$ SD of earthworms per  $m^2$  in grassland-, interrow- and vineyard soils in the Nietvoorbij area (\*significantly different from grassland,  $p < 0.05$ ).

### 3.3.3. Mean number of collected earthworms

The mean number of earthworms per  $m^2$ , collected in the Nietvoorbij area, was  $47.11 \pm 41.24$  in the grassland soils ( $n=18$ ),  $1.88 \pm 7.76$  in the interrow soils ( $n=18$ ) and  $9.78 \pm 18.31$  in the vineyard soils ( $n=17$ ), (Figure 15; Table 14). The mean number of earthworms per  $m^2$  in the grassland soils was significantly higher ( $p < 0.05$ ) than in the interrow- and vineyard soils. There was no significant difference ( $p > 0.05$ ) between the mean number of earthworms per  $m^2$  in the interrow- and vineyard soils.

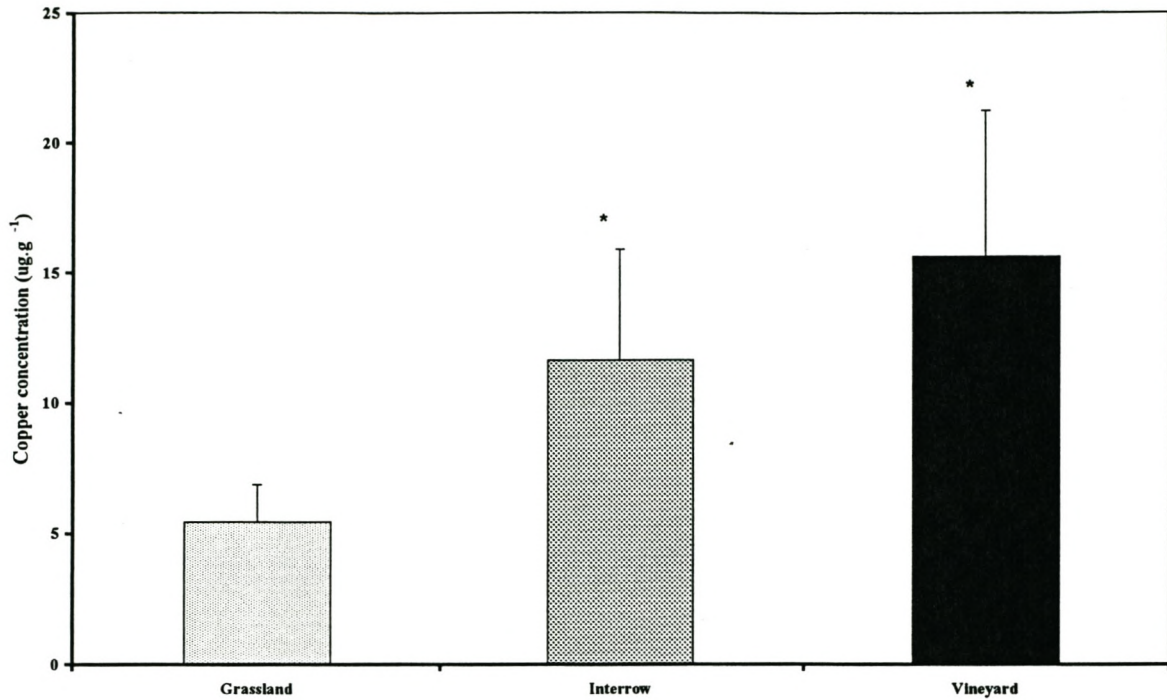


**Figure 15** Mean number  $\pm$ SD of earthworms per  $m^2$  in grassland-, interrow- and vineyard soils in the Nietvoorbij area (\*significantly different from grassland,  $p < 0.05$ ).

#### 3.3.4. Mean copper content in Nietvoorbij soils

The mean copper concentration in soils collected from the Nietvoorbij area, at the same sites where the earthworms were sampled, was  $5.44 \pm 1.43 \mu\text{g.g}^{-1}$  in the grassland soils,  $11.62 \pm 4.26 \mu\text{g.g}^{-1}$  in the interrow soils and  $15.60 \pm 5.60 \mu\text{g.g}^{-1}$  in the vineyard soils (Figure 16; Table 14).

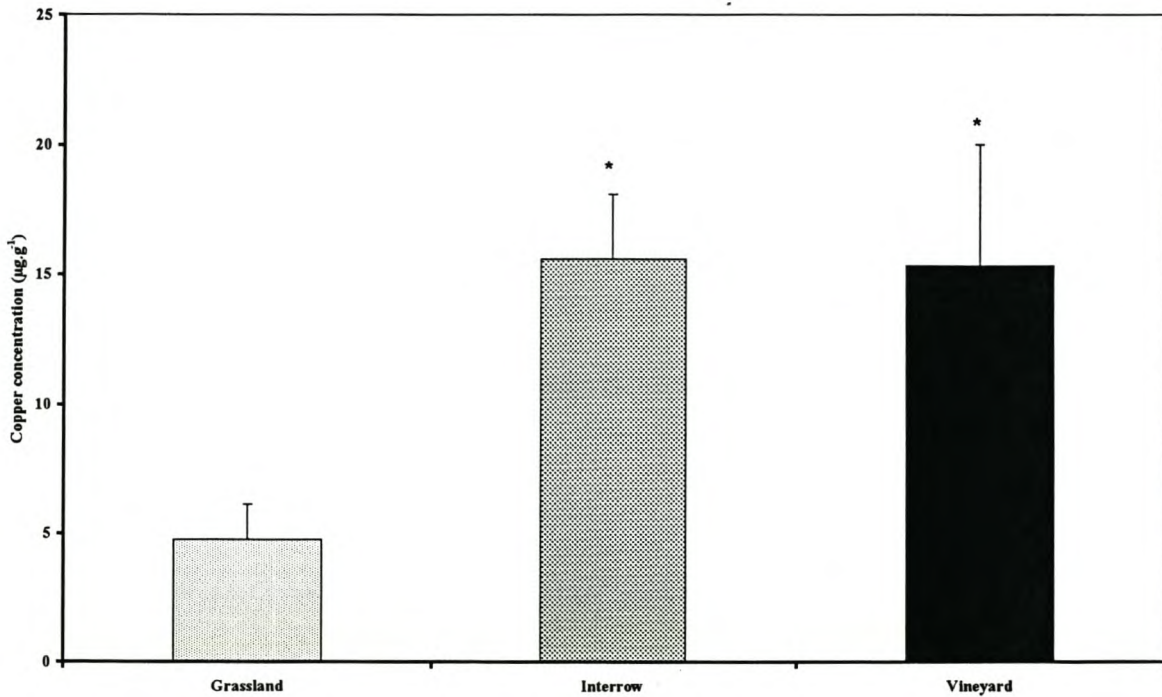
The mean copper concentration in the grassland soils was significantly lower ( $p < 0.05$ ) than in the interrow- and vineyard soils. There was no significant difference ( $p > 0.05$ ) between the mean copper content in the interrow- and vineyard soils.



**Figure 16** Mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in grassland-, interrow- and vineyard soils in the Nietvoorbij area (\*significantly different from grassland,  $p < 0.05$ ;  $n=10$ ).

### 3.3.5. Mean copper content of earthworm body tissues

The mean copper concentrations in earthworm body tissues collected from the Nietvoorbij area, were  $4.75 \pm 1.35 \mu\text{g.g}^{-1}$  in the grassland soils,  $15.60 \pm 2.51 \mu\text{g.g}^{-1}$  in the interrow soils and  $15.25 \pm 4.65 \mu\text{g.g}^{-1}$  in the vineyard soils (Figure 17; Table 14). The mean copper concentration in the grassland soils was significantly lower ( $p < 0.05$ ) than in the interrow- and vineyard soils. There was no significant difference ( $p > 0.05$ ) between the mean copper content in the interrow- and vineyard soils.



**Figure 17** Mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in earthworm body tissues collected from grassland-, interrow- and vineyard soils in the Nietvoorbij area (\*significantly different from grassland,  $p < 0.05$ ;  $n=6$ ).

**Table 14** Mean biomass (grams)  $\pm$ SD and number  $\pm$ SD of earthworms per  $m^2$  in comparison to the copper concentrations ( $\mu\text{g}\cdot\text{g}^{-1}$ )  $\pm$ SD found in soils ( $n=10$ ) and earthworm body tissues ( $n=10$ ) from the grassland- ( $n=18$ ), interrow- ( $n=18$ ) and vineyard soils ( $n=17$ ) in the Nietvoorbij sampling area (\*significantly different from grassland soils,  $p<0.05$ ).

	Mean biomass/ $m^2$ (g)	Mean number/ $m^2$	Mean [Cu] in soil ( $\mu\text{g}\cdot\text{g}^{-1}$ )	Mean [Cu] in earthworms ( $\mu\text{g}\cdot\text{g}^{-1}$ )	BCF
<b>Grassland soil</b>	46.00 $\pm$ 33.45	47.11 $\pm$ 41.24	5.44 $\pm$ 1.43	4.75 $\pm$ 1.35	0.87
<b>Interrow soil</b>	4.53 $\pm$ 10.13*	1.88 $\pm$ 7.76*	11.62 $\pm$ 4.26*	15.60 $\pm$ 2.51*	1.34
<b>Vineyard soil</b>	7.31 $\pm$ 15.82*	9.78 $\pm$ 18.31*	15.60 $\pm$ 5.60*	15.25 $\pm$ 4.65*	0.98

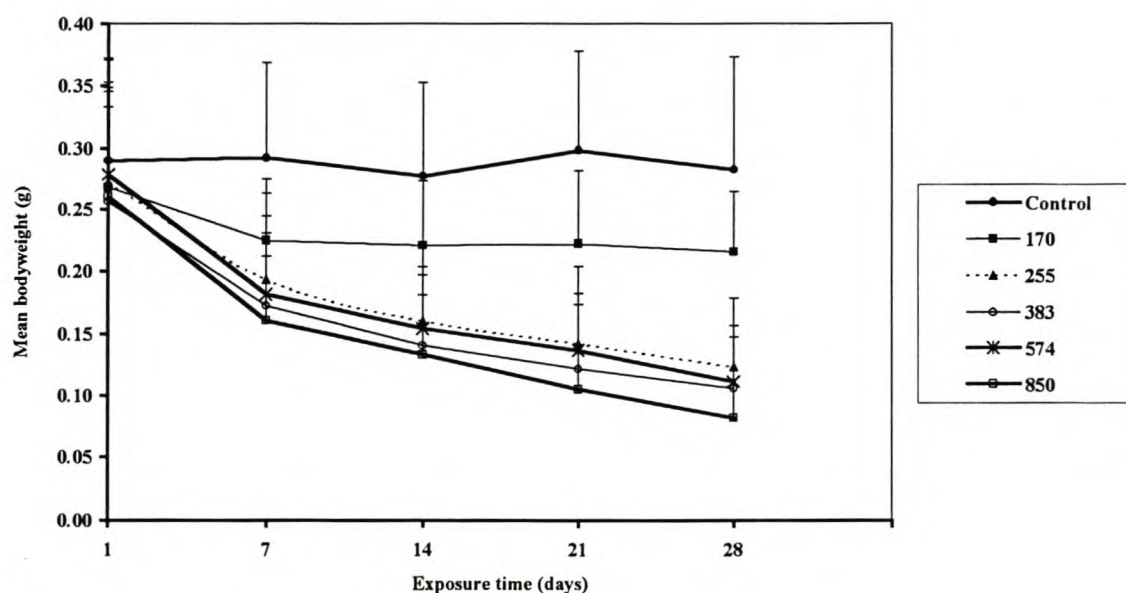
### 3.3.6. Mean copper content in the Robertson and Worcester soils

The mean copper concentrations in soils from the Robertson area were 16.64 $\pm$ 2.20  $\mu\text{g}\cdot\text{g}^{-1}$  in grassland soil, 28.12 $\pm$ 7.61  $\mu\text{g}\cdot\text{g}^{-1}$  in the vineyard soil and 28.80 $\pm$ 3.91  $\mu\text{g}\cdot\text{g}^{-1}$  in the interrow soils. At Worcester mean copper concentrations in soils were 15.36 $\pm$ 2.95  $\mu\text{g}\cdot\text{g}^{-1}$  in grassland soil, 30.52 $\pm$ 3.71  $\mu\text{g}\cdot\text{g}^{-1}$  in the vineyard soil and 28.32 $\pm$ 2.09  $\mu\text{g}\cdot\text{g}^{-1}$  in the interrow soils. At both localities soil copper concentrations in the vineyard and interrow soils were significantly higher ( $p<0.05$ ) than in the grassland soils.

### 3.4. Acute toxicity tests

#### 3.4.1. Change in mean biomass

At the beginning of the experiment there was no statistically significant difference ( $p > 0.05$ ) between the biomasses of the control group and the different exposure groups (Figure 1). The biomass of earthworms exposed to  $170 \mu\text{g.g}^{-1}$  copper oxychloride did not differ significantly ( $p > 0.05$ ) from those in the control group for the duration of the study, except on day seven when it was significantly lower ( $p < 0.05$ ) (Figure 18; Table 15). The earthworms exposed to  $255 \mu\text{g.g}^{-1}$  -  $850 \mu\text{g.g}^{-1}$  copper oxychloride started to show a significant ( $p < 0.05$ ) decrease in mean biomass, seven days after exposure until termination of the experiment and had a significantly lower ( $p < 0.05$ ) mean biomass than the control group (Figure 18; Table 15). There was no statistically significant difference ( $p > 0.05$ ) between the mean biomass of the  $255 \mu\text{g.g}^{-1}$  -  $850 \mu\text{g.g}^{-1}$  exposure groups from day seven until the end of the experiment. The control group earthworms showed a biomass decrease of 3.12% and the  $170 \mu\text{g.g}^{-1}$ -treatment group a biomass decrease of 19.35%. Biomass decrease in the earthworms exposed to  $255 \mu\text{g.g}^{-1}$  -  $850 \mu\text{g.g}^{-1}$  copper oxychloride ranged from 55.01 - 66.26%.



**Figure 18** Mean bodyweight (gram)  $\pm$ SD of earthworms (*E. fetida*) over 28 days in groups exposed to different copper oxychloride concentrations (n= 60).

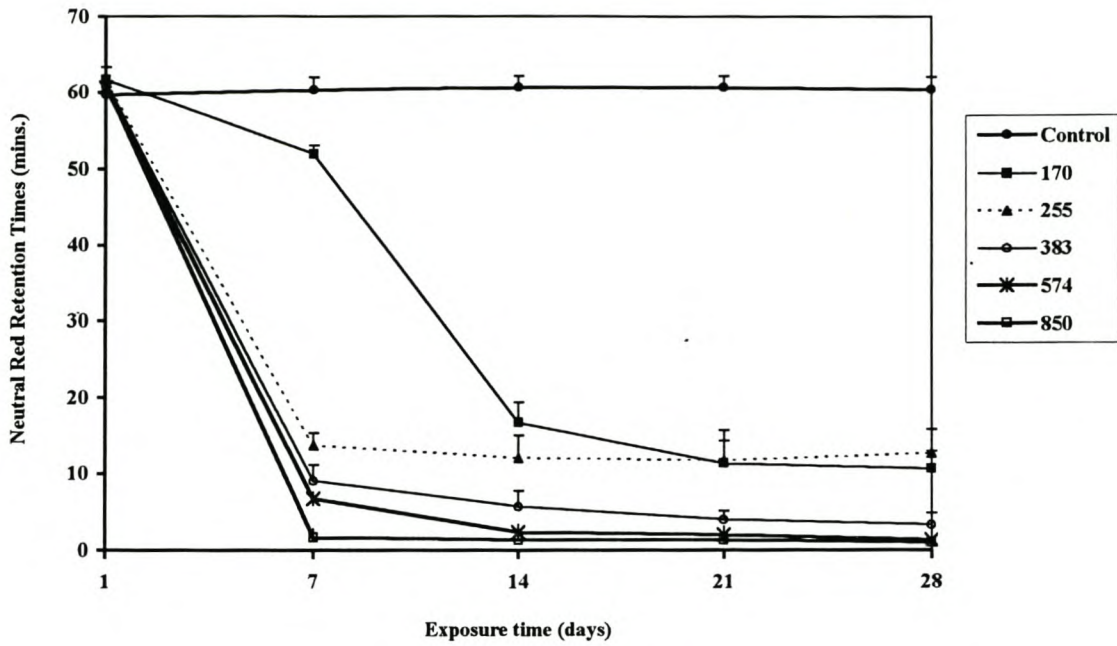


**Table 15** Mean growth (gram)  $\pm$ SD of earthworms (*E. fetida*) over 28 days in groups exposed to different copper oxychloride concentrations ( $\mu\text{g}\cdot\text{g}^{-1}$ ) and % change in bodyweight ("-" = decrease) after 28 days; (\*significantly different from control,  $p < 0.05$ ).

Time (days)	n	Mean biomass (gram) $\pm$ SD of different exposure concentration groups					
		Control	170	255	383	574	850
1	60	0.29 $\pm$ 0.06	0.27 $\pm$ 0.07	0.27 $\pm$ 0.10	0.26 $\pm$ 0.09	0.28 $\pm$ 0.09	0.26 $\pm$ 0.09
7	54	0.29 $\pm$ 0.08	0.22 $\pm$ 0.05	0.19 $\pm$ 0.07*	0.17 $\pm$ 0.06*	0.18 $\pm$ 0.06*	0.16 $\pm$ 0.05*
14	48	0.28 $\pm$ 0.07	0.22 $\pm$ 0.05	0.16 $\pm$ 0.06*	0.14 $\pm$ 0.05*	0.15 $\pm$ 0.06*	0.13 $\pm$ 0.05*
21	42	0.30 $\pm$ 0.08	0.22 $\pm$ 0.06	0.14 $\pm$ 0.06*	0.12 $\pm$ 0.05*	0.13 $\pm$ 0.05*	0.10 $\pm$ 0.04*
28	36	0.28 $\pm$ 0.09	0.21 $\pm$ 0.05	0.12 $\pm$ 0.06*	0.11 $\pm$ 0.05*	0.11 $\pm$ 0.04*	0.08 $\pm$ 0.03*
%Change		-3.12 $\pm$ 11.19	-19.35 $\pm$ 2.12	-55.01 $\pm$ 4.01	-58.83 $\pm$ 3.47	-60.29 $\pm$ 2.79	-66.26 $\pm$ 8.36

#### 3.4.2. Changes in lysosomal membrane stability of coelomocytes: Neutral red retention times

There was a positive correlation ( $r^2=0.96$ ) between a decrease in NRRT with an increase in the mean soil copper concentration, after 28 days of exposing *E. fetida* to different copper oxychloride concentrations. NRRT of earthworms for the duration of the study are depicted in Figure 19 and Table 16. At the start of the experiment, before copper oxychloride exposure, there was no statistically significant difference ( $p > 0.05$ ) between the mean NRRT of the earthworms in the control and copper oxychloride exposed groups and it ranged from 59.67 - 61.67 min. From day seven until the end of the experiment on day 28 the NRRT in the control group earthworms was significantly ( $p < 0.05$ ) higher than in the exposure groups. On day seven the NRRT in the control group was significantly ( $p < 0.05$ ) higher than in all the exposure groups. The NRRT between all the exposure groups were significantly different from each other except between the 383- and 574  $\mu\text{g}\cdot\text{g}^{-1}$  exposure groups at this stage. On day 14 the NRRT between all the exposure groups were significantly different ( $p < 0.05$ ) from each other except between the 383- and 574  $\mu\text{g}\cdot\text{g}^{-1}$  and between the 574- and 850  $\mu\text{g}\cdot\text{g}^{-1}$  exposure groups. The NRRT between all the exposure groups differed significantly ( $p < 0.05$ ) from each other except between the 170- and 255-; and the 574- and 850  $\mu\text{g}\cdot\text{g}^{-1}$  exposure groups on days 21 and 28 respectively.



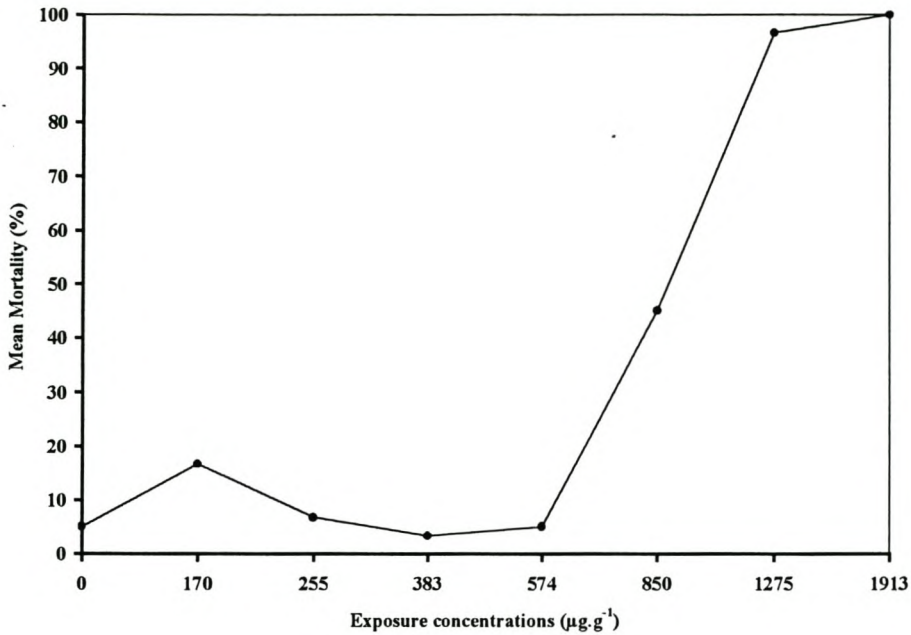
**Figure 19** Mean NRRT (min) ±SD of earthworms (*E. fetida*) over 28 days in groups exposed to different copper oxychloride concentrations (n= 6)

**Table 16** Mean NRRT (min) ±SD of earthworms over 28 days in groups exposed to different copper oxychloride concentrations ( $\mu\text{g.g}^{-1}$ ); (\*significantly different from control,  $p < 0.05$ ; n=6)

Mean neutral retention times (mins.) ±SD of groups in different exposure concentrations						
Time (days)	Control	170	255	383	574	850
1	59.67 ± 1.03	61.67 ± 1.63	61.33 ± 1.97	61.33 ± 1.97	60.67 ± 1.97	61.00 ± 1.79
7	60.33 ± 1.63	52.00 ± 1.10*	13.67 ± 1.63*	9.00 ± 2.19*	6.67 ± 0.82*	1.67 ± 1.03*
14	60.67 ± 1.51	16.67 ± 2.66*	12.00 ± 3.03*	5.67 ± 2.07*	2.33 ± 1.03*	1.33 ± 0.82*
21	60.67 ± 1.51	11.33 ± 2.94*	11.67 ± 3.93*	4.00 ± 1.10*	2.00 ± 1.10*	1.33 ± 0.82*
28	60.33 ± 1.63	10.67 ± 2.33*	12.67 ± 3.20*	3.33 ± 1.51*	1.33 ± 0.82*	1.00 ± 0.00*

### 3.4.3. Mortality ( $LC_{50}$ )

The calculated  $LC_{50}$  for copper oxychloride (Spearman-Kärber Trim) was  $882.78\mu\text{g.g}^{-1}$  and  $519.40\mu\text{g.g}^{-1}$  for copper. The mean mortality rate in the control and exposure groups, after 28 days is depicted in Figure 20 and Table 17.



**Figure 20.** Mean percentage of mortality in earthworms (*E. fetida*) exposed to different copper oxychloride concentrations ( $\mu\text{g.g}^{-1}$ ) after 28 days (n=60 per group).

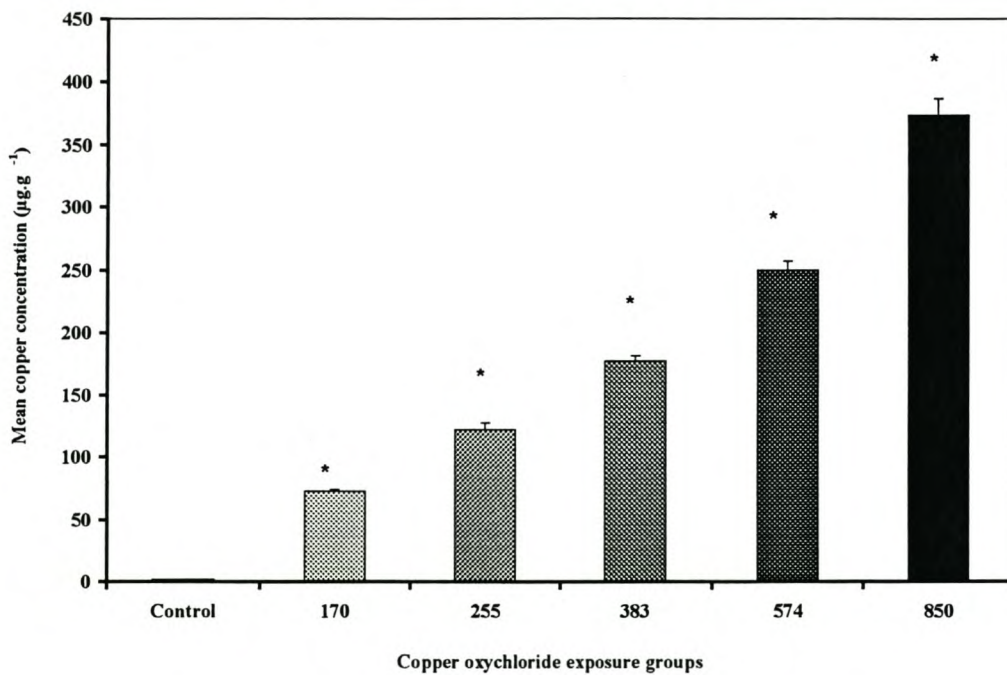
**Table 17** The mean percentage of mortality of earthworms (*E. fetida*) exposed to different concentrations for the duration of the experiment.

Exposure groups	Number in each group	Mean % mortalities
Control	60	5.00
170	60	16.67
255	60	6.67
383	60	3.33
574	60	5.00
850	60	45.00
1275	30	96.67
1913	30	100

#### 3.4.4. Copper content of substrate

The mean copper concentration in the substrate from the control group was  $1.66 \pm 0.20 \mu\text{g.g}^{-1}$ ,  $73.17 \pm 1.35 \mu\text{g.g}^{-1}$  in the 170-,  $121.56 \pm 5.93 \mu\text{g.g}^{-1}$  in the 255-,  $177.27 \pm 3.88 \mu\text{g.g}^{-1}$  in the 383-,  $249.73 \pm 7.18 \mu\text{g.g}^{-1}$  in the 574- and  $372.75 \pm 13.63 \mu\text{g.g}^{-1}$  in the 850 copper oxychloride exposure group (Figure 21).

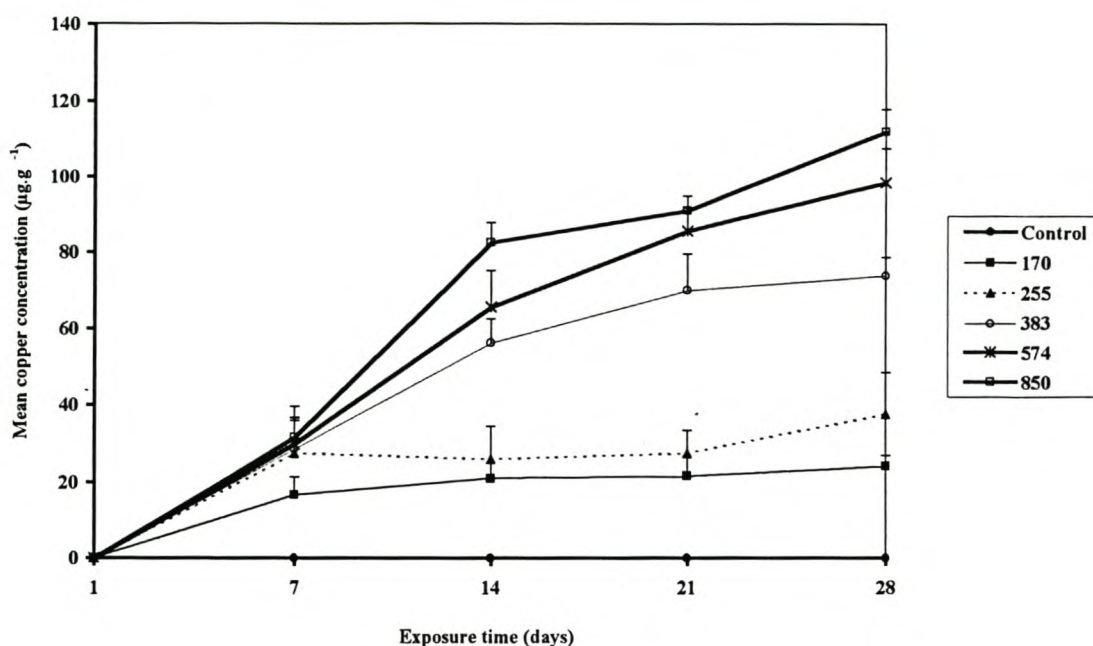
The differences in the median values among all the different treatment groups was significantly ( $p < 0.05$ ) different from each other and higher than the control group.



**Figure 21** Mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in the substrates from the control and copper oxychloride exposed groups in acute toxicity tests (\*significantly different from control,  $p < 0.05$ ;  $n=10$ ).

#### 3.4.5. Copper content of earthworm body tissues

The copper content of earthworm body tissues of the control and different exposure groups are depicted in Figure 21 and Table 18. At the start of the experiment the copper in the body tissues of all the earthworms in the control and exposure groups were below the detection limit. On day seven until day 28 the copper concentration in body tissues of all the exposure group earthworms were significantly higher ( $p < 0.05$ ) than that in the control group earthworms. The mean copper concentration in earthworms from the  $170 \mu\text{g.g}^{-1}$  exposure group on day seven was significantly lower ( $p < 0.05$ ) than the rest of the exposure groups ( $383 - 850 \mu\text{g.g}^{-1}$ ), except from the  $255 \mu\text{g.g}^{-1}$  exposure group. For the same period there was a significant ( $p < 0.05$ ) difference between the copper concentrations in the body tissues of all the exposure groups. On day 14 there was a significant difference in the copper concentrations between all the exposure groups except between the  $170-$  and  $255 \mu\text{g.g}^{-1}$  and between the  $383-$  and  $574 \mu\text{g.g}^{-1}$  exposure groups. On day 21 there was a significant difference in the copper concentrations between all the exposure groups except between the  $170-$  and  $255 \mu\text{g.g}^{-1}$  and between the  $574-$  and  $850 \mu\text{g.g}^{-1}$  exposure groups. At the termination of the experiment, on day 28, the mean copper concentration in earthworm body tissues between all the exposure groups differed statistically significant ( $p < 0.05$ ) from each other and were significantly ( $p < 0.05$ ) higher than in the control group. At this stage no copper was detected in the body tissues of the control group, and the mean copper concentration in the body tissues of the exposure groups was  $24.21 \pm 2.68 \mu\text{g.g}^{-1}$  in the  $170-$ ,  $37.70 \pm 10.86 \mu\text{g.g}^{-1}$  in the  $255-$ ,  $74.03 \pm 4.80 \mu\text{g.g}^{-1}$  in the  $383-$ ,  $98.22 \pm 8.96 \mu\text{g.g}^{-1}$  in the  $574-$  and  $111.79 \pm 5.89 \mu\text{g.g}^{-1}$  in the  $850 \mu\text{g.g}^{-1}$  copper oxychloride exposure group. The bioconcentration factors (BCF) of copper in the body tissues of earthworms ranged between  $0.30 - 0.42$  in the exposure groups (Table 19). Since the copper concentrations in the body tissues of the control group earthworms were below the detection limit a BCF could not be determined.



**Figure 22** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*E. fetida*) from control and copper oxychloride exposed groups in acute toxicity tests (\*significantly different from control,  $p < 0.05$ ;  $n = 6$ ).

**Table 18** Change over time in the mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*E. fetida*) from control and copper oxychloride exposed groups in acute toxicity tests (\*significantly different from control,  $p < 0.05$ ;  $n = 6$ ; nd=not detected).

Time (days)	Mean copper concentration ( $\mu\text{g.g}^{-1}$ ) $\pm$ SD					
	Control	170	255	383	574	850
1	nd	nd	nd	nd	nd	nd
7	nd	16.69 $\pm$ 4.66*	27.40 $\pm$ 9.17*	28.55 $\pm$ 7.98*	29.79 $\pm$ 6.07*	31.46 $\pm$ 7.93*
14	nd	20.93 $\pm$ 3.93*	25.89 $\pm$ 8.38*	56.20 $\pm$ 6.22*	65.73 $\pm$ 9.40*	82.61 $\pm$ 5.09*
21	nd	21.53 $\pm$ 5.01*	27.41 $\pm$ 5.92*	70.12 $\pm$ 9.49*	85.51 $\pm$ 6.03*	90.91 $\pm$ 3.91*
28	nd	24.21 $\pm$ 2.68*	37.70 $\pm$ 10.86*	74.03 $\pm$ 4.80*	98.22 $\pm$ 8.96*	111.79 $\pm$ 5.89*

**Table 19** Comparative concentrations ( $\mu\text{g.g}^{-1}$ ) of Effekto Virikop<sup>®</sup>, a.i. copper oxychloride, effective copper, measured copper content of substrates and earthworm body tissues and the bioconcentration factors (BCF) for copper after 28 days. [nd = not detected].

Effekto Virikop	Copper oxychloride	Copper	Copper: Substrates	Copper: Earthworms	BCF
0	0	0	1.66±0.20	nd	-
200	170	100	73.17±1.35	24.21±2.61	0.33
300	255	150	121.56±5.93	37.70±10.86	0.31
450	383	225	177.27±3.88	74.03±4.80	0.42
675	574	338	249.73±7.18	98.22±8.96	0.39
1000	850	500	372.75±13.63	111.79±5.89	0.30
1500	1275	750	-	-	-
2250	1913	1125	-	-	-

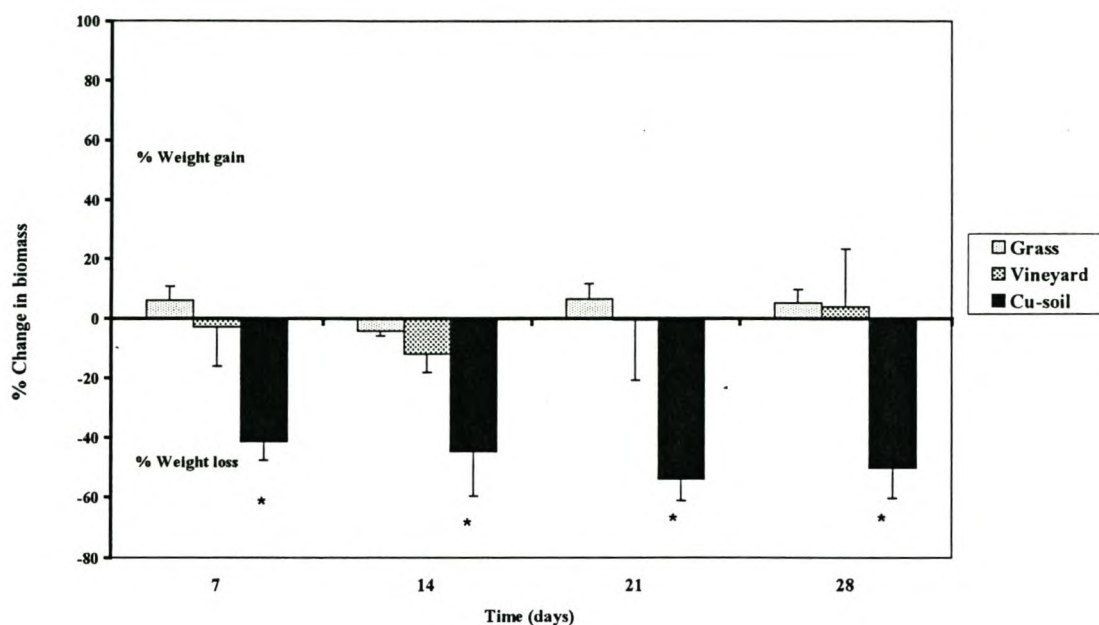
### 3.5. Bioassay

#### 3.5.1. Change in biomass

The mean change in biomass (mean weight loss/gain) of *A. caliginosa* over time in the grassland-, vineyard- and copper oxychloride contaminated soils for the duration of the study is depicted in Figure 23 and Table 19. On day seven of the experiment the earthworms exposed to the grassland soil showed an increase in biomass of  $5.63 \pm 4.67$  %. These worms showed a decrease in biomass of  $4.21 \pm 1.91$  % on day 14 of the experiment. On days 21 and 28 the earthworms exposed to grassland soil showed an increase in biomass of respectively  $6.33 \pm 5.19$  % and  $5.03 \pm 4.62$  %. At no stage during the experiment was the change in biomass significantly different ( $p > 0.05$ ) from the initial weight of the earthworms.

The earthworms exposed to the vineyard soil showed a decrease in biomass from day seven to day 21 of the experiment. The decrease in biomass was respectively  $2.89 \pm 13.09$  % on day seven,  $12.01 \pm 6.26$  % on day 14 and  $0.68 \pm 20.15$  % on day 21. At the end of the experiment on day 28 these earthworms had an increase in biomass of  $3.79 \pm 19.51$  %. At no stage during the experiment was the change in biomass significantly different ( $p > 0.05$ ) from the initial weight of the earthworms. Earthworms exposed to copper in the form of copper oxychloride showed a statistically significant ( $p < 0.05$ ) decrease in biomass from day seven until the end of the experiment on day 28. On day seven the decrease in biomass was  $41.57 \pm 6.10$  %,  $44.89 \pm 14.81$  % on day 14,  $54.20 \pm 6.95$  % on day 21 and  $50.14 \pm 10.26$  % on day 28.





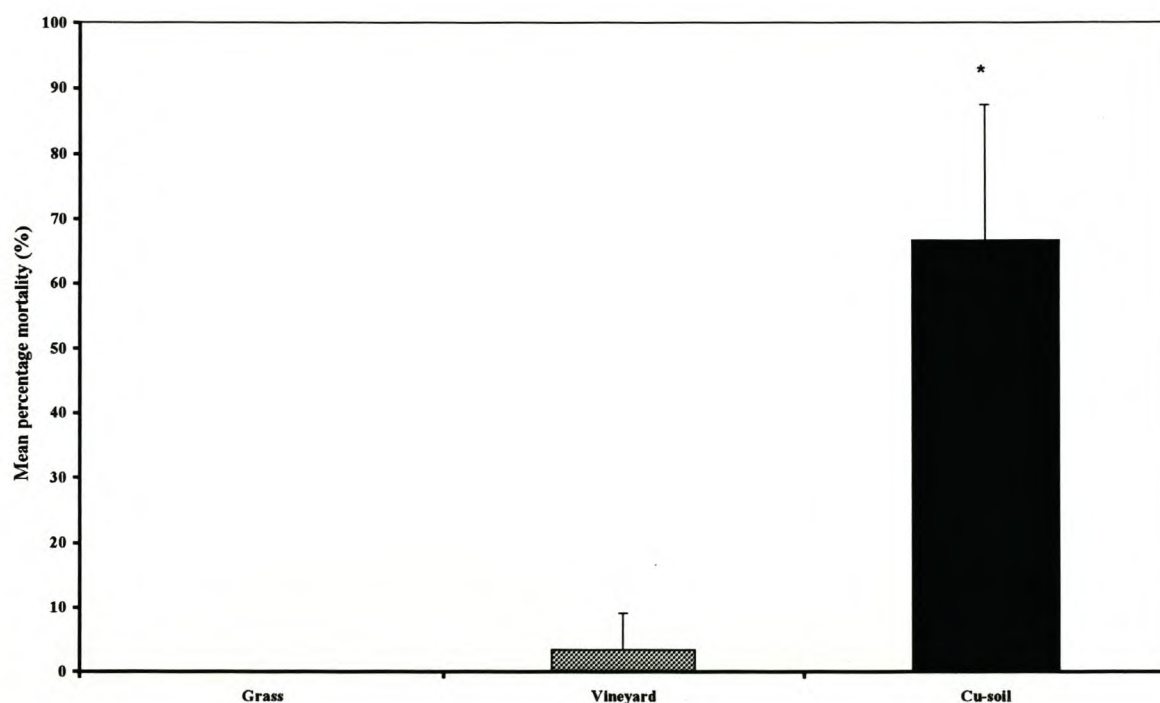
**Figure 23** Mean percentage change in biomass (weight gain/loss) over time of earthworms (*A. caliginosa*) exposed to grassland-, vineyard- and copper oxychloride ( $60\mu\text{g.g}^{-1}$  copper) treated soil for the duration of the study (\*significantly different from initial bodyweight,  $p < 0.05$ ;  $n = 30$ ).

**Table 20** Mean percentage change in biomass (weight gain/loss) over time of earthworms (*A. caliginosa*) exposed to grassland-, vineyard- and copper oxychloride ( $60\mu\text{g.g}^{-1}$ ) treated soil for the duration of the study (\*significantly different from initial bodyweight,  $p < 0.05$ ).

Time (days)	n	Mean % weight gain/loss $\pm$ SD		
		Grassland	Vineyard	Copper oxychloride
7	30	$5.63 \pm 4.57$	$-2.89 \pm 13.09$	$-41.57 \pm 6.10^*$
14	30	$-4.21 \pm 1.92$	$-12.01 \pm 6.26$	$-44.89 \pm 14.81^*$
21	30	$6.33 \pm 5.19$	$-0.68 \pm 20.15$	$-54.20 \pm 6.95^*$
28	30	$5.03 \pm 4.62$	$3.79 \pm 19.51$	$-50.14 \pm 10.26^*$

### 3.5.2. Mortality

There was no mortality observed in earthworms exposed to grassland soil on termination of the experiment after 28 days (Figure 24; Table 21). At the end of the experiment the mean percentage mortality in the earthworms exposed to vineyard soil was  $3.33 \pm 5.77\%$  and  $66.67 \pm 20.82\%$  in the soil treated with copper oxychloride (Figure 22; Table 21). The percentage mortality in copper oxychloride treated soil was significantly higher ( $p < 0.05$ ) than in both the grassland and vineyard soils.

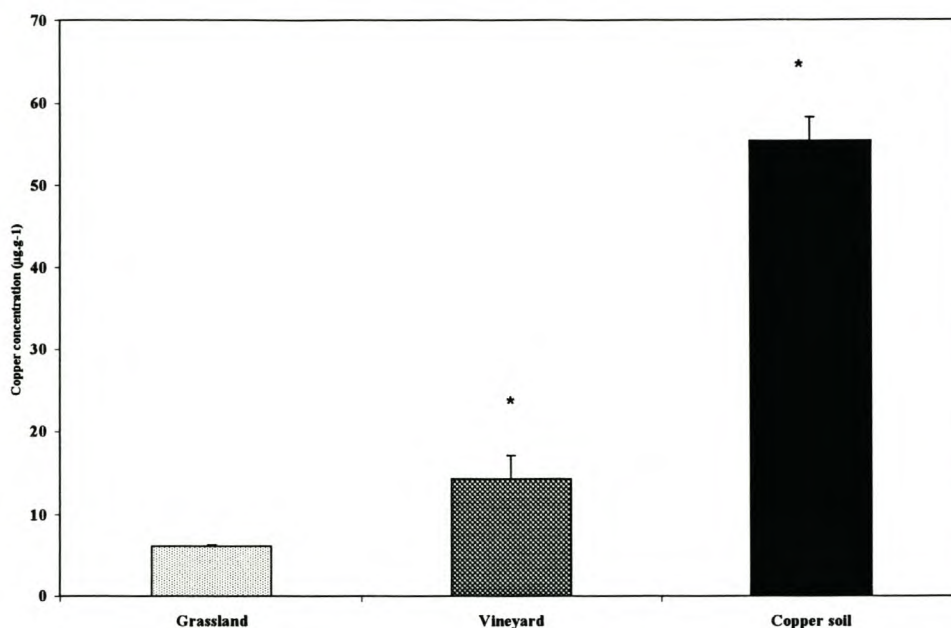


**Figure 24** Mean mortality (%) of earthworms (*A. caliginosa*) in the grassland-, vineyard- and copper oxychloride ( $60 \mu\text{g.g}^{-1}$ ) treated soil after 28 days (\*significantly different from grassland soil,  $p < 0.05$ ;  $n = 30$ ).

### 3.5.3. Mean copper content of substrates

At the end of the experiment the mean copper content was  $6.10 \pm 0.16 \mu\text{g.g}^{-1}$  in the grassland soil,  $14.38 \pm 2.78 \mu\text{g.g}^{-1}$  in the vineyard soil and  $55.43 \pm 2.85 \mu\text{g.g}^{-1}$  in the copper oxychloride contaminated soil, to which the earthworms (*A. caliginosa*) were exposed (Figure, 25; Table 21). The mean copper content in the vineyard and copper oxychloride contaminated soils were significantly higher ( $p < 0.05$ ) than in the grassland soils. The copper

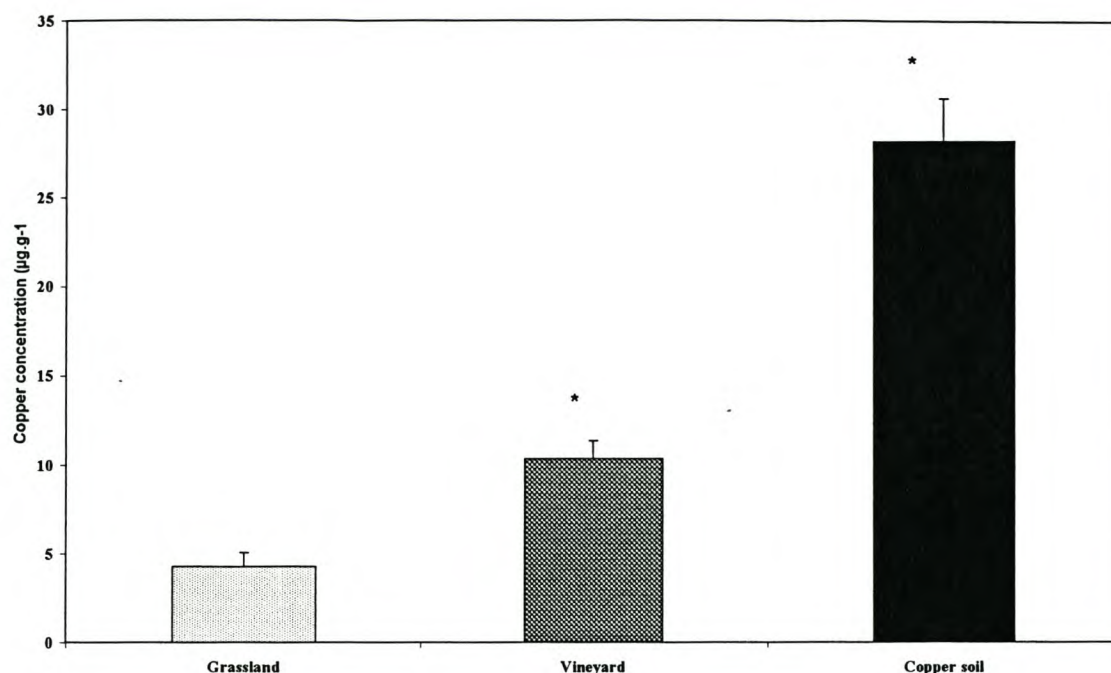
content in the copper contaminated soils were also significantly higher ( $p < 0.05$ ) than in the vineyard soils.



**Figure 25** Mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in substrates from the grassland, vineyard and copper oxychloride exposed groups in the bioassay tests after 28 days (\*significantly different from control,  $p < 0.05$ ,  $n=6$ )

#### 3.5.4. Mean copper content of earthworm body tissues

The copper concentration in the body tissues of the earthworms was determined after termination of the experiment on day 28 (Figure, 26; Table 21). The mean copper content of the earthworms body tissues was  $4.28 \pm 0.77 \mu\text{g.g}^{-1}$  exposed to grassland soil,  $10.36 \pm 1.02 \mu\text{g.g}^{-1}$  in earthworms exposed to vineyard soil and  $28.19 \pm 2.49 \mu\text{g.g}^{-1}$  in earthworms exposed to copper oxychloride contaminated soil (Figure, 26; Table 21). The mean copper content of earthworm body tissues exposed to the vineyard and copper oxychloride contaminated soils were significantly higher ( $p < 0.05$ ) than in the grassland soils. The copper content of earthworm body tissues in the copper oxychloride contaminated soil was also significantly higher ( $p < 0.05$ ) than in the vineyard soil.



**Figure 26** Mean copper concentration ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in earthworm body tissues (*A. caliginosa*) from the grassland, vineyard and copper oxychloride exposed groups in the bioassay tests after 28 days (\*significantly different from control,  $p < 0.05$ ,  $n=6$ ).

**Table 21** Mean percentage mortality  $\pm$ SD of earthworms (*A. caliginosa*) in comparison to copper concentrations ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD in soils and earthworm body tissues exposed to grassland-, vineyard- and copper oxychloride contaminated soil (effective [Cu] of  $60\mu\text{g.g}^{-1}$ ) over 28 days and the bioconcentration factors (BCF) for copper in earthworm body tissues ( $n=10$ ; \*significantly different from grassland soils,  $p < 0.05$ ).

	% Mortality	[Cu] in soil	[Cu] in worms	BCF
<b>Grassland soil</b>	0	6.10 $\pm$ 0.16	4.28 $\pm$ 0.77	0.70
<b>Vineyard soil</b>	3.33 $\pm$ 5.77	14.38 $\pm$ 2.78*	10.36 $\pm$ 1.02*	0.72
<b>Cu oxychloride contaminated soil</b>	66.67 $\pm$ 20.82*	55.43 $\pm$ 2.85*	28.19 $\pm$ 2.49*	0.51

### 3.6. Burrowing activity and avoidance response of *Aporrectodea caliginosa*

#### 3.6.1. Burrow rate of earthworms

The mean burrow distance, at the end of the experiment, of earthworms in the grassland soil was  $48.31 \pm 12.23$  cm,  $21.72 \pm 9.99$  cm in the vineyard soil and  $28.67 \pm 11.56$  cm in soil with a calculated copper concentration of  $60 \mu\text{g.g}^{-1}$  in the form of copper oxychloride (Table 22). The burrow distance of earthworms in the grassland soil was significantly higher ( $p < 0.05$ ) than in the vineyard and copper oxychloride contaminated soil. There was no significant difference ( $p > 0.05$ ) between the burrow distances of earthworms from the vineyard and copper oxychloride contaminated soil. The mean copper content of the grassland soil *A. caliginosa* was exposed to was  $4.79 \pm 1.37 \mu\text{g.g}^{-1}$  and was significantly lower ( $p < 0.05$ ) than that found in the vineyard soil ( $14.37 \pm 1.46 \mu\text{g.g}^{-1}$ ) and copper oxychloride contaminated soil ( $49.20 \pm 1.97 \mu\text{g.g}^{-1}$ ), (Table 22). Further the mean copper content in the vineyard soil was also significantly lower ( $p < 0.05$ ) than found in the copper oxychloride contaminated soil.

#### 3.6.2. Soil avoidance by earthworms

##### 3.6.2.1. Grassland- vs. Vineyard soil

The mean burrowing distance of earthworms on the grassland soil section of the soil profile, as measured in terms of the burrowing distance over time, was  $37.75 \pm 22.54$  cm and  $5.49 \pm 7.52$  cm on the vineyard soil section (Table 22). The mean burrow rate of earthworms on the grassland soil section of the soil profile was significantly higher ( $p < 0.05$ ) than on the vineyard soil section. The mean copper content of  $4.98 \pm 0.65 \mu\text{g.g}^{-1}$  in the grassland soil was significantly lower ( $p < 0.05$ ) than the mean copper content of  $15.47 \pm 1.12 \mu\text{g.g}^{-1}$  in the vineyard soil (Table 22).

##### 3.6.2.2. Grassland- vs. Copper oxychloride contaminated soil

The mean burrow distance of earthworms in the grassland soil section of the soil profile was  $44.95 \pm 22.65$  cm and  $10.11 \pm 8.87$  cm on the copper oxychloride contaminated soil section (Table 22). The mean burrow rate of earthworms on the grassland soil section of the soil profile was significantly higher ( $p < 0.05$ ) than in the copper oxychloride contaminated soil section.

The mean copper content of  $4.48 \pm 1.48 \mu\text{g.g}^{-1}$  in the grassland soil was significantly ( $p < 0.05$ ) lower than the mean copper content of  $49.24 \pm 1.20 \mu\text{g.g}^{-1}$  in the copper oxychloride contaminated soil (Table 22).

**Table 22** Mean tunnelling activity (cm)  $\pm$ SD of earthworms in the burrow activity experiments (n=18) in comparison to the mean copper concentrations ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD (n=6) in grassland-, vineyard- and copper oxychloride contaminated soils (effective [Cu] of  $60\mu\text{g.g}^{-1}$ ) over a period of 4 days (\*significantly different from grassland soils,  $p<0.05$ ).

	Mean distance tunnelled (cm)	Soil [Cu] $\mu\text{g.g}^{-1}$
<b>Grassland soil</b>	48.31 $\pm$ 12.23	4.79 $\pm$ 1.38
<b>Vineyard soil</b>	21.72 $\pm$ 9.99*	14.37 $\pm$ 1.46*
<b>Cu oxychloride soil</b>	28.67 $\pm$ 11.56*	49.20 $\pm$ 1.97*

**Table 23** Mean tunnelling activity (cm)  $\pm$ SD of earthworms in the soil avoidance experiments (n=12) in comparison to the mean copper concentrations ( $\mu\text{g.g}^{-1}$ )  $\pm$ SD (n=6) in grassland-, vineyard- and copper oxychloride contaminated soils (effective [Cu] of  $60\mu\text{g.g}^{-1}$ ) over a period of 4 days (\*significantly different from grassland soils,  $p<0.05$ ).

	Mean distance tunnelled (cm)	Soil [Cu] $\mu\text{g.g}^{-1}$
<b>Grassland soil vs.</b>	37.75 $\pm$ 22.54	4.98 $\pm$ 0.65
<b>Vineyard soil</b>	5.49 $\pm$ 7.52*	15.47 $\pm$ 1.12
<b>Grassland soil vs.</b>	44.95 $\pm$ 22.65	4.48 $\pm$ 1.48
<b>Cu oxychloride soil</b>	10.11 $\pm$ 8.87*	49.24 $\pm$ 1.20

## CHAPTER 4

### *DISCUSSION...*

#### **4.1. Field experiments at Nieuwoudtville and Vergenoegd**

##### *4.1.1. Changes in biomass and numbers in relation to copper content of soils and earthworms*

Initially, before spraying with copper oxychloride there was no significant difference between the mean biomass and number of earthworms per m<sup>2</sup> at both Nieuwoudtville and Vergenoegd. At Nieuwoudtville the mean biomass of earthworms per m<sup>2</sup> decreased significantly ( $p < 0.05$ ) after two spraying applications (Figure 4; Table 3) which resulted in a soil copper concentration of  $21.01 \pm 1.59 \mu\text{g}\cdot\text{g}^{-1}$  (Figure 7; Table 6). The mean number of earthworms per m<sup>2</sup> decreased significantly ( $p < 0.05$ ) after four spraying applications (Figure 5; Table 4) at a soil copper concentration of  $29.76 \pm 2.28 \mu\text{g}\cdot\text{g}^{-1}$ . At Vergenoegd the mean biomass (Figure 9; Table 9) and number (Figure 10; Table 10) of earthworms per m<sup>2</sup> decreased significantly ( $p < 0.05$ ) after eight spraying applications at a soil copper concentration of  $46.74 \pm 18.89 \mu\text{g}\cdot\text{g}^{-1}$  (Figure 12; Table 12). Since neither of the areas has previously been polluted with copper it can be assumed that any difference between the control and exposure plots was due to the spraying with copper oxychloride.

At both areas the copper concentrations in the soils of the exposure plots were significantly higher ( $p < 0.05$ ) than in the control plots after two spraying applications (Figures 7 and 12; Tables 6 and 12). The mean copper content of earthworm body tissues started to increase significantly after eight spraying applications in Nieuwoudtville (Figure 8; Table 7) and after two at Vergenoegd (Figure 13; Table 13). The bioconcentration factor (BCF);  $([\text{Cu}]^{\text{e/worm}}/[\text{Cu}]_{\text{soil}})$  for copper ranged from 0.28-1.06 (Nieuwoudtville) and 0.03-0.29 (Vergenoegd) in earthworms from the control plots. In the exposure plots it ranged from 0.11-0.45 (Nieuwoudtville) and 0.03-0.53 (Vergenoegd). This is in agreement with studies done by Neuhauser *et al.* (1995) and Pizl and Josens (1995) who concluded that copper is only moderately concentrated in earthworms body tissues since it is a micronutrient that can be regulated by the earthworms. There was a correlation between the increased copper concentrations in soils and the increase in the copper concentrations in earthworm

body tissues. This has also been found in numerous other studies (Abdul Rida and Bouché, 1997; Ma, 1982; Morgan and Morgan 1998; Neuhauser *et al.*, 1995). Ma (1982) concluded that the body concentration of copper in earthworm tissues is determined by the concentration of copper present in the soil. Some recent studies also confirmed the negative effects of high soil copper concentrations on earthworms. Paoletti (1999) stated that fungicides are highly toxic to earthworms, especially copper and zinc residues. Spurgeon and Hopkin (1995) found that cocoon production was significantly reduced in copper contaminated soils. The threshold for the impact of copper oxychloride on growth and reproduction for juveniles of *E. fetida* was reported to be  $8.92 \mu\text{g}\cdot\text{g}^{-1}$  copper in exposure substrates (Helling *et al.*, 2000). This can provide an explanation for the negative effects on population biomass and numbers found in the field in the current study, although a direct comparison cannot be made. The substrate used in the above mentioned study was organic and the experiment was conducted under controlled physical conditions.

The mean biomass of the earthworms in Nieuwoudtville field plots was a more sensitive endpoint than numbers of earthworms. Since biomass is a reflection of growth in the population and numbers a reflection of reproduction and mortality, this would indicate that earthworm growth was initially detrimentally affected before effects on mortality and reproduction became noticeable. The effect on biomass coincided with increased soil copper concentrations in the exposure plots. These negative effects were observed before significantly higher ( $p < 0.05$ ) copper concentrations were detected in the earthworm body tissues. Klok *et al.* (1997) concluded that if earthworms are under environmental stress, energy requirements for maintenance of bodily functions always takes precedence over growth and reproduction, with extinction of earthworm populations as a result. Since the maximum growth rate of earthworm populations is most sensitive to changes in adult earthworms (Klok *et al.*, 1997), it might provide a possible explanation for the effects observed at the population level in the present study.

The significant decrease in worm numbers seven months after spraying had stopped in Vergenoegd was surprising, especially since the copper levels detected in the soil on the sampling dates were relatively low ( $8.48 \pm 1.3 \mu\text{g}\cdot\text{g}^{-1}$ ). The body burdens of copper were also relatively low at this stage ( $4.36 \pm 1.8 \mu\text{g}\cdot\text{g}^{-1}$ ). The NOEC and  $\text{EC}_{50}$  for the effect of copper on growth was reported as respectively  $56 \mu\text{g}\cdot\text{g}^{-1}$  and  $> 100 \mu\text{g}\cdot\text{g}^{-1}$  Cu (Van Gestel *et al.*, 1991) for *Eisenia andrei*. Species differences



could partly account for these differences. These copper levels would normally be considered as non-toxic for earthworms. The soil copper level at the end of the spraying period in January 1999 ( $46.74 \pm 18.9 \mu\text{g.g}^{-1}$ ) was however high enough to cause considerable mortality if the results of Svendsen and Weeks (1997b) are taken into account. These authors found that the percentage survival in a mesocosm in the field was only about 20% in "spiked" soil copper concentrations of  $26.0\text{-}43.5 \mu\text{g.g}^{-1}$ .

One of the physical characteristics of the soil influenced by the spraying of copper oxychloride at both experimental field sites was the soil pH. The pH in soils from the exposure plots was significantly lower ( $p < 0.05$ ) than in the control plots after spraying with copper oxychloride started (Tables 2 and 8). Because spraying with copper oxychloride decreased the pH in soils significantly, it could also have increased the bioavailability of copper. Various authors have commented on the effect of pH on copper bioavailability in soils. Ash and Lee (1980) found that copper differences in earthworms were more significant in acid soils than in alkaline soils. According to Ma (1988) the activity of  $\text{Cu}^{2+}$  ions increase with decreasing pH with the result that the reproduction of *A. caliginosa* exposed to different copper concentrations was lower in soils with a low pH. Abdul Rida and Bouché (1997) found a correlation between Ca and Cu in earthworm body tissues in relation to silt and high pH soils. It has also been reported that pH can act as a stress agent, causing a decrease in reproduction in oligochaetes (*Enchytraeus albidus*) at a pH of 4.5 (Amorim *et al.*, 1999). Hence, a decrease in pH not only increases copper bioavailability to earthworms but can also affect reproduction negatively. This might have negative effects on the physiological processes and manifest as a decrease in NRRT, in earthworms at cellular level and consequently on the individual and population level as was found in the present study. This might provide a possible explanation for the negative effects observed at the population level at both the study areas.

#### *4.1.2. Changes in neutral red retention times in relation to copper content of soils and earthworms, and the link of these changes to population changes*

The neutral red retention times (NRRT) of earthworm coelomocytes decreased significantly ( $p < 0.05$ ) after one month of spraying with copper oxychloride at both Nieuwoudtville and Vergenoegd (Figures 6 and 11; Tables 5 and 11). This coincided with the significant increase in soil copper concentrations (Figures 7 and 12; Tables 6

and 12). In a study by Svendsen and Weeks (1997a) earthworms (*E. andrei*) exposed to  $\text{CuCl}_2$  ( $20 \mu\text{g.g}^{-1}$ ) also had lower NRRT than control group earthworms. The lower NRRT in the present study could be indicative of physiological stress associated with growth (biomass of the earthworm population) as an observed effect. It coincided with the statistically significant ( $p < 0.05$ ) decrease in the mean biomass of earthworms per  $\text{m}^2$  in Nieuwoudtville (Figure 4; Table 5). This implies that changes at the cellular level manifest at the same time as changes in growth impacts on earthworm populations as was discussed previously (refer 4.1.1). These cellular and growth effects preceded the decline in earthworm abundance that manifested one month later (Figure 5; Table 4). At Vergenoegd the cellular effects were evident at an even earlier stage. This is in agreement with Morgan *et al.* (1999) who, in an overview of biomarkers, concluded that biomarkers could provide an early indication of toxicant impacts on field populations of earthworms.

The response in NRRT was therefore indicative of the observed effects on growth which had an impact at the population level on the long term. NRRT utilised as biomarker in ecotoxicological studies can therefore be useful if used with the necessary controls allowing for quick (one month after spraying started in the present study) assessment of the impact of copper-based agrochemicals on earthworm populations. It is however essential to verify these cellular responses further with organismal and population responses, especially with growth as a measured endpoint.

#### 4.2. A comparison between cellular and population responses at both localities

From the results obtained on mean number and biomass of earthworms per  $\text{m}^2$  it would seem that *Microchaetus* sp. displayed a more sensitive response to copper oxychloride than *A. caliginosa*. Environmental conditions between the two localities however differed, making a direct comparison to explain these differences difficult.

The pH of exposure soils at both localities decreased significantly ( $p < 0.05$ ) after being sprayed with copper oxychloride (Table 24). *Microchaetus* might possibly be more sensitive to pH fluctuations than *A. caliginosa* thus accounting for the earlier response at population level. There is no information available on the general biology and physiology *Microchaetus* sp. making it difficult to compare the observed differences in response.

**Table 24** Comparative table of measured parameters and % change from the control in the copper oxychloride sprayed plots (4250  $\mu\text{g}\cdot\text{g}^{-1}$  per application) at the two different study areas (Nieuwoudtville and Vergenoegd) for *Microchaetus sp.* and *A. caliginosa* for the duration of the study [arrows = significant ( $p < 0.05$ ) increase ( $\uparrow$ ) or decrease ( $\downarrow$ ) in comparison to values from the control plots]; (OM = Organic Matter). X = Long-term assessment of earthworm populations 12 months (Nieuwoudtville) and 6 months (Vergenoegd) after spraying was stopped.  $p > 0.05$  = no statistically significant difference between control and exposure plots.

		Number of spraying applications		2	4	6	8	X
Soil physical parameters	Species	Nieuwoudt.	<i>Microchaetus sp.</i>					
		Vergenoegd	<i>A. caliginosa</i>					
	% Soil OM	Nieuwoudt.	6.4					
		Vergenoegd	6.2					
	% Silt	Nieuwoudt.	8.8					
		Vergenoegd	15.3					
	Mean rainfall (mm)	Nieuwoudt.	131					
		Vergenoegd	242					
	pH	Nieuwoudt.	4.9 (7% $\downarrow$ )	4.8 (12% $\downarrow$ )	4.7 (14% $\downarrow$ )	4.5 (18% $\downarrow$ )	5.5 ( $p > 0.05$ )	
		Vergenoegd	6.0 (7% $\downarrow$ )	5.8 (9% $\downarrow$ )	5.8 (9% $\downarrow$ )	-	6.4 ( $p > 0.05$ )	
$\text{Cu}_{\text{soil}}$ ( $\mu\text{g}\cdot\text{g}^{-1}$ )	Nieuwoudt.	21.0	29.8	46.2	58.6	21.6		
	Vergenoegd	(133% $\uparrow$ )	(259 $\uparrow$ )	(643% $\uparrow$ )	(623% $\uparrow$ )	(285% $\uparrow$ )		
Response level	Sub-organismal	NRRT (min)	Nieuwoudt.	56.6 (6% $\downarrow$ )	57.0 (5% $\downarrow$ )	-	-	-
			Vergenoegd	50.3 (19% $\downarrow$ )	50.0 (20% $\downarrow$ )	-	-	-
	Organismal	$[\text{Cu}]_{\text{worms}}$ ( $\mu\text{g}\cdot\text{g}^{-1}$ )	Nieuwoudt.	4.3	3.4	-	10.0	9.8
			Vergenoegd	( $p > 0.05$ )	( $p > 0.05$ )	-	(152% $\uparrow$ )	(65% $\uparrow$ )
	Population	Biomass/ $\text{m}^2$ (g)	Nieuwoudt.	101.9	43.6	-	2.95	37.5
			Vergenoegd	(35% $\downarrow$ )	(52% $\downarrow$ )	-	(78% $\downarrow$ )	(70% $\downarrow$ )
		Number/ $\text{m}^2$	Nieuwoudt.	92.0	51.2	-	9.6	32.0
			Vergenoegd	( $p > 0.05$ )	(57% $\downarrow$ )	-	(63% $\downarrow$ )	(57% $\downarrow$ )
			Nieuwoudt.	41.6	64.0	48.0	-	102.4
			Vergenoegd	( $p > 0.05$ )	( $p > 0.05$ )	( $p > 0.05$ )	-	(57% $\downarrow$ )

Copper concentrations in the soils from the two study areas were not significantly different ( $p > 0.05$ ) from each other (Table 24). The bioavailability of soil concentrations of copper could have differed due to the environmental differences. *Microchaetus sp.* from exposure plots accumulated significantly higher ( $p < 0.05$ ) copper concentrations in their body tissues after eight spraying applications (Figure 8; Table 7) and *A. caliginosa* after only two spraying applications (Figure 13, Table 13). Earlier accumulation of copper in *A. caliginosa* might possibly be explained by the fact that copper usually accumulates in the top 0-5 cm of grassland (Ma, 1988).

Although there is little known about the behaviour and ecology of *Microchaetus sp.* it was concluded that it is a deep burrower as judged from the depth at which they were found in the present study. *A. caliginosa* occurs closer to the soil surface, bringing the species closer to the soil surface and therefore to the copper oxychloride that was sprayed on the surface.

The silt content in Nieuwoudtville (8.8%) was lower than in Stellenbosch (15.3%) which also might have had an effect on copper bioavailability (Table 24). Abdul Rida and Bouché (1997) found a positive correlation ( $p < 0.001$ ) between silt content in soils and copper availability to earthworms. This might be another reason for the earlier accumulation of copper in the body tissues of *A. caliginosa*. The mean rainfall of 131 mm in Nieuwoudtville was also lower than the 242 mm in Stellenbosch during the study period (Table 24). Since earthworm populations are positively affected by high rainfall, earthworms in Nieuwoudtville may have experienced added stress in addition to toxic effects of copper oxychloride further explaining the earlier response at population level. It was however reported by Marinussen and Van der Zee (1997) that soil moisture has no effect on the copper uptake of the earthworm *Lumbricus rubellus*. The authors also concluded that soil temperature influences copper uptake, but this relationship has not been quantified.

There was no difference in the NRRT response of the worms from the two localities, as was found with earthworm biomass and numbers (Table 24). The reduction in neutral red retention times observed in the earthworms during the present study indicated stress resulting from exposure to copper oxychloride in the field. Although a reduction in earthworm numbers did not occur simultaneously, such a reduction did eventually occur. According to Moore et al (1982), the response of the neutral red retention assay to environmental contaminants occurs sooner at the subcellular level than at the physiological or other levels, making it a useful biomarker to serve as an early warning of environmental stress. That the reduction in neutral red retention times observed during the present study preceded the decline in worm numbers is an indication that exposure to copper oxychloride may have had longer term implications at the population level. Since the reduction in neutral red retention times could, in this case at least, be attributed to the presence of increased levels of copper, they were predictive of changes at the population level under field conditions. The results therefore confirm the findings of Svendsen and Weeks (1997a; 1997b) that the neutral red retention assay could be useful in environmental

risk assessment and through routine monitoring could provide a possible warning of impending ecological damage, at least at the population level. This could make it a reliable parameter for quick assessment of the impact of copper-based agrochemicals on earthworm populations. It is still necessary to verify these cellular responses with organismal and population responses since there is a paucity of data in ecotoxicological studies. The response in NRRT times however indicative of observed effects at the population level over the long term for both populations.

#### **4.3. Field experiments at Nietvoorbij (Stellenbosch), Robertson and Worcester**

To validate the data from the field experiments an assessment of earthworm populations in vineyards was done in Stellenbosch. Because these vineyards have been sprayed with copper oxychloride over many years, the results obtained could give an indication of the long-term effects that this chemical may have had on earthworms. It was found that the mean number and biomass of earthworms per m<sup>2</sup> in the grassland soils was significantly higher ( $p < 0.05$ ) than in the vineyard- and interrow soils (Figure 14; Table 14). The mean biomass of earthworms in the grassland soils was almost nine times that in the vineyard- and interrow soils. These results on biomass and numbers were related to the mean copper concentrations found in the soils and in the earthworm body tissues. The copper concentration found in grassland soils was 5.44  $\mu\text{g}\cdot\text{g}^{-1}$  which was significantly lower ( $p < 0.05$ ) than those found in the vineyard- and interrow soils which were 15.60  $\mu\text{g}\cdot\text{g}^{-1}$  and 11.62  $\mu\text{g}\cdot\text{g}^{-1}$  respectively (Figure 16; Table 14).

The mean copper concentrations in soils from vineyards in the Robertson and Worcester area were  $\pm 30 \mu\text{g}\cdot\text{g}^{-1}$  in vineyard and interrow soils and  $\pm 15 \mu\text{g}\cdot\text{g}^{-1}$  in grassland soils (refer 3.3.6). These are relatively low copper concentrations when compared to the results found in the acute toxicity tests (refer 3.4) and also those of Svendsen and Weeks (1997a) reported to have an effect on earthworm growth. These authors concluded that the threshold for short-term effects of copper lies between 80-121  $\mu\text{g}\cdot\text{g}^{-1}$  copper in substrates. The threshold for the effects of copper oxychloride on growth and reproduction of juvenile *E. fetida* was reported to be 8.92  $\mu\text{g}\cdot\text{g}^{-1}$  copper in substrates (Helling *et al.*, 2000). This latter finding can provide a possible explanation for the lower population densities and biomass in the vineyard- and interrow soils.

The mean copper concentrations found in the earthworm body tissues were linearly related to those found in the soils thus confirming the results of Ma (1982, 1988). Significantly lower ( $p < 0.05$ ) body copper concentrations were found in earthworms collected from grassland soils in comparison to those from the vineyard- and interrow soils (Figure 17; Table 14). The BCF of copper in earthworms from the grassland soil was 0.87, 0.97 in earthworms from the vineyard soil and 1.34 in earthworms from the interrow soils (Table 14). The pH values and organic matter content of the different soils (grassland vs. interrow vs. vineyard) did not differ significantly from each other (refer to 3.1.1). These parameters could therefore be excluded as factors having a severe influence on the studied populations.

It can be concluded from the presented data that the presence of elevated levels of copper, probably due to spraying of copper oxychloride, had detrimental effects on earthworm populations on the long term. It therefore supports the findings from the two field experiments in which earthworm populations were shown to be detrimentally affected (refer to 4.1 and 4.2). These results can give an indication of the possible effects spraying with copper oxychloride might have on the fertility and sustainability of soils, since soil fertility is closely linked to soil biota activity. These detrimental effects might have serious implications for sustainability of vineyard soils in Southern Africa. Because of the paucity of data on soil biota in South African agricultural soils, there is an urgent need for further studies on the implications of using pesticides, such as copper oxychloride.

#### 4.4. Acute toxicity tests (*Eisenia fetida*)

Substrates with a copper content of  $73.17 \mu\text{g.g}^{-1}$  ( $170 \mu\text{g.g}^{-1}$  copper oxychloride a.i.) used in the acute toxicity tests had no effect on the biomass of *E. fetida* (Figure 18, Table 15). This supports results of Svendsen and Weeks (1997a) who found that soil copper concentrations of up to  $80 \mu\text{g.g}^{-1}$  did not inhibit worm growth of adult *E. andrei* exposed to  $\text{CuCl}_2$ . Biomass was negatively affected by copper exposure concentrations of  $121.56\text{--}372.75 \mu\text{g.g}^{-1}$  (Figure 18, Table 15). This suggests that the threshold for short-term effects of copper on biomass of adult earthworms of this species lies between 80 (Svendsen and Weeks, 1997a) and  $121.56 \mu\text{g.g}^{-1}$  (present study) copper in substrates. However, these results were obtained in laboratory experiments under controlled conditions using substrates that are quite

different from field soils and different earthworm species. The results of the field studies (refer to 3.1 and 3.2) seem to indicate that other earthworm species (*A. caliginosa* and *Microchaetus*) may be affected at much lower concentrations.

NRRT of earthworm coelomocytes decreased significantly ( $p < 0.05$ ) from day seven onwards for copper substrate concentrations ranging from 73.17-372.75  $\mu\text{g}\cdot\text{g}^{-1}$  (Figure 19, Table 16). Svendsen and Weeks (1997a) reported that the threshold for toxic effects of copper contaminated soils on lysosomal membrane stability lies between 40-80  $\mu\text{g}\cdot\text{g}^{-1}$  for *E. andrei* after 28 days of  $\text{CuCl}_2$  exposure. The data obtained in the present study indicates that this effect already manifests itself in less than seven days after exposure in *E. fetida*. NRRT gave an earlier indication of the effects of copper exposure (Svendsen and Weeks, 1997a) in comparison to biomass change. Our data supports this conclusion since the biomass (Figure 18; Table 15) of earthworms with body burdens of copper between 16.69-24.21  $\mu\text{g}\cdot\text{g}^{-1}$  (Figure 22, Table 18) showed no reduction, while lysosomal membrane stability was negatively affected (Figure 19; Table 16).

The calculated  $\text{LC}_{50}$  for copper oxychloride of 882.78  $\mu\text{g}\cdot\text{g}^{-1}$  is in agreement with Heimbach (1985) who tested the toxicity of Cu-oxychloride (45% a.i.) on *E. fetida* over a period of 14 days in an artificial substrate. It was concluded that the  $\text{LC}_{50}$  of "about 900  $\mu\text{g}\cdot\text{g}^{-1}$ " was "unclear". Kokta (1992) reported that pesticides with an  $\text{LC}_{50}$  higher than 1000  $\mu\text{g}\cdot\text{g}^{-1}$  should be considered as harmless to earthworms in the field. If this was the case, the conclusion might be drawn that copper oxychloride has little or no effect on earthworm populations in the field. From the previous field studies (refer 4.1 and 4.2) it was experimentally shown that spraying with copper oxychloride at recommended dosages had a negative effect on earthworm populations in the field. Helling *et al.* (2000) exposed juveniles of *E. fetida* over a period of eight weeks to a range of copper oxychloride concentrations and concluded that it might have an effect on earthworm populations because of the effects on growth and reproduction at relatively low concentrations.

The reasons for the standardised acute toxicity test having "low" ecological relevance with regard to copper oxychloride are manifold. One reason may be the lack of sensitivity of *E. fetida*, although it is prescribed as test species by the OECD (1984). In a review on the sensitivity of different earthworm species to contaminants Edwards and Coulson (1992) found *E. fetida* to be the least sensitive. They proposed that a correction factor of 10 would bring *E. fetida* in line with more sensitive species.

If this factor would be applied to the copper oxychloride results, copper levels of 52  $\mu\text{g}\cdot\text{g}^{-1}$  in the form of copper oxychloride would affect more sensitive earthworm species. In the field studies conducted on the effect of copper oxychloride on earthworm populations, copper in soils was respectively  $\pm 60 \mu\text{g}\cdot\text{g}^{-1}$  (Nieuwoudtville) and  $\pm 50 \mu\text{g}\cdot\text{g}^{-1}$  (Stellenbosch). In both these studies it was found that spraying with copper oxychloride had negative effects on earthworm populations. The presented data supports the conclusion that a safety factor of 10 would bring *E. fetida* in line with more sensitive earthworm species, and should therefore be considered in risk assessment studies.

Another reason for the "low" ecological relevance of acute toxicity tests may be the physical composition of the substrates used which differed from soils in the field. The texture, organic matter content and seasonal parameters are all factors that can contribute to these differences. The pH of the substrates utilised in the acute toxicity tests was adjusted to  $\pm 7$  which is not ecologically realistic since the pH of soils in the field tests could not be adjusted homogeneously. This may be important, since pH influences copper bioavailability to earthworms (Ash and Lee, 1980; Ma, 1988; Abdul Rida and Bouché, 1997). The pH of  $\pm 7$  in the acute toxicity tests was higher than that observed in the field experiments. In view of the fact that copper availability decreases with increasing pH (Ma, 1988) this may have resulted in an underestimation of the effects of copper based agrochemicals thus lowering the ecological relevance of these findings. It should therefore be considered to conduct standardised acute toxicity tests using a range of pH values for replicate concentrations in order to obtain greater relevancy.

Other factors that might contribute to the difference between results obtained in the laboratory and the field are temperature, moisture content and organic matter content. The acute toxicity tests were conducted under optimum conditions for growth and reproduction regarding *E. fetida* as test species. Moisture content and temperature were at optimal levels as suggested by Reinecke and Venter (1985) and Reinecke *et al.* (1992). Marinussen and Van der Zee (1997) concluded that soil moisture content has no influence on copper uptake by *L. rubellus*. On the other hand the authors found that soil temperature does affect copper uptake, although there is no data available to quantify this effect. There is a strong relationship between organic matter content of substrates and copper availability. Because soil organic matter binds  $\text{Cu}^{2+}$  ions strongly, an increase in organic matter will decrease copper



availability (Ma, 1982). The standardised organic matter content of 10% used in the acute toxicity tests was higher than in the field experiments (6.4% in Nieuwoudtville and 6.2% in Vergenoegd) with the result that copper availability in the laboratory might have been lower than in the field.

Earthworms in the field are also exposed to low concentrations of copper in soils over a longer period than earthworms in laboratory tests. Since acute toxicity tests do not take the duration of exposure and organismal uptake of contaminants into consideration, it makes it more difficult to link these results to those from the field. In view of this, acute toxicity tests should be followed with sublethal tests over a longer period (more than one life cycle) to take this temporal variable into account. Care should also be taken in interpreting of  $LC_{50}$  test results obtained in the laboratory using artificial soil and using *E. fetida* as test species. The  $LC_{50}$  in the present study could not be considered to be ecologically relevant for copper oxychloride unless a correction factor of 10 or more is applied to extrapolate to other more sensitive species. Changes in biomass appear to be a more ecologically sensitive parameter than  $LC_{50}$ , since reproduction and bodyweight are closely related (Kokta, 1992). However, changes in biomass using adult earthworms could be misleading since populations in the field consists of earthworms from different age groups, which will be affected differently.

NRRT results seems to have higher ecological relevance than mortality and growth, when viewed against the background of previous findings on responses in field populations. Svendsen and Weeks (1997a) reported that the threshold for effects on lysosomal membrane stability lay between 40-80  $\mu\text{g}\cdot\text{g}^{-1}$  copper in soils. In the present study copper concentrations in the soils ranged from 21.01-58.67  $\mu\text{g}\cdot\text{g}^{-1}$  in Nieuwoudtville and 8.48-46.74  $\mu\text{g}\cdot\text{g}^{-1}$  in Vergenoegd respectively, and these resulted in altered NRRT values as well as population declines. This gives further support to the conclusion that an early threshold of effects can be obtained with the NRRT assay. It is therefore suggested that NRRT assays be undertaken when testing the toxicity of agrochemicals (containing copper) and that these be done in conjunction with sublethal tests on the effects on reproduction. This would give more ecologically relevant results than acute toxicity tests with mortality as the main endpoint.

#### 4.5. Bioassays of soils with *Aporrectodea caliginosa*

From the results it is evident that earthworms exposed to copper oxychloride contaminated soils ( $60 \mu\text{g}\cdot\text{g}^{-1}$  copper) showed a significant decrease ( $p < 0.05$ ) in biomass ( $-50.14 \pm 10.26 \%$ ) after 28 days of exposure (Figure 23, Table 20). Earthworms exposed to grassland- and vineyard soils both showed an increase in biomass ( $5.03 \pm 4.62 \%$  and  $3.79 \pm 19.51 \%$  respectively) although the increase was not statistically significantly different ( $p > 0.05$ ) from the initial weight of the earthworms (Figure 23, Table 20). This supports the conclusion drawn from the acute toxicity tests which indicated that change in biomass as measured endpoint in adult earthworms is not ecologically relevant. No mortality was observed in *A. caliginosa* exposed to grassland soil and only a  $3.33 \pm 5.77 \%$  mortality rate in earthworms exposed to vineyard soil (Figure 24; Table 21). In earthworms exposed to copper oxychloride contaminated soil a mortality rate  $66.67 \pm 20.87 \%$  was observed which is significantly higher ( $p < 0.05$ ) than that found in both the grassland- and vineyard soils (Figure 24; Table 21).

The responses of *A. caliginosa* using changes in biomass and percentage mortality as endpoints corresponded closely with the copper concentrations found in the soils and earthworm body tissues. The mean copper concentration in the copper oxychloride contaminated soil was  $55.43 \pm 2.85 \mu\text{g}\cdot\text{g}^{-1}$ , which was significantly higher ( $p < 0.05$ ) than the  $6.10 \pm 0.16 \mu\text{g}\cdot\text{g}^{-1}$  of copper in the grassland soil and the  $14.38 \pm 2.78 \mu\text{g}\cdot\text{g}^{-1}$  in the vineyard soil (Figure 25; Table 21). A similar trend was observed in the copper concentrations found in earthworm body tissues. Earthworms exposed to copper oxychloride contaminated soil had a mean copper concentration of  $28.19 \pm 2.49 \mu\text{g}\cdot\text{g}^{-1}$  in their body tissues. This was significantly higher ( $p < 0.05$ ) than the  $4.28 \pm 0.77 \mu\text{g}\cdot\text{g}^{-1}$  of copper found in earthworms exposed to grassland soil and  $10.36 \pm 1.02 \mu\text{g}\cdot\text{g}^{-1}$  in earthworms exposed to vineyard soil (Figure 26; Table 21). There may be a possible relationship between the significant ( $p < 0.05$ ) decrease in biomass and the increase in observed mortality. The significant increase in copper concentrations of soils and earthworm body tissues affected growth which, in turn, decreases populations as suggested by Klok *et al.* (1997). This might explain the effects of copper oxychloride on earthworm populations in the field studies conducted in Nieuwoudtville and Vergenoegd. At both these locations the mean biomass- and number of earthworms per  $\text{m}^2$  decreased significantly ( $p < 0.05$ ) after soils were sprayed with copper oxychloride. The copper concentrations found in the

Nieuwoudtville soils (max. of  $58.67 \pm 2.49 \mu\text{g.g}^{-1}$ ) and Vergenoegd (max. of  $46.74 \pm 18.89 \mu\text{g.g}^{-1}$ ) are similar to that found in the copper oxychloride contaminated soils in the bioassay ( $55.43 \pm 2.85 \mu\text{g.g}^{-1}$ ), (see also Table 24).

Changes in biomass and mortality as endpoints in bioassays using *A. caliginosa* seems to be ecologically relevant, since they could be related to responses observed in the field. Change in biomass, as endpoint in bioassays could therefore give a possible indication of biomass responses of field populations of earthworms. In the same regard mortality could give an indication of the density of field populations of earthworms.

#### 4.6. Burrow activity and avoidance response

The burrowing activity of earthworms exposed to vineyard soils containing increased copper concentrations as well as copper oxychloride contaminated soil was significantly lower ( $p < 0.05$ ) than the burrowing activity of worms in grassland soils (Table 22). The tunnelling activity of earthworms, measured as the length of the burrows of earthworms in the vineyard soil, was 44% of that in the grassland soil (set at 100%); the burrowing activity of earthworms in the copper oxychloride soil was 58%. A possible reason for the lower burrowing activity observed in earthworms exposed to soils with elevated copper levels may be due to copper accumulation in the nervous tissues of earthworms (Bengtsson *et al.*, 1986) which could, in turn, lead to behavioural disturbances.

The soil avoidance experiments showed that *A. caliginosa* could "detect" and avoid vineyard soil containing increased copper concentrations. The same was true when earthworms were given a choice between grassland- and copper oxychloride contaminated soil. The burrow distance of earthworms was significantly higher ( $p < 0.05$ ) in grassland soil ( $37.75 \pm 22.54$  cm) than in vineyard soil ( $5.49 \pm 7.52$  cm); (Table 22). The burrow distance of earthworms given a choice between grassland- ( $44.95 \pm 22.65$  cm) and copper oxychloride contaminated soil ( $10.11 \pm 8.87$  cm) was significantly higher ( $p < 0.05$ ) in the grassland soil (Table 22).

The mechanisms by which earthworms can detect metals in soil are not very well understood. Hopkin (1989) concluded that some terrestrial invertebrates cannot detect the levels of metals in their food. Chemoreceptors that respond to specific metals have not been discovered on the mouthparts or in the gut of any animal.

Instead, physiological mechanisms have evolved which are able to regulate the concentrations of essential and non-essential metals. The palatability of contaminated soils has been mentioned by Depta *et al.* (1999) who found that *Lumbricus terrestris* preferred unpolluted leaves to those contaminated with copper and zinc. There is thus a need to examine the physiological basis on how earthworms are able to detect and avoid certain contaminants.

The lower burrowing activity and soil avoidance of earthworms exposed to the vineyard and copper oxychloride contaminated soils correlated with the significantly higher ( $p < 0.05$ ) copper concentrations in these soils. (Table 22). The soil copper concentrations in the vineyard soils used in the burrowing activity and soil avoidance experiments were similar to that found in vineyard soils in the field inventories at Nietvoorbij ( $\pm 15 \mu\text{g.g}^{-1}$ ), Robertson ( $\pm 30 \mu\text{g.g}^{-1}$ ) and Worcester ( $\pm 30 \mu\text{g.g}^{-1}$ ). Further, the soil copper concentrations used in copper oxychloride contaminated soil in the burrowing activity and soil avoidance experiments were similar to those found in soils from the field exposure plots at Nieuwoudtville (max.  $58.67 \pm 2.49 \mu\text{g.g}^{-1}$ ) and Vergenoegd ( $46.74 \pm 18.89 \mu\text{g.g}^{-1}$ ). This would suggest that should earthworms in the field be exposed to elevated copper levels in soils ( $\pm 50 \mu\text{g.g}^{-1}$ ) resulting from spraying, they would have much lower burrowing activities and could be expected to display avoidance behaviour towards these soils. In the field this would affect migration and colonisation by earthworms from adjacent soils (Ma, 1988), as well as soil turnover. The combined effects of soil avoidance and decreased burrowing activity would cause a strong and sustained decline of lumbricid populations at copper polluted sites (Ma, 1988).

Earthworms play several ecological roles such as the upkeep of soil fertility by fragmenting and mixing organic and inorganic matter, promotion of microbial activity, maintenance of soil structure, aeration and drainage (Ash and Lee, 1980; Bengtsson *et al.*, 1988; Edwards and Bate, 1992; Edwards and Bohlen, 1996). A reduction in earthworm feeding activity leads to an accumulation of undecomposed leaf litter (Hopkin, 1989) and this phenomenon is coupled to the beginning of soil degeneration as observed by Van de Westering (1972) in worm free orchard soils. Copper oxychloride contamination at the levels that was observed in this study could therefore have significant effects on decomposition processes and soil fertility which in turn will impact on the sustainable use of agricultural soils.

Taking the field observations into account, it can be concluded that a decrease in the burrowing activity of field populations of earthworms in the Nieuwoudtville and Vergenoegd sites exposed to copper oxychloride, might have occurred before effects at the population level were manifested. Burrowing activity could be an ecologically relevant parameter when assessing the effects of copper based agrochemicals on field populations of earthworms. The benefit of using the burrowing activity of *A. caliginosa* as an ecotoxicological tool in risk assessment lies in the fact that the method is relatively quick (four days) and easy to apply. Burrowing activity and soil avoidance responses were more sensitive than on growth and mortality data obtained in the bioassay experiments. The reason for this is that earthworms exposed to vineyard soil in the bioassay did not show significant ( $p > 0.05$ ) responses (with change in biomass and mortality as endpoints), while those observed in burrowing activity and avoidance response did. This is in agreement with the findings of Wentsel and Guelta (1988) who concluded that avoidance could be a sensitive measure of the sublethal effects of chemicals applied to soils.

Taking all these factors into consideration, it can be concluded that decreased burrowing activity and soil avoidance due to copper oxychloride spraying might have important implications for soil fertility and structure. Since earthworms play such an important ecological role in many soils, an impact on their presence and activity will affect important functions in the soil ecosystem. The challenge for land users in the agricultural sector is to take these factors into consideration when making management decisions on agricultural practices if long term sustainable use of soils is to be achieved.

## CHAPTER 5

---

### *CONCLUSIONS...*

- ❶ It can be concluded that spraying with copper oxychloride at recommended application rates caused a decrease in soil pH, which increased copper availability in soils. These increased copper concentrations in soils resulted in increased copper uptake in earthworms which, in turn, decreased earthworm population numbers in the studied South African soils. Further, NRRT is a reliable biomarker of exposure and effect if used with the necessary controls. It provides a quick assessment of stress resulting from the use of copper-based agrochemicals and is indicative of long-term effects observed at the population. It could also have a role in environmental risk assessment and routine monitoring because it could provide a sufficiently accurate warning of impending ecological damage, at least at the population level.
- ❷ Field populations of earthworms in vineyards that have been exposed to copper oxychloride spraying over the long term, were detrimentally affected. It is concluded that the results obtained from the field inventory of earthworms in vineyards also validated the data obtained from the experimental field studies in Nieuwoudtville and Vergenoegd.
- ❸ The determination of an  $LC_{50}$  for *E. fetida* in the present study cannot be considered ecologically relevant for copper oxychloride exposure unless a safety factor of 10 or more is applied to extrapolate to other more sensitive species. Further, NRRT results generated during the laboratory and field tests were of higher ecological relevance than results obtained on mortality and growth in the laboratory when viewed against the background of responses of field populations of earthworms.
- ❹ Results obtained from the bioassays indicated that change in biomass and mortality as measured endpoints could be ecologically relevant. These responses were indicative of population and other responses observed in the field studies.

- ⑤ Increased soil copper concentrations resulting from copper oxychloride contamination caused a decrease in burrowing activity of earthworms and also resulted in soil avoidance behaviour. Burrowing activity and soil avoidance were ecologically relevant endpoints, since they corresponded with population responses in the field.

## CHAPTER 6

---

### *REFERENCES...*

- Abdul Rida, A.M.M., and Bouché, M.B. (1997) Heavy metal linkages with mineral, organic, and living soil compartments. *Soil Biol. Biochem.* **29**(3), 649-655.
- Amorim, M.J., Sousa, J.P., Nogueira, A.J.A., and Soares, A.M.V.M. (1999) Comparison of chronic toxicity of Lindane ( $\gamma$ -HCH) to *Enchytraeus albidus* in two soil types: the influence of soil pH. *Pedobiologia* **43**, 635-640.
- Arendse, W., Den Braber, K., Van Halder, I., Hoogerbrugge, I., Kramer, M. and Van der Valk Wageningen, H. (1989) Pesticides. Compounds use and hazards. Agrimosa, The Netherlands.
- Ash, C.P.J., and Lee, D.L. (1980) Lead, cadmium and iron in earthworms from roadside sites. *Environ. Pollut. Ser. A* **22**, 59-67.
- Babich, H., and Borenfreund, E. (1990) Applications of the neutral red cytotoxicity assay to in vitro toxicology. *ATLA* **18**, 129-144.
- Bembridge, J.D. (1998) Recommendations from the Second International Workshop on Earthworm Ecotoxicology, Amsterdam, Netherlands (April 1997). In *Advances in earthworm ecotoxicology* (S. Sheppard, J. Bembridge, M. Holmstrup, and L. Posthuma, Eds.), pp. 389-398. SETAC Press.
- Bengtsson, G., Gunnarsson, T., and Rundgren, S. (1986) Effects of metal pollution on the earthworm *Dendrobaena rubida* (Sav.) in acidified soils. *Water Air Soil Pollution* **28**, 361-383.
- Bengtsson, G., Berden, M., and Rundgren, S. (1988) Influence of soil animals and metals on decomposition processes: A microcosm experiment. *J. Environ. Qual.* **17**(1), 113-119.



- Bouché, M.B. 1992. Earthworm species and ecotoxicological studies. In *Ecotoxicology of earthworms* (P.W. Greig-Smith, H. Becker, P.J. Edwards and F. Heimbach, eds.), pp. 20-35, Intersept, UK.
- Cook, N. and Hendershot, W.H. (1996) The problem of establishing ecologically based soil quality criteria: The case of lead. *Can. J. Soil Sci.* **76**(3), 335-342.
- De Klerk, C.A. (1988) Programbespuiting vir die beheer van witroes, donsskimmel en Botrytis. *Wynboer Junie*, 8-12.
- De Peyster, A., and Long, W.F. (1993) Fathead minnow optomotor response as a behavioral endpoint in aquatic toxicity testing. *Bull. Environ. Contam. Toxicol.* **51**, 88-95.
- Depledge, M.H., and Fossi, M.C. (1994) The role of biomarkers in environmental assessment. *Ecotoxicology* **3**, 161-172.
- Depledge, M.H., Weeks, J.M., and Bjerregaard, P. 1994. Heavy metals. In *Handbook of ecotoxicology* (P. Calow, ed.), Vol. II Ch. 5, Blackwell Scientific Publications, Oxford.
- Depta, B., Koscielniak, A., and Rozen, A. 1999. Food selection as a mechanism of heavy metal resistance in earthworms. *Pedobiologia* **43**, 608-614.
- Edwards, C.A. (1980) Interactions between agricultural practice and earthworms. In *Soil biology as related to and use practices. Proceedings of the VII International Colloquium of Soil Zoology* (D.L., Dindal, ed), pp. 3-12. Office of Pesticide and Toxic Substances, EPA, Washington, D.C.
- Edwards, C.A. (1992) Testing the effects of chemicals on earthworms-the advantages and limitations of field tests. In *Ecotoxicology of earthworms* (P.W. Greig-Smith, H. Becker, P.J. Edwards and F. Heimbach, eds.), pp. 75-84, Intersept, UK.

- Edwards, C.A. (1993) The impact of pesticides on the environment. In *The pesticide question. Environment, Economics and Ethics* (D. Pimental, and H. Lehman, eds.), Chapman and Hall, New York.
- Edwards, C.A., and Bater, J.E. (1992) The use of earthworms in environmental management. *Soil. Biol. Biochem.* **24**(12), 1683-1689.
- Edwards, C.A., and Bohlen, P.J. (1992) The effects of toxic chemicals on earthworms. *Rev. Environ. Contam. Toxicol.* **125**, 23-99.
- Edwards, C.A., Bohlen, P.J., Linden, D.R., and Subler, S. (1995) Earthworms in agroecosystems. In *Earthworm ecology and biogeography* (P.H. Hendrix, ed.), pp. 185-213, Lewis Publishers, Boca Raton.
- Edwards, C.A., and Bohlen, P.J. (1996) *Biology and ecology of earthworms*. 3rd edn, Chapman and Hall, London.
- Edwards, P.J., and Coulson, J.M. (1992) Choice of earthworm species for laboratory tests. In *Ecotoxicology of earthworms* (P.W. Greig-Smith, H. Becker, P.J. Edwards and F. Heimbach, eds.), pp. 36-43, Intercept, UK.
- Eijsackers, H. (1987) The impact of heavy metals on terrestrial ecosystems: Biological adaptation through behavioural and physiological avoidance. In: *Ecological assessment of environmental degradation, pollution and recovery* (O. Ravera, ed.), pp. 245-259, Elsevier Science, The Netherlands.
- Everts, J. (1990) Why are field trials necessary? In *Pesticide effect on terrestrial wildlife* (L. Somerville, C.H. Walker, eds.), pp. 1-3, Taylor and Francis, London.
- Giamberini, L., and Pihan, J.P. (1997) Lysosomal changes in the hemocytes of the freshwater mussel *Dreissena polymorpha* experimentally exposed to lead and zinc. *Diseases of Aquatic Organisms* **28**, 221-227.

- Heimbach, F. (1985) Comparison of laboratory methods, using *Eisenia foetida* and *Lumbricus terrestris*, for the assessment of the hazard of chemicals to earthworms. *J. Plant Diseases and Protection* **92**(2), 186-193.
- Heimbach, F. (1992) Effects of pesticides on earthworm populations: Comparison of results from laboratory and field tests. In: Greig-Smith, P.W., Becker, H., Edwards, P.J., Heimbach F. (Eds.), *Ecotoxicology of earthworms*. Intersept, UK, pp. 100-106.
- Heimbach, F. (1998) Comparison of the sensitivities of an earthworm (*Eisenia foetida*) reproduction test and a standardized field test on grassland. In: *Advances in earthworm ecotoxicology* (S. Sheppard, J. Bembridge, M. Holmstrup, and L. Posthuma, Eds.), pp. 235-245. SETAC Press.
- Helling, B., Reinecke, S.A., and Reinecke, A.J. (2000) Effects of the fungicide copper oxychloride on the growth and reproduction of *Eisenia fetida* (Oligochaeta). *Ecotoxicol. Environ. Saf.* **46**, 108-116.
- Hendrix, P.H. (1998) Earthworms in agroecosystems: A summary of current research. In: *Earthworm ecology* (C.A. Edwards, ed.) pp . CRC Press, USA.
- Hopkin, S.P. (1989) *Ecophysiology of metals in terrestrial invertebrates*. Elsevier Applied Science, London.
- Katz, S.A., and Jennis, S.W. (1983) *Regulatory compliance monitoring by atomic absorption spectroscopy*. Verlag Chemie International, Florida.
- Klok, C., De Roos, A.M., Marinissen, J.C.Y., Baveco, H.M. and Ma, W. (1997) Assessing the effects of abiotic environmental stress on population growth in *Lumbricus rubellus* (Lumbricidae, Oligochaeta). *Soil Biol. Biochem.* **29**, 287-293.

- Kokta, C. (1992) Measuring effects of chemicals in the laboratory: Effect criteria and endpoints. In *Ecotoxicology of earthworms* (P.W. Greig-Smith, H. Becker, P.J. Edwards and F. Heimbach, eds.), pp. 55-62. Intersept, UK
- Krause, M., Nel, A., and Ramautar, N. (1996) A guide to the use of pesticides and fungicides in the Republic of South Africa. Department of Agricultural Development, Pretoria.
- Kula, H. (1992) Measuring the effects of pesticides on earthworms in the field-test design and sampling methods. In *Ecotoxicology of earthworms* (P.W. Greig-Smith, H. Becker, P.J. Edwards and F. Heimbach, eds.), pp. 90-99. Intersept, UK
- Kula, C. (1994) A prolonged laboratory test on sublethal effects of pesticides on *Eisenia fetida*. In *Ecotoxicology of soil organisms* (M.H. Donker, H. Eijsackers, and F. Heimbach, eds.), pp 257-262. Lewis, Boca Raton, FL.
- Kula, C. (1998) Endpoints in laboratory testing with earthworms: experience with regard to regulatory decisions for plant protection products. In *Advances in earthworm ecotoxicology* (S. Sheppard, J. Bembridge, M. Holmstrup, and L. Posthuma, Eds.), pp. 3-14, SETAC Press.
- Kula, H., and Larink, O. (1998) Tests on the earthworms *Eisenia fetida* and *Aporrectodea caliginosa*. In *Handbook of soil invertebrate toxicity tests* (H. Lokke, and C.A.M. van Gestel, eds.), pp , John Wiley and Sons, New York.
- Lee, K.E. (1985) Earthworms: Their ecology and relationships with soils and land use, Academic Press, Sydney.
- Lofs, A. (1992) Measuring effects of earthworms in the field: Effect criteria and endpoints. In: *Ecotoxicology of earthworms* (P.W. Greig-Smith, H. Becker, P.J. Edwards and F. Heimbach eds.), pp. 85-89. Intersept, UK.

- London, L., and Meyers, J. (1995) General patterns of agrichemical usage patterns in the southern region of South Africa. *S. Afr. J. Sci.* **91**, 509-514.
- Lowe, D.M., Moore, M.N., and Evans, B.M. (1992) Contaminant impact on the interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. *Marine Ecology Progress Series* **91**, 135-140.
- Lukens, R.J. (1971) Molecular biology, biochemistry and biophysics-chemistry of fungicidal action, Vol. 10. Springer-Verlag, Berlin.
- Ma, W. (1982) The influence of soil properties and worm-related factors on the concentration of heavy metals in earthworms. *Pedobiologia* **24**, 109-119.
- Ma, W. (1988) Toxicity of copper to lumbricid earthworms in sandy agricultural soils amended with Cu- enriched organic waste materials. *Ecological Bulletins* **39**, 53-56.
- Malkomes, H.P. (1997) Applications of ecotoxicity tests to assess side effects of pesticides in soils. In *Soil Ecotoxicology* (J. Tarradellas, G. Bitton, and D. Rossel, eds.), pp 319-343. Lewis Publishers, Boca Raton.
- Marinussen, M.P.J.C. and Van der Zee, S.E.A.T.M. (1997) Cu accumulation by *Lumbricus rubellus* as affected by total amount of Cu in soil, soil moisture and soil heterogeneity. *Soil Biol. Biochem.* **29**, 641-647.
- Martin, H. (1973) The scientific principles of crop protection. 6th edition, Edward Arnold (Publishers) Ltd., London.
- Moore, M.N. (1985) Cellular responses to pollutants. *Mar. Poll. Bull.* **16**, 134-139.
- Moore, M.N. (1990) Lysosomal cytochemistry in marine environmental monitoring. *Histochemical Journal* **22**, 187-191.

- Moore, M.N., Pipe, R.K., Farrar, S.V. (1982) Lysosomal and microsomal responses to environmental factors in *Littorina littorea* from Sullom Voe. *Mar. Poll. Bull.* **13**, 340-345.
- Morgan, J.E., and Morgan, A.J. (1998) Earthworms as biological monitors of cadmium, copper, lead and zinc in metalliferous soils. *Environ. Pollut.* **54**, 123-128.
- Morgan, J.E., Morgan A.J., and Corp, N. (1992) Assessing soil metal pollution with earthworms: Indices derived from regression analyses. In *Ecotoxicology of earthworms* (P.W. Greig-Smith, H. Becker, P.J. Edwards and F. Heimbach, eds.), pp. 233-237, Intersept, UK.
- Morgan, A.J., Stürzenbaum, S.R., and Kille, P. (1999) A short overview of molecular biomarker strategies with particular regard to recent developments in earthworms. *Pedobiologia* **43**, 574-584.
- Neuhauser, E.F., Cukic, Z.V., Malecki, M.R., Loehr, R.C., and Durkin, P.R. (1995) Bioconcentration and biokinetics of heavy metals in the earthworm. *Environ. Poll.* **89**(3), 293-301.
- Nimmo, D.R., and McEwen, L.C. (1994) Pesticides. In *Handbook of ecotoxicology* (P. Calow, ed.), pp 155-203. Blackwell Scientific Publications, London.
- OECD, 1984. Guideline for the testing of chemicals no. 207. Earthworm acute toxicity tests.
- Paoletti, M.G. (1999) The role of earthworms for assessment of sustainability and as bioindicators. *Agric. Ecosys. Environ.* **79**, 137-155.

- Pascoe, D., Gower, D.E., McCahon, C.P., Poulton, M.J., Whiles, A.J. and Wulffhorst, J. (1991) Behavioural responses to pollutants - Application in freshwater bioassays. In *Bioindicators and environmental management* (D.W. Jeffrey, and B. Madden, eds.), pp. 45-254, Academic Press.
- Peakall, D. (1994) Animal biomarkers as pollution indicators. (M.H. Depledge, and B. Sanders, Eds.). Chapman and Hall, London.
- Pizl, V., and Josens, G. (1995) Earthworm communities along a gradient of urbanization. *Environ. Pollut.* **90**(1), 7-14.
- Pugh, G.J.F., Williams, J.I., and Wainwright, M. (1975) The effects of fungicides on microbial activities in the soil. In *Progress in Soil Zoology* (J. Vanek, ed.), pp. 489-496, Academia Publishing House, Prague.
- Reinecke, A.J. (1992) Review of ecotoxicological test methods using earthworms. In: *Ecotoxicology of earthworms*, (P.W. Greig-Smith, H. Becker, P.J. Edwards and F. Heimbach, eds.), pp. 7-19. Intercept, UK.
- Reinecke, A.J., and Venter, J.M. (1985) The influence of moisture on the growth and reproduction of the compost worm *Eisenia fetida* (Oligochaeta). *Rev. Ecol. Biol. Soil* **22**, 473-481.
- Reinecke, A.J., Viljoen, S.A. and Saayman, R.J. (1992) The suitability of *Eudrilus eugeniae*, *Perionyx excavatus* and *Eisenia fetida* (Oligochaeta) for vermicomposting in Southern Africa in terms of their temperature requirements. *Soil Biol. Biochem.* **24**(12), 1295-1307.
- Reinecke, S.A., and Reinecke, A.J. (1999) Lysosomal response of earthworm coelomocytes induced by long-term experimental exposure to heavy metals. *Pedobiologia* **43**, 585-593.

- Scott-Fordsmand, J.J., and Weeks, J.M. (1998) Review of selected biomarkers in earthworms. In: *Advances in earthworm ecotoxicology* (S. Sheppard, J. Bembridge, M. Holmstrup, and L. Posthuma, Eds.), pp. 173-189. SETAC Press.
- Shane, B.S. (1994) Principles of ecotoxicology. In: *Basic environmental toxicology* (L.G. Cockerham and B.S. Shane, eds.), Ch. 2, CRC Press, Florida.
- Simms, R.W. and Gerard, B.M. (1985) Earthworms. Brill and Backhuys, London.
- Slimak, K.M. (1997) Avoidance response as a sublethal effect of pesticides on *Lumbricus terrestris* (Oligochaeta). *Soil. Biol. Biochem.* **29**, 713-715.
- Somerville, L. (1990) Introduction. In *Pesticide effect on terrestrial wildlife* (L. Somerville, C.H. Walker, eds.), pp. 1-3, Taylorand Francis, London.
- Spurgeon, D.J., and Hopkin, S.P. (1995) Extrapolation of the laboratory-based OECD earthworm toxicity test to metal-contaminated field sites. *Ecotoxicology* **4**, 190-205.
- Stenersen, J., Brekke, E., and Engelstad, F. (1992) Earthworms for toxicity testing; species differences in response towards cholinesterase inhibiting insecticides. *Soil Biol. Biochem.* **24**(12), 1761-1764.
- Stephenson, G.L., Kaushik, A., Kaushik, W.K., Solomon, K.R., Steele, T., and Scroggins, R.P. (1998) Use of an avoidance-response test to assess the toxicity of contaminated soils to earthworms. In: *Advances in earthworm ecotoxicology* (S. Sheppard, J. Bembridge, M. Holmstrup, and L. Posthuma, Eds.), pp. 67-81. SETAC Press.
- Sternlieb, I., and Goldfischer, S. (1976) Heavy metals and lysosomes. In *Lysosomes in biology and pathology* (J.T. Dingle, and R.T. Dean, eds.), Vol. 5, North Holland Publishing Company, Amsterdam.



- Stürzenbaum, S.R., Kille, P., and Morgan, A.J. (1998) Identification of new heavy-metal-responsive biomarkers in the earthworm. In: *Advances in earthworm ecotoxicology* (S. Sheppard, J. Bembridge, M. Holmstrup, and L. Posthuma, Eds.), pp. 215-224. SETAC Press.
- Svendsen, C., and Weeks, J.M. (1997a) Relevance and applicability of a simple earthworm biomarker of copper exposure. I. Links to ecological effects in a laboratory study with *Eisenia andrei*. *Ecotoxicol. Environ. Saf.* **36**, 72-79.
- Svendsen, C., and Weeks, J.M. (1997b) Relevance and applicability of a simple earthworm biomarker of copper exposure. II. Validation and applicability under field conditions in a mesocosm experiment with *Lumbricus rubellus*. *Ecotoxicol. Environ. Saf.* **36**, 80-88.
- Tarradellas, J., and Bitton, G. (1997) Chemical pollutants in soils. In *Soil Ecotoxicology* (J. Tarradellas, G. Bitton, and D. Rossel, eds.), pp 3-32. Lewis Publishers, Boca Raton.
- Thompson, W.T. (1978) *Agricultural chemicals, Book IV. Fungicides*. Thompson, Fresno.
- Tomlin, A.D. (1992) Behaviour as a source of earthworm susceptibility to ecotoxicants. In *Ecotoxicology of earthworms* (P.W. Greig-Smith, H. Becker, P.J. Edwards and F. Heimbach, eds.), pp. 116-125, Intercept, UK.
- Turner, L.W. (1990) Objectives of terrestrial field studies. In *Pesticide effect on terrestrial wildlife* (L. Somerville, C.H. Walker, eds.), pp. 1-3, Taylor and Francis, London.
- Van de Westeringh, W. (1972) Deterioration of soil structure in worm free orchard soils. *Pedobiologia* **12**, 6-15.
- Van der Merwe, H. (1991) Ou swamdoder nou doeltreffender. *Landbouweekblad*. **9 Augustus**, 40.

- Van Gestel CAM, Van Dis WA, Van Breemen EM, Sparenberg PM, Baurelman R. (1991) Influence of cadmium, copper and pentachlorophenolon growth and sexual development of *Eisenia andrei* (Oligochaeta: Annelida). *Biol Fertil Soils* **12**, 117-121
- Van Gestel, C.A.M., and Van Brummelen, T.C. (1996) Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. *Ecotoxicology* **5**, 217-225.
- Van Gestel, C.A.M. (1997) Scientific basis for extrapolating results from soil ecotoxicity tests to field conditions and the use of bioassays. In *Ecological risk assessment of contaminants* (N.M. van Straalen, and H. Lokke, eds), pp , Chapman and Hall, London.
- Van Rhee, J.A. (1975) Copper contamination effects on earthworms by dispersal of pig wastes in pastures. In *Progress in Soil Zoology* (J. Vanek, ed.), pp. 451-457, Academia Publishing House, Prague.
- Venter, J.M. and Reinecke, A.J. (1988) The life-cycle of the compost worm *Eisenia fetida* (Oligochaeta). *S. Afr. J. Zool.* **23**, 161-165.
- Walker, G.R. (1992) Pesticides and our environment. In *Basic guide to pesticides-their characteristics and hazards* (S.A. Briggs, ed.), pp 267-275, Hemisphere Publishing Company, Washington.
- Walker, C.H., Hopkin, S.P., Sibly, R.M., and Peakall, D.B. (1997) Principles of ecotoxicology. Taylor and Francis, London.
- Weeks, J.M., and Svendsen, C. (1996) Neutral-red retention by lysosomes from earthworm (*Lumbricus rubellus*) coelomocytes: a simple biomarker of exposure to soil copper. *Environ. Toxicol. Chem.* **15**, 1801-1805.

Wentzel, R.S. and Guelta, M.A. (1988) Avoidance of brass powder-contaminated soil by the earthworm, *Lumbricus terrestris*. *Environ. Toxicol. Chem.* **7**, 241-243.

Yeardeley, R.B., Lazorchak, J.M. and Gast, L.C. (1996) The potential of an earthworm avoidance test for evaluation of hazardous waste sites. *Environ. Toxicol. Chem.* **15**(9), 1532-1537.