Stress responses of *Eisenia andrei* and *Enchytraeus doerjesi* (Oligochaeta) to combined effects of temperature and metal contamination

by

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Dissertation presented for the degree of Doctor of Zoology at the University of Stellenbosch

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March 2011

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Abstract

The terrestrial Oligochaete species *Eisenia andrei* and *Enchytraeus doerjesi* were exposed to different concentration series of Cd and Zn, both separately and in mixtures for 28 days in artificial OECD soil at 15, 20 and 25^{o}C. At the end of the four week exposure period, survival and reproduction were assessed in *E. doerjesi* and survival, reproduction, biomass change, metal uptake and biomarker responses (MTT and comet assays) in *E. andrei*.

Survival results for both *E. andrei* and *E. doerjesi* indicated that the lethality of Cd increased at higher temperatures, whereas the opposite was observed for Zn. Cadmium LC_{50} values were the highest at 15^{o}C and the lowest at 25^{o}C. In the Zn exposures LC_{50} increased with increasing temperature. Mixture results in both test organisms indicated that mixtures were less lethal than the metals separately. Effects of mixtures on survival, nonetheless, increased with increasing temperature.

In the Cd experiment, reproduction in *E. andrei* was only recorded in the control treatments at the three temperatures investigated. However, the deleterious effect of Zn on the reproduction of both *E. andrei* and *E. doerjesi* decreased with increasing temperature and Zn EC_{50} for reproduction increased with increasing temperature. Results for exposures to mixtures indicated in both test organisms that the interaction between Cd and Zn were antagonistic. In both *E. andrei* and *E. doerjesi,* the effect of mixture exposures on reproduction decreased with increasing temperatures. The highest mixture EC_{50} values for reproduction were found at higher temperature.

In *E. doerjesi* (using reproduction results in MixToxModules) Cd and Zn interactions were dose level dependent at the three temperatures investigated. Antagonism was the predominant interaction at lower mixture concentrations whereas synergism occurred at mixture concentrations equal to or higher than the mixtures' EC_{50} values.

Biomass loss increased with increasing temperature in the Cd exposures ($p \leq 0.05$) but not in the Zn exposures in *E. andrei.* In this species mixture results indicated

antagonistic interactions between Cd and Zn at all temperatures investigated. The deleterious effect of mixtures on the biomass of *E. andrei* increased with increasing temperature.

When Cd and Zn interactions were further investigated in *E. andrei* (using biomass results in MixToxModules) it was found that they were dose level dependent at the three temperatures investigated. Antagonism was the predominant interaction at lower mixture concentrations whereas synergism occurred at mixture concentrations higher than the mixtures EC_{50} values.

The assessment of metal uptake in *E. andrei* revealed a temperature dependent Cd uptake with higher Cd body burdens occurring at higher exposure concentrations and temperatures ($p \leq 0.05$). In the case of Zn, although uptake was lower at higher temperature, there was no statistical difference in uptake between exposure concentrations and between temperatures. Mixture results however indicated that in mixture exposures less Cd was accumulated by *E. andrei* than in single Cd exposures ($p \leq 0.05$). Inversely, in mixture exposures more Zn was accumulated by *E. andrei* than in single Zn exposures ($p \leq 0.05$).

Biomarker studies revealed that Cd and Zn were both cytotoxic and genotoxic whether in single or mixture exposures. Factorial ANOVA analyses of the effects of temperature and metals on the reduction of MTT by *E. andrei* indicated that temperature rather than the metals was the most important factor controlling mitochondrial activity ($p < 0.001$). In both Cd and Zn exposures significant deleterious metal effects on mitochondrial processes were found to increase with temperature ($p \leq 0.01$). Mixture exposures indicated decreasing cytotoxicity with increasing temperature ($p \leq 0.05$) and possible antagonism between Cd and Zn at cellular level.

Results of the comet assay showed that the genotoxic profile of Cd was the opposite of the genotoxic profile of Zn. Cd was less genotoxic at lower temperature and increasingly deleterious at higher temperature while Zn was more genotoxic at lower than higher temperature ($p \leq 0.05$). The results of mixture exposures indicated

decreasing mixture genotoxicity with increasing temperature and suggested that the interactions between Cd and Zn at molecular level were probably antagonistic.

Opsomming

Eisenia andrei en *Enchytraeus doerjesi* is aan verskillende konsentrasiereekse van Cd en Zn, afsonderlik en in mengsels, vir 28 dae in OECD kunsmatige grond onderskeidelik by 15, 20 en 25 ^{o}C blootgestel Die volgende eindpunte is aan die einde van die vier weke blootstellingsperiode gemeet: oorlewing en voortplanting (by *E. doerjesi)* en oorlewing, voortplanting, biomassaverandering, metaalopname, MTT en komeettoetse (by *E. andrei)*.

Oorlewingsresultate by beide *E. andrei* en *E. doerjesi* het getoon dat toenemende temperatuur die letale toksisiteit van Cd laat toeneem terwyl die teenoorgestelde waar was vir Zn. By die Cd blootstellings was die LK_{50} waardes die hoogste by 15^{o}C en die laagste by 25^{o}C. By die blootstellings aan Zn het die LK_{50} waardes toegeneem by hoër temperatuur. Resultate by die mengsels by beide toestspesies het aangetoon dat die mengsels minder letaal was as die afsonderlike metale. Effekte van mengsels op oorlewing het nietemin toegeneem met toenemende temperatuur.

By die Cd blootstellings is voortplanting slegs by die kontroles en in die geval van *E. andrei* by die drie onderskeie temperature ondersoek. Die nadelige uitwerking van Zn op voortplanting by beide *E. andrei* en *E. doerjesi* het afgeneem met stygende temperatuur en die Zn LK_{50} vir voortplanting het toegeneem met toenemende temperatuur. Resultate van die blootstellings aan mengsels het getoon dat die wisselwerking tussen Cd en Zn by beide spesies antagonisties was. By beide spesies het die invloed van die mengsels op voortplanting afgeneem met stygende temperatuur. Die hoogste mengsel LK_{50} waardes vir voortplanting is by hoër temperature gevind.

By *E. doerjesi* was Cd en Zn wisselwerkings by blootstelling aan mengsels (voortplantingsresultate ondersoek deur van MixToxModules gebruik te maak) dosisvlak verwant by die drie temperature wat ondersoek is. Antagonisme was die oorwegende wisselwerking by laer mengsel konsentrasies terwyl sinergisme

voorgekom het by mengsel konsentrasies gelyk aan of hoër as die LK_{50} waardes van die mengsels.

In die geval van die Cd blootstellings by *E. andrei* het biomassaverlies toegeneem met toenemende temperatuur ($p \leq 0.05$) maar nie by die Zn blootstellings nie. Resultate van blootstellings aan mengsels het getoon dat die uitwerking van mengsels op die biomassa van *E. fetida* toegeneem het met toenemende temperatuur.

By die verdere ondersoek van Cd en Zn wisselwerkings, waar gekyk is na dosis verhouding of dosisvlak antagonisme (deur van biomassa resultate in MixToxModules gebruik te maak), is gevind dat Cd en Zn wisselwerkings dosisvlak afhanklik was by die drie temperature wat ondersoek is. Antagonisme was die oorwegende wisselwerking by laer mengselkonsentrasies terwyl sinergisme voorgekom het by mengselkonsentrasies hoër as die mengsel EK_{50} konsentrasies.

Die bepaling van metaalopname deur *E. andrei* het 'n temperatuurafhanklike opname van Cd getoon met hoër Cd liggaamskonsentrasies by hoër blootstellingskonsentrasies en temperature ($p \leq 0.05$). Alhoewel Zn opname laer was by hoër temperatuur was daar geen statisties betekenisvolle verskille in opname tussen blootstellingskonsentrasies of temperature nie. Die bepaling van metaalopname by wurms wat aan mengsels blootgestel is, het getoon dat minder Cd deur *E. andrei* opgeneem is as waneer die wurms aan Cd as enkelmetaal blootgestel is ($p \leq 0.05$), Daarteenoor het die teenoorgestelde gebeur in die geval van Zn, Meer van die metaal is opgeneem wanneer *E. andrei* aan mengsels blootgestel is as aan die enkelmetaal.

Biomerkerstudie het getoon dat Cd en Zn beide sito- en genotoksies kan wees ongeag of dit as enkelmetale of in mengsels toegedien is. Faktoriale ANOVA analises van die effekte van temperatuur en metale op die verlaging van MTT by *E. andrei* het getoon dat temperatuur 'n belangriker faktor was as metaalbesoedeling by die kontrole van mitochondriale aktiwiteit. ($p \leq 0.001$). By beide Cd en Zn blootstellings was daar in elk geval statisties betekenisvolle metaaleffekte op mitochondriale

prosesse met toename in temperatuur ($P \leq 0.01$). By blootstellings aan mengsels is gevind dat sitotoksisiteit afgeneem het met toenemende temperatuur ($p \leq 0.05$) asook ’n moontlike antagonisme tussen Cd en Zn op sellulêre vlak.

Resultate van die komeettoets het getoon dat die genotoksiese profiel van Cd die teenoorgestelde was as die van Zn. Cd was minder genotoksies by laer temperature en meer en meer skadelik by hoër temperature terwyl Zn meer genotoksies was by laer as by hoër temperature ($p \leq 0.05$). Die resultate van blootstlling aan mengsels het laer genotoksisiteit getoon met toename in temperatuur. Dit dui daarop dat wisselwerkings tussen Cd en Zn op molekulêre vlak moontlik antagonisties was.

Acknowledgements

Special thanks to:

Prof S.A. Reinecke and Prof. A.J. Reinecke for their guidance, diligence, encouragements and constant support.

SETAC/Procter & Gamble for awarding me the 2010 Procter & Gamble fellowship for doctoral research in environmental science.

Dr. Thomas Delfosse (Procter & Gamble) for insightful discussions on this project.

My wife for her invaluable support, patience and sacrificial love.

My occasional laboratory assistants: M. Bayimbi, J.P. Koura Mbadinga, A.M. Bakoussou Mbadinga, J. Avika Mondavwandi, G.G. Angoue Ndoutoume, M. Bouanga, B. Nsi Ovono, F. Ntahinta Mboumba and L.A. Moussavou Mbadinga. Your contribution to this project will never be forgotten.

M. Van Wyk & F. Fourie for true camaraderie and insightful suggestions on various laboratory and statistical issues.

Mr. Jonathan Williams for his availability and reliable technical assistance.

Bourses et Stages (Gabon) for their long lasting financial support.

My parents, siblings and friends for their support and encouragements.

Dedication

To my father Mr. Georges Voua and son Ryan Georges Tali. To the older for believing in me and teaching me to always give my best. To the younger for the strength and motivation that I have found in his smiles and amusing ways.

A mon père M. Georges Voua et mon fils Ryan Georges Tali. Au plus vieux pour avoir cru en moi et pour m'avoir appris à toujours donner le meilleur de moi. Au plus jeune pour la force et motivation que j'ai trouvées dans ses sourires et voix amussantes.

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1. General introduction

Pesticides, litter, oil, domestic and industrial waste and heavy metals are the main pollution threats to marine, freshwater and terrestrial ecosystems (Forbes & Forbes 1994). Heavy metal pollution especially has become a great source of concern because subsequent to metal mining and industrial processing, metals often find their way to the environment (Nriagu & Pacyna 1988). Cadmium (Cd), mercury (Hg) and lead (Pb), amongst many other metals, have been listed as the most hazardous heavy metals to humans and ecosystems (Forbes & Forbes 1994).

1.1. Essential and non enssential metals

Most hazardous metals fall in the category of non-essential metals. These are metals such as Hg; Barium (Ba); silver (Ag); antimony (Sb) and Pb. They have no known biological functions (Table 1). Cd previously was thought to be of no biological importance but has a role in carbonic anhydrase activity in the diatom species *Thalassiosira weissflogii* (Lane & Morel 2000; Lane *et al*. 2005). In the typically Zinc (Zn) depleted seawater, *T. weissflogii* has evolved the ability to functionally substitute Zn with Cd in its carbonic anhydrase enzyme (Lane & Morel 2000). This enzyme enables marine phytoplankton to acquire inorganic carbon for photosynthesis thus enhancing their growth rate (Lane & Morel 2000; Lane *et al*. 2005).

Essential metals such as Manganese (Mn), Iron (Fe), copper (Cu) and Zn (Table 1) are needed by humans and other animals in very small quantities (< 100 mg/day). In the organism, essential metals bind to proteins to form metalloproteins, and other molecules such as phosphates, phytates, polyphenols and other chelating compounds (Fraga 2005). Nevertheless, at concentrations higher than what the body requires for biological functions, these essential metals can become toxic (Fraga 2005).

1.2. Metal sources

Both essential and non-essential metals are discarded into the environment through anthropogenic activities. Nriagu & Pacyna (1988) listed coal, oil and wood combustion; metal and cement production, refuse incineration, mining, and phosphate fertilizers as the main sources of metals in air, water and soil. They reported that urban refuse and coal combustion were the two main sources of metals such as Cu, Hg, Pb, Cd, vanadium (V) and Zn found in soils. In the United Kingdom, Hutton & Symon (1986) identified the major metal sources in air, water and soil: (1) Major atmospheric sources were non-ferrous metal production, iron and steel production, fossil fuel combustion and refuse incineration; (2) Major sources in costal waters were the direct discharge of sewage and industrial effluents; and (3) the largest inputs to landfill and agricultural land emanated from the disposal of municipal waste and the use of phosphate fertilizers respectively. Plachy (1997) stated that approximately 2 600 tons of Cd were globally released into the soil through the use of fertilizers alone.

In urban settings, new pollution sources are emerging with the demands of the current lifestyle. Haus *et al*. (2007) reported that the combined occurrence of Sb and platinum (Pt) in ecosystems could be an indication of automobile related pollution. Once in the environment, metals have the potential to accumulate in soil, water and sediments where they could be readily available to plants and animals.

1.3. The fate of metals in soil

Some metals accumulated in soils find their way into plants and animals to whom they might be toxic. The toxicity of a metal in soil depends on its bioavailability, which in turn depends on both the solubility of the metal and its interactions with soil particles (Clemens 2006). Lanno *et al*. (2004) stated that metal bioavailability is the result of the interactions between soil physical and chemical characteristics and the physiology and behavior of soil biota. (Bio) availability according to

Lanno *et al*. (2004) is a threefold concept including *environmental availability* (referring to the portion of total chemical in soil that is not sequestered and thus rendered possible for soil biota to interact with); *environmental bioavailability* (referring to the portion of total chemical in soil that is eventually taken up by soil biota) and finally *toxicological bioavailability* (referring to the portion of absorbed chemical that reach and interact with the site(s) of toxic action). Due to their inherent characteristics certain metals tend to be more or less bioavailable than others. Metals such as Cr, Ag and Pb are seldom taken up by plants because of their low solubility (Clemens 2006). On the contrary, earthworms for instance, that feed on the organic fraction of the soil, show the tendency to accumulate a metal such as Cd that tends to bind to soil organic matter (Li & Shuman 1996). McBride (1994) reported that Hg, Cd and Pb are the most toxic metals known to animals whereas Cu, nickel (Ni); and cobalt (Co) are particularly toxic to plants.

1.4. Effects of metals on organisms

In the eventuality of metal uptake by an animal or a plant, both essential and non-essential metals have the potential to become detrimental (Fraga 2005). Essential and non-essential metals have been associated with human pathologies such as cancer and Alzheimer's disease. There are several lines of evidence linking Al and Mn to three different neurodegenerative conditions, which are respectively Alzheimer's disease, manganism and Parkinson's disease (Zatta *et al*. 2003). Another neurodegenerative condition involves the essential metal Cu. Kuo *et al*. (2007) have established that the consumption of grass pea (*Lathyrus sativus*) seeds (particularly rich in Cu) as staple food causes a deprivation of methionine which in turn leads to a disruption in Cu homeostasis in some populations of rural Ethiopia. This ultimately causes the development of an ancient neurodegenerative disease that could affect up to 6% of the populations concerned.

Table 1. Common metals and their biological function(s)[1]

Metal	Biological function (s)
Aluminum (Al)	May activate succinic dehydrogenase
Barium (Ba)	None known
Beryllium (Be)	None known
Cadmium (Cd)	Non essential to most organisms. Can substitute Zn in carbonic anhydrase in diatoms (Lane & Morel 2000; Lane *et al*. 2005)
Cobalt (Co)	Essential for mammals. Cofactor in numerous enzymes. Role in symbiotic N_2 fixation
Chromium (Cr)	May be involved in sugar metabolism in mammals
Copper (Cu)	Essential to all organisms. Cofactor in redox enzyme, O_2-transport pigments
Iron (Fe)	Essential to all organisms. Cofactor in many enzyme, heme proteins
Lead (Pb)	None known
Manganese (Mn)	Essential for mammals. Cofactor in numerous enzymes. Involved in H_2O-splitting reaction of photosythesis
Mercury (Hg)	None known
Molybdenum (Mo)	Essential to almost all organisms. Enzymes cofactor. Role in symbiotic N_2 fixation and NO_3^-
Nickel (Ni)	None known in mammals. May be essential to plants. Found in urease enzyme
Silver (Ag)	None known
Vanadium (V)	Required by green algae; may be involved in N_2 fixation. Porphyrin and heme constituent
Zinc (Zn)	Essential to all organisms. Cofactor in numerous enzymes

[1] Table adapted from McBride (1994)

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Some metals have also been associated with the development of other health complications such as cancer, anaemia and sterility in humans. Nawrot *et al*. (2006) investigated the occurrence of lung cancer in Noorderkempen (Belgium), a Cd-contaminated area. They reported that individuals living in the metal contaminated area were more prone to developing lung cancer than their counterpart living on a nearby reference site. An investigation of the effects of Pb on the haematological system of 75 Indian children aged 1-7 years by Ahamed *et al*. (2007) has indicated that elevated blood Pb levels (≥ 10 µg/dl) is associated with risk of anaemia. Lead is also reported to affect the absorption and metabolism of essential trace metals (Johnson 1998). This author reviewed the main biological effects of Pb poisoning. These included together with anaemia, a neuromuscular syndrome causing paralysis of the peripheral motor nerve, kidney damage, sterility (in males and females), abnormal foetal development, and abnormal neurological development and function. Lead is also known to cause or increase risks of cancer in both humans and animals (Johnson 1998).

In the environment, some essential and non essential metals have the potential to affect a wide range of organisms. Son *et al*. (2007) reported that in soil metal concentrations of 129 mg/kg Cd, 2 mg/kg Hg and 1312 mg/kg Pb respectively, were potentially lethal to the Koran springtail *Paronychiurus kimi*. Similarly, Cd and Pb have been reported to cause the same effect on two other collembolan species *Sinella coeca* and *Folsomia candida* (Menta *et al*. 2006). Concentrations nearing 50 mg/kg soil for Cd and 1000 mg/kg for Pb were found sufficient to significantly affect both reproduction and survival in *F. candida* (Menta *et al*. 2006). In the Norwegian lobster *Nephrops norvegicus*, exposure to Mn has been reported to cause suppression of fundamental immune mechanisms (Hernroth *et al*. 2004). In fish such as the common carp *Cyprinus carpio* and in the gibel carp *Carassius auratus gibelio* Cu has been reported to cause respiratory stress (De Boeck *et al*. 2007). Similarly, relatively high doses of both Cu and Mn have been linked to oxidative stress and changes in chloroplast proteins in barley plants *Hordeum vulgare* (Demirevska-Kepova *et al*. 2004). Research suggests that an

excess of Cu, Zn superoxide dismutase (Cu_2Zn_2SOD) (whose physiological role is to protect cells from oxidative stress) could be genotoxic and consequently detrimental to cells (Han *et al*. 2007).

Essential metals do, however, often show the tendency of being regulated by organisms without causing apparent toxic effects. Sierra *et al*. (1998) after exposing feral pigeons to 239 mg/kg of Mn for 7 h/day, 5 days/week for up to 13 consecutive weeks, did not find significant toxic effects. Similarly, Bartoskewitz *et al*. (2007) fed captive white-tailed male deer a diet of up to 236 mg/kg Cu and 1135 mg/kg Zn *ad libitum* for over a year and observed no indication of Cu or Zn toxicity.

The effects of both essential and non essential metals on terrestrial oligochaetes have also been widely studied. Maboeta *et al*. (1999) conducted a study on the sub-lethal effects of Pb on the growth and reproduction of *Perionyx excavatus* and reported that Pb hampered normal growth rate and caused the production of non-viable cocoons. Reinecke *et al*. (2001) similarly reported that although metal contaminated (0.1% $Pb(NO_3)_2$, 0.1% $ZnSO_4$, 0.01% $CdSO_4$) cultures of *Eudrilus eugeniae*, *Perionyx excavatus* and *Eisenia fetida* could still normally lay cocoons; the latter were not always able to hatch. Helling *et al.* (2000) stated that a Cu concentration of 8.92 mg/kg was enough to hamper the growth and maturation of juvenile *Eisenia fetida* specimens significantly and that 15.92 mg/kg Cu had a similar effect on reproduction. Lock & Janssen (2002) found that the reproduction of *Enchytraeus albidus* in OECD soil could be affected by different quantities of metals such as Cu (42 day EC_{50} = 305 mg/kg) and Pb (42 day EC_{50} = 320 mg/kg).

Metal effects have also been assessed in terrestrial oligochaetes at the sub-individual level. Reinecke & Reinecke (1997) for instance reported that a sub-lethal dietary supply of Pb (29.6 µmol g^{-1}) and Mn (4.3 µmol g^{-1}) caused ultra-structural damages on the spermatozoa of *E. fetida*. Similarly Siekierska & Urbanska-Jasik (2002) reported that Cd concentrations as low as 10 and 50

mg/kg caused ovarian damages in the earthworm *Dendrobaena veneta*. Saint-Denis *et al*. (2001) reported dose response inhibitions of selected enzymes such as acetylcholinesterase, glutathione-S-transferase and glutathione reductase after exposing *E. andrei* to Pb in artificial soil. Maboeta *et al*. (2002 & 2004) reported disrupting effects of Cu on the lysosomal membrane of *Microchaetus sp* and *E. fetida* respectively. Similarly, Maleri *et al*. (2008) linked lysosomal membrane damage and mitochondrial impairment to Cd exposure in *E. andrei* using the Neutral Red and the MTT assays respectively.

Even at population level in the field, Abdul Rida & Bouché (1995) ascertained that the earthworm genus *Scherotheca* has been eradicated from southern France because of environmental Pb (from mining and car exhausts) and Cu (from pesticides).

According to Tolcin (2006), Cu together with Fe, Al, and Zn are the four most used metals by humans. Zn is further important because one of the main byproducts of its smelting and refining is Cd, the metal causing the most environmental concern (McBride 1994; Plachy 1997).

1.5. Zinc

Zinc occurs naturally as the sulphide mineral, sphalerite (ZnS) in rocks. Sphalerite is the most commonly mined zinc-containing ore since it can contain up to 62% Zn (Lehto 1968). In soils, the only soluble from of Zn is Zn^{2+}, which is mostly mobile in acid soils (McBride 1994). In 2006, China, Australia and Peru were the world's top Zn producers, producing 2.6, 1.3 and 1.2 million tons of Zn respectively (Tolcin 2006). Zinc is most commonly used as an anti-corrosion agent through galvanization, which is the process of coating Fe and steel with Zn (Greenwood & Earnshaw 1997). Other common applications are found in the battery, paint, rubber and brass industries (Lehto 1968; Besenhard 1999; Emsley 2001; Wiaux & Waefler 2009) in communication equipment, hardware, musical instruments water valves, and agricultural fungicides (Lehto 1968). In medicine,

Zn is included in the list of mineral supplements and vitamins (DiSilvestro 2004) and recommended as an antimicrobial agent (McCarthy *et al*. 1992; Valko *et al*. 2005).

As an essential trace element, Zn plays various roles in organisms. Zinc is involved in the activity of numerous enzymes and is present in Zn-fingers involved in DNA replication and transcription (Fraga 2005; Greenwood & Earnshaw 1997). Zinc and Fe are the two most abundant metals in organisms, with Zn being the only one present in all enzyme classes (Broadley *et al*. 2007). The recommended dietary allowance for Zn in the U.S. is 11 mg/day for men and 8 mg/day for women (United States National Research Council- USNRC 2000).

In humans, Zn is essential for normal growth and development in pregnancy and plays a significant role in strengthening the immune system (Fraga 2005). Zinc deficiency has been associated with the emergence of oxidative damage in the airways (Zalewski 2006), the weakening of the immune system which affects the sense of smell and taste and impairs DNA synthesis (Fraga 2005). Other symptoms of Zinc deficiency include delayed growth and sexual maturation, and altered cognition (USNRC 2000).

Although essential, Zn has been shown to be toxic to several taxa including oligochaetes. Van Gestel *et al*. (1993) found that Zn concentrations above 560 mg/kg in artificial soil significantly reduced reproduction and induced the production of malformed cocoons in *E. andrei*. Reinecke *et al*. (2001) similarly found that long-term exposure to sub-lethal quantities of Zn causes a decrease in hatchling success in *E. fetida*. Lock & Janssen (2002) found an EC_{50} (reproduction) of 345 mg/kg and an LC_{50} of 610 mg/kg for Zn in *E. albidus*. In *E. fetida* exposed to Zn in artificial soil, Spurgeon *et al*. (1994) recorded 14 day and 56 day LC_{50} values of 1010 and 745 mg/kg respectively.

Zinc and Cd are likely to occur simultaneously in the same environment for Cd is recovered during the smelting of Zn and other complex ores (Plachy 1997; OECD 1984).

1.6. Cadmium

In rocks, Cd is found bound to Zn in sphalerite and other sulphide ore minerals. In soils, due to weathering, Cd is mainly found in its soluble form Cd^{2+}, which is particularly mobile at low pH (McBride 1994).

During its early production in the 1930s-1940s, Cd was used mainly as an anti-corrosion agent (Lansche 1956). In 1997, most of the cadmium produced was utilized in nickel-cadmium batteries (55%), cadmium pigments (20%), cadmium-bearing stabilizers (10%), cadmium coatings (8%), cadmium-containing alloys (3%) and for miscellaneous purposes (4%) (Plachy 1997). According to the Cadmium Market and Trends report of 2005 (Morrow 2005) more than 80% of the Cd produced in 2004 was still used for the making of Nil-Cd batteries. Although Cd production is decreasing, 16 500 tons of Cd per year were used in the mid 90s (Plachy 1997). Cd production has indeed been in a steady decline in the West, but its worldwide production in the 2004 still exceeded 15 000 tons due to Asian based production (Morrow 2005).

In humans, Cd contamination occurs mainly through occupational exposure and it mainly affects the renal and respiratory systems and increases risks of bone fractures and cancer developing (Nawrot *et al*. 2006). Cadmium has also been shown to be harmful to several other organisms including: plants (Carpena *et al.* 2003; Nouairi *et al.* 2006); insects (Cervera *et al*. 2004), amphibians (Loumbourdis *et al*. 1999) and rats (Kim *et al*. 1998; Lafuente & Esquifino 2002).

Several studies have investigated the effects of Cd on various oligochaete species such as *E. fetida, E. andrei,* and *Dendrobaena veneta* (Bengtsson & Rundgren 1992; Spurgeon *et al.* 1994; Reinecke & Reinecke 1996; Reinecke *et al.* 1999). These studies have reported a spectrum of effects ranging from

disturbance in water and homeostatic balance (Reinecke *et al.* 1999); changes in the ovarian structure (Siekierska & Urbanska-Jasik 2002), nephridial degeneration (Prinsloo 1999) to reduction in cocoon production and hatching (Bengtsson & Rundgren 1992; Spurgeon *et al.* 1994; Reinecke & Reinecke 1996).

Research suggests that Cd and Zn are metabolic antagonists with Cd toxicity being significantly influenced by Zn (Brzóska & Moniuszko-Jakoniuk 2001). Torreblanca *et al*. (1992), for instance, indicated that human trophoblasts (cells forming a large part of the placenta and involved in placental transport of Zn) exposed to Cd tend to reduce their transport of Zn, thus showing how Cd could prevent this trace element from reaching the foetus. Ishitobi & Watanabe (2005) stated that Cd exposure affects the concentrations of both Zn and Cu in mice neonates.

Apart from the effects caused by these interactions between metals, metal toxicity is also controlled by several other factors, many of which are inherent to the environment where the metal might occur.

1.7. Environmental factors and metal effects

In the environment, the extent of negative effects caused by metal pollution could be influenced by various factors. The testing of chemicals in the laboratory usually involves experimental designs where the effects of toxicants are tested under optimal conditions. In laboratory settings, abiotic factors such as temperature, moisture, pH, salinity, nutrient availability etc. are usually kept constant while different doses of toxicants are applied to the selected test organisms. Even though this approach helps to single out the specific effects of the toxicant under investigation, it does not give an insight as to how environmental factors influence the final effects of toxicants in nature. For instance, it is well known that pH influences the toxicity of metals (van Gestel & Hoogerwerf 2001; Sivakumar & Subbhuraam 2005; Ownby *et al*. 2005). Both

Zn^{2+} and Cd^{2+} for example are more soluble and mobile at lower pH values (McBride 1994). Crommentuijn *et al*. (1997), looking at the bioavailability of Cd to *F. candida* in an artificial substrate, reported that water soluble Cd increased with decreasing pH values. Anderson & Christensen (1998) stated that pH was the key factor influencing the distribution of Cd, Co, Ni and Zn in soils. Janssen *et al*. (1997b) acknowledged that both abiotic and biotic characteristics of the soil work together to determine the bioavailability (thus the toxicity) of toxicants to soil organisms.

Under extreme environmental conditions on one hand, the physiology of an organism could be altered and these internal changes may influence the capability of that organism to cope with a toxic insult. On the other hand, toxic stress has the potential to affect the ability of soil organisms to deal with changes in the environment. Sørensen & Holmstrup (2005) assessed the effects of various contaminants on the ability of *F. candida* to withstand drought. They reported that previous exposure to toxicants can increase drought vulnerability in that species.

Temperature has also been reported to influence metal toxicity in soil-dwelling organisms such as earthworms. Spurgeon *et al.* (1997) found that Zn toxicity increases with temperature in *E. fetida*. Marinussen & Van der Zee (1997) reported that field temperature affects Cu accumulation in the earthworm *Lumbricus rubellus*. Spurgeon *et al*. (2005) also found that higher temperature increases Cd tissue accumulation in *L. rubellus*. To earthworms, moisture and temperature are the two most important environmental factors influencing growth, survivorship and reproduction (Presley *et al*. 1996; Berry & Jordan 2001; Wever *et al*. 2001). As far as temperature is concerned, Stoate *et al*. (2001) stated that global warming was increasingly impacting on terrestrial and aquatic ecosystems. Van Jaarsveld & Chown (2001) for instance predicted a temperature increase of 1 to 3°C and a reduction of 5 to 10 % in mean annual precipitation if the current South African level of atmospheric CO_2 was to double in the future. This would cause an alteration of the geographical ranges of up to 44% of plant and 80% of

animal species. This specific change will enhance extinction risk. Such a change will greatly affect soil-dwelling organisms such as earthworms that have a very poor dispersal ability (Van der Werff *et al*. 1998).

1.8. Soil metal pollution and the threats of Climate Change

The global increase in temperature as estimated by the Intergovernmental Panel on Climate Change (IPCC) is predicted to be between 1.4 and 5.8°C by 2100 (IPCC 2001). According to the European Environment Agency (EEA), European annual mean air temperatures have already risen by 0.3 to 0.6°C since 1900 (EEA 1998).

Global warming, potentially, could render some metals more available for uptake by organisms. Johnson (1998), for instance, said that sediments contain substantial amounts of Pb that could become progressively bioavailable due to global warming. Monitoring the effects of an environmental factor such as temperature on metal stressed soil organisms might help foresee the fate of terrestrial ecosystems in the current critically warming environment.

According to Noyes *et al*. (2009), global warming will act on animals as a co-stressor with chemical toxicants, affecting their physiological processes. A rise in the mean global temperature will increase the chances of the occurrence of heat shock stresses, which could significantly affect soil ecosystems especially in polluted areas. Soil organisms play both a structural and a functional role in terrestrial ecosystems. Collembola for instance can be responsible for up to 30% of soil invertebrate respiration (Hopkin 1997). According to Reinecke & Reinecke (2004a) soil organisms such as earthworms are important in soils, where they positively contribute by enhancing the decomposition of organic matter, soil aeration, water transport and soil structure. Earthworm burrows under pasture act as an efficient buffer against runoff and erosion (Chan 2004). Enchytraeids, for their part, help to improve the pore structure of the soil and are also involved in the degradation of organic matter (Amorim *et al.* 2008). These oligochaetes

constitute an important component of soil biomass and terrestrial food web, as they are a protein source for small mammals, reptiles and birds (Peijnenburg *et al*. 1999). Given the importance of these soil-dwelling organisms, the effects of environmental metal pollution coupled with global warming could ultimately alter both the structure and functioning of soil ecosystems with repercussions that could affect several other organisms including men.

1.9. Relevance of the present study

There is evidence of the role temperature could play in the toxicity of metals to soil organisms (Spurgeon *et al*. 2005; Marinussen & Van der Zee 1997; Spurgeon *et al.* 1997). Moreover, because of global warming, temperature will be one of the two main parameters controlling the fate of chemicals in the environment (Noyes *et al*. 2009). It is therefore relevant to attempt to assess both the separate and the combined effects of temperature and metal ions on different soil organisms. Such an endeavour could provide an understanding as to how temperature will interplay with metal pollution to impact on soil ecosystems. This may help to develop mitigating strategies against possible higher impact of metal pollution in the near future. This study may also provide a theoretical understanding of the consequences of metal pollution in less studied warmer tropical soil ecosystems.

To investigate the potential threats presented by the combined occurrence of metal pollution and increasing temperature to soil ecosystems, it is important to use ubiquitous soil species that are sensitive to changes in the environment. Because of their relatively high sensitivity to environmental pollution, oligochaetes among many other invertebrates would be the ideal candidate for such an enterprise. Many, such as the earthworm species *E. andrei* and the enchytraeid *Enchytraeus albidus* are also used as standard test organisms in laboratory assays (OECD 1984; OECD 2004; Römbke & Moser 2002).

Such organisms could be exposed to metal ions at different temperatures and different endpoints such as growth, reproduction and survival could be assessed**.** This could be combined with sub-organism tests called biomarkers (Svendsen & Weeks 1997). Within the organism, a biomarker is a biochemical, cellular or molecular response that is triggered before the whole organism is irremediably affected (Dallinger *et al*. 2000). Biomarkers such as the comet assay that measures the genotoxicity of substances (Singh *et al*. 1988; Fairbairn *et al*. 1995; Reinecke & Reinecke 2004b) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay that measures cell activity via their mitochondrial activity (Mosmann 1983) could be useful.

1.10. General aim and specific objectives

The main aim of the present study was to assess the separate and combined effects of various levels of temperature and metal ions on the growth, reproduction, survival and selected biomarkers of the earthworm *E. andrei,* and the enchytraeid *Enchytraeus doerjesi*. A secondary aim was to derive data that could be used to model these effects and predict future trends in stressed environments.

The specific objectives were:

1. To assess the effects of Cd and Zn (separately and in mixtures) on the reproduction, growth and survival of *E. andrei* and *E. doerjesi* at three different temperatures.
2. To assess the genotoxicity of Cd and Zn (separately and in mixtures) in *E. andrei* at three different temperatures using the comet assay.
3. To assess cellular activity in *E. andrei* following exposure to Cd and Zn (separately and in mixtures) at three different temperatures, using the MTT assay.

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2. General materials & methods

This chapter provides a general overview of the materials and methods used during this study. The protocols of specific assays and bioassays are presented fully in the relevant chapters.

2.1. Experimental animals

The experimental animals chosen for this study are from the class Oligochaeta. Oligochaetes are segmented worms (annelids), found in freshwater and terrestrial habitats (Wallwork 1983). Terrestrial oligochaetes collectively called Terricolae include families such as the Moniligastridae, the Megascolidae, the Eudrilidae, the Glossoscolecidae and the Lumbricidae (Stephenson 1930). According to Blakemore (2006), the Lumbricidae family contains 42 recognized genera. Of these, the genus *Eisenia* contains fifteen species, one of them being *Eisenia andrei* (Bouché 1972).

The family Enchytraeidae contains genera belonging to both the Terricolae and to their mud-water dwelling counterparts the Limicolae (Stephenson 1930). This family has 17 genera. Of these, the genus *Enchytraeus* contains more than 40 known species, one of them being the fairly recently described *Enchytraeus doerjesi* (Westheide & Graefe 1992). *E. andrei* and *E. doerjesi* were used as model organisms for the present study.

The soft body of oligochaetes and their lack of body armour make them ideal candidates as test species in studies investigating the adverse effects of substances to soil organisms. In the soil, these organisms are in direct contact with all soil phases (solid, gas and liquid) and the potentially harmful substances they may contain. Both *E. andrei* and *E. doerjesi* are reared in our laboratories in the Stress Ecology Research Group at the University of Stellenbosch. The original stock culture of *E. andrei* was established in 1994 from cocoons donated by Professor O. Graff (Braunschweig, Germany). *E. doerjesi* has only been

cultured since 2006. The culture was started with specimens obtained from Dr P. Kramarz from Krakow, Poland. Both these species are kept in a climate control room with an ambient temperature of 20^{o}C and a relative humidity (Rh) of 60%. *E. andrei* is maintained on a clean natural soil and cattle manure substrate and fed weekly with clean fresh cattle manure. *E. doerjesi* is reared on a clean sandy substrate and fed once a week with raw oats (Jungle oats®).

2.1.1. *Eisenia andrei*

2.1.1.1. Classification

According to Sims and Gerard (1985), the classification of *E. andrei* is as follows:

Phylum: Annelida
Subphylum: Clitellata
Class: Oligochaeta
Order: Haplotaxida
Suborder: Lumbricina
Superfamily: Lumbricodea
Family: Lumbricidae
Subfamily: Lumbricinae
Genus: *Eisenia*
Species: *Eisenia andrei* (Bouché 1972)

2.1.1.2. Morphology

Apart from differences in pigmentation *E. andrei* is morphologically indistinguishable from *E. fetida* (Sims and Gerard 1985). According to these authors, *E. andrei* has a more intensely red-brown uniform colouration and lacks the whitish cream intersegmental stripes that are present in *E. fetida*. Haimi (1990) determined that *E. andrei* has more body segments than *E. fetida* and can weigh up to 1.2g. In adulthood, the saddle-shaped clitellum covers 6-8 segments (from segment 26 to 32). The tubercula pubertatis is found on the ventral side of the clitellum, covering three segments (Sims and Gerard 1985).

2.1.1.3. Biology and ecology of *E. andrei*

In artificial OECD soil, at 18 ^{o}C, *E. andrei* has an average longevity of 63 months, although a life span of over 104 months has been recorded in one specimen (Mulder *et al*. 2007). Adult specimens produce 0.8 to 2 cocoons per week (Mulder *et al*. 2007). Although information on the duration of incubation of the cocoons is still lacking, *E. andrei* is known to have a higher reproduction rate than *E. fetida* (Reinecke & Viljoen 1991; Haimi 1990), whose cocoon incubation period lasts ± 23 days (Venter & Reinecke 1988). Hatchlings are fully clitellate 35 days after hatching (Reinecke & Viljoen 1991).

E. andrei is a peregrine species although the species is known to have originated in Europe (Stephenson 1930; Edwards and Bohlen 1996). This species has become ubiquitous with a worldwide distribution because of its resilience and fairly wide temperature and moisture tolerance ranges (Dominguez *et al.* 2005). *E. andrei*, according to Sims & Gerard (1985), occurs mostly in the same habitat as *E. fetida*. Dominguez *et al.* (2005) confirmed that both species are syntopic (sharing the same habitat) but *E. andrei* is predominant in indoor cultures whereas *E. fetida* abounds in natural environments. *E. andrei* is a standard test species for the testing of chemicals (OECD 1984; OECD 2004). This species is also economically important due to its usefulness in the vermicomposting of organic wastes and as a protein source for small mammals, reptiles and birds (Peijnenburg *et al*. 1999, Tajbakhsh *et al.* 2008).

2.1.2. *Enchytraeus doerjesi*

2.1.2.1. Classification

The classification of *E. doerjesi*, from information gathered both from the US National Center for Biotechnology Information (NBCI) and Westheide & Graefe (1992) is as follows:

Phylum: Annelida
Subphylum: Clitellata

Class: Oligochaeta
Order: Haplotaxida
Suborder: Tubificina
Family: Enchytraeidae
Genus: *Enchytraeus*
Species: *Enchytraeus doerjesi* Westheide & Graefe 1992

2.1.2.2. Morphology

E. doerjesi has a milky white translucid body. As described by Westheide & Graefe (1992), this species mean body length is 5.3 mm divided in up to 37 body segments in adulthood. This species has a comparatively smaller body size than other common *Enchtryaeus spp.* The clitellum comprises segments 12 and 13.

2.1.2.3. Biology and ecology of *E. doerjesi*

At 21^{o}C, *E. doerjesi* has a mean time of embryological development of 6.8 days and hatchlings take 8.5 days to reach maturity. A life span of up to 127 days has been recorded for this species, although average estimates are around 80-90 days. At 21^{o}C, adult specimens of this species can produce 4.3 eggs per day (Westheide & Graefe 1992).

Being a fairly recently described species, the distribution *E. doerjesi* has not yet been clearly established. However, species from the same genus have been found across the globe from Alaska to India in littorals and fresh water bodies (Stephenson 1930). Westheide & Graefe (1992) reported discovering *E. doerjesi* in garden mould and earthworm substrate from France, Holland and the Philippines, suggesting that this species could be found in Europe and Asia. Its origin, however, still needs to be investigated. Observations from our laboratory culture suggest that this species thrives on moist sandy substrates and is resilient to short periods of drought.

2.2. Substrate

To standardize the influence of the interactions between soil components, all exposures were performed in OECD soil (OECD 1984). The OECD soil consisted of 10% sphagnum peat moss (Les Tourbes Nirom Peat, Canada), 20% kaolin clay (Serina Kaolin, South Africa), and 70% silica (made of 2/3 fine grains and 1/3 coarse grains) (Consol Limited Industrial Minerals, South Africa). To prepare the soil, dry weights of these ingredients were thoroughly mixed together and the pH adjusted to 6 ± 0.5 by the addition of $CaCO_3$ (± 0.4%) (Merck, Germany). pH was measured using a CRISON micro pH 2001 apparatus.

In order to obtain the desired metal concentrations for the exposure experiment, the artificial soil was spiked with the relevant metals by diluting the metal salts ($CdSO_4$ and/or $ZnSO_4$) with enough distilled water to attain 50% water holding capacity (WHC) in the artificial soil. To determine the WHC of the artificial soil, soil samples (contained in plastic pipes sealed on one end with filter paper No. 1) were saturated with water during a three-hour immersion in water. Soil samples were thereafter left to drain on moist sand for two hours. Then, using a moisture meter (Sartorius MA 45), the quantity of water necessary to attain 100% WHC was measured. From this information, the volume of water needed to dilute the metals for a final WHC of 50% was determined. The moist spiked substrate was left to incubate at room temperature for 2 days before adding the test organisms to be exposed.

2.3. Feeding

During the experiments, *E. doerjesi* was fed with finely ground Jungle oats®, while *E. andrei* was provided with laboratory processed cattle manure. This manure was collected on a university farm (Welgevallen) where animals are not treated with growth hormones or similar veterinary products. The raw fresh manure was dried at 70-90^{o}C for two days, re-wet (washed) and dried again

before being finely ground. In this dried form, the manure could be conserved in sealed containers until the time of use.

2.4. Endpoints

The endpoints assessed in this project can be divided into two categories: life cycle parameters (measured at individual level) and biomarkers (measured within the organism). Another parameter of interest was the total metal accumulated by the test species at the end of the exposure period.

2.4.1. Life cycle parameters

At the individual level, the endpoints of interest were biomass, reproduction and survival. *E. doerjesi*, being a fairly small species (± 5.3 mm), could not be accurately weighed. Consequently, only reproduction and survival were assessed for this species.

2.4.2. Biomarkers

Biomarkers are cellular, biochemical or molecular responses to an insult by a toxicant (Dallinger *et al*. 2000). These below-individual levels effects can be determined long before they manifest in the whole organism. The responses assessed at the appropriate time can provide early warnings of potential deleterious effects that may later manifest in the whole organism (Svendsen & Weeks 1997). The biomarkers selected for the present study were the comet and the MTT assays.

2.4.2.1. The comet assay

This assay measures the genotoxicity of substances by measuring DNA strand breaks. It was originally developed by Östling & Johanson (1984) as an electrophoretic technique for the visualization and quantification of DNA damage in individual cells. The comet assay is used extensively in the medical field (Kan *et al*. 2002, Le *et al*. 2003, Lou *et al*. 2007; Shahidi *et al*. 2007) and increasingly

as a tool in ecotoxicology (Reinecke & Reinecke 2004; Fourie *et al*. 2007, Voua Otomo *et al.* 2010).

2.4.2.2. The MTT assay

Mosmann (1983) developed the MTT assay as a means of measuring the activity/viability of cells via their mitochondrial activity. As live mitochondria reduce the yellow tetrazolium salt MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) by cleaving its tetrazolium ring, a blue product (formanzan) is released. The spectrophotometric estimation of the blue formanzan is an indication of cell survival and proliferation. The MTT test has been successfully used in ecotoxicology (Maleri *et al*. 2008*,* Voua Otomo *et al*. 2010).

2.4.3. Total metal body burden

In order to compare metal accumulation patterns across concentrations and temperatures, total metal body burden was assessed in selected specimens. Since bodyweight could not be accurately measured for *E. doerjesi*, total metal body burden was only assessed in *E. andrei*. The worms were acid digested as described by Katz & Jeniss (1983) and the metal contents were estimated by flame atomic absorption spectroscopy using a Varian AA-1275 machine.

2.5. Statistical Analysis

Descriptive statistics on the data during this study were performed using ToxRat® 2.09 (Toxicity Response Analysis and Testing; GMBH, Germany). Endpoints assessed at each temperature and metal/mixture concentrations were tested for normality using the range-to-standard-deviation ratio (R/s). Normally distributed data were tested further for the homogeneity of variances using the Cochran's test. Normally distributed data with homogenous variances were analyzed using a parametric multiple test (One way-ANOVA, with Bonferroni posttest). Normally distributed data with non-homogenous variances were analyzed using the parametric Bonferroni t-test for non-homogenous variances.

Non-parametric data were analyzed using the Kruskal-Wallis ANOVA followed by Dunns' test.

Factorial ANOVA followed by Bonferroni posttest was also performed to assess the interaction between temperature and metal on the selected endpoints at all three temperatures and metal/mixture concentrations. The level of significance was $p < 0.05$. These analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

EC_{50s} indices were estimated using non-linear regression analyses. The following log-logistic equation model was used:

$$y = \frac{y\max}{1+\left(\frac{Log[c]}{EC_{50}}\right)^{b}} \qquad (1)$$

where $y$max is the response (reproduction/biomass) at dose zero, c is the concentration (dose) of the toxicant under investigation, EC_{50} is the median effect concentration and b the slope parameter.

Equation (1) was programmed in PASW (Predictive Analytics softWare) statistics version 18 (SPSS, Inc, 2009, Chicago, Illinois, www.spss.com) and non-linear analyses of the endpoints of interest measured at different exposure regimes were performed. LC_{50} indices were generated when possible using the trimmed Spearman-Karber Program version 1.5.

Mixture data were analyzed using the toxic unit (TU) approach. Bliss (1939) was the firsts to suggest that the "toxicity of poisons applied jointly" could be conveniently assessed after expressing the concentration of each of the contributing "poisons" as ratios of their LD50. This approach is based on the concept of concentration additivity (Newman & Unger 2003). It assumes that the

toxicity of a mixture of *n* toxicants can be predicted by adding up the relative toxicity of each of the toxicants (Hermens & Leeuwangh 1982; Kraak *et al*. 1994). The expected toxicity of the mixture, expressed as the sum of the ratios of actual toxicant concentrations to their effective concentrations (EC_{50} or LC_{50}), representing the sum of toxic units can be expressed as follows:

$$\sum TU_i = \sum_{i=1}^{n} \frac{c_i}{ECx_i} \qquad (2)$$

Where c_i is the nominal concentration of toxicant i and EC_x the concentration of toxicant i that causes a reduction of x percent of the endpoint being measured as compared to the control.

The sum of TU at 50% inhibition (EC_{50mix}) was also estimated using equation (1). EC_{50mix} was subsequently used to assess the type of metal interactions in the mixtures: concentration additive (EC_{50mix} = 1TU), synergistic (EC_{50mix} < 1TU), or antagonistic (EC_{50mix} > 1TU) (Spehar and Fiandt 1986; Newman & Unger 2003; An *et al*. 2004).

Mixture data, nonetheless, were further analyzed following the method of Jonker *et al*. (2005). These authors stated that more complex interactions than synergism and antagonism are often overlooked during mixture analyses. Mixtures are routinely analyzed, using either the concentration addition (CA) or the independent action (IA) reference models (De March 1987). Simply stated, the CA model seeks to find out whether the relative toxicity of a mixture correlates to the relative toxicity of its individual chemicals. The IA model however seeks to establish whether the chemicals involved in a mixture could still cause independent responses (De March 1987).

Jonker *et al*. (2005) argued that the CA and IA models are not necessarily mutually exclusive and proposed a stepwise statistical approach that could help to establish which of the two reference models describes a chosen set of data

(explains the variance) the best . Moreover, Jonker *et al*. (2005) have ascertained four chemical interactions that could fall within either the CA or the IA reference models. These are listed in Table 1 together with the algorithms that define them.

The presence of these interactions was investigated using MixToxModules, which are Microsoft Office Excel spreadsheets already programmed with the algorithms in Table 1 based on the stepwise analysis approach used by Jonker *et al.* (2005). MixToxModules were downloaded from http://www.ceh.ac.uk/products/stats/MixtureToxicity-AnalysisTools.html.

Table 1. Common mixture interactions and their inherent model algorithms. These algorithms are the product of an extensive process of mathematical manipulations that begins with the single dose response model and the mixture model functions (See Jonker *et al*. [2005] for more details).

Type of interaction	Meaning	Algorithm
(1) Synergism or (2) Antagonism (S/A)	The chemicals involved in the mixture cause toxic effect either in a cohesive (1) or a competitive (2) way	$G(z_1,...,z_n) = a\prod_{i=1}^{n} z_i$ G = deviation function from either CA or IA reference models z_i = relative amount of toxic units of each chemical involved Parameter a indicates either synergism or antagonism. For interpretation, see Table 2
(3) Dose ratio dependent Synergism/Antagonism (DR)	The nature of the interaction between the chemicals involved changes from synergism to antagonism, or the other way around, depending on the chemical ratio	$G(z_1,...,z_n) = a\,(a + b_1 z_1 + ... + b_{n-1} z_{n-1})\prod_{i=1}^{n} z_i$ Parameter a indicates either synergism or antagonism. Parameter b_i indicates the chemicals that contribute the most to the synergism or antagonism. For interpretation, see Table 2
(4) Dose level dependent Synergism/Antagonism (DL)	The nature of the interaction between the chemicals involved changes from synergism to antagonism, or the other way around, depending on the concentrations of the chemicals.	$G(z_1,...,z_n) = a\left(1 - b_{DL}\sum_{i=1}^{n} TU50_i\right)\prod_{i=1}^{n} z_i$ Parameter a indicates whether synergism or antagonism occurs at low or higher dose. Parameter b_{DL} indicates at which dose the switch between synergism and antagonism occurs. For interpretation, see table 2.

Table 2 is provided as an interpretation guide for the equation parameters (a, b_i and $b_{DL,}$) after MixToxModules data analyses. After these analyses 3D surface plots of the models were generated in Statistica (Data analysis software system), version 9. (Statsoft, Inc, 2009, Tulsa, Oklahoma, www.statsoft.com)

Table 2. Interpretation of equation parameters a, b_i and $b_{DL,}$ under each reference model (CA and IA). Table reproduced integrally from Jonker *et al*. (2005).

Parameter	Value		Meaning
	CA	IA	
		Synergism/antagonism	
a	>0	>0	Antagonism
	<0	<0	Synergism
		Dose ratio dependence	
a	>0	>0	Antagonism, except for those mixture ratios where significant negative b_is indicate synergism
	<0	<0	Synergism, except for those mixture ratios where significant positive b_is indicate antagonism
b_i	>0	>0	Antagonism where the toxicity of the mixture is caused mainly by toxicant i
	<0	<0	Synergism where the toxicity of the mixture is caused mainly by toxicant i
		Dose level dependence	
a	>0	>0	Antagonism low dose level and synergism high dose level
	<0	<0	Synergism low dose level and antagonism high dose level
b_{DL}	>1	>2	Change at lower dose level than the EC50
	$=1$	$=2$	Change at the EC50 level
	$0 < b_{DL} < 1$	$1 < b_{DL} < 2$	Change at higher dose level than the EC50
	<0	<1	No change, but the magnitude of synergism/antagonism is dose level (CA) or effect level (IA) dependent

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3. Survival, reproduction and biomass change of *Eisenia andrei* in single and mixture exposures of cadmium and zinc at different temperatures

3.1. Introduction

Research in the field of terrestrial ecotoxicology has shown that metal toxicity to earthworms can affect a wide range of parameters including growth (biomass), reproduction and survival.

Ma (1984) reported that Cu concentrations of 100-150 mg/kg in sandy or loamy soils could cause significant decrease in cocoon production in *Lumbricus rubellus*. This author also found that although biomass seemed not to be affected at Cu concentrations in the order of 300 mg/kg, mortality had started to occur. Spurgeon & Hopkin (1999) reported that the total abundance and biomass of earthworms collected along two transects from a primary Pb/Zn/Cd smelting works (at Avonmouth, UK) decreased with proximity to the smelter. Panda *et al.* (1999) assessed the effects of Zn on the growth, reproduction and life cycle of the Indian oligochaete *Drawida willsi.* They found that although at the highest tested metal concentration (400mg/kg) there was still no significant changes in biomass with the control treatment, reproduction in *D. willsi* was significantly reduced in treatments exceeding 200 mg/Kg Zn. Results found by Helling *et al*. (2000) showed that Cu concentrations from 15.92 mg/kg significantly reduced reproduction success in *E. fetida*. Reinecke *et al.* (2001), after exposing three earthworm species (*Eudrilus eugeniae, Perionyx excavatus* and *E. fetida*) to sublethal concentrations of Pb, concluded that although cocoon production was not impaired by the metal, cocoon viability was different between the control and the Pb contaminated treatments. Reinecke & Reinecke (1997) had previously shown that metals such as Pd and Mn could affect reproduction in *E. fetida* by inflicting structural damage of spermatozoa to contaminated earthworms. Such damage included breakage and loss of nuclear and flagellar membranes, thickening of membranes, malformed acrosomes and loss of nuclear material.

In the specific case of the earthworm *E. andrei*, van Gestel *et al.* (1989) assessed the influence of Cu on the cocoon production in this species. The no-effect level for this specific endpoint was reported to lie between 60 and 120 mg/kg Cu. Van Gestel *et al.* (1992) established, while assessing the effects of nine different chemicals (including Cd, Cr, benomyl and carbendazim) on *E. andrei*, that Cd was the most toxic and that it particularly affected reproduction and growth (given the concentrations tested 0-100 mg/kg Cd). Van Gestel *et al*. (1993) subsequently established that metal concentrations in the order of 10mg/kg for Cd, 100mg/kg for Cr and 560 mg/kg for Zn could significantly reduce cocoon production in *E. andrei* when exposed in artificial soil for three weeks. Similarly, exposure of *E. andrei* to Al (0-1000 mg/kg) in artificial soil at neutral pH for 6 weeks was also found to affect cocoon production at the highest concentrations (Van Gestel *et al*. 2001).

Given the results found by the authors above, it seems that as an endpoint, reproduction is the most sensitive compared to both growth and survival. Even for an essential metal such as Cu, although the EC_{50} for reproduction would be fairly high, reproduction would usually be critically affected before growth and survival (Pasteris *et al.* 2008).

Apart from the direct deleterious effects of metal contamination on the life-cycle characteristics of earthworms, growth, reproduction and biomass variation in earthworms can also be influenced by ambient conditions.

According to Wever *et al.* (2001) and Eriksen-Hamel & Whalen (2006), soil moisture and temperature are the key factors controlling growth and survival in earthworms. Both these factors are reported to account for up to 68% of variation in growth in the endogeic earthworm species, *Aporrectodea tuberculata* (Wever *et al.* 2001). Eriksen-Hamel & Whalen (2006) have established that growth rates in *Aporrectodea caliginosa* are a function of both temperature and moisture.

Viljoen & Reinecke (1992) reported that the epigeic earthworm species, *Eudrilus eugeniae* was unable to survive temperatures below 12^{o}C, and could not withstand a temperature of 30^{o}C for more than 50 days. Similarly, Berry & Jordan (2001) found that *Lumbricus terrestris* was unable to survive at 30^{o}C and 25^{o}C for more than 14 and 182 days respectively. Daniel *et al.* (1996) had previously established in the same species that maturation time decreased with increasing temperature from 27.7 weeks at 7.5^{o}C to 9.4 weeks at 20^{o}C.

As far as reproduction is concerned, Viljoen *et al.* (1992) have established, in the case of *Dendrobaena veneta*, that although higher temperature (25^{o}C vs. 15^{o}C) favours higher cocoon production, hatching however is higher at lower temperature.

Although it has been established that metal pollution and temperature variation could independently affect the growth, reproduction and survival of earthworms, studies investigating the combined influence of both these stressors are very few.

The following authors have investigated this question. Spurgeon *et al.* (1997) assessed the influence of temperature on the toxicity of Zn on the survival and reproduction of *E. fetida* at 15, 20 and 25^{o}C in a 21-day toxicity test conducted in OECD soil. Results indicated that the LC_{50} decreased (from 1598 to 1131 mg/Kg) as temperature increased (from 15 to 25^{o}C). Similarly, the EC_{50} for reproduction decreased from 382 mg/kg (at 15^{o}C) to 234 mg/kg (at 25^{o}C). These results indicated that Zn toxicity in *E. fetida* increases with increasing temperature. Spurgeon *et al*. (2005) assessed the response of *L. rubellus* to Cd and Cu under fluctuating environmental conditions and concluded that although for Cu there seemed to be no interactions, higher temperature seemed to increase Cd tissue accumulatio,n. Concerning Cu toxicity to *L. rubellus*, Marinussen *et al.* (1997), in contrary, reported that variation in field temperature (in an agricultural field in Wageningen, The Netherlands) were positively correlated to Cu accumulation in that earthworm species: increasing field temperature led to increased accumulation of Cu in *L. rubellus*.

In light of these findings, the extent of the influence heat stress might have on metal toxicity in earthworms merits further investigation. Temperature can be expected to vary daily, seasonally and at a more global scale due to climate change and the predicted global warming. Daily soil temperature amplitudes of up to 30^{o}C, for example, have been documented during the spring of 1999 in Skukuza, South Africa (Pinheiro *et al*. 2001). In Cape Town, current seasonal maximum mean air temperatures vary from 26^{o}C in summer to 22^{o}C in autumn and 17.6^{o}C in winter to 20^{o}C in spring (BBC weather center). Due to the current rate of global warming, a 4.5 to 5.8^{o}C increase of global mean temperature is expected by the year 2100 (IPCC 2001). Moreover, differences in temperature caused by geographical location and variation in altitude calls for the combined effects of metal pollution and temperature on selected organisms to be investigated in order to assess risks realistically.

The aim of this study was to assess the separate and combined effects of Cd and Zn on the survival, reproduction and biomass of the earthworm species *E. andrei* at three different temperatures, namely 15, 20 and 25^{o}C.

3.2. Materials & Methods

3.2.1. Acclimation to OECD soil

Two days prior to the start of the experiment, 10 to 12 week old (adult) *E. andrei* specimens were selected and placed in clean moist (50% WHC) OECD soil in order to allow the test organisms to acclimate to the exposure substrate. Earthworms were fed clean dried and ground cattle manure during that time (5g per 10 worms).

3.2.2. Cadmium and zinc exposures

Acclimated earthworms were thereafter taken out of the acclimation soil, rinsed with distilled water, weighed individually and exposed in artificial OECD soil to Cd and Zn separately or in mixtures. The metals were provided in the form of $CdSO_4$ and $ZnSO_4$. Ten specimens per treatment were exposed in 500 g (wet weight) of soil for each replicate. For single metal exposures, concentrations were 0, 250, 500, 750, and 1000 mg/kg for both Cd and Zn. These concentrations were decided upon after a careful study of the literature and chosen to be largely sub-lethal. In the present study, the experimental design for mixtures was such that the fix ratio of metals was 1:1. Mixture concentrations were therefore: 0+*0*, 50+*50*,100+*100*; 250+*250*, 500+*500*, and 750+*750* mg/kg Cd+*Zn*. Exposures were carried out in round glass containers (75 [diameter] x 160 [height] mm) with perforated plastic lids. Similar experiments each with three replicates were run separately at 15, 20 and 25^{o}C for four weeks.

During the experiments, earthworms were fed clean dried and ground cattle manure on a weekly basis, starting 24 hours after the start of the exposure period. Five grams (dry weight) of manure were moistened with distilled water and placed on top of the substrate in each glass container. During the following four weeks, that portion of food was either maintained or reduced depending on the feeding ability of the earthworms.

Earthworm reproduction (cocoon number), survival and biomass were assessed weekly. The cocoons collected were kept at their respective temperature of origin in Petri dishes filled with tap water. These cocoons were further monitored daily to determine hatching success for up to 12 weeks.

3.2.3. Body burdens of metals in the earthworms

At the end of the exposure period, the metal concentration in the tissues of the earthworms was estimated. Three worms per treatment were randomly selected and acid digested (Katz & Jenniss 1983). The selected worms were weighed and

placed in test tubes overnight at room temperature in 5 ml (55%) nitric acid. Thereafter the samples were heated (in a heating block) between 40^{o}C-60^{o}C for 2 hours and then the temperature was increased to 120^{o}C and kept constant until brown fumes were release from the test tubes. Samples were then left to cool down for 1 hour at room temperature before 0.5 ml perchloric acid (70%) was added to each tube. Thereafter tubes were again heated to 120^{o}C until the release of brown fumes. After cooling down for another hour, 5 ml of distilled water were added to each sample before they were reheated to 120^{o}C until the appearance of white fumes. Samples were then left to cool down overnight before they were filtered through Whatman no. 6 filter paper, made up to 10 ml with distilled water and filtered again through 0.45-lm Sartorius cellulose nitrate microfilters. All samples were kept in dark plastic tubes until metal contents (Cd and/or Zn) were assessed with a Varian AA-1275 flame atomic absorption spectrophotometer (Varian Inc., Palo Alto, California, USA). Precision data were as follows: For Cd average percent relative standard deviation (% RSD) = 0.1; while average % RSD for Zn = 3.5.

3.2.4. Statistical Analysis

Descriptive statistics were performed using ToxRat® 2.09 (Toxicity Response Analysis and Testing; GMBH, Germany). Normally distributed data were tested further for the homogeneity of variances using the Cochran's test. Normally distributed data with homogenous variances were analyzed using a parametric multiple test (One-way ANOVA, followed with Bonferroni posttest). Normally distributed data with non-homogenous variances were analyzed using the parametric Bonferroni t-test for non-homogenous variances. Non-parametric data were analyzed using the Kruskal-Wallis ANOVA followed by Dunns' test. Factorial ANOVA followed by Bonferroni posttest was performed to assess the interaction between temperature and metal on the selected endpoints at all three temperatures and metal/mixture concentrations. These analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). LC_{50} values were estimated using

the trimmed Spearman-Karber Program version 1.5. EC_{50} indices were estimated using non-linear regression analyses in PASW (Predictive Analytics softWare) statistics version 18 (SPSS, Inc, 2009, Chicago, Illinois, www.spss.com). Mixture data were analyzed using the toxic unit (TU) approach (Bliss 1939). Mixture interactions were further investigated using MixToxModules spreadsheets (Jonker *et al*. 2005). The level of significance was $p < 0.05$. After these analyses 3D surface plots of the models were generated in Statistica (Data analysis software system), version 9. (Statsoft, Inc, 2009, Tulsa, Oklahoma, www.statsoft.com)

3.3. Results

3.3.1. Survival

3.3.1.1. Cd exposures

Mortality in the single Cd exposures was between 0-7% in all Cd concentrations at both 15 and 20°C. At 15°C, mortality (3.3%) only occurred at 1000 mg/kg Cd (Fig. 1). At 20°C mortality was recorded at 1000 (6.6%) and 750 mg/kg Cd (6.6%; Fig. 1). At 25°C, mortality rates were above 55% in the two highest Cd treatments 56.6% at 750 mg/kg and 66.6% at 1000 mg/kg and also occurred at 500 mg/kg Cd (13.3%; Fig. 1). Consequently, an LC_{50} (with 95% confidence intervals) of 725.46 (579.12 - 908.79) mg/Kg could only be estimated at 25°C.

Two-way ANOVA followed by Bonferroni posttests, revealed that there was a highly significant interaction between Cd concentrations and temperature which affected the survival of *E. andrei* after exposure to Cd at 15, 20 and 25°C ($p < 0.0001$; Table 1).

Table 1. Two-way ANOVA results of the analysis of the effects of temperature and Cd on the survival of *E. andrei* after exposure in artificial OECD soil for four weeks at 15, 20 and 25^{o}C**.**

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	33.76	8	73.6	9.2	10.62	$p < 0.0001$
Temperature	31.07	2	67.73	33.87	39.08	$p < 0.0001$
Cd concentrations	23.24	4	50.67	12.67	14.62	$p < 0.0001$
Residual		30	26	0.867		

Fig. 2 represents a comparison of survival rates between the three temperatures. There was no significant difference in survival between 15 and 20^{o}C. Between both these temperatures and 25^{o}C however, survival was significantly lower at 25^{o}C, in the 750 and 1000 mg/kg treatments ($p < 0.001$; Fig. 2).

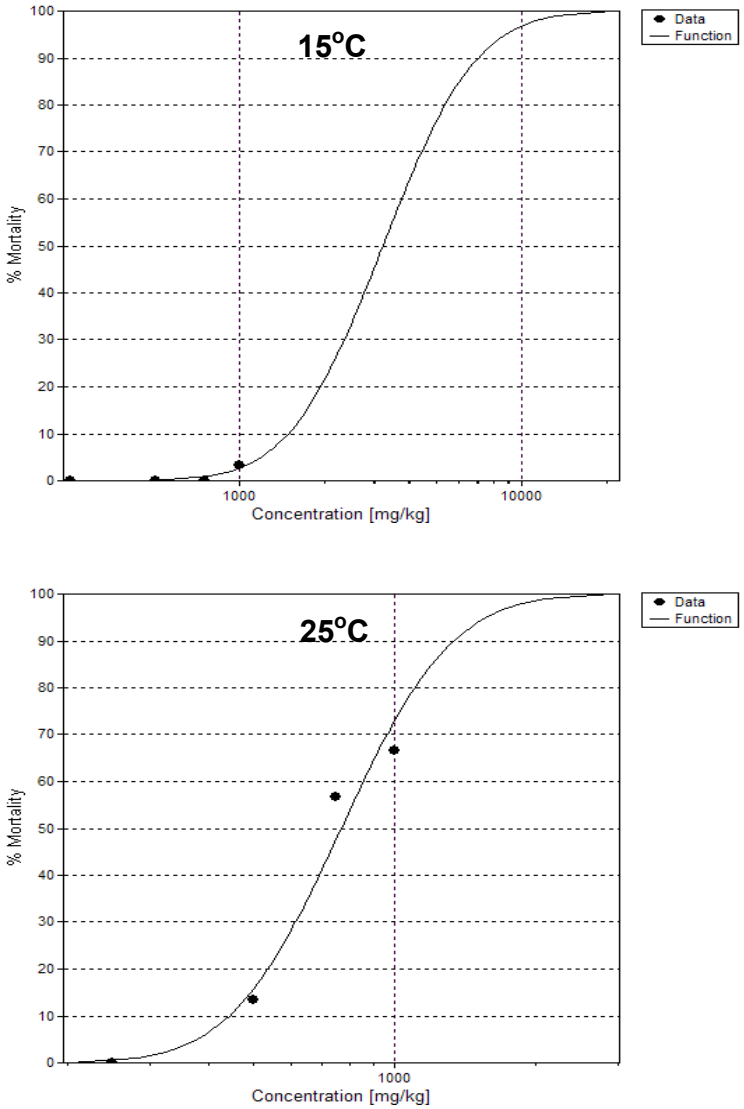


Fig. 1. Survival curves of *E. andrei* after exposure to Cd in artificial OECD soil for four weeks at 15, 20 and 25°C.

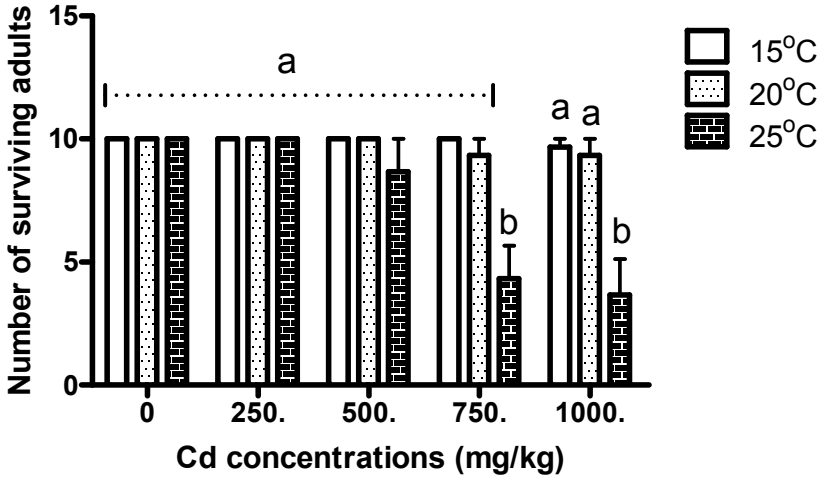


Fig. 2. Comparison of the survival rates of *E. andrei* at 15, 20 and 25°C after exposure to Cd in artificial OECD soil for four weeks. Error bars represent standard error. Different letters on bars represent statistical differences ($p < 0.001$).

3.3.1.2. Zn exposures

In the Zn single exposures at 15°C, no mortality was recorded. At 20°C, 30% mortality occurred in 1000 mg/kg. At 25°C, only 16.6% mortality was recorded in 1000 mg/kg (Fig. 3). Consequently, an LC_{50} for Zn could not be estimated because at all temperatures and treatments, less than 50% mortality was recorded.

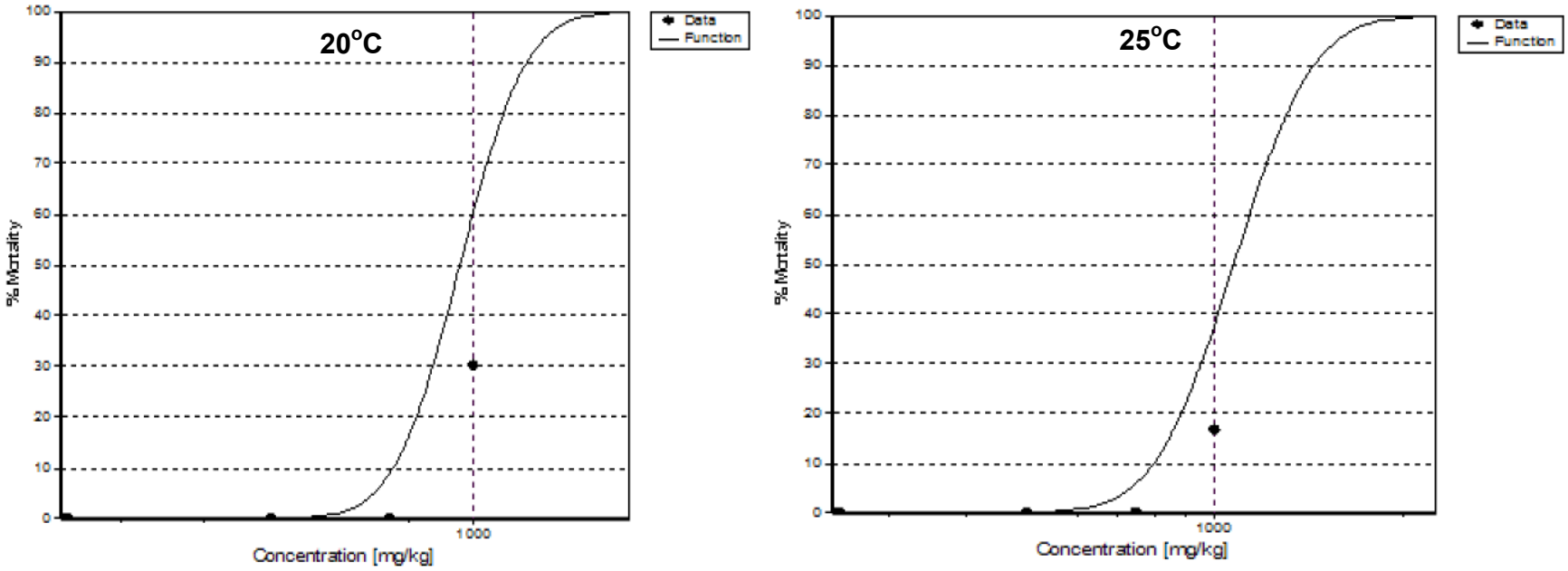


Fig. 3. Survival curves of *E. andrei* after exposure to Zn in artificial OECD soil for four weeks at 20 and 25°C. A survival curve could not be computed for exposures at 15°C since no mortality occurred in any of the Zn treatments at this temperature.

Two-way ANOVA followed by Bonferroni posttests, revealed that there was a highly significant interaction between Zn concentrations and temperature which affected the survival of *E. andrei* after exposure to Zn at 15, 20 and 25^{o}C ($P < 0.0001$; Table 2).

Table 2. Two-way ANOVA results of the analysis of the effects of temperature and Zn on the survival of *E. andrei* after exposure in artificial OECD soil for four weeks at 15, 20 and 25^{o}C.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	30.42	8	10.84	1.356	8.714	p<0.0001
Temperature	7.61	2	2.711	1.356	8.714	0.001
Zn concentrations	48.88	4	17.42	4.356	28.00	p<0.0001
Residual		30	4.667	0.1556		

Fig. 4 represents a comparison of survival rates between all three temperatures. Survival was significantly lower only in the 1000 mg/kg treatment at both 20 and 25^{o}C ($p < 0.001$ and $p < 0.01$; respectively; Fig. 4).

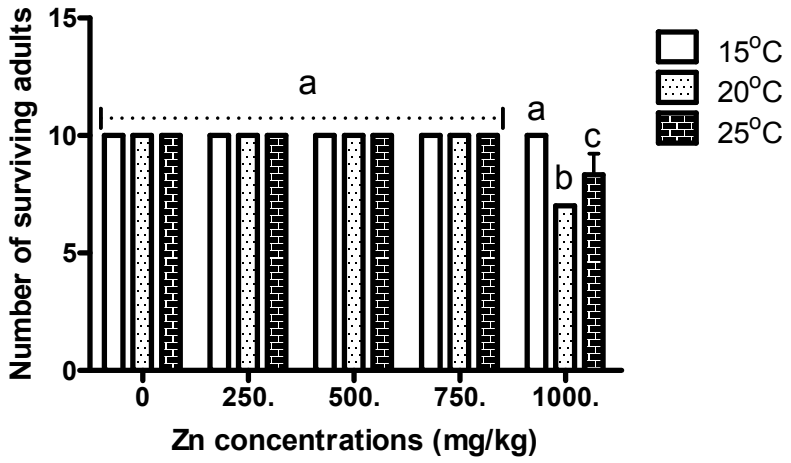


Fig. 4. Comparison of the survival rates of *E. andrei* at 15, 20 and 25°C after exposure to Zn in artificial OECD soil for four weeks. Error bars represent standard error. Different letters on bars represent statistical differences ($p \leq 0.01$).

3.3.1.3. Mixture exposures

The mixture concentration range of Cd+Zn (0+*0*, 50+*50*,100+*100*; 250+*250*, 500+*500*, and 750+*750* mg/kg) was equivalent to 0, 1.26, 2.52, 6.31, 12.61 and 18.92 TU (See details in section 3.3.2.3.1.).

At 15^{o}C, mortality only occurred in the two highest mixture concentrations, 12.61 TU (3.3%) and 18.92 TU (30%; Fig. 5). At 20^{o}C, mortality similarly only occurred at 12.61 TU (10%) and 18.92 TU (53.33%; Fig 5). At 25^{o}C, mortality occurred at 6.31 TU (6.66%), at 12.61 TU (3.3%) and 18.92 TU (80%; Fig. 5).

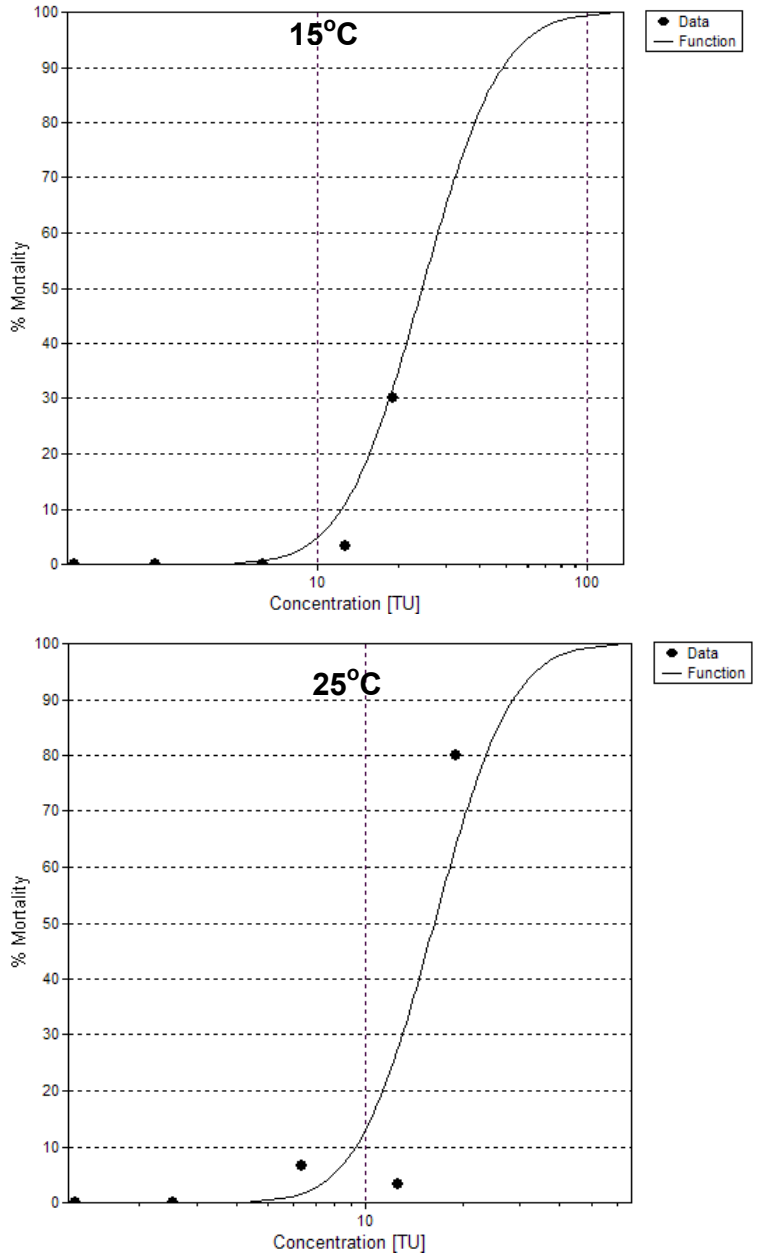
LC_{50} (with 95% confidence intervals) estimated only at 20 and 25^{o}C were 18.34 (15.66-21.47) TU and 16.09 (15.29-16.92) TU respectively.

Two-way ANOVA between temperature and mixture treatments revealed a highly significant interaction (p = 0.0002; Table 3)

Table 3. Two-way ANOVA results of the analysis of the effects of temperature and mixtures of Cd and Zn on the survival of *E. andrei* after exposure in artificial OECD soil for four weeks at 15, 20 and 25^{o}C.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	11.43	10	31.3	3.13	4.97	0.0002
Temperature	2.93	2	8.04	4.02	6.38	0.0042
Mixture concentrations	77.37	5	212	42.4	67.3	p<0.0001
Residual		36	22.7	0.630	4.97	

Fig. 6 represents a comparison of survival rates between all three temperatures. In the mixture treatments, survival was significantly different only in the 18.92 TU treatment between all temperatures (p ≤ 0.01; Fig. 6).



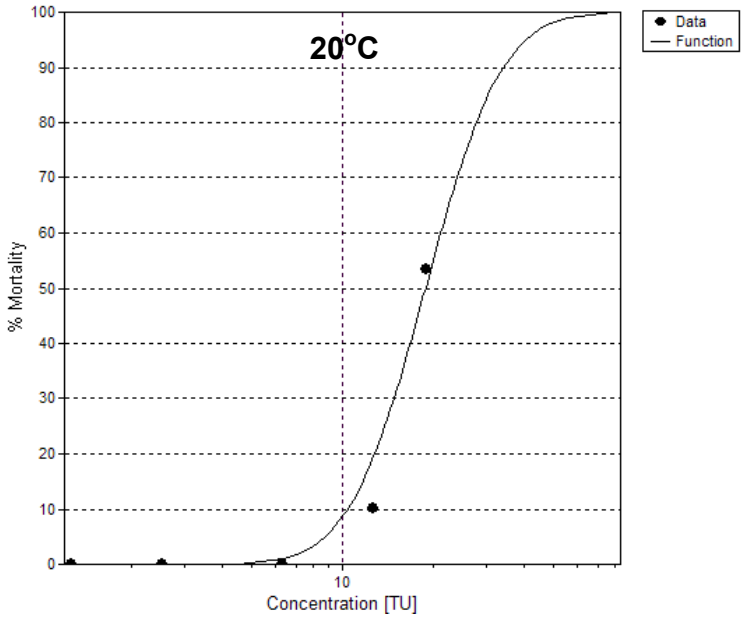


Fig. 5. Survival curves of *E. andrei* after exposure to mixtures of Cd and Zn in artificial OECD soil for four weeks at 15, 20 and 25°C.

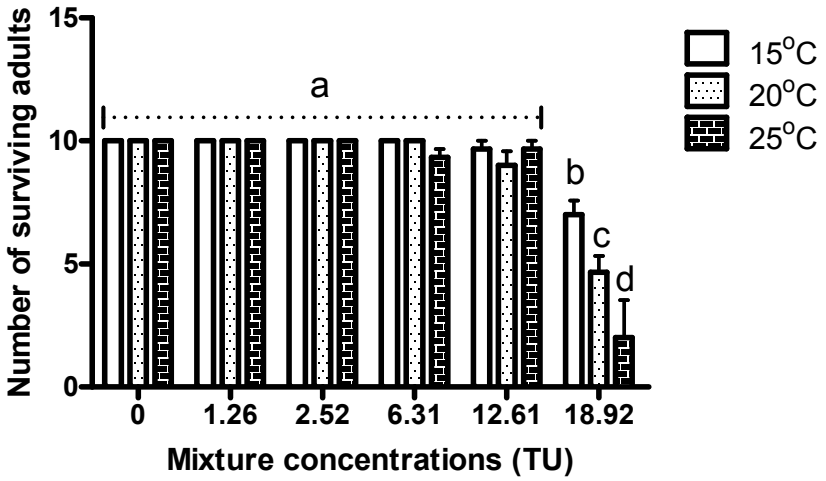


Fig. 6. Comparison of the survival rates of *E. andrei* at 15, 20 and 25°C after exposure to mixture concentrations of Cd and Zn in artificial OECD soil for four weeks. Error bars represent standard error. Different letters on bars represent statistical differences ($p \leq 0.01$).

3.3.2. Reproduction

3.3.2.1. Cd exposures

In the Cd exposures, reproduction, as evidenced by cocoon production, was only recorded in the control treatments (0 mg/kg Cd). No cocoons were found in the Cd contaminated substrates. The cocoon production per week is presented in Fig. 7 for each of the exposure temperatures. Between 15 and 20°C, the number of cocoons produced was significantly different only at week 2 ($p < 0.05$) with higher reproduction occurring at the higher temperature. Between 15 and 25°C, significantly higher reproduction only occurred at week 1 with higher cocoon production at 25°C ($p < 0.05$). There was no difference in cocoon production between 20 and 25°C except at week 1 where reproduction was significantly higher at 25°C ($p < 0.001$; Fig. 7).

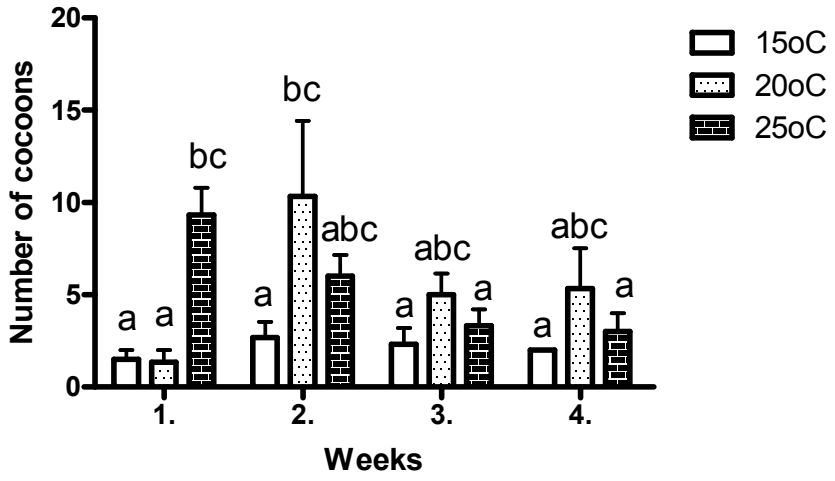


Fig. 7. Mean cocoon production of *E. andrei* in the controls (0 mg/kg) of the Cd experiment over four weeks at 15, 20 and 25°C in artificial OECD soil. Error bars represent standard error. Different letters above bars represent statistical differences ($p < 0.05$). n = 30 / temperature.

Although the total number of cocoons produced in the exposures incubated at 20°C exceeded the number of cocoons produced at 25°C, there were more hatchlings at 25°C than at 20°C (Table 4). The mean cocoon number per worm per week at each temperature was 0.166 at 15°C, 0.55 at 20°C and 0.51 at 25°C. Thus, for four weeks, the mean cocoon number per worm was 0.66 at 15°C, 2.2 at 20°C and 2.06 at 25°C (Table 4).

Table 4. Total and mean number of cocoons and hatchlings of *E. andrei* in the controls (0 mg/kg) of the Cd experiment after four weeks at 15, 20 and 25°C in artificial OECD soil. n = 30 / temperature.

	15°C	20°C	25°C
Total number of cocoons	20.00	66.00	62.00
Mean number of cocoons /worm	0.67± 0.2	2.20 ± 0.5	2.06± 0.8
Total number of hatchlings	29.00	103.00	111.00
Mean number of hatchlings /cocoon	1.45	1.56	1.79

Cocoon incubation time decreased consistently as temperature increased from 15 to 20 and 25^{o}C. At 15^{o}C, cocoons required an incubation time of 80 ± 1.6 days before hatching. At 20 and 25^{o}C, the incubation periods were 20 ± 1 and 13 ± 0.33 days respectively. These durations were significantly different from one temperature to another ($p \leq 0.01$).

The mean number of hatchlings per cocoon increased from 1.45 at 15^{o}C, to 1.56 at 20^{o}C and 1.79 at 25^{o}C (Table 4). These numbers, however, did not differ statistically

3.3.2.2. Zn exposures

In the Zn treatments, reproduction was recorded in 0, 250 and 500 mg/kg (Fig. 8). Cocoon production increased as temperature increased but decreased with increasing Zn concentrations. Between 15 and 20^{o}C, cocoon production was statistically different only in the control (0 mg/kg) with higher reproduction occurring at 20^{o}C ($p < 0.05$). Between 15 and 25^{o}C, statistically higher cocoon production occurred at 25^{o}C in both the control ($p < 0.001$) and 250 mg/kg ($p < 0.01$). Between 20 and 25^{o}C, similarly, statistically higher cocoon production was recorded at 25^{o}C in both the control ($p < 0.05$) and 250 mg/kg ($p < 0.01$; Fig. 8). Table 5 shows the number of cocoons per week and per worm at all three temperatures.

Table 5. Cocoon production rates of *E. andrei* after exposure to Zn in artificial OECD soil for four weeks at 15, 20 and 25^{o}C. n = 90 / temperature.

Zn concentrations (mg/kg)	Cocoons/week/worm		
	15	20	25
0	0.16	0.4	0.65
250	0.15	0.08	0.46
500	0.041	0.041	0.21

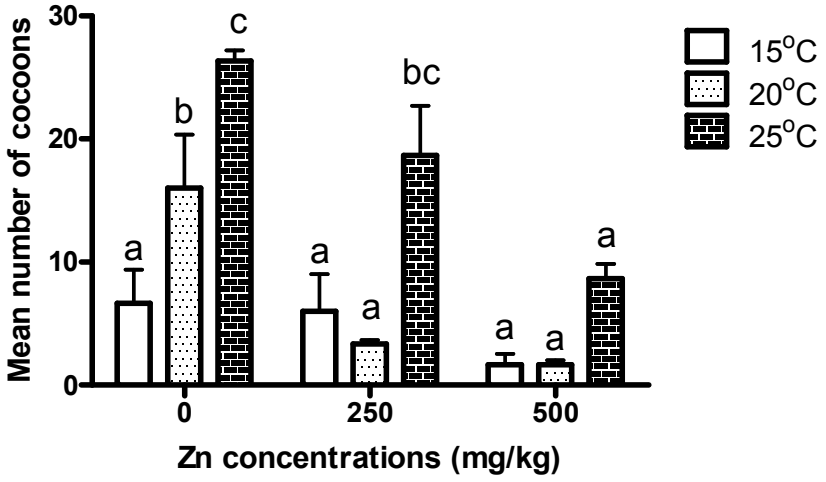


Fig. 8. Cocoon production of *E. andrei* after exposure to Zn for four weeks in artificial OECD soil at 15, 20 and 25°C. Error bars represent standard error. Different letters on bars represent statistical differences ($p \leq 0.05$). n = 90 / temperature.

Two-way ANOVA followed by Bonferroni posttests showed that there was no interaction between Zn and temperature and that temperature alone explained 42.19% of the recorded reproduction (Table 6).

Table 6. Two-way ANOVA results of the analysis of the effects of temperature and Zn on the reproduction of *E. andrei* after exposure in artificial OECD soil for four weeks at 15, 20 and 25°C. The abbreviation ns means not significant.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	9.29	4	195.1	48.78	2.655	0.0668^{ns}
Temperature	42.19	2	886.2	443.1	24.12	P<0.0001
Zn concentrations	32.78	2	688.7	344.3	18.74	P<0.0001
Residual		18	330.7	18.37		

Fig. 9 showed that incubation times in the Zn experiment were temperature dependent rather than Zn dependent. Cocoon incubation time was found to

decrease with increasing temperature. However, as metal concentration increased, the incubation duration of the cocoons collected at each respective concentration remained fairly constant. In all Zn treatments where cocoons were collected, incubation times decreased from 43 ± 3 days at 15°C, to 21 ± 1 day at 20°C and 15 ± 2 days at 25°C (Fig. 9). These durations were highly different from one another ($p < 0.001$).

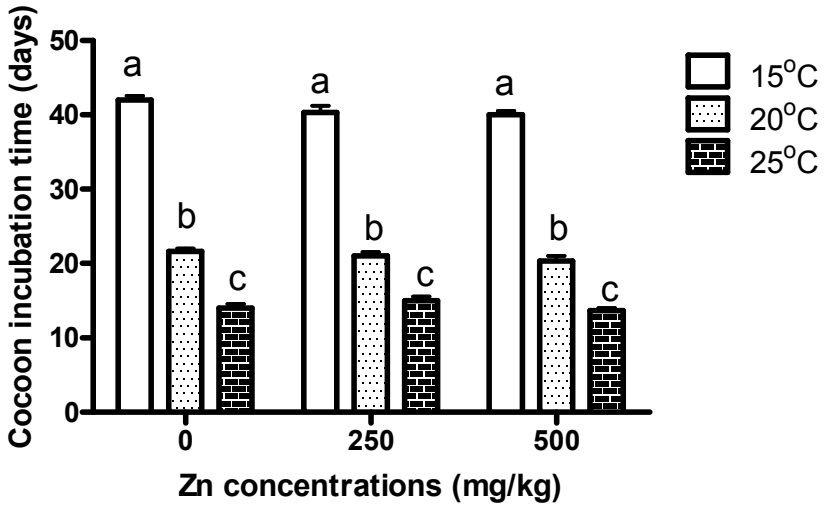


Fig. 9. Cocoon incubation time (in days) of *E. andrei* after exposure to Zn for four weeks in artificial OECD soil at 15, 20 and 25°C. Error bars represent standard error. Different letters on bars represent statistical differences ($p < 0.001$).

Hatchling numbers increased with increasing temperature but decreased with increasing Zn concentration (Fig. 10). Between 15 and 20°C, the number of hatchlings was statistically different only in the control with higher numbers occurring at 20°C ($p < 0.05$). Between 15 and 25°C, the number of hatchlings was statistically higher at 25°C in both the control ($p < 0.001$) and the 250 mg/kg treatment ($p < 0.01$). Between 20 and 25°C, similarly, the number of hatchlings was statistically higher at 25°C in both the control ($p < 0.05$) and the 250 mg/kg treatment ($p < 0.01$; Fig. 10).

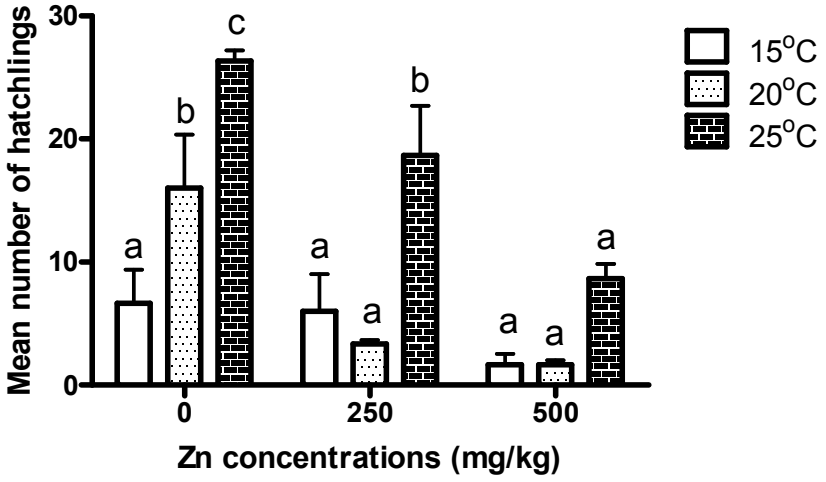


Fig. 10. Mean hatchling numbers of *E. andrei* after exposure to Zn in artificial OECD soil for four weeks at 15, 20 and 25°C. Error bars represent standard error. Different letters on bars represent statistical differences ($p \leq 0.05$).

The estimation of Zn EC_{50} for cocoon production using non-linear regression analyses revealed that the EC_{50} for cocoon production decreased between 15 and 20°C (from 185.22 to 94.62 mg/kg) and increased to 363.91 (241.37-548.02) between 20 and 25°C (Table 7).

Table 7. Estimation of Zn EC_{50} (mg/kg) for cocoon production after exposure of *E. andrei* in artificial OECD soil for four weeks at 15, 20 and 25°C. The numbers in brackets indicate 95% confidence intervals.

	15°C	20°C	25°C
logEC_{50}	2.67*	1.97*	2.56 (2.38-2.73)
EC_{50}	185.22	94.62	363.91 (241.37-548.02)
Model R^2	0.54	0.76	0.80

*95% confidence intervals could not be estimated at 15 and 20°C

3.3.2.3. Mixture exposures

3.3.2.3.1. Determining Toxic Units

Since median effective concentrations (EC_{50}) for cocoon production could not be estimated for Cd in the present study (due to the lack of reproduction in Cd treatments), in order to work out mixture concentrations and subsequent exposures, a Cd EC_{50} was selected after a literature search. Only a 56 day Cd EC_{50} for cocoon production in *E. fetida* of 46.3 mg/kg (determined at 20^{o}C) was found in Spurgeon *et al*. (1994). In order to avoid any experimental discrepancies, since all the Zn EC_{50}s estimated in the present study were 28 day EC_{50}s estimated in *E. andrei*, a 56 day Zn EC_{50} of 279 mg/kg was also selected from the same source. Both these EC_{50}s were used to express the mixture concentration series in toxic unit using equation (2) (See Chapter 2: General Materials & Methods; Section 2.5.). The same mixture concentration series was used at all three temperatures. As stated previously, the mixture concentration series 0+*0*, 50+*50*,100+*100*; 250+*250*, 500+*500*, and 750+*750* mg/kg (using equation [2]) was converted into the following concentration gradient in toxic units: 0, 1.26, 2.52, 6.31, 12.61 and 18.92 TU.

3.3.2.3.2. Reproduction in the mixture treatments

Reproduction in the mixture experiment, as estimated by cocoon production, occurred in the control and the three lowest mixture treatments (Fig. 11). Table 8 represents the cocoon production rates of *E. andrei* after exposure to mixtures of Cd and Zn for four weeks in artificial OECD soil. The comparison of the reproductive output between the temperatures revealed that between 15 and 20^{o}C, reproduction was significantly higher at 20^{o}C in the control ($p < 0.01$) and in the 1.26 TU treatment ($p < 0.01$). Between 15 and 25^{o}C, significantly higher cocoon production was recorded at 25^{o}C, in both the 1.26 ($p < 0.001$) and 2.52 TU mixture treatments ($p < 0.05$). Between 20 and 25^{o}C, no difference in cocoon production was found in any of the treatments (Fig. 11). In the 6.31 TU treatment, only one cocoon was recorded in the highest temperature.

Table 8. Cocoon production rates of *E. andrei* after exposure to mixtures of Cd and Zn in artificial OECD soil for four weeks at 15, 20 and 25°C. n = 120 / temperature.

Mixture concentrations (TU)	Cocoons/week/worm		
	15	20	25
0	0.28	0.59	0.46
1.26	0.04	0.38	0.60
2.52	0.08	0.26	0.33
6.31	0	0	0.008

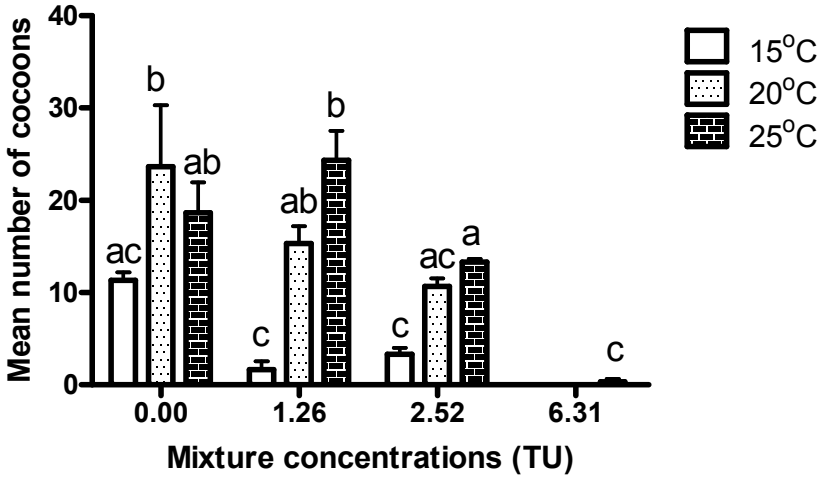


Fig. 11. Cocoon production of *E. andrei* after exposure to mixtures of Cd and Zn for four weeks in artificial OECD soil at 15, 20 and 25°C. Error bars represent standard error. Different letters on bars represent statistical differences ($p \leq 0.05$). n = 120 / temperature.

Two-way ANOVA followed by Bonferroni posttests indicated that there was a very significant interaction between temperature and mixture concentrations, which determined cocoon production ($p = 0.0037$; Table 9).

Table 9. Two-way ANOVA results of the analysis of the effects of temperature and mixtures of Cd and Zn on the reproduction of *E. andrei* after exposure in artificial OECD soil for four weeks at 15, 20 and 25°C.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	15.01	6	476.8	79.47	4.449	0.0037
Temperature	21.94	2	696.7	348.4	19.50	p<0.0001
Mixture concentrations	49.56	3	1574	524.7	29.37	p<0.0001
Residual		24	428.7	17.86		

In the mixture treatments, cocoon incubation time varied with both temperature and mixture concentrations (Fig.12). In general, cocoon incubation times were the longest at 15°C and the shortest at 25°C. At 15°C, incubation time was significantly longer for the cocoons collected from the 1.26 TU treatment (53 ± 3 days), than in the ones collected from both the control (38 ± 1 days) and the 2.52 TU treatment (35 ± 2 days; $p < 0.001$). There was no difference in incubation duration between the cocoons collected from the control and the 2.52 TU at 15°C (Fig. 12).

At 20°C, there was no significant difference in incubation time between the cocoons from the control (20 ± 2 days) and the ones from the 1.26 TU treatment (22 ± 1 days). However, both these durations were significantly longer than the incubation time of the cocoons from the 2.52 TU treatment (14 ± 1 days; $p < 0.01$; Fig. 12).

At 25°C, the incubation time of the cocoons from the control (5 ± 1 days) was shorter than the incubation times of the cocoons collected from the other treatments ($p < 0.01$). There was no difference in incubation duration between the cocoons collected at 1.26 (11 ± 2 days) and 2.52 TU (9 ± 1 days) at 25°C. The single cocoon collected at 6.31 TU incubated for a significantly longer period (19 days) than the ones collected in the other treatments ($p < 0.001$; Fig. 12).

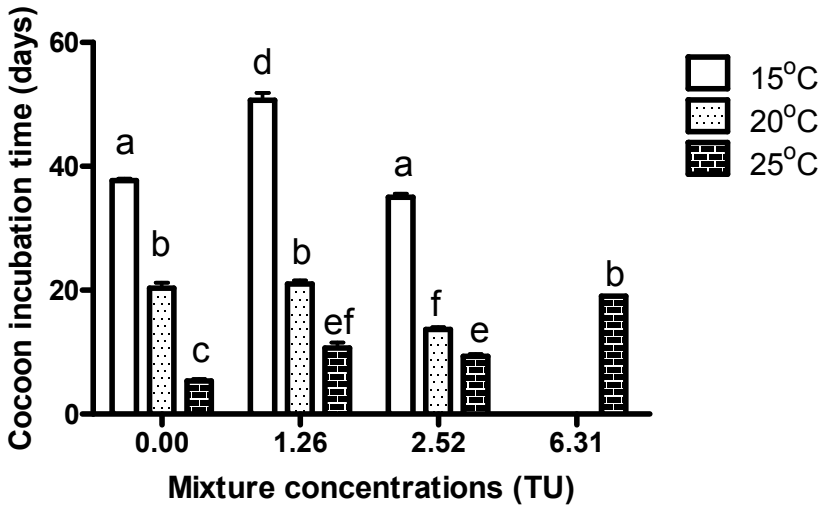


Fig. 12. Cocoon incubation time (in days) of *E. andrei* after exposure to mixtures of Cd and Zn for four weeks in artificial OECD soil at 15, 20 and 25°C. Error bars represent standard error. Different letters on bars represent statistical differences ($p \leq 0.01$).

In mixture treatments, hatchling numbers increased with high temperature but decreased with increasing mixture concentrations (Fig. 13). Between 15 and 20°C, the number of hatchlings was statistically lower at 15°C in the control and the 1.26 TU treatment ($p \leq 0.01$). Between 15 and 25°C, the number of hatchlings was statistically higher at 25°C in both the 1.26 and the 2.52 TU treatments ($p \leq 0.05$; Fig. 13).

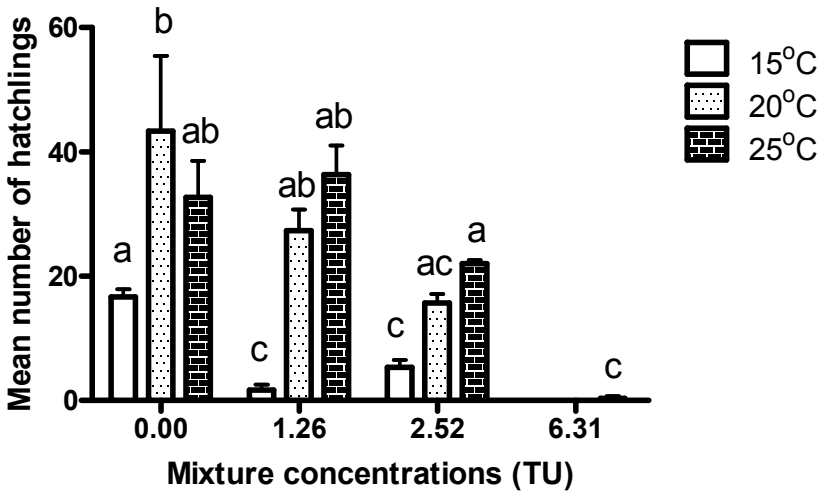


Fig. 13. Mean hatchling numbers of *E. andrei* after exposure to mixtures of Cd and Zn in artificial OECD soil for four weeks at 15, 20 and 25°C. Error bar represent standard error. Different letters on bars represent statistical differences ($p \leq 0.05$).

The estimation EC_{50mix}[†] for cocoon production using non-linear regression analyses revealed that the EC_{50mix} for cocoon production increased consistently with temperature (Table 10). These EC_{50mix} were all greater than one ($EC_{50mix} > 1$), which indicated antagonistic interactions between Cd and Zn in mixtures. These interactions however, needed to be further investigated using MixToxModules (Jonker *et al*. 2005) in order to test whether these antagonistic interactions were dose ratio (DR) or dose level (DL) dependent.

Table 10. Estimation of EC_{50mix} (TU) for cocoon production after exposure of *E. andrei* to mixtures of Cd and Zn in artificial OECD soil for four weeks at 15, 20 and 25°C. The numbers in brackets indicate 95% confidence intervals.

	15°C	20°C	25°C
$logEC_{50mix}$	0.394*	0.406*	0.432 (0.293-0.570)
EC_{50mix}	2.47	2.54	2.70 (1.96-3.71)
Model R^2	0.54	0.66	0.83

*95% confidence intervals could not be estimated at 15 and 20°C

[†] $EC_{50mix} = EC_{50}$ for the indicated endpoint computed after mixture exposures.

3.3.3. Biomass change

3.3.3.1. Cd exposures

In the Cd exposures, weight gain, although not statistically significant, was only observed in the control treatments incubated at 15°C. Although weight loss occurred in the controls incubated at 20 and 25°C, it was not statistically different from the initial starting weight at week 0 (Fig. 14). In the Cd exposures, weight loss occurred in a dose response manner and increased with increasing temperature. Weight loss in these treatments and at all temperatures was statistically different from the initial weight at week 0 (Fig. 14, $p \leq 0.01$).

Two-way ANOVA followed by Bonferroni posttests, revealed a highly significant interaction between Cd concentrations and temperature on the biomass variation of *E. andrei* during the experiment ($p < 0.0001$; Table 11).

Table 11. Two-way ANOVA results of the analysis of the effects of temperature and Cd on the biomass of *E. andrei* after exposure in artificial OECD soil for four weeks at 15, 20 and 25°C.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	17.29	56	18990	339.0	10.06	p<0.0001
Temperature	42.51	4	46690	11670	346.3	p<0.0001
Cd concentrations	35.60	14	39100	2793	82.87	p<0.0001
Residual		150	5056	33.71		

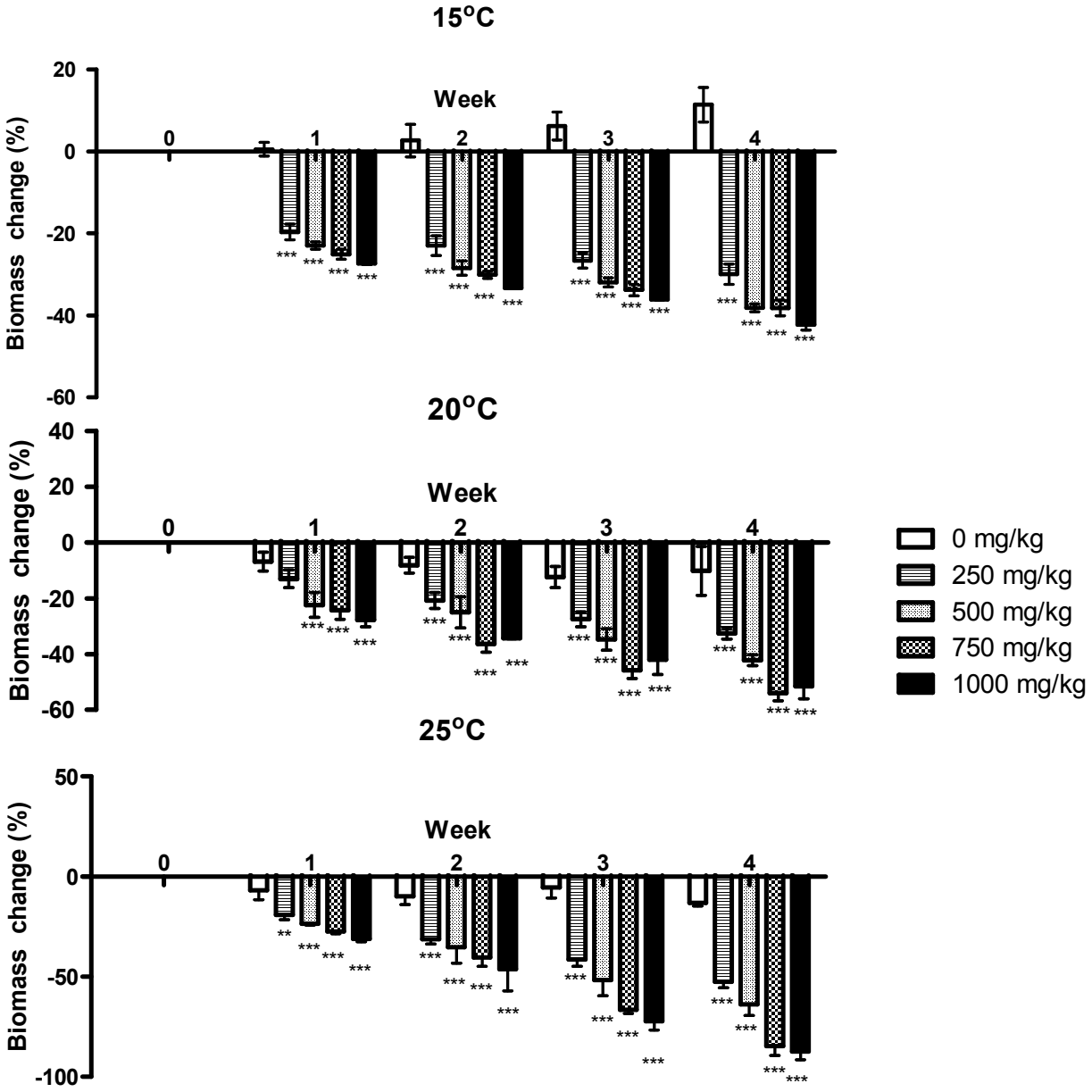


Fig. 14. Biomass change of *E. andrei* after exposure to Cd in artificial OECD soil for four weeks at 15, 20 and 25°C. Error bars represent standard error. Week 0 represents the initial starting weight. Stars represent statistical differences with initial biomass at week 0. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

Using non-linear analysis, an EC_{50} value (with 95% confidence intervals) of 307.60 (231.26-409.54) mg/kg for biomass ($R^2 = 0.93$) could only be estimated at

25°C. At the other temperatures, the biomass loss in the Cd treatments was less than 50% of the biomasses of the worms in their respective controls.

3.3.3.2. Zn exposures

As in the Cd experiment weight gain in the Zn exposures, although not statistically significant, was only observed at 15°C (Fig. 15). At all three temperatures however, biomass change was significantly lower from the initial biomass in the 1000 mg/kg treatments throughout the exposure period ($p < 0.001$; Fig 14).

Two-way ANOVA followed by Bonferroni posttests, revealed a highly significant interaction between Zn concentrations and temperature on the biomass variation of *E. andrei* during the experiment ($p < 0.0001$; Table 12).

Table 12. Two-way ANOVA results of the analysis of the effects of temperature and Zn on the biomass of *E. andrei* after exposure in artificial OECD soil for four weeks at 15, 20 and 25°C.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	17.78	56	13390	239.1	3.508	P<0.0001
Temperature	11.77	4	8864	2216	32.51	P<0.0001
Zn concentrations	56.87	14	42830	3059	44.88	P<0.0001
Residual		150	10220	68.16		

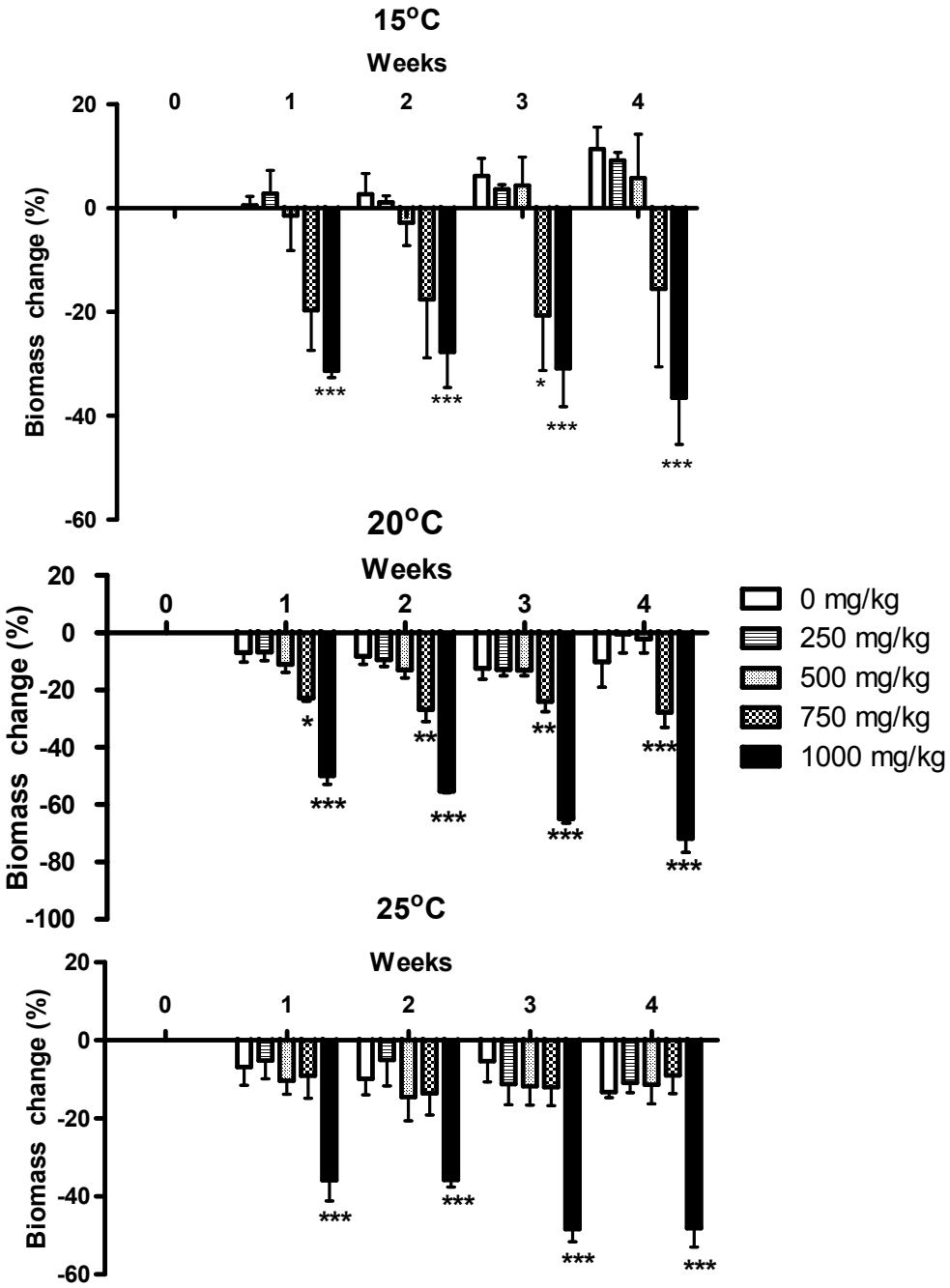


Fig. 15. Biomass change of *E. andrei* after exposure to Zn in artificial OECD soil for four weeks at 15, 20 and 25°C. Error bars represent standard error. Week 0 represents the initial starting weight. Stars represent statistical difference with initial biomass at week 0. * = P < 0.05; ** = P < 0.01, *** = P < 0.001.

Using non-linear analysis, an EC_{50} value (with 95% confidence intervals) of 880.03 (813.20-952.36) mg/kg for biomass (R^2 = 0.88) could only be estimated at 20°C. At the 15 and 25°C, the biomass loss in the Zn treatments was less than 50% of the biomasses of the worms in their respective controls.

3.3.3.3. Mixture exposures

In the mixture experiment as in Zn exposures, non significant weight gain was recorded at 15°C in earthworms exposed to lower mixture concentrations (Fig. 16). Moreover, non-significant weight gain was also recorded at 25°C in the 1.26 and 2.52 TU treatments from week 2 (Fig. 16). At all three temperatures, significantly higher biomass losses compared to the initial biomass mostly occurred in the two highest mixture treatments ($p \leq 0.5$; Fig. 16).

Two-way ANOVA followed by Bonferroni posttests, revealed a highly significant interaction between mixture concentrations and temperature on the biomass variation of *E. andrei* during the experiment ($p < 0.0001$; Table 13).

Table 13. Two-way ANOVA results of the analysis of the effects of temperature and mixtures of Cd and Zn on the biomass of *E. andrei* after exposure in artificial OECD soil for four weeks at 15, 20 and 25°C.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	21.11	68	31290	460.1	6.250	p<0.0001
Temperature	5.91	4	8763	2191	29.76	p<0.0001
Mixture concentrations	64.04	17	94900	5582	75.83	p<0.0001
Residual		180	13250	73.62		

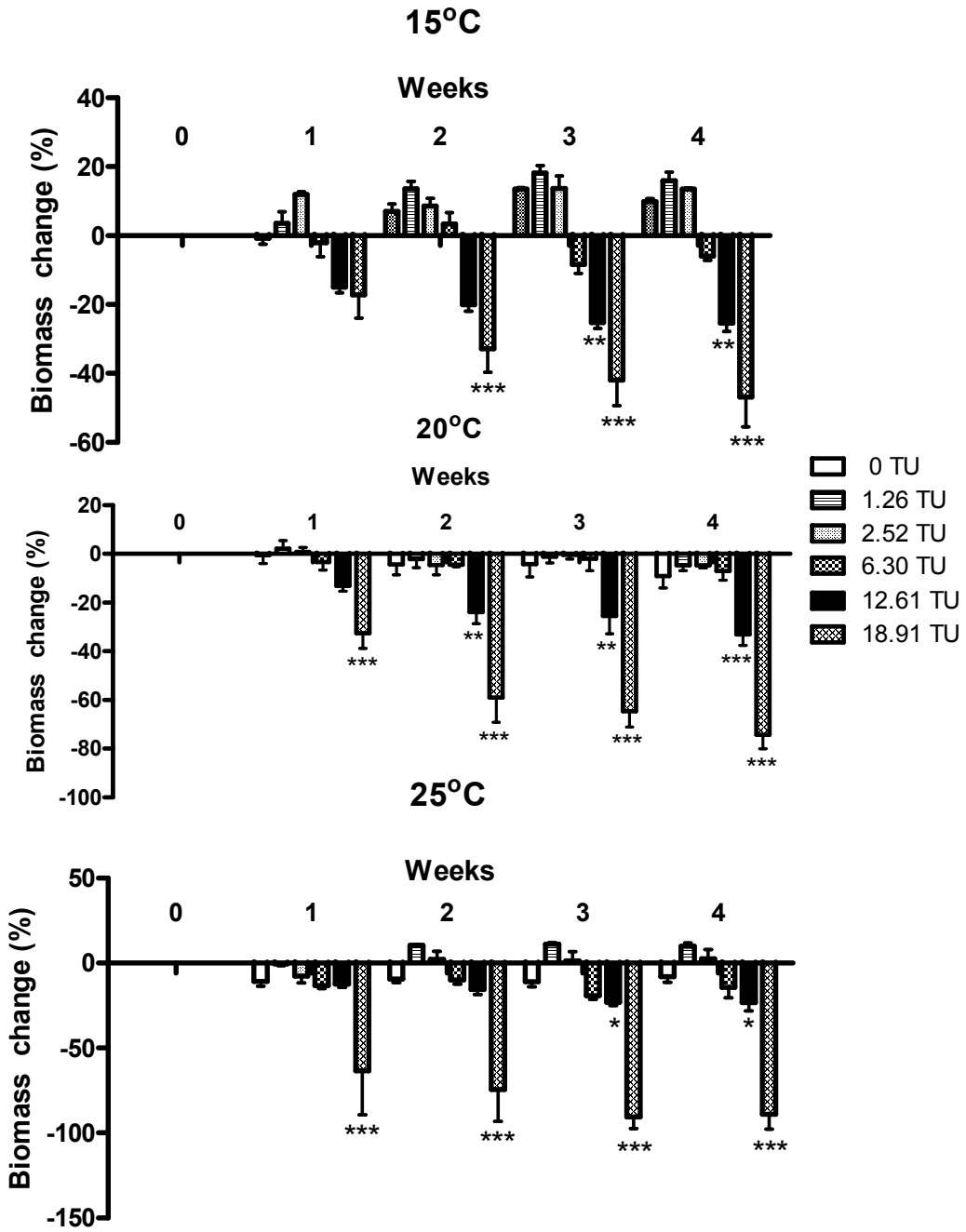


Fig. 16. Biomass change of *E. andrei* after exposure to mixtures of Cd and Zn in artificial OECD soil for four weeks at 15, 20 and 25°C. Error bars represent standard error. Week 0 represents the initial starting weight. Stars represent statistical differences with initial biomass at week 0. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

The estimation EC_{50mix} for biomass variation using non-linear regression analyses revealed that the EC_{50mix} for biomass decreased consistently (Table 14).

Table 14. Estimation of EC_{50mix} (TU) for biomass variation after exposure of *E. andrei* to mixtures of Cd and Zn in artificial OECD soil for four weeks at 15, 20 and 25°C. The numbers in brackets indicate 95% confidence intervals.

	15°C	20°C	25°C
$logEC_{50mix}$	1.26 (1.17-1.34)	1.18 (1.15-1.21)	1.16 (1.12-1.20)
EC_{50mix}	18.23 (15.09-22.03)	15.22 (14.23-16.29)	14.65 (13.26-16.18)
Model R^2	0.93	0.94	0.89

The estimated EC_{50mix} were all greater than one ($EC_{50mix} > 1$), which indicated antagonistic interactions between Cd and Zn in mixtures. These interactions however, needed to be further investigated and this was done using MixToxModules (Jonker *et al*. 2005) in order to test whether these antagonistic interactions were dose ratio (DR) or dose level (DL) dependent.

3.3.4. Metal analysis

3.3.4.1. Cd exposures

The analyses of metal body burden indicated that the tissue contents of Cd in *E. andrei* increased with both metal concentration and temperature (Fig. 17). There was no significant difference in accumulation between the worms exposed to Cd at 15 and 20°C. Except for the control treatments, the Cd body burden of the worms incubated at 25°C was greater than the body burden of those incubated at both 15 and 20°C ($p < 0.001$ and $p \leq 0.01$ respectively).

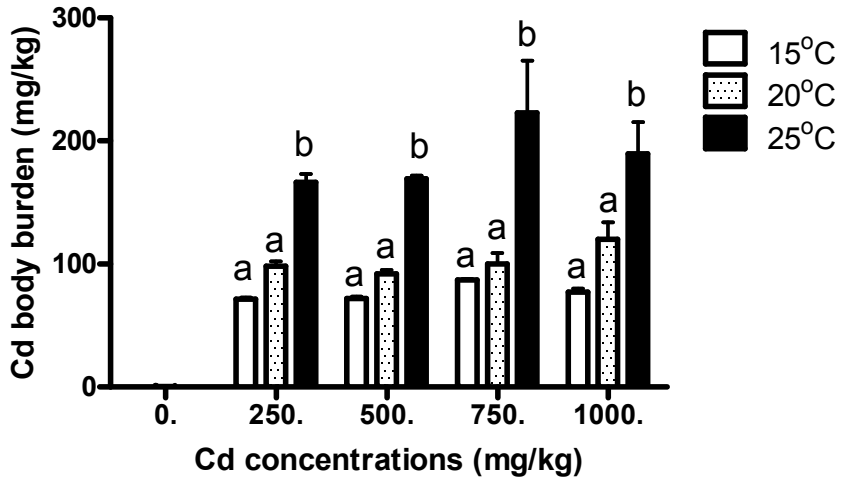


Fig. 17. Body burdens of Cd in *E. andrei* after exposure in artificial OECD soil for 4 weeks at 15, 20 and 25°C. Error bars represent standard errors. Different letters above bars represent statistical differences ($p \leq 0.05$).

Two-way ANOVA followed by Bonferroni posttests revealed a very significant interaction between Cd concentrations and temperature on the body concentration of Cd in *E. andrei* ($p = 0.0016$; Table 15).

Table 15. Two-way ANOVA results of the analysis of the effects of temperature and Cd on the body concentration of Cd in *E. andrei* after exposure in artificial OECD soil at 15, 20 and 25°C.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	9.19	8	19470	2434	4.283	0.0016
Temperature	30.04	2	63640	31820	55.98	p < 0.001
Cadmium concentrations	52.72	4	111700	27920	49.12	p < 0.001
Residual		30	17050	568.4		

3.3.4.2. Zn exposures

The analyses of Zn body burdens indicated that Zn tissue contents in *E. andrei* were generally higher in lower temperatures and tended to decrease with increasing temperature (Fig. 18). However, these variations were not significantly different from one temperature to another. Thus, within each Zn treatment, the amount of Zn taken up was more or less the same at all temperatures.

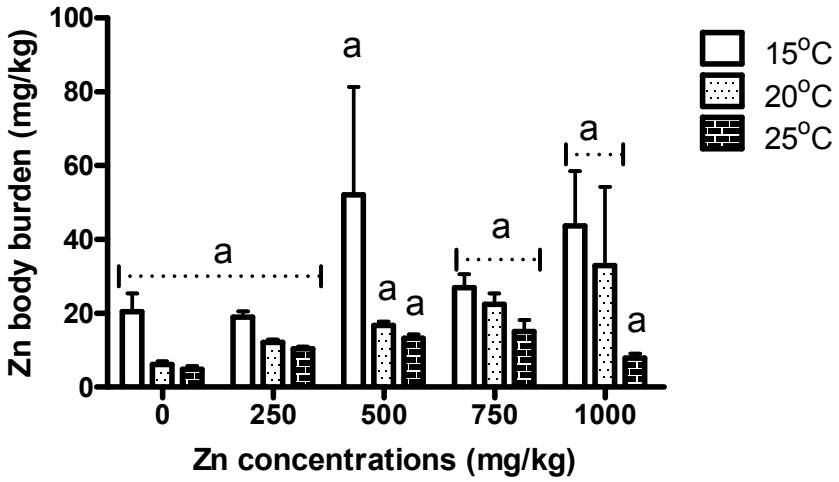


Fig. 18. Body burden of Zn in *E. andrei* after exposure in artificial OECD soil for 4 weeks at 15, 20 and 25°C. Error bars represent standard errors. Different letters above bars represent statistical differences ($p \leq 0.05$).

Two-way ANOVA followed by Bonferroni posttests revealed that there was no significant interaction between Zn concentrations and temperature in determining the body concentration of Zn in *E. andrei* (Table 16).

Table 16. Two-way ANOVA results of the analysis of the effects of temperature and Zn on the body concentration of Zn in *E. andrei* after exposure in artificial OECD soil at 15, 20 and 25°C. The abbreviation ns means not significant.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	10.41	8	1811	226.4	0.7125	0.6786^{ns}
Temperature	21.81	2	3796	1898	5.974	0.0065
Zinc concentrations	13.01	4	2264	565.9	1.781	0.1587^{ns}
Residual		30	9533	317.8		

3.3.4.3. Mixture exposures

In the mixture treatments, Cd and Zn body burdens were measured separately. Fig. 19a depicts the accumulation of Cd_{mix}‡. It was found that Cd_{mix} was accumulated in both a dose response and temperature dependent manner. Higher accumulation was recorded at higher mixture concentrations and at higher temperatures. The comparison of these body burdens revealed that between 15 and 20°C, Cd_{mix} body burdens were significantly higher at 20 than 15°C in 18.92 TU only ($p < 0.05$). Between 15 and 25°C, significantly higher body burdens occurred at 25°C in the 6.31, 12.61 and 18.92 TU treatment ($p \leq 0.01$). Between 20 and 25°C, a significantly higher body burden was recorded at 25°C only at 18.92 TU ($p < 0.001$; Fig. 19a).

Two-way ANOVA followed by Bonferroni posttests revealed that there was a highly significant interaction between mixture concentrations and temperature on the uptake of Cd_{mix} *E. andrei* ($p = 0.0002$; Table 17).

‡ Cd_{mix} = proportion of Cd accumulated in *E. andrei* after exposure to mixtures of Cd and Zn for four weeks at 15, 20 and 25°C

Table 17. Two-way ANOVA results of the analysis of the effects of temperature and mixtures of Cd and Zn on the uptake of Cd_{mix} in *E. andrei* after exposure in artificial OECD soil for four weeks at 15, 20 and 25°C.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	10.05	10	9434	943.4	4.839	0.0002
Temperature	13.92	2	13070	6534	33.51	P<0.0001
Mixture concentrations	74.70	5	70140	14030	71.94	P<0.0001
Residual		35	6824	195.0		

Fig. 19b depicts the body burdens of Zn_{mix}[§]. It was found that Zn_{mix} accumulation was neither dose nor temperature dependent. There was no significant difference in Zn_{mix} accumulation in mixture treatments at all three temperatures, except in the 18.92 TU treatment where significantly greater accumulation was recorded at 25°C (Fig. 19b).

Two-way ANOVA followed by Bonferroni posttests revealed that there was a very significant interaction between mixture concentrations and temperature on the uptake of Zn_{mix} in *E. andrei* ($p = 0.017$; Table 18).

Table 18. Two-way ANOVA results of the analysis of the effects of temperature and mixtures of Cd and Zn on the uptake of Zn_{mix} in *E. andrei* after exposure in artificial OECD soil for four weeks at 15, 20 and 25°C. The abbreviation ns means not significant.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	34.95	10	507.3	50.73	2.623	0.0170
Temperature	19.26	2	279.6	139.8	7.230	0.0024
Mixture concentrations	2.68	5	38.97	7.793	0.4030	0.8434^{ns}
Residual		35	676.8	19.34		

[§] Zn_{mix} = proportion of Zn accumulated in *E. andrei* after exposure to mixtures of Cd and Zn for four weeks at 15, 20 and 25°C.

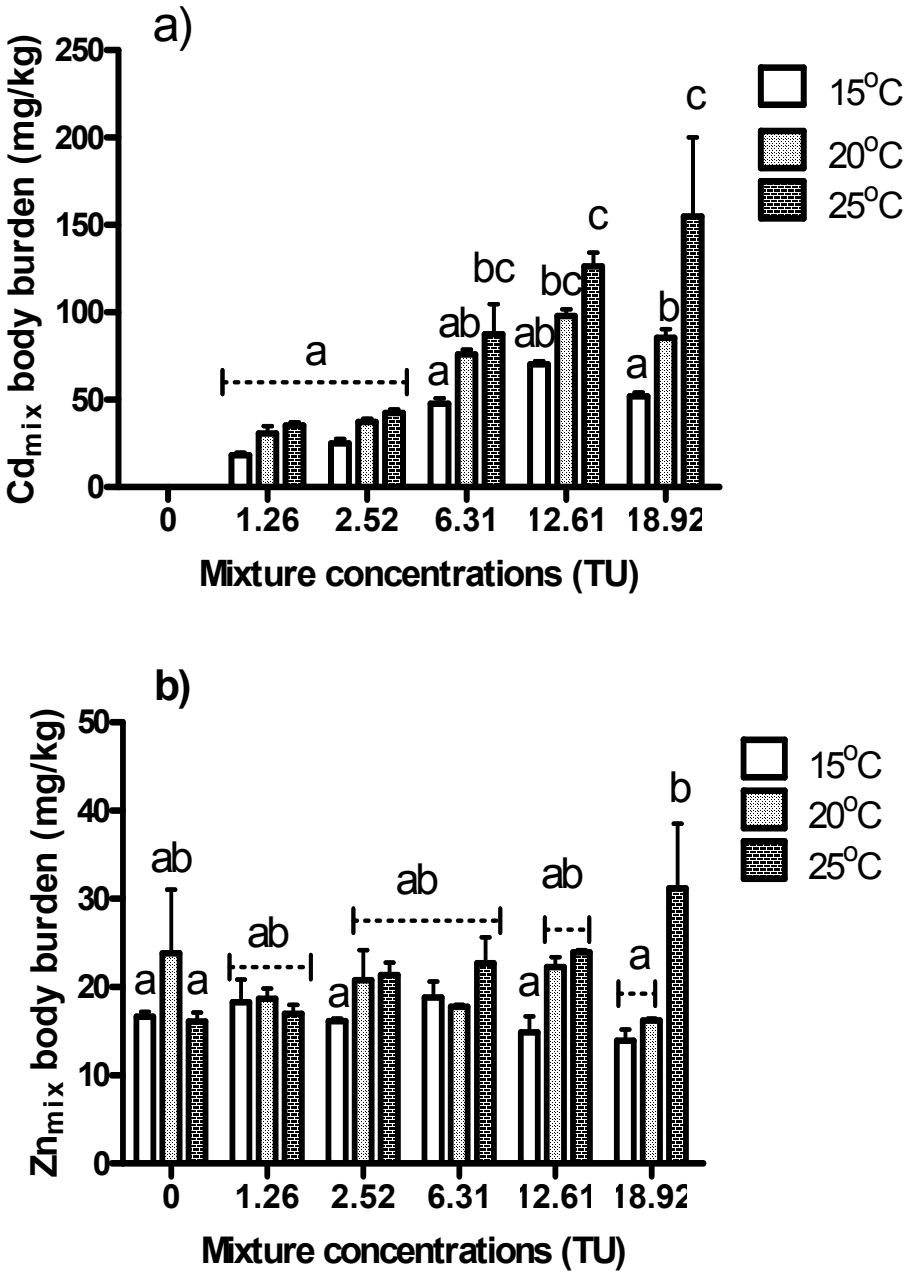


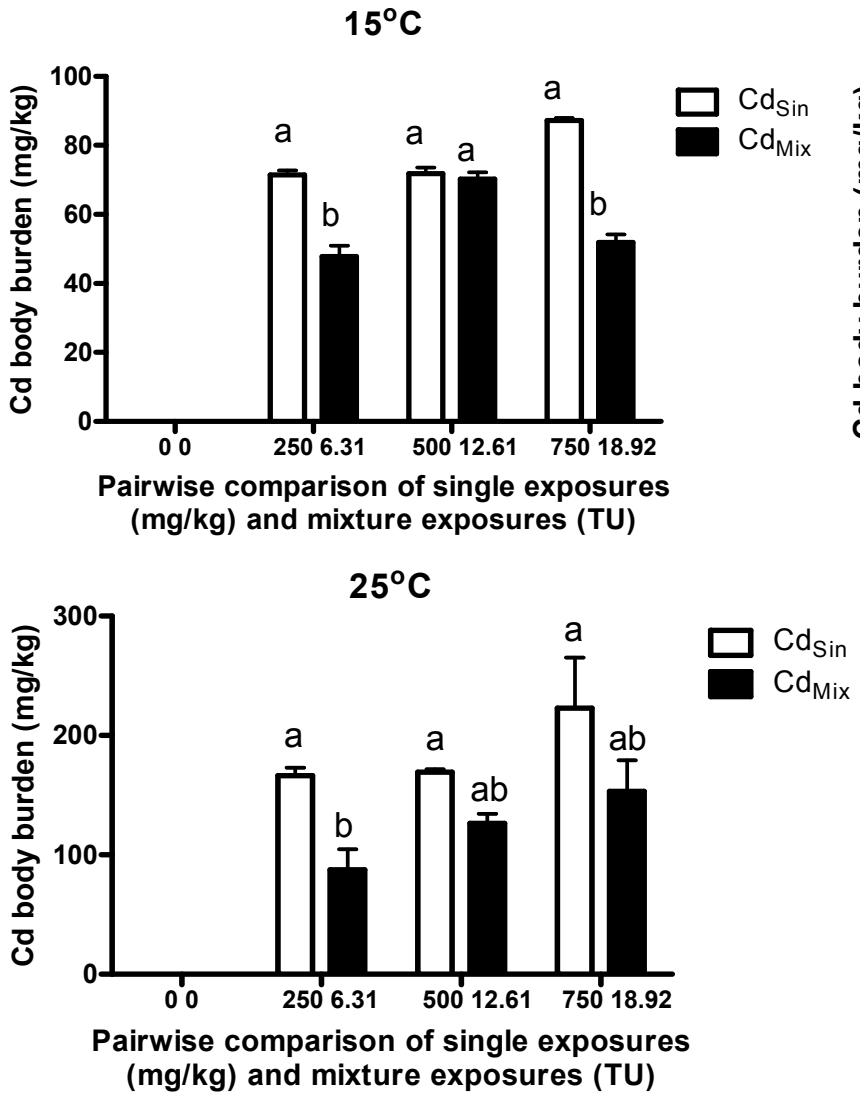
Fig. 19. Body burdens of Cd_{mix} (a) and Zn_{mix} (b) in *E. andrei* after exposure to mixtures of Cd and Zn for four weeks at 15, 20 and 25°C in artificial OECD soil. Error bars represent standard errors. Different letters above bars represent statistical differences ($p \leq 0.05$).

In order to compare the difference between Cd and Zn body burdens from both single (Cd_{Sin} & Zn_{Sin})** and mixture (Cd_{Mix} & Zn_{Mix}) exposures, selected metal body burdens were compared in a pair wise manner.

Cd_{Sin} & Zn_{Sin} in worms exposed to 0; 250; 500 & 750 mg/kg in the single metal exposures were compared to the values measured in worms exposed to 0; 6.31; 12.61 & 18.92 TU respectively. In these selected pairs of exposure concentrations, *E. andrei* was exposed to the same nominal concentrations of either of the metals, alone (expressed in mg.kg^{-1}) or in mixtures (expressed in TU). These pairs of exposure concentrations were 0/0; 250/6.31; 500/12.61 and 750/18.9 mg.kg^{-1}/TU. Metal tissue concentrations were measured in mg/kg regardless of the exposure regime.

Fig. 20 depicts the comparison between Cd_{Sin} and Cd_{Mix} at all three temperatures after separate exposures of *E. andrei* to either Cd alone or mixtures of Cd and Zn for four weeks at 15, 20 and 25^{o}C. At 15^{o}C, Cd_{Mix} was significantly lower than Cd_{Sin} in both the 250/6.31 and the 750/18.9 mg.kg^{-1}/TU pairs of exposure concentrations ($p < 0.001$). At 20^{o}C, Cd_{Mix} was significantly lower than Cd_{Sin} in the 250/6.31 mg.kg^{-1}/TU pair of exposure concentrations only ($p < 0.01$). At 25^{o}C, similarly, Cd_{Mix} was significantly lower than Cd_{Sin} only in the 250/6.31 mg.kg^{-1}/TU pair of exposure concentrations ($p < 0.05$). No case of $Cd_{Mix} > Cd_{Sin}$ was found.

** Cd_{Sin} & Zn_{Sin} = proportions of Cd & Zn accumulated in *E. andrei* after single exposure to Cd or Zn for four weeks at 15, 20 and 25^{o}C.



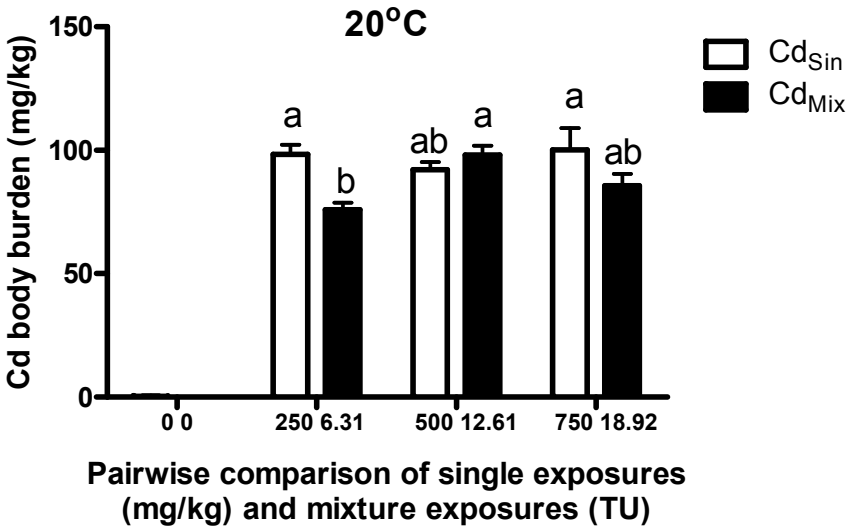
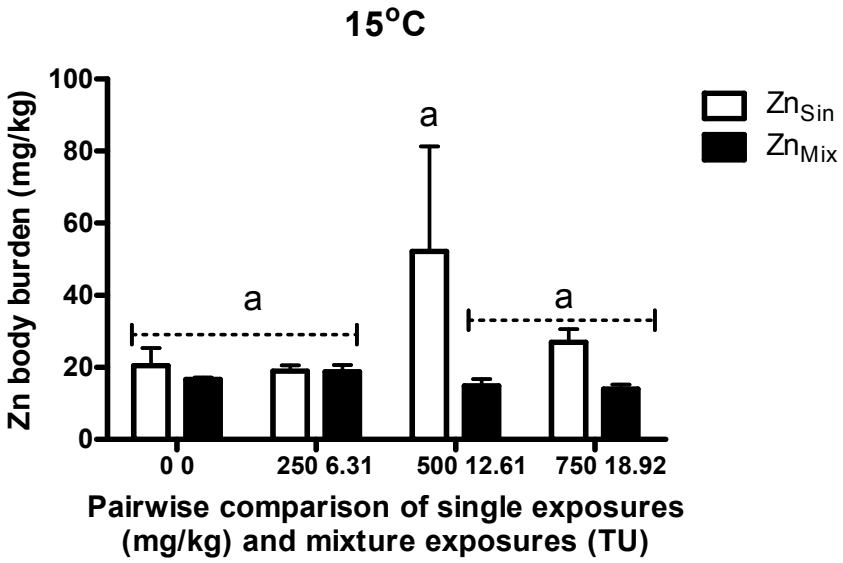
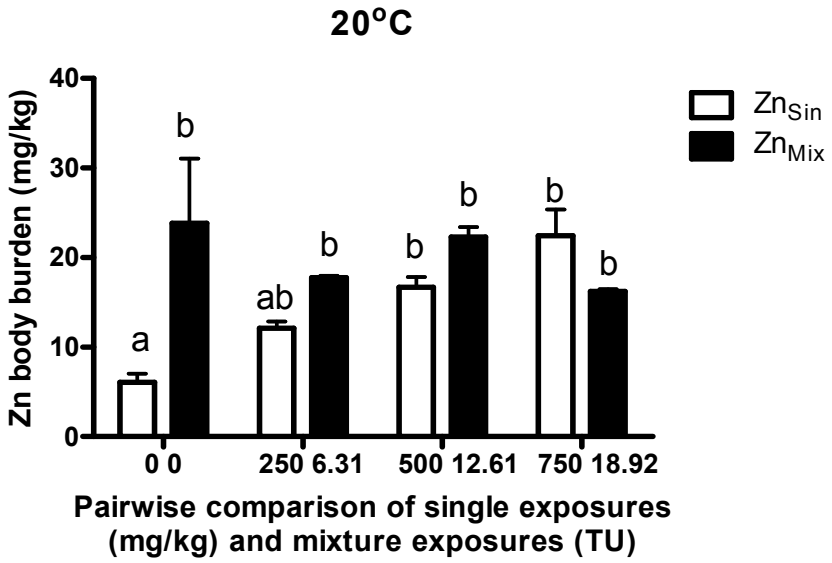


Fig. 20. Comparison between Cd body burdens from single Cd exposures (Cd_{Sin}; white bars) and mixture exposures (Cd_{Mix}; black bars) in *E. andrei* after separate exposure to either Cd alone or mixtures of Cd and Zn for four weeks at 15, 20 and 25°C in artificial OECD soil. Different letters above bars represent statistical differences ($p \leq 0.05$).

Fig. 21 depicts the comparison between Zn_{Sin} and Zn_{Mix} at all three temperatures after separate exposures of *E. andrei* to either Zn alone or mixtures of Cd and Zn for four weeks at 15, 20 and 25^{o}C.

At 15^{o}C, no significant difference between Zn_{Mix} and Zn_{Sin} was observed in all pairs of exposure concentrations. At 20^{o}C, Zn_{Mix} was significantly higher than Zn_{Sin} in the control pair of exposure concentrations only ($p < 0.01$). At 25^{o}C, Zn_{Mix} was significantly higher than Zn_{Sin} in all pairs of exposure concentrations ($p \leq 0.05$). No significant case of $Zn_{Sin} > Zn_{Mix}$ was found.





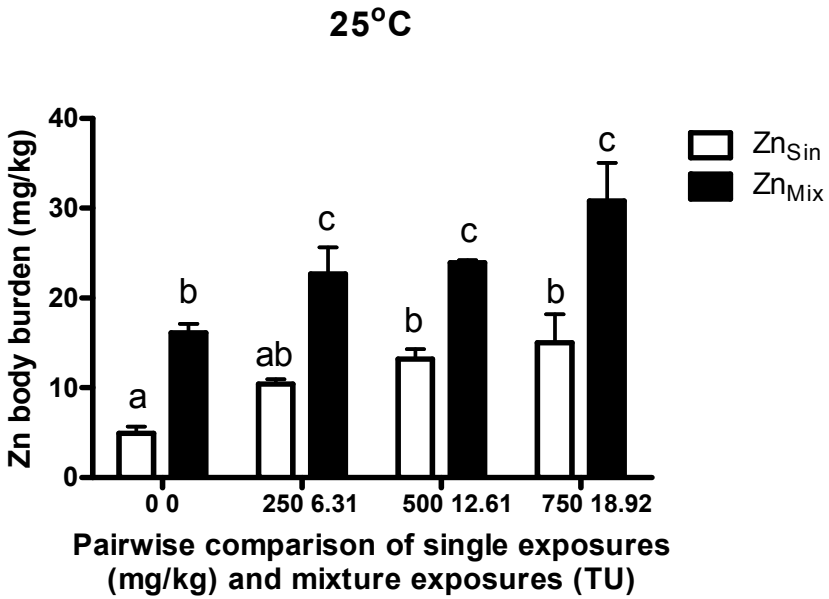


Fig. 21. Comparison between Zn body burdens from single Zn exposures (Zn_{Sin}; white bars) and mixture exposures (Zn_{Mix}; black bars) in *E. andrei* after separate exposure to either Zn alone or mixtures of Cd and Zn for four weeks at 15, 20 and 25°C in artificial OECD soil. Different letters above bars represent statistical difference ($p \leq 0.05$).

3.3.5. Modelling antagonistic interactions using MixToxModules

The results of all endpoints (survival, reproduction, and biomass change) could potentially be used to assess metal interactions in mixtures. However, not all were suitable as explained below:

Survival data were not suited for investigating dose ratio (DR) or dose level (DL) antagonistic dependency because the presence of antagonistic interactions between Cd and Zn would not be reflected in the number of surviving adults (by an increase in the number of adults for instance). This was in part because of the duration of the exposure period. In addition, a lack of mortality in the mixture treatments could be due to mixture concentrations lower than the NOEC for this endpoint (regardless of the presence or not of metal interactions) and could not always be interpreted as antagonism.

As for reproduction, in the single metal experiments reproduction did not occur in any of the Cd contaminated treatments (see section 3.3.2.1) causing a lack of necessary data for the investigation of DR or DL antagonistic dependency using reproduction as endpoint.

Biomass change however, was recorded in every treatment of every experiment and generally decreased with increasing metal or mixture concentrations. The investigation of DR or DL antagonistic dependency was consequently carried out using biomass data.

3.3.5.1. Modelling antagonistic interactions at 15^{o}C

When *E. andrei* biomass data from single and mixture exposures at 15^{o}C were modeled using the MixToxModules, antagonism in the mixture treatments was confirmed under both the concentrations addition (CA) and the independent action (IA) assumptions (parameter $a > 0$ when testing for Synergism/Antagonism [S/A]; Table 19). Further tests investigating DR or DL dependency showed that

DL was the most significant interaction ($p[\chi^2] < 0.001$) under the CA assumption. Under the IA assumption, no model could explain the variation further.

The interpretation of the DL model parameters under the CA reference model (using Table 2 from Chapter 2: General Materials & Methods, Section 2.5.) showed the following: a[††] = 19.78 indicated antagonism at low mixture concentrations and synergism at high mixture concentrations. b_{DL}[‡‡] = 0.71 (from Table 19) indicated that the change from antagonism to synergism occurred at higher dose level than the EC_{50mix}, precisely at $\frac{1}{b_{DL}} \cdot EC_{50mix}$ (Jonker *et al.* 2005). At 15°C, EC_{50mix} biomass was 18.23 TU (Table 14). Thus, the switch occurred at $\frac{1}{0.71}$ x 18.23 = 25.67 TU.

Fig. 22 depicts 3D surface plots of *E. andrei* biomass change in mixture and single metal treatments at 15°C. The CA reference model is reported with the most significant deviation model; DL. Model fitness was highly significant ($p[\chi^2] < 0.001$; Table 19).

[††] Parameter a indicates whether synergism or antagonism occurs at low or higher dose c.f. table 2, chapter 2.

[‡‡] Parameter b_{DL} indicates at which concentration the switch from antagonism to synergism occurs c.f. table 2, chapter 2.

Table 19. Summary of the modelling of the effect of Cd and Zn on the biomass of *E. andrei* after exposure in artificial OECD soil for four weeks at 15°C. b is the slope of the individual dose response curve; EC_{50} (in mg/kg) is the median effect concentration; a, b_{DL}, and b_{Zn} are parameters in the deviation functions; SS is the sum of squares; χ^2 is the test statistic; Df is the degrees of freedom; and $p(\chi^2)$ indicates the outcome of the likelihood ratio test. S/A is synergism/antagonism, DL is dose level dependent deviation from the reference, and DR is dose ratio dependent deviation from the reference. The abbreviation na means that the quantity is not applicable.

	Concentration addition				Independent action			
	Reference	S/A	DR	DL	Reference	S/A	DR	DL
EC_{50Cd}	1063.62	817.73	817.73	916.19	928.13	792.35	792.35	792.35
EC_{50Zn}	1306.01	1227.40	1227.40	1258.04	1296.11	1241.87	1241.87	1241.87
a	na	2.29	2.29	19.78	na	122.06	122.06	122.06
b_{DL}	na	na	na	0.71	na	na	na	2
b_{Zn}	na	na	$-3.64 \cdot 10^{-8}$	na	na	na	0	na
SS	1109.637	674.81	674.81	276.61	31084.25	30487.94	30487.94	30487.94
χ^2	na	8.95	$1.33 \cdot 10^{-10}$	16.05	na	0.34	$4 \cdot 10^{-15}$	$4 \cdot 10^{-15}$
Df	na	1	2	1	na	1	2	1
$p(\chi^2)$	na	0.002	0.99	<0.001	na	0.55	1	1

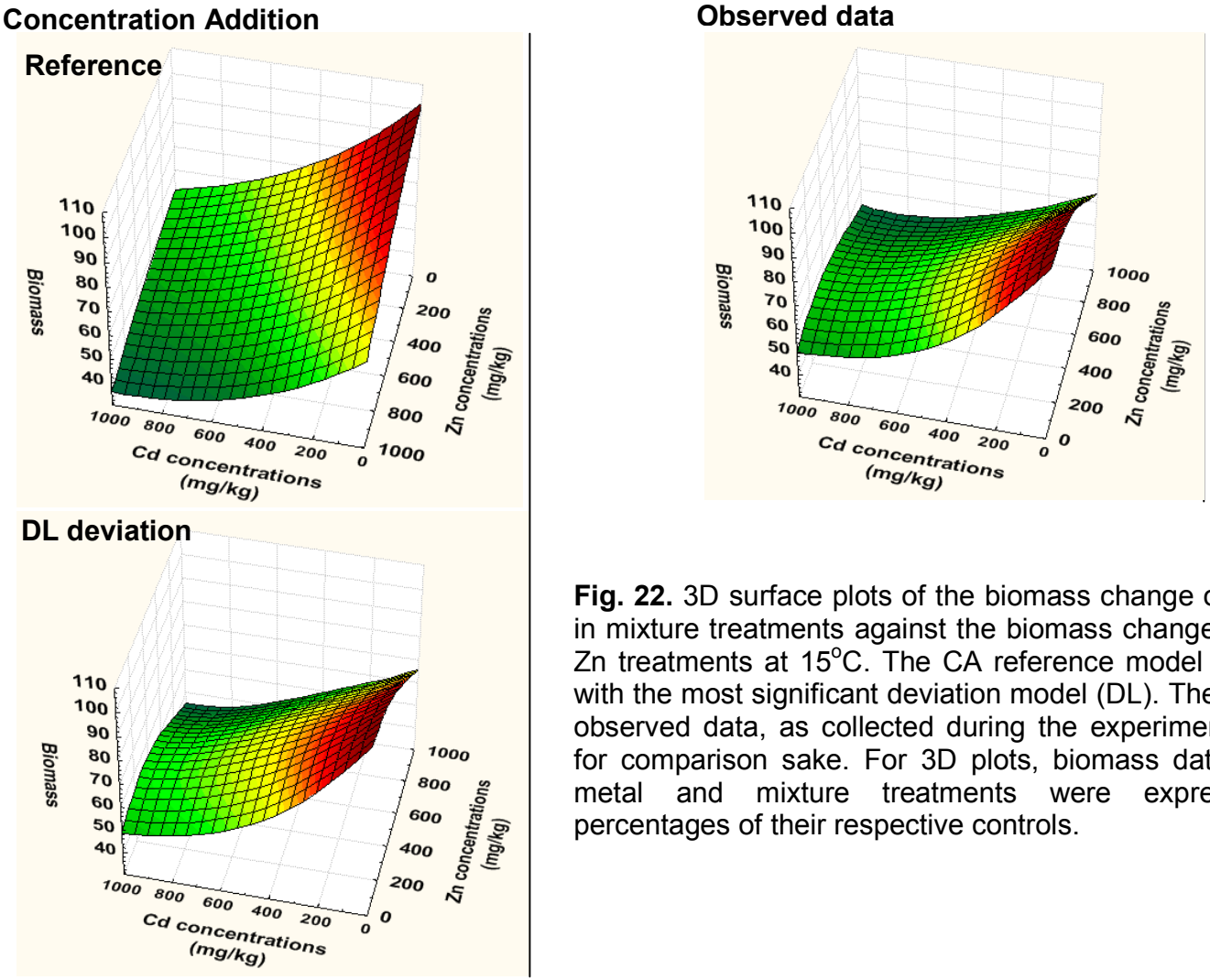


Fig. 22. 3D surface plots of the biomass change of *E. andrei* in mixture treatments against the biomass change in Cd and Zn treatments at 15°C. The CA reference model is reported with the most significant deviation model (DL). The plot of the observed data, as collected during the experiment, is given for comparison sake. For 3D plots, biomass data in single metal and mixture treatments were expresssed as percentages of their respective controls.

3.3.5.2. Modelling antagonistic interactions at 20°C

When *E. andrei* biomass data from single and mixture exposures at 20°C were modeled using the MixToxModules, antagonism in the mixture treatments was confirmed under both the CA and the IA assumptions (parameter $a > 0$ when testing for S/A; Table 20). Further tests investigating DR or DL dependency showed that DL was the most significant interaction ($p[\chi^2] < 0.001$) under both the CA and the IA assumptions.

The interpretation of the DL model parameters under the CA reference model (using Table 2 from Chapter 2: General Materials & Methods, Section 2.5.) showed the following: a = 13.75 indicated antagonism at low mixture concentrations and synergism at high mixture concentrations. b_{DL} = 0.61 (from Table 20) indicated that the change from antagonism to synergism occurred at higher dose level than the EC_{50mix}; precisely at $\frac{1}{b_{DL}} \cdot EC_{50mix}$. At 20°C, EC_{50mix} biomass was 15.22 TU (Table 14). Thus, under the CA reference model, the switch occurred at $\frac{1}{0.61}$ x 15.22 = 24.95 TU.

The interpretation of the DL model parameters under the IA reference model (using Table 2 from Chapter 2: General Materials & Methods, Section 2.5.) showed the following: a = 9.28 indicated antagonism at low mixture concentrations and synergism at high mixture concentrations. b_{DL} = 2.10 (from Table 20) indicated that the change from antagonism to synergism occurred at lower dose level than the EC_{50mix}, more precisely at $\frac{1}{b_{DL}} \cdot EC_{50mix}$. At 20°C, EC_{50mix} biomass was 15.22 TU (Table 14). Thus, under the IA reference model, the switch occurred at $\frac{1}{2.10}$ x 15.22 = 7.24TU.

Fig. 23 depicts 3D surface plots of *E. andrei* biomass change in mixture and single metal treatments at 20°C. The CA and IA reference models are reported

with the most significant deviation model; DL. Model fitness was highly significant in both cases ($p[\chi^2] < 0.001$; Table 20).

Table 20. Summary of the modelling of the effect of Cd and Zn on the biomass of *E. andrei* after exposure in artificial OECD soil for four weeks at 20°C. b is the slope of the individual dose response curve; EC_{50} (in mg/kg) is the median effect concentration; a, b_{DL}, and b_{Zn} are parameters in the deviation functions; SS is the sum of squares; χ^2 is the test statistic; Df is the degrees of freedom; and $p(\chi^2)$ indicates the outcome of the likelihood ratio test. S/A is synergism/antagonism, DL is dose level dependent deviation from the reference, and DR is dose ratio dependent deviation from the reference. The abbreviation na means that the quantity is not applicable.

	Concentration addition				Independent action			
	Reference	S/A	DR	DL	Reference	S/A	DR	DL
EC_{50Cd}	1168.34	866.21	866.21	916.75	850.67	829.78	829.78	931.81
EC_{50Zn}	904.56	886.19	886.19	888.88	865.71	861.40	861.40	893.34
a	na	1.40	1.40	13.75	na	0.30	0.30	9.28
b_{DL}	na	na	na	0.61	na	na	na	2.10
b_{Zn}	na	na	$1.16 \cdot 10^{-6}$	na	na	na	0	na
SS	1621.26	957.55	957.55	239.52	990.04	997.91	997.91	221.17
χ^2	na	8.95	$1.27 \cdot 10^{-11}$	23.55	na	0.20	$3.77 \cdot 10^{-15}$	25.26
Df	na	1	2	1	na	1	2	1
$p(\chi^2)$	na	0.002	0.99	<0.001	na	0.64.	1	<0.001

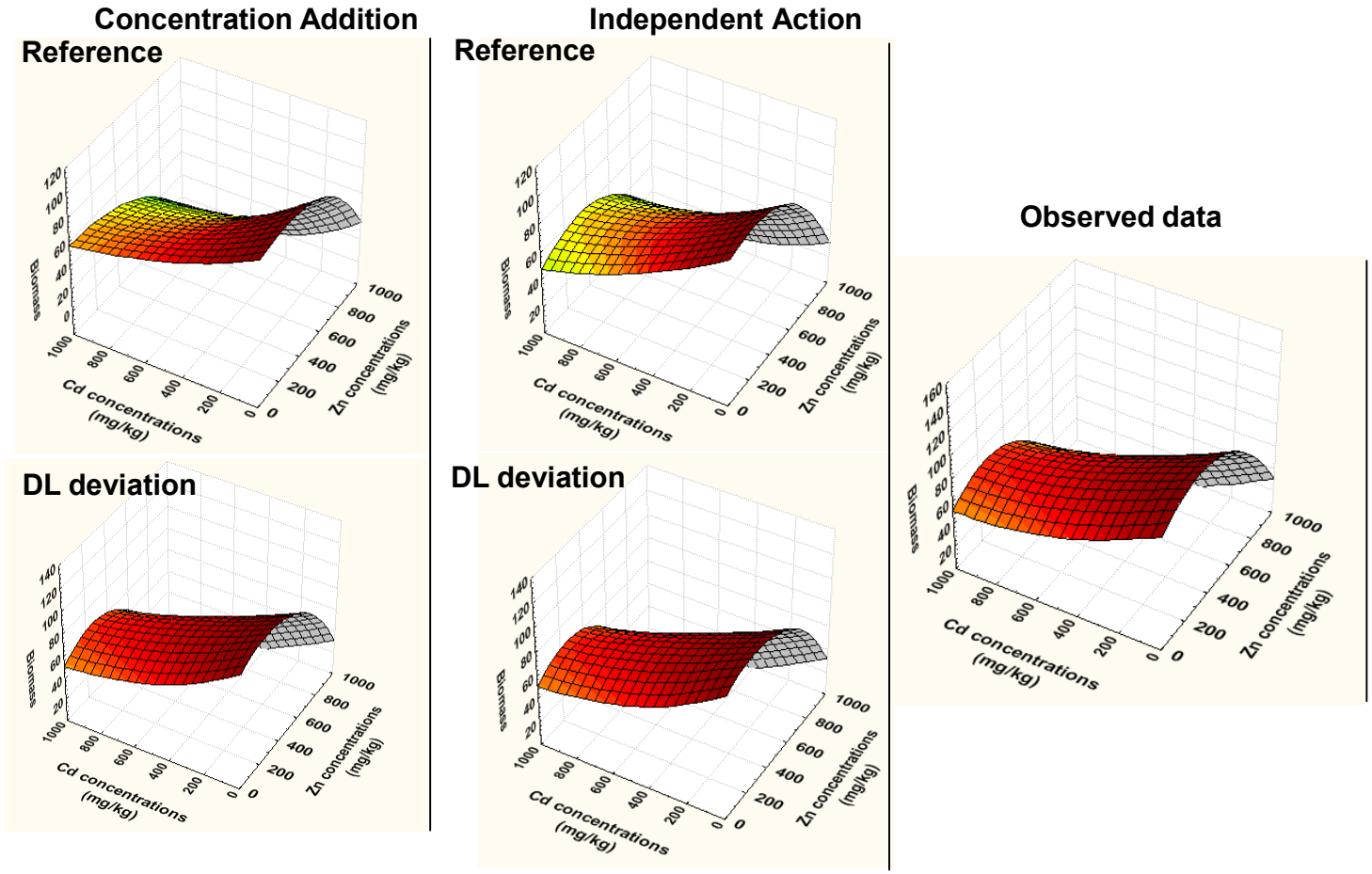


Fig. 23. 3D surface plots of the biomass change of *E. andrei* in mixture treatments against the biomass change in Cd and Zn treatments at 20°C. The reference models are reported with the most significant deviation model (DL). The plot of the observed data, as collected during the experiment, is given for comparison sake. For 3D plots, biomass data in single metal and mixture treatments were expresssed as percentages of their respective controls.

3.3.5.3. Modelling antagonistic interactions at 25°C

When *E. andrei* biomass data from single and mixture exposures at 25°C were modeled using the MixToxModules, antagonism in the mixture treatments was confirmed under both the CA and the IA assumptions (parameter $a > 0$ when testing for S/A; Table 21). Further tests investigating DR or DL dependency showed that DL was the most significant interaction ($p[\chi^2] < 0.001$) under both the CA and the IA assumptions.

The interpretation of the DL model parameters under the CA reference model (using Table 2 from Chapter 2: General Materials & Methods, Section 2.5.) showed the following: a = 39.24 indicated antagonism at low mixture concentrations and synergism at high mixture concentrations. b_{DL} = 0.33 (from Table 21) indicated that the change from antagonism to synergism occurred at higher dose level than the EC_{50mix}; precisely at $\frac{1}{b_{DL}} \cdot EC_{50mix}$. At 25°C, EC_{50mix} biomass was 14.65 TU (Table 14). Thus, under the CA reference model, the switch occurred at $\frac{1}{0.33}$ x 14.65 = 44.39 TU.

The interpretation of the DL model parameters under the IA reference model (using Table 2 from Chapter 2: General Materials & Methods, Section 2.5.) showed the following: a = 75.23 indicated antagonism at low mixture concentrations and synergism at high mixture concentrations. b_{DL} = 1.27 (from Table 21) indicated that the change from antagonism to synergism occurred at lower dose level than the EC_{50mix}, more precisely at $\frac{1}{b_{DL}} \cdot EC_{50mix}$. At 25°C, EC_{50mix} biomass was 14.65 TU (Table 14). Thus, under the IA reference model, the switch occurred at $\frac{1}{1.27}$ x 14.65 = 11.53TU.

Fig. 24 depicts 3D surface plots of *E. andrei* biomass change in mixture and single metal treatments at 25^{o}C. The CA and IA reference models are reported with the most significant deviation model; DL. Model fitness was highly significant in both cases ($p[\chi^2] < 0.001$; Table 21).

Table 21. Summary of the modelling of the effect of Cd and Zn on the biomass of *E. andrei* after exposure in artificial OECD soil for four weeks at 25°C. b is the slope of the individual dose response curve; EC_{50} (in mg/kg) is the median effect concentration; a, b_{DL}, and b_{Zn} are parameters in the deviation functions; SS is the sum of squares; χ^2 is the test statistic; Df is the degrees of freedom; and $p(\chi^2)$ indicates the outcome of the likelihood ratio test. S/A is synergism/antagonism, DL is dose level dependent deviation from the reference, and DR is dose ratio dependent deviation from the reference. The abbreviation na means that the quantity is not applicable.

	Concentration addition				Independent action			
	Reference	S/A	DR	DL	Reference	S/A	DR	DL
EC_{50Cd}	492.33	333.52	333.52	290.26	479.35	334.71	334.71	292.56
EC_{50Zn}	1667.85	1379.91	1379.91	1431.42	1413.03	1380.81	1380.81	1435.84
a	na	4.83	4.83	39.24	na	4.57	4.57	75.23
b_{DL}	na	na	na	0.33	na	na	na	1.27
b_{Zn}	na	na	$3.89 \cdot 10^{-7}$	na	na	na	$-2.52 \cdot 10^{-7}$	na
SS	4631.96	2384.78	2384.78	1067.47	3455.06	2347.34	2347.34	1086.28
χ^2	na	11.28	$8.94 \cdot 10^{-12}$	13.66	na	6.57	$3.65 \cdot 10^{-12}$	13.09
Df	na	1	2	1	na	1	2	1
$p(\chi^2)$	na	<0.001	0.99	<0.001	na	0.01	1	<0.001

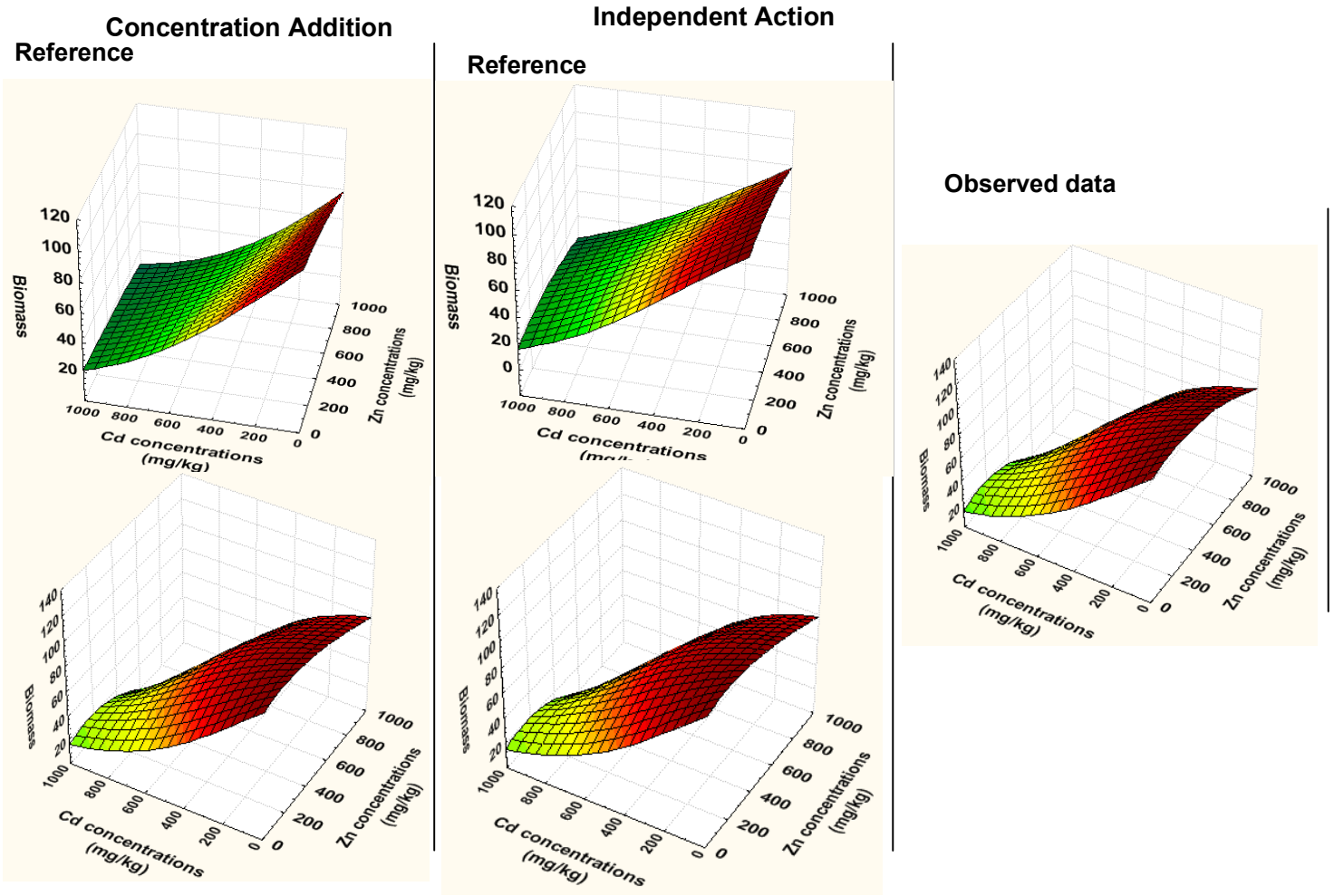


Fig. 24. 3D surface plots of the biomass change of *E. andrei* in mixture treatments against the biomass change in Cd and Zn treatments at 25°C. The reference models are reported with the most significant deviation model (DL). The plot of the observed data, as collected during the experiment, is given for comparison sake. For 3D plots, biomass data in single metal and mixture treatments were expresssed as percentages of their respective controls.

3.4. Discussion

3.4.1. Survival and reproduction in single metal exposures

The effects of Cd on the survival of *E. andrei* in artificial OECD soil increased consistently with the increase in temperature, indicating a clear temperature-dependent toxicity pattern. An LC_{50} could only be computed at 25°C when sufficient mortality had occurred in the higher Cd concentrations. At this particular temperature, more *E. andrei* individuals reached the lethal critical Cd body burden thus increasing mortality rates. According to Conder & Lanno (2003), the estimation of lethal critical body residues of Cd (inducing 50% mortality) in *E. fetida* is 5.72 (3.54-7.91) mmol/kg (equivalent to 642 (397.32-887.80) mg/kg). In the present study, Cd body concentrations of up to 460 mg/kg (4.09 mmol/Kg) were recorded at higher temperature and metal concentrations. These values fall within the 95% confidence value range of concentrations found to induce 50% mortality in *E. fetida* by Conder & Lanno (2003). The Cd LC_{50} (with 95% confidence intervals) of 725.46 (579.12 - 908.79) mg/Kg, calculated in the present study for *E. andrei,* is lower (yet not significantly different) than the 900 mg/Kg estimated by Song *et al.* (2002) at 25°C for *E. fetida.*

Zinc toxicity on the survival of *E. andrei* within the range of concentrations and temperatures investigated in the present study, was mostly sublethal. A median lethal concentration (LC_{50}) could not be reached in any of the Zn treatments at all three temperatures. When mortality was recorded, in the 1000 mg/kg treatment at both 20 and 25°C, it was statistically higher at 20 than 25°C ($p < 0.01$). This indicated that from 20°C, Zn lethality could decrease with increasing temperature. According to Spurgeon *et al.* (1997) who assessed the toxicity of Zn to *E. fetida* at the same temperatures used in the present study, the LC_{50} values were 1598 mg/kg Zn at 15°C, 1235 mg/kg Zn at 20°C and 1131 mg/kg Zn 25°C. These values were all higher than the highest Zn treatment used in the present study. Moreover, these LC_{50}s decreased with increasing temperature, suggesting an increase of Zn lethality to *E. fetida* with increasing temperature.

In terms of reproduction, the lack thereof in the Cd treatments made it impossible to calculate EC_{50}s for reproduction at all thee temperatures within the range of Cd concentrations investigated. Cocoon numbers in the controls nevertheless increased consistently with increasing temperature (as did hatchling numbers, Table 4). The mean numbers of cocoon per worm per week found in the control treatments (0.166 at 15^{o}C, 0.55 at 20^{o}C and 0.51 at 25^{o}C) were lower than the 0.8 to 2 cocoons per worms per week reported by Mulder *et al*. (2007) at 18^{o}C. This difference may be the result of differences in the source of nutrients. Haimi (1990) found slight differences in cocoon numbers after rearing *E. andrei* in different waste materials. Mulder *et al*. (2007) however did not divulge what they used to feed the worms during their study.

Interestingly, the presently recorded increase in the number of hatchlings per cocoon with increasing temperature could imply that temperature may influence both cocoon production and fertilization. Reinecke & Kriel (1981) reported that temperature influenced the number of hatchlings per cocoon in *E. fetida*, although they found evidence for fewer hatchlings per cocoon at 25 than at 20^{o}C.

In the Zn experiment, EC_{50}s for cocoon production were 185.22 mg/kg at 15^{o}C, 94.62 mg/kg at 20^{o}C and 363.91 mg/kg at 25^{o}C (Table 7). As in the case of survival, for reproduction, Zn toxicity overall seemed to decrease between 15 and 25^{o}C. At 20^{o}C, however Zn toxicity was the highest (as it was the case for survival as well) as evidenced by the lowest EC_{50} value. Spurgeon *et al.* (1997) found Zn EC_{50}s for reproduction of 382 mg/kg at 15^{o}C, 308 at mg/kg at 20^{o}C and 234 mg/kg at 25^{o}C. Their findings again suggested that Zn toxicity to *E. fetida* consistently increased with increasing temperature. This was not consistent with our findings that seem to indicate decreasing Zn toxicity with increasing temperature.

This apparent discrepancy could be explained by the fact that while Spurgeon *et al.* (1997) used zinc nitrate ($ZnN0_3$), zinc sulfate ($ZnS0_4$) was used in the present study. It has previously been reported that metal salts could differ in their toxicity

(Pavlica *et al*. 2009; Reinecke & Reinecke 1996). Reinecke & Reinecke (1996) especially found, while assessing the influence of different metal salts on the growth and reproduction of *E. fetida*, that $ZnSO_4$ compared to $ZnCl_2$ tended to be more deleterious to cocoon viability and to be the most accumulated. The possibility of such a difference between $ZnSO_4$ and $ZnN0_3$ still needs to be investigated.

Regarding cocoon incubation times, in all the controls there was a consistent decrease in incubation time as temperature increased. The lack of reproduction in Cd treatments did not allow for monitoring Cd effects on cocoon incubation. However, as far as Zn is concerned, it was found that Zn did not have an effect on incubation time (Fig. 9) and that temperature was the main factor controlling that particular parameter. Shorter incubation periods associated with temperature increases have been reported in other earthworm species such as *Dendrobaena veneta* (Viljoen *et al.*1992) and *E. fetida* (Reinecke & Kriel 1981).

3.4.2. Survival and reproduction in mixture exposures

Mortality in the mixture exposures occurred in the higher treatments at all three temperatures although LC_{50mix}[8] could only be estimated at 20 (18.34 TU) and 25^{o}C (16.09 TU). These values suggested that mixture lethality increased with increasing temperature.

EC_{50mix} for reproduction increased slightly from 2.47 TU at 15^{o}C, to 2.54 at 20^{o}C and 2.70 at 25^{o}C (Table 10). Although these values did not differ statistically from one another, they indicated a slight decrease in mixture toxicity to this specific endpoint (cocoon production) as temperature increased.

The fact that the reproduction of *E. andrei* under all exposure regimes tended to be higher at higher temperatures (see Tables 4, 5 & 8) is consistent with other studies where the effects of temperature (alone or with metals) were investigated

[8] LC_{50mix} = median lethal concentrations after exposure of *E. andrei* to mixtures of Cd and Zn at 15, 20 and 25^{o}C.

on the reproduction of earthworms (Ma 1984; Fayolle *et al*. 1997; Spurgeon *et al*. 1997; Reinecke & Kriel 1981).

Fayolle *et al*. (1997) argued that low cocoon production at cooler temperature is caused by prolonged development (perhaps caused by lower metabolic rates, earthworms being ectotherm). Arrillo & Melodia (1991) reported that a moderate increase in temperature improves reproduction in *E. fetida* through a supply of extra energy from a temperature-induced mitochondrial cycle. Presley *et al*. (1996) also noticed that *E. fetida* incubated in moderately high temperatures (20-25^{o}C) increased their reproductive output instead of their growth (biomass).

3.4.3. Biomass change in single and mixture exposures

The latter observation by Presley *et al*. (1996) could be verified using the biomass variation in the Zn treatments where reproduction occurred (Fig. 15). It was found that at the lowest temperature (15^{o}C) where *E. andrei* reproduced the least, the biomass of earthworms increased. At higher temperature however, when reproduction increased, no weight gain was recorded in the worms at both 20 and 25^{o}C (Fig. 15).

The trade-off between weight gain and reproduction in moderately high temperatures was, however, not observed in worms exposed to mixtures. In the mixture treatments, weight gain as observed at 15^{o}C also occurred at 25^{o}C in the same treatments where reproduction was recorded (Fig. 16). This could be due by the antagonistic relationship between Cd and Zn, which may cause less deleterious mixture effects on the reproduction of *E. andrei*.

Uvarov & Scheu (2004) have ascertained why biomass gain and reproduction are inversely correlated in earthworms. According to these authors, respiration rates in epigeic species increased with increasing temperature and this according to Byzova (1965), impedes on biomass gain, as oxygen consumption and increase in biomass are inversely correlated in epigeic earthworms (such as *L. rubellus* and *E. andrei*).

Moreoever, at 15^{o}C, where the worms showed signs of biomass gain, *E. andrei* feeding rates were expected to be at their lowest. Jager *et al*. (2003) assessed the feeding activity of *E. andrei* at 10 and 20^{o}C in OECD artificial soil and reported that the feeding rate of this earthworm species is temperature-dependent. Thus in a colder and more contaminated environment such as Cd, Zn or mixture contamination at 15^{o}C, *E. andrei* would probably ingest less soil/food and contaminant. This may prevent greater toxicant uptake and subsequent weight loss.

Biomass change in the mixture treatments mimicked the ones recorded in Cd treatments. In the Cd experiment, it was found that biomass loss increased consistently with increasing temperature (Fig. 14), while it was the opposite in Zn treatments where weight losses at 20^{o}C, were higher than at 25^{o}C (Fig. 15). This further indicated that Zn became less deleterious when temperature increased above 20^{o}C, probably due to increased Zn excretion at 25^{o}C (Fig. 18). In mixtures, EC_{50mix} for biomass (Table 14) indicated that mixture toxicity to this particular endpoint increased with increasing temperature. No explanation could be provided for this observation as mixture toxicity as influenced by temperature seemed to vary depending on the endpoint.

3.4.4. Metal body burdens in single and mixture exposures

Our findings in terms of Cd body burden at the three incubation temperatures are consistent with the occurrence of lower feeding rates at lower temperatures. At 15^{o}C, Cd body concentrations were the lowest (Fig 17 & 19). Decreased feeding rates at 15^{o}C may explain the low Cd body contents found in *E. andrei* at this particular temperature. Although the primary route of uptake of metals by earthworms was believed to be through passive adsorption via the body wall (Spurgeon & Hopkin 1996), a significant quantity of metal ions could be ingested during feeding (Vijver *et al*. 2003). Vijver *et al*. (2003) suggested that 17-30% of metals could be taken up through ingestion.

Research also shows that metal availability in soils is increased at higher temperature (Hogg *et al.* 1993; Si *et al*. 2006). The increase in Cd body burden with increasing temperature in both single and mixture exposures is also consistent with this understanding. Cd metal analysis results are also consistent with the findings of Spurgeon *et al*. (2005) who reported that higher temperature increased Cd tissue accumulation in another earthworm species (*L. rubellus*).

The comparison of Cd body burdens between single exposures (Cd_{Sin}) and mixture exposures (Cd_{Mix}) showed a prevailing tendency for $Cd_{Sin} > Cd_{Mix}$ (Fig. 20). This indicated that the presence of Zn in the mixtures somehow prevented such Cd_{Mix} uptake matching the levels accumulated in single Cd exposures. This was further verified by the consistent antagonistic interactions found between Cd and Zn in all mixture treatments (see parameter a in Tables 19, 20 and 21 when testing for S/A).

Zinc body burdens in both single and mixture exposures seemed not to be depending on temperature (Figs. 18 & 19). Morgan & Morgan (1991) suggested that because Zn is an essential element, there exist physiological pathways regulating the level of this metal in earthworms. Zinc regulation in earthworms has been previously documented (Lock & Janssen 2001; Demuynck *et al*. 2007).

The comparison in Zn uptake between single exposures (Zn_{Sin}) and mixture exposures (Zn_{Mix}) showed a prevailing tendency for $Zn_{Mix} > Zn_{Sin}$ (Fig. 21). This indicated that the presence of Cd in the mixtures in some way caused increased Zn_{Mix} uptake. This was further verified by the fact that all interactions between Cd and Zn were antagonistic at low concentrations and switched to synergism at higher concentrations (see parameter b_{DL} in Tables 19, 20 and 21). As more Zn_{Mix} was accumulated and inversely less Cd_{Mix} was taken up in worms exposed to higher mixture concentrations, the “antagonistic equilibrium” eventually tilted toward synergism where Zn_{mix} toxicity probably contributed the most.

Previous research not only shows that Cd and Zn are metabolic antagonists but also that Cd toxicity is significantly altered by the presence of Zn (Brzóska & Moniuszko-Jakoniuk 2001). This, according to Brzóska & Moniuszko-Jakoniuk (2001) is primarily because both Cd and Zn ions in the organism bind preferentially to the same proteins (such as albumin, metallothioneins and other proteins in tissues). Moreover, according to these authors, Zn shows the ability to reduce Cd absorption, accumulation and toxicity in the organism. This was observed in the present study when reduced Cd uptake occurred in the presence of Zn (in mixtures) when compared to uptake levels in single Cd exposures (Fig. 20).

Furthermore, according to Sunderman & Barber (1988), Cd toxicity occurs through a disruption of Zn-mediated or Zn-dependent processes in the organism. Such processes may include cellular synthesis of DNA and RNA. This could imply that although in the present study more Zn uptake was observed in mixture exposures when compared to uptake levels in single Zn exposures (Fig. 21), this may still not reflect sound physiological functioning because of Cd interfering with biochemical processes.

3.4.5. Metal interactions in mixture exposures

Although antagonism was already established in mixture exposures when considering EC_{50mix} values (EC_{50mix} >1) for cocoon production (Table 10) and biomass (Table 14) the modelling of biomass data from single metal and mixture exposures using MixToxModules provided more information about the type of antagonistic interactions involved. At 15^{o}C, dose level dependent antagonism (CA reference model) was exclusively found (Fig. 22 & Table 19). At 20^{o}C, dose level dependent antagonism was found for both the CA and IA reference models (Fig. 23 & Table 20). At 25^{o}C, dose level dependent antagonism was equally found in both the CA and the IA reference models (Fig. 24 & Table 21).

These results showed that temperature had no direct effect on the type of antagonistic interactions present as dose level dependent antagonism was present at each temperature investigated in this study.

Based on the reference model assumptions for CA (correlating relative toxicity of a mixture to the relative toxicity of its individual chemicals) and IA (investigating whether chemicals in a mixture could still cause independent responses) MixToxModules analyses could be discussed as follows:

At 15^{o}C (Table 19), the relative toxicity of mixtures correlated to the relative toxicity of Cd and Zn toxicities separately. At that temperature however, both metals interacted in a dose level dependent manner. Antagonism occurred mainly at low mixture concentrations while synergism was more prevalent in high mixture concentrations (a > 0 while testing for DL; Table 19). As previously established, the switch from antagonism to synergism at this temperature occurred at 25.67 TU.

At 20^{o}C (Table 20), the relative toxicity of mixtures correlated to the relative toxicity of Cd and Zn toxicities separately. Moreover, both Cd and Zn at 20^{o}C acted independently and interacted in a dose level dependent manner (as suggested by both the CA and IA reference models). Antagonism occurred mainly at low mixture concentrations while synergism was more prevalent in high mixture concentrations (a > 0 while testing for DL; Table 20). As previously established, the switch from antagonism to synergism at this temperature occurred at 24.95 TU (CA reference model) or 7.24TU (IA reference model).

At 25^{o}C (Table 21), the relative toxicity of mixtures correlated to the relative toxicity of Cd and Zn toxicities separately. Moreover, both Cd and Zn at 25^{o}C acted independently and interacted in a dose level dependent manner (as suggested by both the CA and IA reference models). Antagonism occurred mainly at low mixture concentrations while synergism was more prevalent in high mixture concentrations (a > 0 while testing for DL; Table 21). As previously

established, the switch from antagonism to synergism at this temperature occurred at 44.39TU (CA reference model) or 11.53TU (IA reference model).

The results of these analyses also revealed that overall, between 15 and 25^{o}C, the “switch points” from antagonism to synergism increased with increasing temperature. At 20^{o}C, in the CA and IA reference models however, the switch points were the lowest, indicating that at this temperature, synergistic interactions started earlier than in the other temperatures. The present results showed a tendency for a delayed onset of synergism as temperature increases above 20^{o}C. Moreover, at each temperature, the “switch points” from antagonism to synergism were highly different in both reference models (CA and IA). Although the IA model could not explain de variation of the data at 15^{o}C, the switch points were consistently higher in the CA reference model in both the 20 ^{o}C and 25^{o}C. The occurrence of lower switch points in the IA model indicated that this model predicted early onset of synergistic interactions between Cd and Zn and that this was possibly the worse case scenario.

3.5. Conclusion

Litter dwelling earthworms such as *E. andrei* are exposed to extreme diurnal and seasonal temperature variations. In the present study, we have shown that higher temperature could exacerbate Cd toxicity and uptake in *E. andrei*. The opposite however is observed for Zn toxicity and uptake that are both less dependent on temperature. Important data on the effects of Cd on the survival of *E. andrei* at the two lower temperatures were not conclusively assessed in the present study. Moreover, the specific Cd concentrations used for exposures did not allow for reproduction to occur in Cd contaminated treatments. Similarly, the effects of Zn on the survival of *E. andrei* could not be conclusively assessed at all temperatures due to the fact that most concentrations were sub-lethal. Future studies addressing these concerns could add to the present contribution in providing a greater understanding of the role of temperature on metal toxicity.

Mixture exposures are found to be dynamic processes at many levels. Mixture toxicity to biomass and survival increases with increasing temperature while toxicity to cocoon production seems to decrease with increasing temperature. Within the range of concentrations and temperatures investigated, Cd and Zn in mixtures display antagonism at low mixture concentrations and synergism at higher concentrations. These interactions occur interchangeably depending on the dose of the toxicants involved.

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4. Reproduction and survival of *Enchytraeus doerjesi* in single and mixture exposures of cadmium and zinc at different temperatures

4.1. Introduction

In the predicted current globally warming environment, climate change could have diverse effects on the toxicity of different classes of toxicants (Noyes *et al.* (2009). These authors have stated that increasing temperatures will enhance chemical toxicity, increase concentrations of tropospheric ozone regionally and increase rates of chemical degradation. Bailey (2003) argued that climate change would cause a decrease in herbicide persistence in European soils rendering autumn and winter weed control more difficult. Similarly, Dalla Valle *et al.* (2007) suggested that global warming may, within the next 50 years, cause a decrease in PCBs and PCDFs environmental concentrations. As far as metals are concerned, Augustsson *et al.* (2009) speculated that because of increased precipitation driven by climate change in Scandinavian countries, the hydrological concentrations of trace metals such as U, Cd, Cu and Ni will be reduced. They argued that wetter and warmer conditions would cause a decrease in the redox potential of metals, which will lead to the precipitation of sulfides and consequently reduce metal mobility in water.

Although information is still lacking as to how global warming will ultimately affect the toxicity of metals to oligochaete species, several authors have investigated the role of temperature in the toxicity of metals to selected oligochaetes. Spurgeon *et al*. (2005) reported that higher temperatures might increase Cd tissue accumulation in *Lumbricus rubellus.* Cadmium tissue accumulation was also found to be temperature-dependent in *Dendrobaena veneta* (Wieczorek-Olchawa *et al.* 2003, Olchawa *et al.* 2006). Janssen *et al*. (1996) reported that Cs accumulation in *Eisenia andrei* and *Lumbricus rubellus* increased by 1.6 and 2.1-fold respectively with a 10°C increase in temperature. Spurgeon *et al*. (1997) found that Zn toxicity increases with increasing temperature in *Eisenia fetida*.

Moreover, in nature, metals mostly occur in mixtures (Weltje 1998; Spurgeon *et al*. 1994) and if temperature has an effect on the toxicity of these substances to organisms, it might as well influence their interactions and ultimately their toxicity as a result.

To date, studies investigating the combined effects of temperature and metal toxicity on oligochaetes have mostly focused on lumbricids rather than enchytraeids (Janssen *et al*. 1996; Spurgeon *et al*. 1997; Wieczorek-Olchawa *et al.* 2003; Spurgeon *et al*. 2005; Olchawa *et al.* 2006). Römbke & Moser (2002) argued however, that Enchytraeidae are true terrestrial oligochaetes as opposed to some Lumbricidae such as *E. fetida* and *E. andrei* whose ecological niche is limited to compost heaps (Stephenson 1930). Studies investigating the combined effects of temperature and metals on oligochaetes should also take into account non-lumbricid species that in some instances are more representative of the soil environment. As an enchytraeid, *Enchytraeus doerjesi* is a possible candidate to be used in such a study.

E. doerjesi is a recently described enchytraeid species. Westheide & Graefe (1992) reported discovering *E. doerjesi* in garden mould and earthworm substrates from France, Holland and the Philippines, suggesting that this species could be found in Europe and Asia. Its origin, however, still needs to be investigated. This potworm species has not been used in many ecotoxicological studies but it shows a promising future as an optional test species in ecotoxicology. Kramarz *et al*. (2005) who used *E. doerjesi* to assess the effects of both metal pollution and population density on population growth rate reported that it is easy to culture, fast growing and consequently ideal for population studies. Owojori *et al*. (2009) successfully used *E. doerjesi* in a comparative study looking at the effects of salinity on the reproduction of selected soil-dwelling organisms.

The aim of the present study therefore was to assess the separate and combined effects of Cd and Zn on the survival and reproduction of the potworm *E. doerjesi* at three different temperatures, namely 15, 20 and 25^{o}C.

4.2. Materials & Methods

4.2.1. Metal exposures

Four replicates of ten adult specimens per treatment were each exposed to Cd and Zn separately and in mixtures in 20g (wet weight) of artificial OECD soil (OECD 1984) and at three different temperatures. The metals were provided in the form of $CdSO_4$ and $ZnSO_4$. For single metal exposures, concentration series of 0, 15, 25, 50, 100, 200, 320 mg/kg for Cd and 0, 30, 64, 100, 200 400, 640 mg/kg for Zn were used. For mixture exposures, the Cd concentrations were kept as in the single metal exposures and the Zn proportion was half the nominal value of each respective Cd concentration. Mixture concentrations were therefore 0+*0*, 15+*7.5*, 25+*12.5*, 50+*25*, 100+*50*, 200+*100*, 320+*160* mg/kg Cd +*Zn* (fixed ratio of 2:1).

Exposures were carried out in 50 ml screw cap conical tubes, 29 x 116 mm (Delatalab S.L, Barcelona, Spain). For each exposure, all treatments were incubated for four weeks at one of the selected temperatures namely 15, 20 and 25 °C. Tubes were opened every 48h to allow an inflow of fresh air. During the course of the experiments, worms were fed *ad libitum* with ground oats.

After exposure, specimens were stained using Bengal red according to the method recommended by Römbke & Moser (2002). The staining was done after transferring the soil with worms into shallow containers and adding 5 ml of ethanol. The containers were then filled with distilled water up to a level of 1-2 cm from the bottom thereafter and a few drops (200-300 µl) of Bengal red (1% solution in ethanol) were added and the contents of the containers mixed carefully and kept at room temperature. After 12 h, the enchytraeids were stained red and could be counted easily under an illuminated magnifying lens. The

numbers of both juveniles and adults were determined. Adults were distinguished by the presence of a clitellum on their bodies. The number of juveniles provided an indication of the reproductive output during the experiment, while the number of remaining adults was an indication of survival.

4.2.2. Statistical analysis

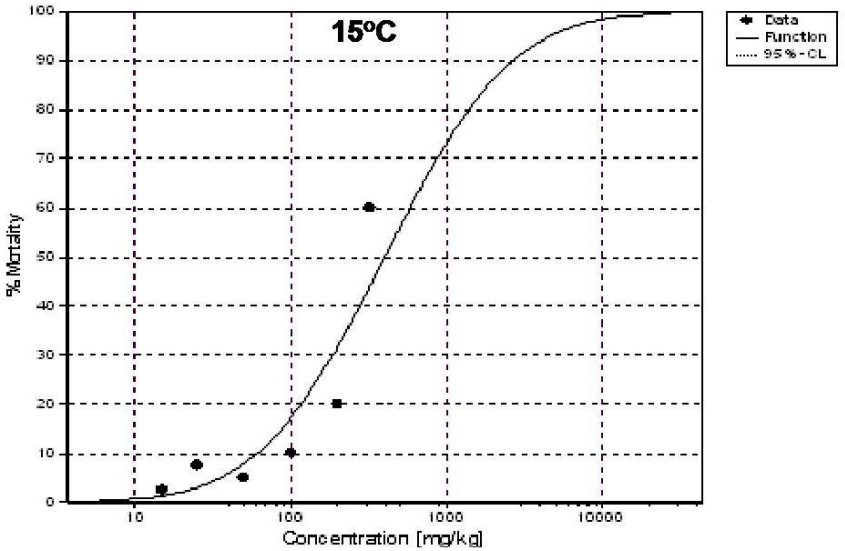
Descriptive statistics on the data during this study were performed using ToxRat® 2.09 (Toxicity Response Analysis and Testing; GMBH, Germany). Juvenile numbers at each temperature and metal/mixture concentrations were tested for normality using the range-to-standard-deviation ratio (R/s). Normally distributed data were tested further for the homogeneity of variances using the Cochran's test. Normally distributed data with homogenous variances were analyzed using a parametric multiple test (One way-ANOVA, with Tukey's posttest). Normally distributed data with non-homogenous variances were analyzed using the parametric Bonferroni t-test for non-homogenous variances. Non-parametric data were analyzed using the Kruskal-Wallis ANOVA followed by Dunns' test. Factorial ANOVA followed by Bonferroni posttest was also performed to assess the interaction between temperature and metals on the recorded juvenile and adult numbers for all three temperatures and metal/mixture concentrations. The level of significance was $p < 0.05$. These analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). LC_{50} values were estimated using the trimmed Spearman-Karber Program version 1.5. EC_{50} indices were estimated using non-linear regression analyses in PASW (Predictive Analytics softWare) statistics version 18 (SPSS, Inc, 2009, Chicago, Illinois, www.spss.com). Mixture data were analyzed using the toxic unit (TU) approach (Bliss 1939). Mixture interactions were further investigated using MixToxModules spreadsheets (Jonker *et al*. 2005). The level of significance was $p < 0.05$. After these analyses 3D surface plots of the models were generated in Statistica (Data analysis software system), version 9. (Statsoft, Inc, 2009, Tulsa, Oklahoma, www.statsoft.com)

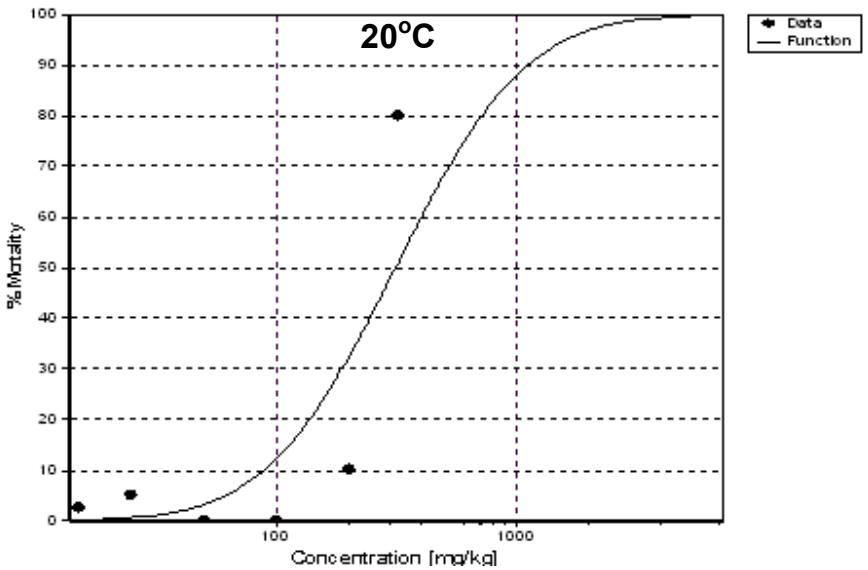
4.3. Results

4.3.1. Survival

4.3.1.1 Cd exposures

Survival data for Cd exposures indicated that the LC_{50} decreased as temperature increased. The highest LC_{50} recorded at 15°C was 293.01 (253.80-338.27) mg/kg. At 20°C, the LC_{50} was 261.62 (247.45-276.60) mg/kg and the lowest value recorded at 25°C was 231.79 (209.60-256.32) mg/Kg. Survival curves at each exposure temperature consequently showed an increase of the percentage mortality as temperature increased (Fig. 1).





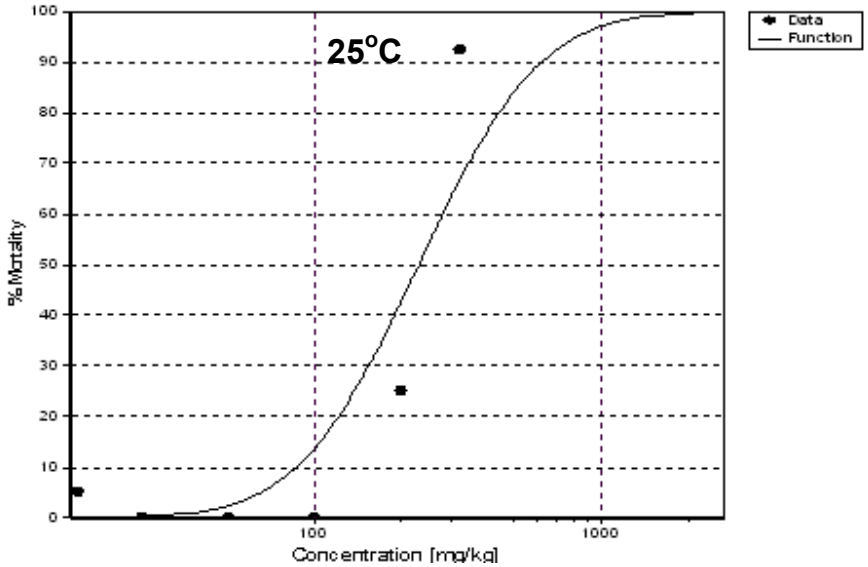


Fig. 1. Survival curves of *E. doerjesi* after exposure to Cd in artificial soil for four weeks at 15, 20 and 25°C.

Factorial ANOVA suggested that there was no significant interaction between the incubation temperature and concentrations of Cd on the mortality of *E. doerjesi*. Cadmium alone explained more than 78% of the observed mortality (Table 1).

Table 1. Two-way ANOVA results of the analysis of the effects of temperature and Cd on the survival of *E. doerjesi* after exposure in artificial soil for four weeks at 15, 20 and 25°C. The abbreviation ns mean not significant.

Source of Variation	Percentage of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	4.17	12	29.43	2.452	1.259	0.2655^{ns}
Temperature	0.36	2	2.571	1.286	0.6599	0.5205^{ns}
Cd concentrations	78.08	6	551.3	91.88	47.16	p<0.0001
Residual		63	122.8	1.948		

A comparison of the number of surviving adults between the three temperatures showed that Cd induced mortality was significantly lower than in the other treatments only in the 320 mg/kg Cd treatments at 25°C ($p < 0.05$; Fig. 2).

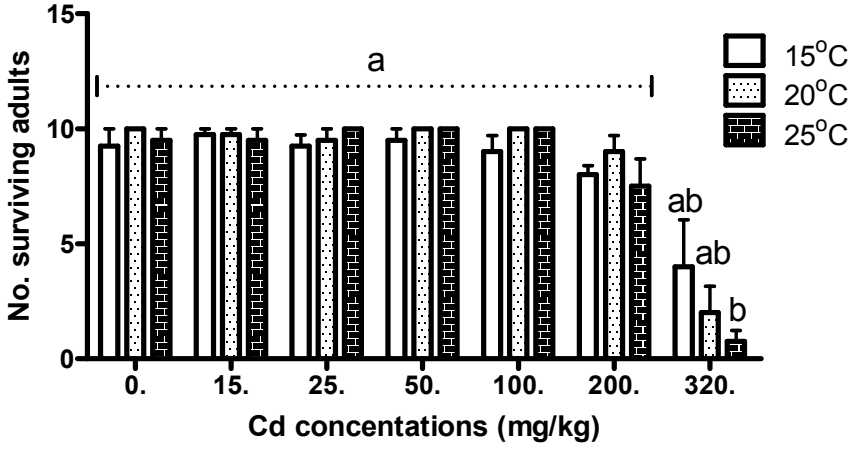


Fig. 2. Comparison of the survival rates of *E. doerjesi* between 15, 20 and 25°C after exposure to Cd in artificial soil for four weeks. Error bars represent standard error. Different letters on bars represent statistical differences ($p < 0.05$).

4.3.1.2 Zn exposures

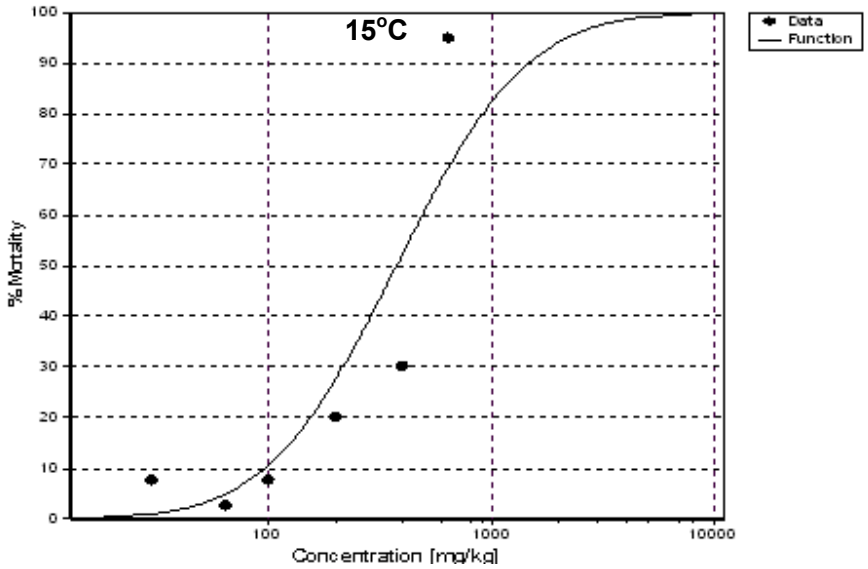
The pattern of survival for the Zn exposures was the reverse of that observed during the Cd experiment. The LC_{50} for Zn increased with increasing temperature so much so that at 25°C a LC_{50} could not be computed due to the lack of sufficient adult mortality. The LC_{50} was 420.21 (371.90-474.80) mg/Kg at 15^{o}C, 518.42 (495.25-542.66) mg/Kg at 20^{o}C and > 640 mg/Kg at 25^{o}C. As a result, survival curves at each exposure temperature showed a decrease of the percentage mortality as the temperature increased (Fig. 3)

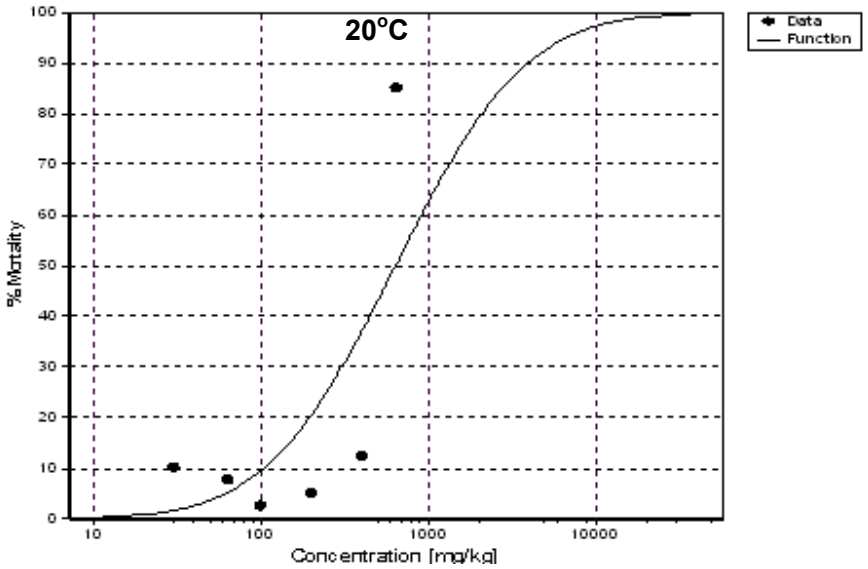
Factorial ANOVA suggested that there was a very significant interaction (p = 0.002) between temperature and Zn although Zn alone explained more than 73% of the mortality observed (Table 2)

Table 2. Two-way ANOVA results of the analysis of the effects of temperature and Zn on the survival of *E. doerjesi* after exposure in artificial soil for four weeks at 15, 20 and 25^{o}C.

Source of Variation	Percentage of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	8.69	12	56.00	4.667	3.015	0.0022
Temperature	3.03	2	19.50	9.750	6.300	0.0032
Zn concentrations	73.15	6	471.3	78.55	50.75	p <0.0001
Residual		63	97.50	1.548		

A comparison of the number of surviving adults between the three temperatures showed that Zn induced mortality was significantly lower only in the 640 mg/kg Zn treatment at 25^{o}C ($p < 0.01$; Fig. 4).





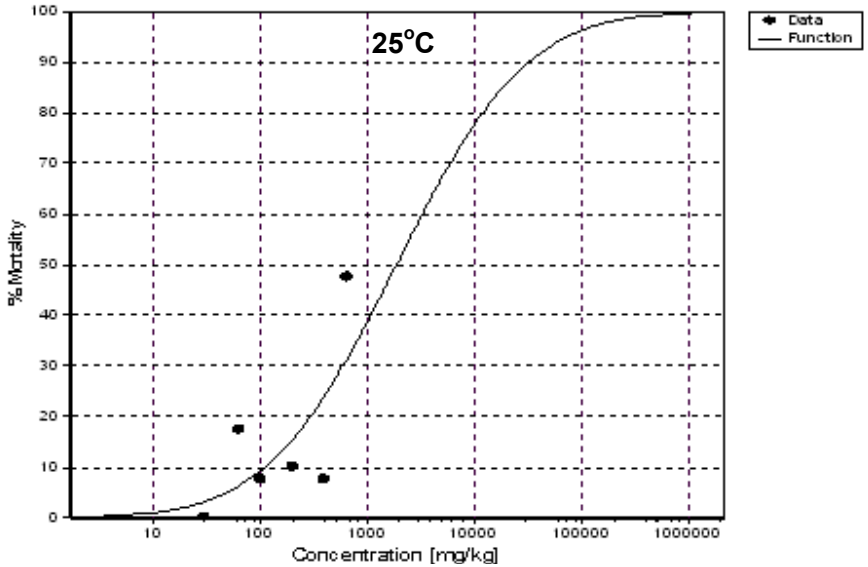


Fig. 3. Survival curves of *E. doerjesi* after exposure to Zn in artificial soil for four weeks at 15, 20 and 25°C.

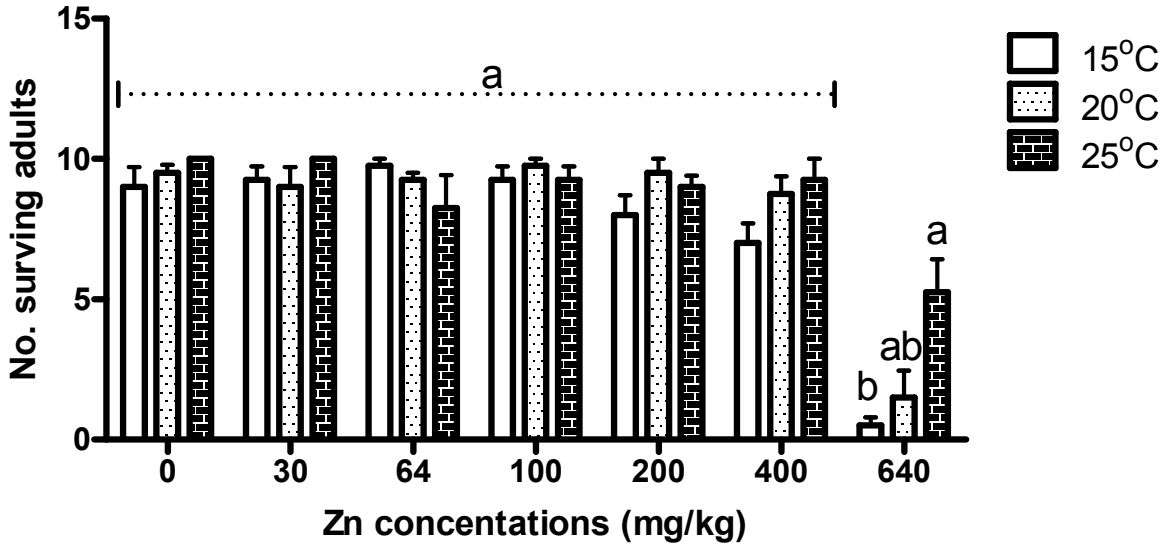


Fig. 4. Comparison of the survival rates of *E. doerjesi* between 15, 20 and 25°C after exposure to Zn in artificial soil for four weeks. Error bars represent standard error. Different letters on bars represent statistical differences ($p < 0.01$).

4.3.1.3 Mixture exposures

The median lethal dose for mixture concentrations could not be estimated at 15 and 20°C, as mortality rates were below 50% in all mixture treatments (Fig. 5). At 25°C, a LC_{50mix} of 9.02 TU was estimated.

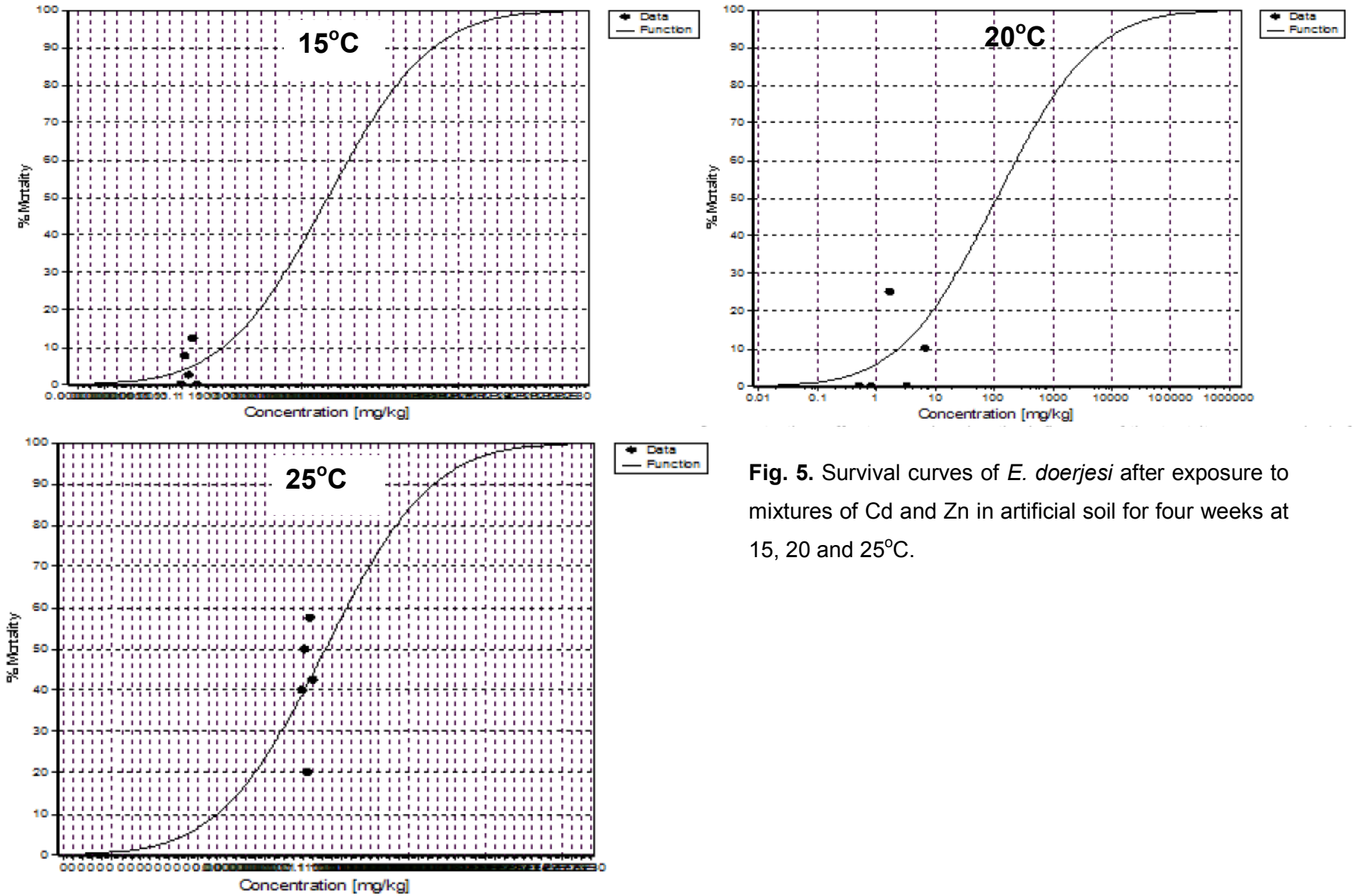


Fig. 5. Survival curves of *E. doerjesi* after exposure to mixtures of Cd and Zn in artificial soil for four weeks at 15, 20 and 25°C.

A comparison of survival rates at temperature level showed that between 15 and 20°C there was no difference in survival rates in the mixture treatments. However, between both these temperatures and 25°C, the difference in survival was very significant ($p < 0.01$), with lower survival occurring at 25°C (Fig. 6).

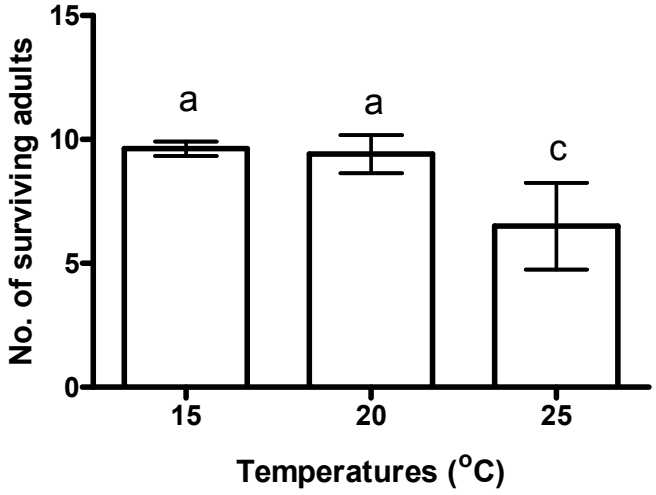


Fig. 6. Comparison of the survival rates of *E. doerjesi* between 15, 20 and 25°C after exposure to mixtures of Cd and Zn in artificial soil for four weeks. Error bars represent standard error. Different letters on bars represent statistical differences ($p < 0.01$).

4.3.2 Reproduction

4.3.2.1 Cadmium exposures

Juvenile numbers in the Cd treatments decreased as metal concentrations increased at all three temperatures (Fig. 7). Although the number of juveniles increased with increasing temperature, at 25°C, the numbers of juvenile in all the Cd treatments were all significantly lower than their control ($p \leq 0.01$). This was not the case at 15 and 20°C, where significantly lower juvenile numbers in the Cd treatments, when compared to their respective controls, were only observed in some treatments (Fig. 7).

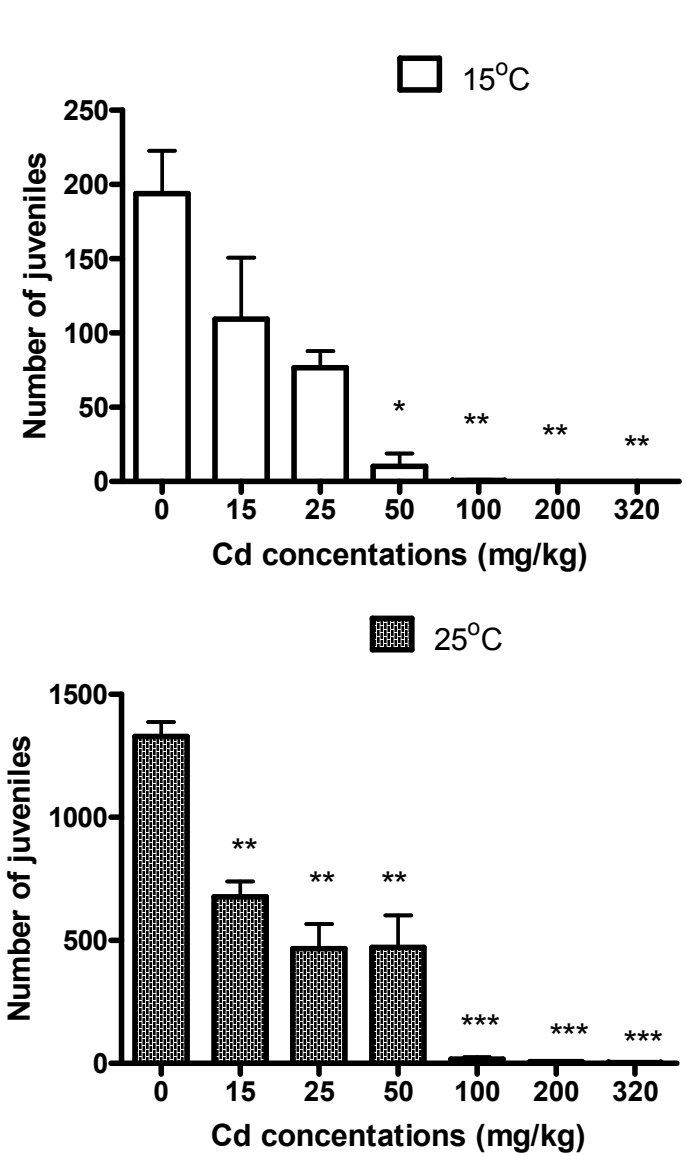


Fig. 7. Mean reproduction of *E. doerjesi* after exposure to Cd in artificial soil for four weeks at 15, 20 and 25°C. Error bars represent standard errors. Stars represent statistical difference from the respective control. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Number of starting adults = 70/temperature/replicate

Nonlinear regression analyses revealed that the Cd EC_{50} for reproduction was the highest 35.114 (12.902-56.296) mg/kg at 20°C and the lowest 16.849 (12.241 -23.192) at 25°C (Fig. 8).

Two-way ANOVA analyses followed by Bonferroni posttests, revealed that there was a highly significant interaction ($p < 0.0001$) between Cd concentrations and temperature on the reproduction of *E. doerjesi* (Table 3). However, temperature and Cd separately were still responsible for 55.84 and 17.61 % of the variation, respectively (Table 3).

Table 3. Two-way ANOVA results of the analysis of the effects of temperature and Cd on the reproduction of *E. doerjesi* after exposure in artificial soil for four weeks at 15, 20 and 25^{o}C.

Source of Variation	Percentage of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	18.42	12	2253000	187700	11.90	p<0.0001
Temperature	55.84	6	6829000	1138000	72.17	p<0.0001
Cd concentrations	17.61	2	2154000	1077000	68.28	p<0.0001
Residual		63	993600	15770		

A comparison of juvenile numbers between all three temperatures indicated that there was no statistical difference between the reproductive output at 20 and 25^{o}C (except in the control where reproduction was significantly higher at 25^{o}C, $p < 0.05$; Fig. 9). Reproduction at 15^{o}C however was significantly lower than the reproduction at both 20 and 25^{o}C ($p \leq 0.05$; Fig. 9).

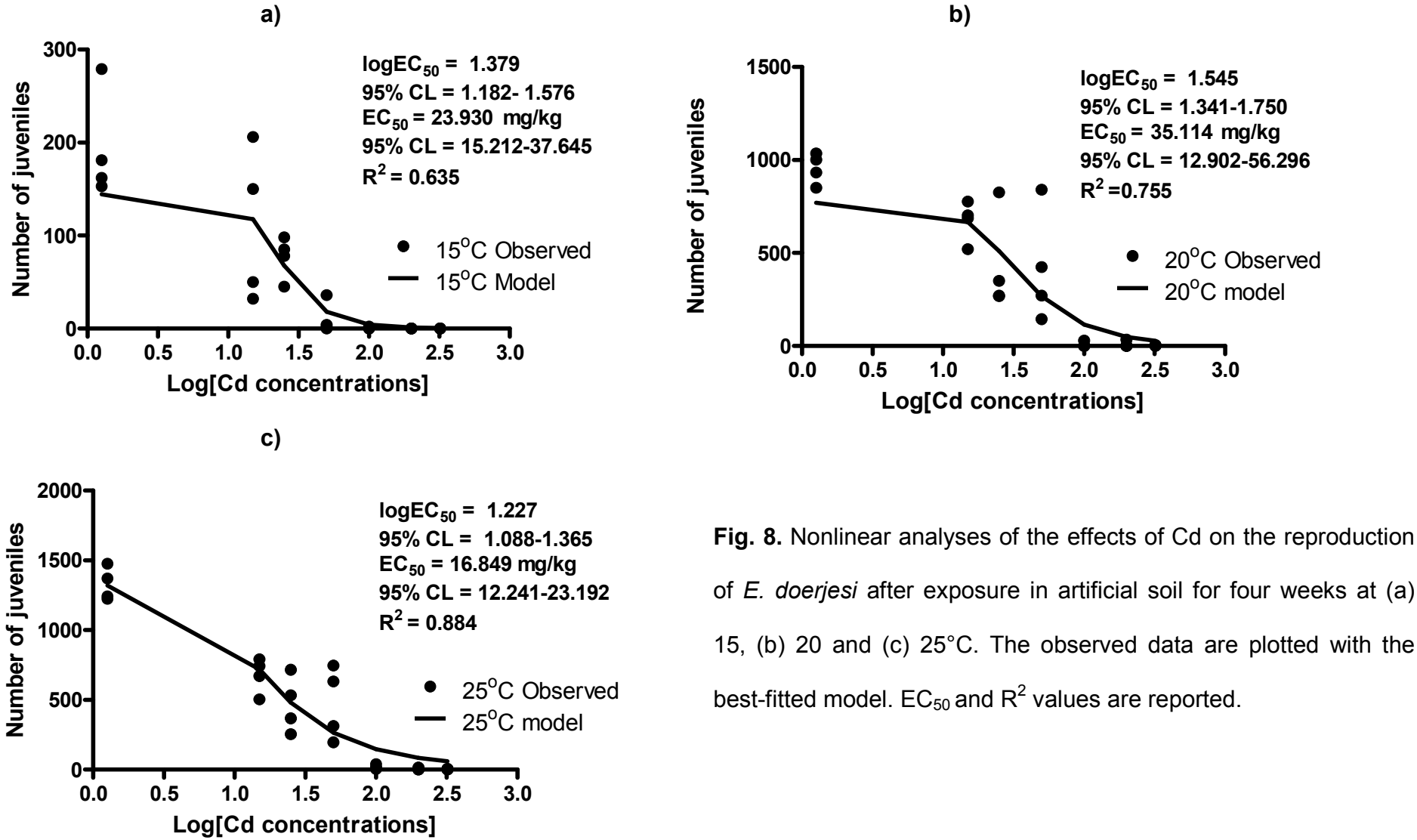


Fig. 8. Nonlinear analyses of the effects of Cd on the reproduction of *E. doerjesi* after exposure in artificial soil for four weeks at (a) 15, (b) 20 and (c) 25°C. The observed data are plotted with the best-fitted model. EC_{50} and R^2 values are reported.

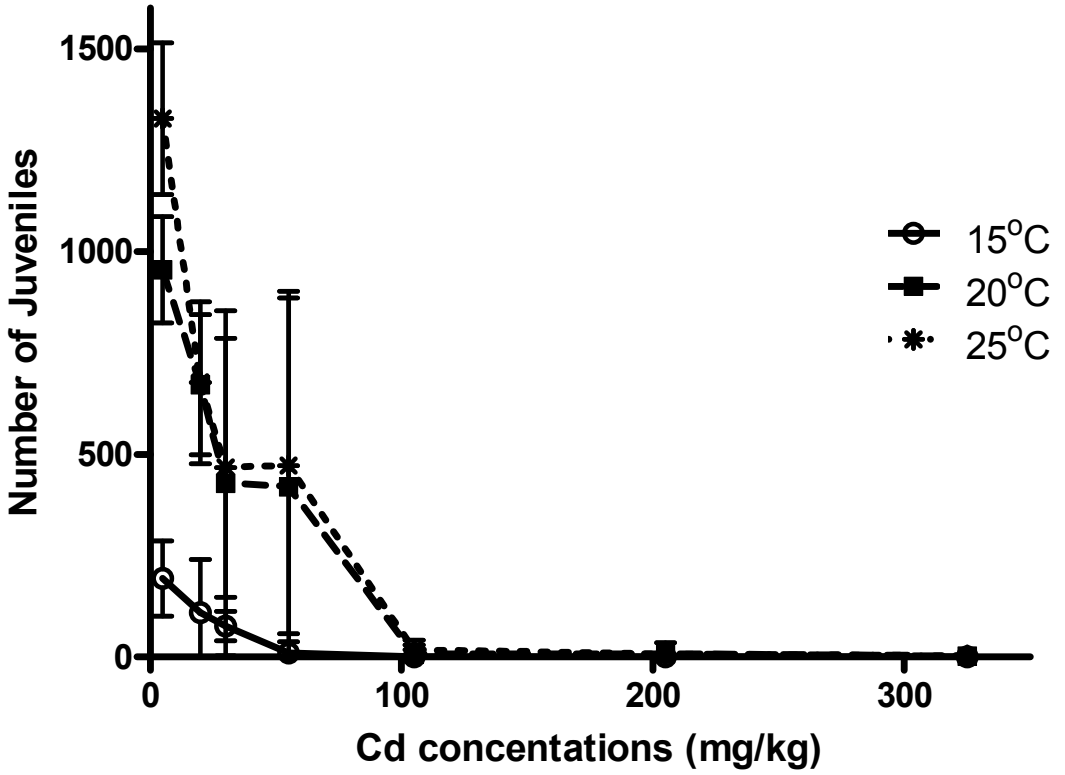
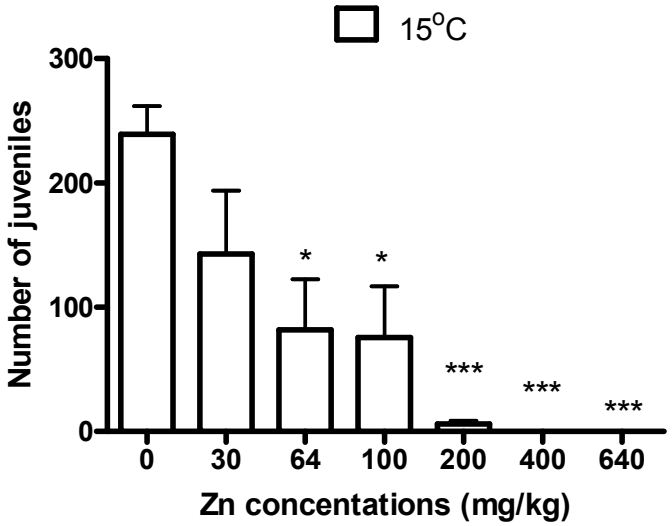


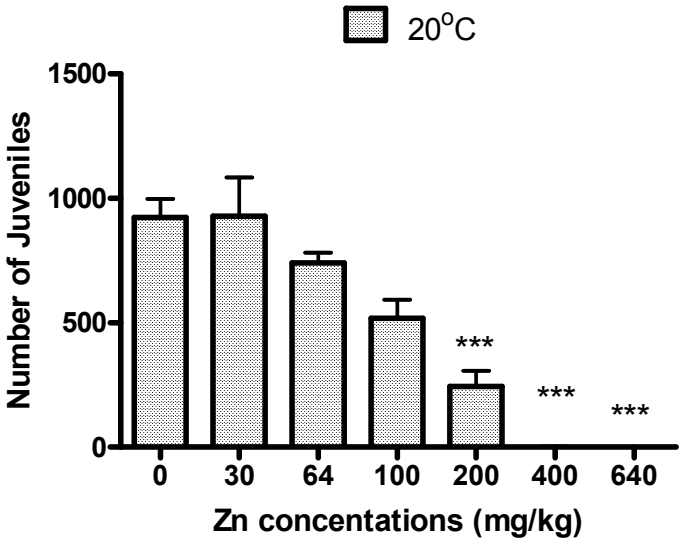
Fig. 9. Comparison of the mean reproductive output of *E. doerjesi* between 15, 20 and 25°C after exposure to Cd in artificial soil for four weeks. Error bars represent 95% confidence intervals. Number of starting adults = 70/temperature/replicate

4.3.2.2 Zinc exposures

Juvenile numbers in the Zn treatments decreased as metal concentrations increased at all three temperatures, except in 30 mg/kg Zn at 20^{o}C where the number of juveniles were the same as in the control (Fig.10).

At 15 and 25^{o}C, juvenile numbers in the Zn treatments were significantly lower than the control from 64 mg/kg Zn and higher concentrations ($p \leq 0.05$, Fig. 10). At 20^{o}C however, significantly lower juvenile numbers than the control were observed from 200 mg/kg Zn and higher concentrations ($p = 0.001$, Fig. 10).





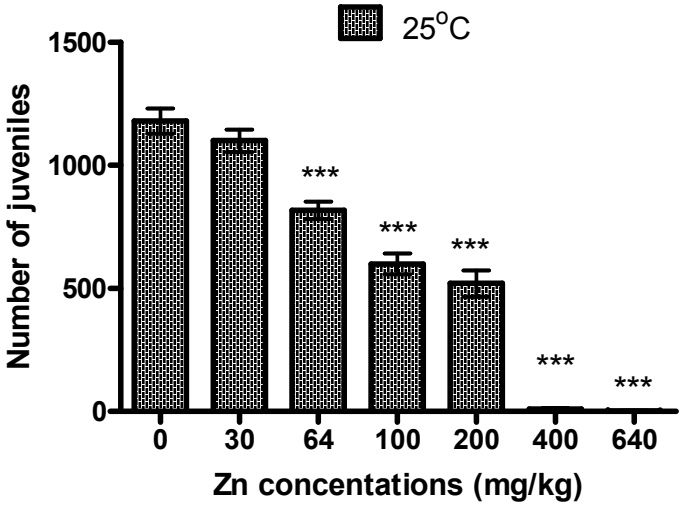


Fig. 10. Mean reproduction of *E. doerjesi* after exposure to Zn in artificial soil for four weeks at 15, 20 and 25°C. Error bars represent standard errors. Stars represent statistical differences from the respective control. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Number of starting adults = 70/temperature/replicate

Nonlinear regression analyses indicated that the EC_{50} for Zn increased with increasing temperature. The lowest value found at 15°C was 41.474 (23.551-73.034) mg/kg and the highest value recorded at 25°C was 112.177 (90.055 - 139.735) (Fig. 11).

Two-way ANOVA analyses followed by Bonferroni posttests, indicated that there was an highly significant interaction ($p < 0.0001$) between Zn concentrations and temperature on the reproduction of *E. doerjesi* (Table 4). However, temperature and Zn on their own were still responsible for 50.46 and 29.34 % of the variation respectively (Table 4).

Table 4. Two-way ANOVA results of the analysis of the effects of temperature and Zn on the reproduction of *E. doerjesi* after exposure in artificial soil for four weeks at 15, 20 and 25°C.

Source of Variation	Percentage of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	15.35	12	2216000	184700	16.60	p<0.0001
Temperature	50.46	6	7286000	1214000	109.1	p<0.0001
Zn concentrations	29.34	2	4237000	2118000	190.4	p<0.0001
Residual		63	701000	11130		

A comparison of juvenile numbers between all three temperatures indicated that there were no statistical differences between the reproductive output at 20 and 25°C (Fig. 12). Reproduction at 15°C however was significantly lower than the reproduction at both 20 and 25°C ($p \leq 0.05$, Fig. 12).

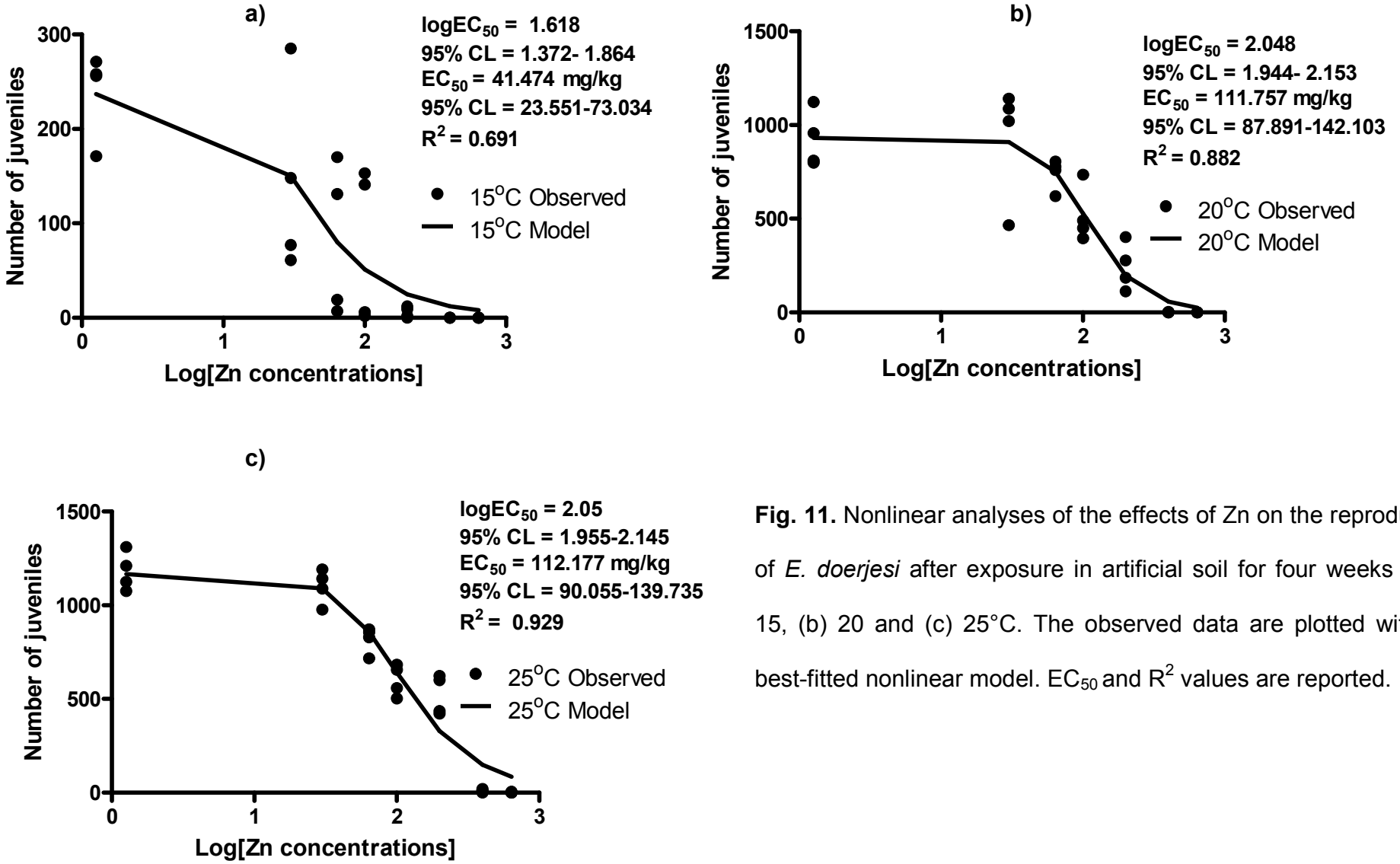


Fig. 11. Nonlinear analyses of the effects of Zn on the reproduction of *E. doerjesi* after exposure in artificial soil for four weeks at (a) 15, (b) 20 and (c) 25°C. The observed data are plotted with the best-fitted nonlinear model. EC_{50} and R^2 values are reported.

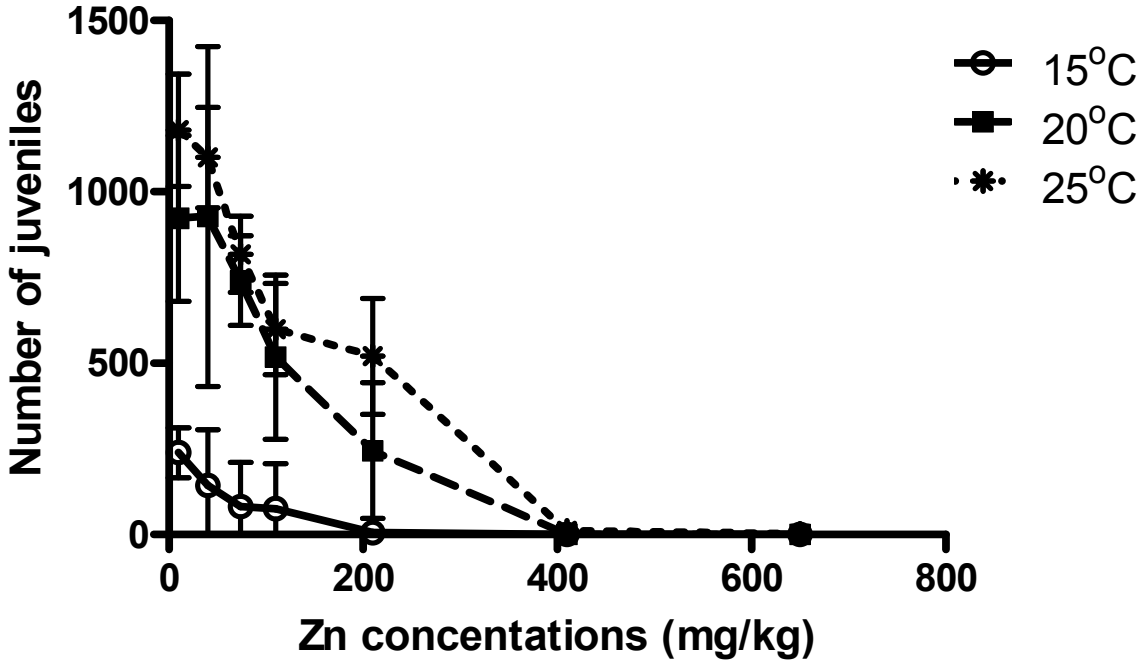
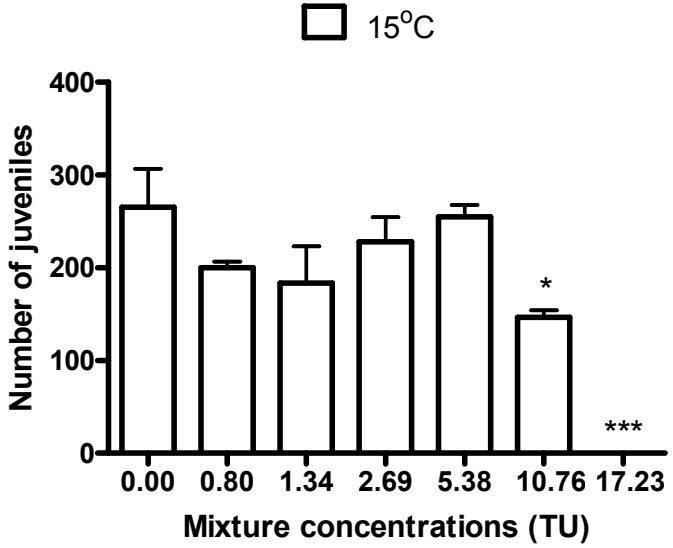


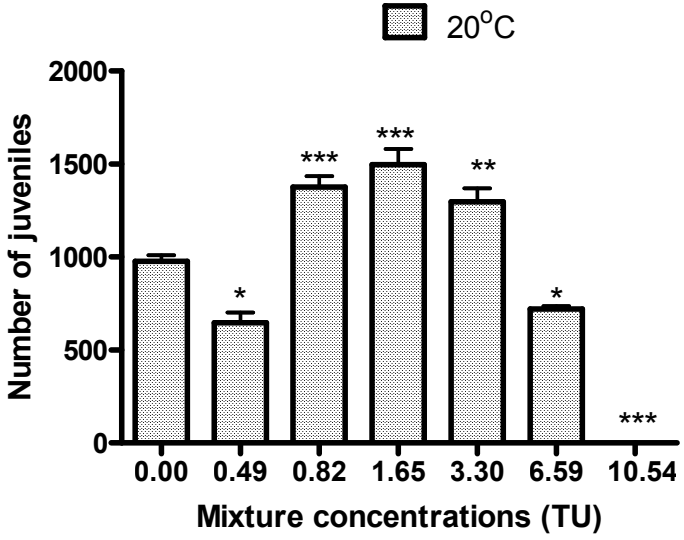
Fig. 12. Comparison of the mean reproductive output of *E. doerjesi* between 15, 20 and 25°C after exposure to Zn in artificial soil for four weeks. Error bars represent 95% confidence intervals. Number of starting adults = 70/temperature/replicate.

4.3.2.3 Mixture exposures

Juvenile numbers in the mixture treatments at all temperatures did not follow a dose response pattern but rather displayed bell-shaped distributions (Fig. 13). At 20^{o}C, reproduction at the median concentrations was significantly higher ($p \leq 0.01$) than in the control. At 25^{o}C, there was a similar trend but the numbers were not statistically different from the control.

Nonlinear regression analyses indicated that between 15 and 20^{o}C, the EC_{50mix} decreased from 9.779 TU to 5.354 TU, which was the lowest EC_{50mix} recorded (Fig. 14). Between 20 and 25 ^{o}C, the EC_{50mix} increased to 11.454 (8.406-15.606) TU, which was the highest EC_{50mix} recorded (Fig. 14).





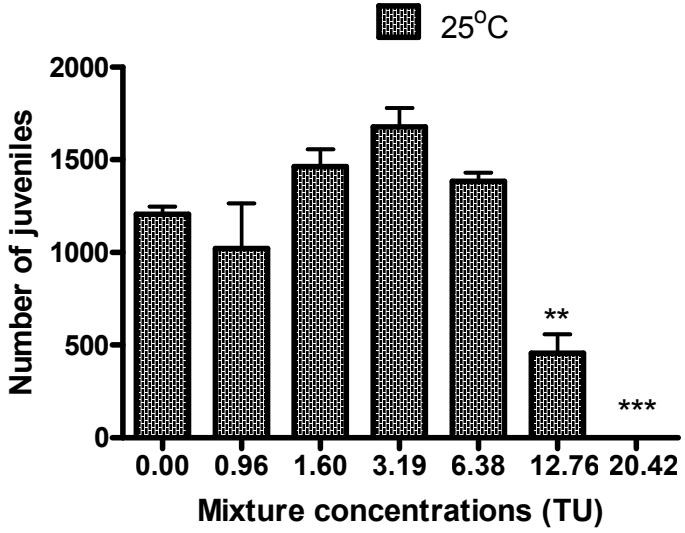


Fig. 13. Mean reproduction of *E. doerjesi* after exposure to mixture concentrations of Cd and Zn in artificial soil for four weeks at 15, 20 and 25°C. Error bars represent standard errors. Stars represent statistical difference from the respective control. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Number of starting adults = 70/temperature/replicate.

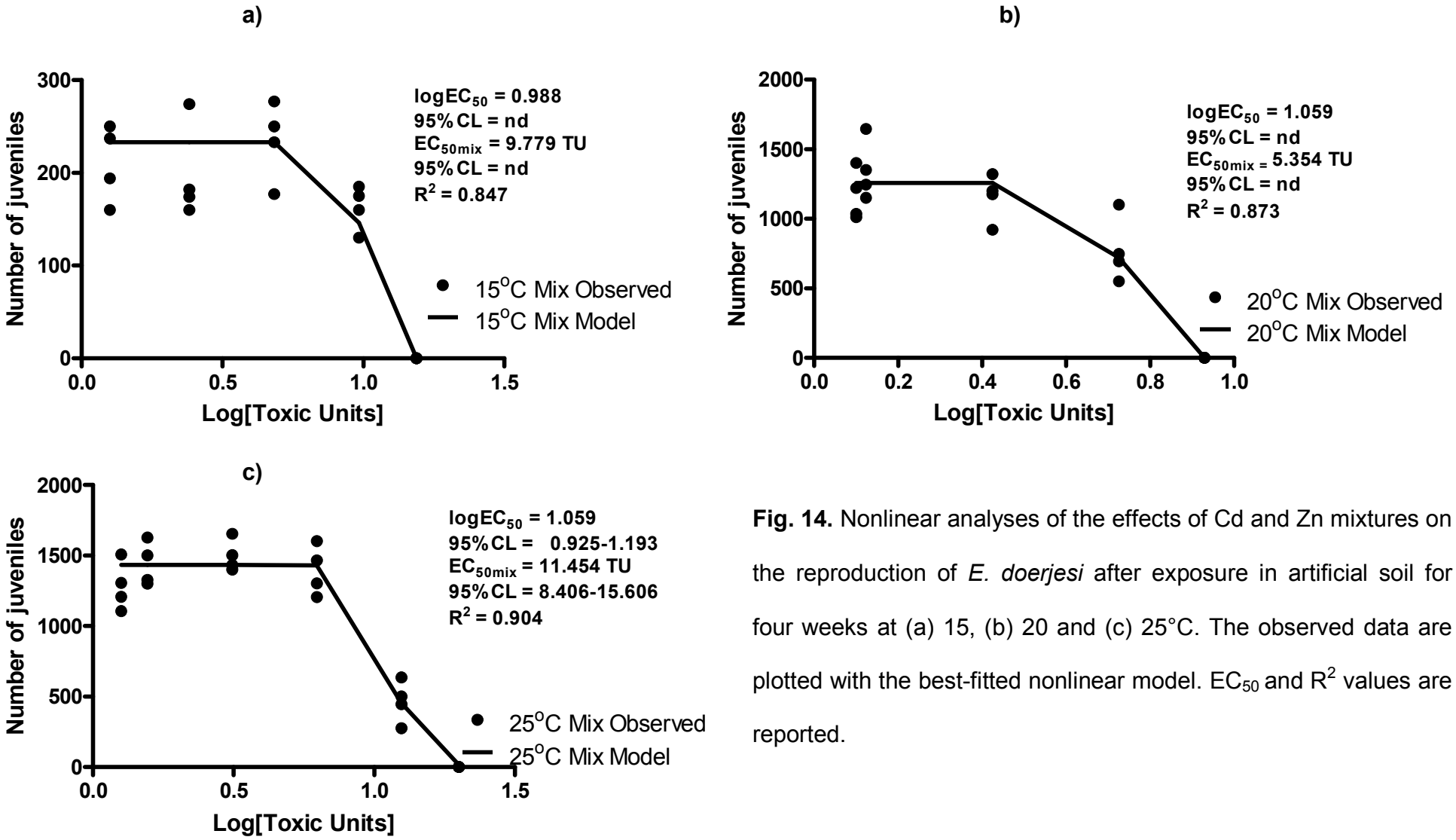


Fig. 14. Nonlinear analyses of the effects of Cd and Zn mixtures on the reproduction of *E. doerjesi* after exposure in artificial soil for four weeks at (a) 15, (b) 20 and (c) 25°C. The observed data are plotted with the best-fitted nonlinear model. EC_{50} and R^2 values are reported.

Because EC_{50} for reproduction for both Cd and Zn varied from one temperature to another, mixture concentration ranges (in toxic units) were different at each temperature (see Fig. 14). It was consequently impossible to compare juvenile numbers at concentration level. A comparison of juvenile numbers nonetheless could be performed at temperature level (Fig. 15). It was found that while the reproduction at 15°C was significantly lower than at the two other temperatures ($p < 0.001$), there was no statistical difference in juvenile numbers between 20 and 25°C (Fig. 15).

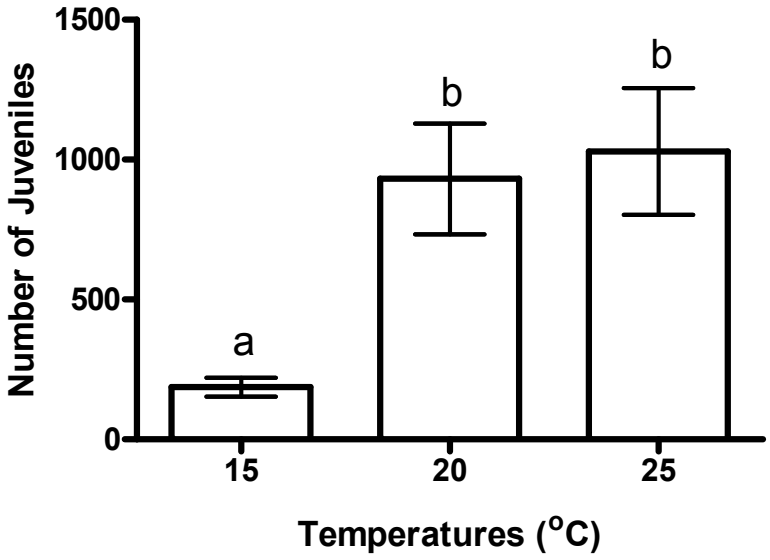


Fig. 15. Comparison of the reproductive output of *E. doerjesi* between 15, 20 and 25°C after exposure to mixtures of Cd and Zn in artificial soil for four weeks. Error bars represent standard error. Different letters on bars represent statistical differences ($p < 0.001$). Number of starting adults = 70/temperature/replicate.

The EC_{50mix} values generated in the present study were all greater than one (EC_{50mix} > 1, Fig. 14) indicating antagonistic interactions between Cd and Zn. These antagonistic interactions were further analyzed in order to find out whether they were dose ratio (DR) dependent or dose level (DL) dependent.

4.3.3 Modeling antagonistic interactions using MixToxModules

4.3.3.1. Modeling antagonistic interactions at 15°C

When reproduction data from single metal and mixture exposures at 15°C were modeled using the MixToxModules (Jonkers *et al*. 2005), antagonism in the mixture treatments was confirmed under both the concentration addition (CA) and the independent action (IA) assumptions (parameter $a^{***} > 0$ when testing for S/A; Table 5). Further tests investigating dose ratio (DR) or dose level (DL) dependency showed that DL was the most significant interaction ($p[\chi^2] < 0.001$) under both the CA assumption and the IA assumption ($p[\chi^2] < 0.001$; Table 5).

The interpretation of the DL model parameters under the CA reference model (using Table 2 from Chapter 2; section 2.5) showed the following: a = 15.24 indicated antagonism at low mixture concentrations and synergism at high mixture concentrations. $b_{DL}^{\dagger\dagger\dagger}$ = 1 (from Table 5) indicated that the change from antagonism to synergism occurred at EC_{50mix} level. Thus, this switch occurred at 9.779TU, which was the EC_{50mix} at 15°C (Fig. 14).

The interpretation of the DL model parameters under the IA reference model using (using Table 2 from Chapter 2; section 2.5) showed the following: a = 161.50 indicated antagonism at low mixture concentrations and synergism at high mixture concentrations. b_{DL} = 2 indicated that the change from antagonism to synergism occurred at EC_{50mix} level. Thus, this switch occurred at 9.779TU, which was the EC_{50mix} at 15°C (Fig. 14).

*** Parameter a indicates whether synergism or antagonism occurs at low or higher dose c.f. table 2, chapter 2.
††† Parameter b_{DL} indicates at which concentration the switch from antagonism to synergism occurs c.f. table 2, chapter 2.

Fig. 16 depicts 3D surface plots of the number of juveniles produced in mixture and single metal treatments. The reference models are reported with the most significant deviation models. Model fitness in both cases was extremely significant ($p[\chi^2] < 0.001$; Table 5).

Table 5. Summary of the modeling of the effect of Cd and Zn on the reproduction of *E. doerjesi* after exposure in artificial soil for four weeks at 15°C. b is the slope of the individual dose response curve; EC_{50} (in mg/kg) is the median effect concentration; a, b_{DL}, and b_{Zn} are parameters in the deviation functions; SS is the sum of squares; χ^2 is the test statistic; Df is the degrees of freedom; and $p(\chi^2)$ indicates the outcome of the likelihood ratio test. S/A is synergism/antagonism, DL is dose level dependent deviation from the reference, and DR is dose ratio dependent deviation from the reference. The abbreviation na means that the quantity is not applicable.

	Concentration addition				Independent action			
	Reference	S/A	DR	DL	Reference	S/A	DR	DL
EC_{50Cd}	43.333	25.06	25.04	25.06	69.82	25.06	25.05	25.04
EC_{50Zn}	55.841	52.68	48.54	51.70	85.36	51.70	51.69	48.54
a	na	15.24	15.24	62.78	na	161.50	166.15	161.50
b_{DL}	na	na	na	1	na	na	na	2
b_{Zn}	na	na	0	na	na	na	10.30	na
SS	13160.42	1467.74	1427.08	649.85	13790.07	1407.27	1406.88	649.85
χ^2	na	46.06	0.59	17.10	na	47.92	0.005	16.22
Df	na	1	2	1	na	1	2	1
$p(\chi^2)$	na	< 0.001	0.44	<0.001	na	<0.001	0.93	<0.001

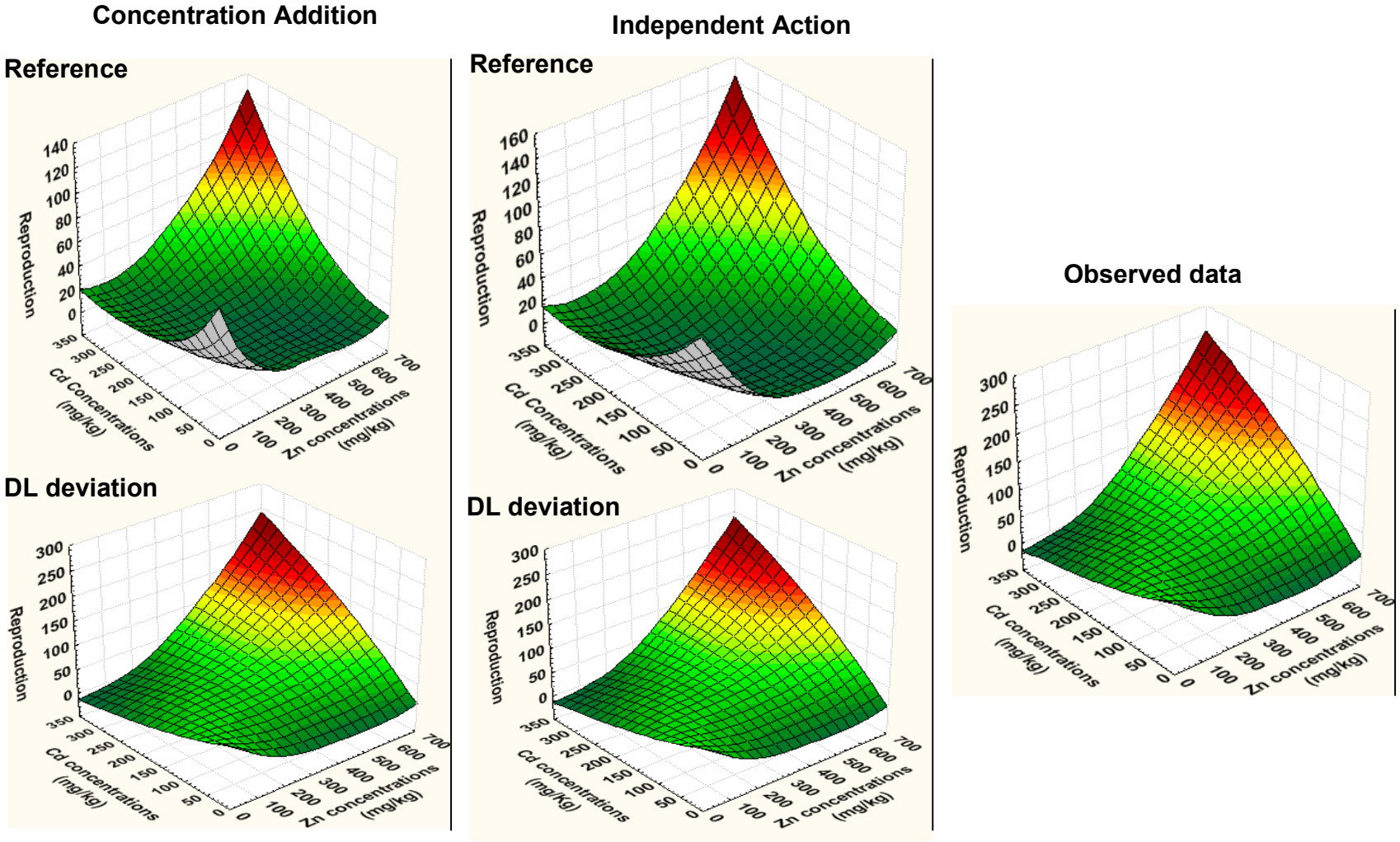


Fig. 16. 3D surface plots of the number of juveniles in the mixtures against the number of juveniles in Zn and Cd treatments at 15^{o}C. The reference models are reported with the most significant deviation model (DL). The plot of the observed data, as collected during the experiment, is given for comparison sake. For 3D plots, the numbers of juveniles (reproduction) in single metal and mixture treatments were expresssed as percentages of their respective controls.

4.3.3.2. Modeling antagonistic interactions at 20°C

When reproduction data from single metal and mixture exposures at 20°C were modeled using the MixToxModules, antagonism in the mixture treatments was confirmed under both the CA and the IA assumptions (parameter $a > 0$ when testing for S/A; Table 6).

Further tests investigating DR or DL dependency showed that DL was the most significant interaction ($p[\chi^2] < 0.001$) under the CA assumption, while no model could further explain the variance in the data under the IA assumption (Table 6).

The interpretation of the DL model parameters under the CA reference model (using Table 2 from Chapter 2; section 2.5) showed the following: $a = 19.96$ indicated antagonism at low mixture concentrations and synergism at high mixture concentrations. $b_{DL} = 1$ indicated that the change from antagonism to synergism occurred at EC_{50mix} level. Thus, the switch from antagonism to synergism occurred at 5.354TU, which was the EC_{50mix} at 20°C.

Fig. 17 shows 3D surface plots of the number of juveniles in mixture and single metal treatments. The reference model CA is reported with DL the most significant deviation model. DL Model fitness was extremely significant ($p[\chi^2] < 0.001$; Table 6).

Table 6. Summary of the modeling of the effect of Cd and Zn on the reproduction of *E. doerjesi* after exposure in artificial soil for 4 weeks at 20°C. b is the slope of the individual dose response curve; EC_{50} (in mg/kg) is the median effect concentration; a, b_{DL}, and b_{Zn} are parameters in the deviation functions; SS is the sum of squares; χ^2 is the test statistic; Df is the degrees of freedom; and $p(\chi^2)$ indicates the outcome of the likelihood ratio test. S/A is synergism/antagonism, DL is dose level dependent deviation from the reference, and DR is dose ratio dependent deviation from the reference. The abbreviation na means that the quantity is not applicable.

	Concentration addition				Independent action			
	Reference	S/A	DR	DL	Reference	S/A	DR	DL
EC_{50Cd}	171.62	28.97	28.97	35.83	156.68	33.31	39.47	32.09
EC_{50Zn}	192.73	102.30	102.30	115.42	105.93	99.96	101.12	100.13
a	na	19.96	19.96	19.96	na	23.81	23.81	204.68
b_{DL}	na	na	na	1	na	na	na	0.922
b_{Zn}	na	na	$9.28 \cdot 10^{-6}$	na	na	na	0	na
SS	27551.87	8764.67	8764.67	704.53	23448.77	8326.03	7705.83	7839.01
χ^2	na	24.05	$1.84 \cdot 10^{-10}$	52.93	na	21.74	1.62	1.26
Df	na	1	2	1	na	1	2	1
$p(\chi^2)$	na	< 0.001	0.99	< 0.001	na	< 0.001	0.20	0.26

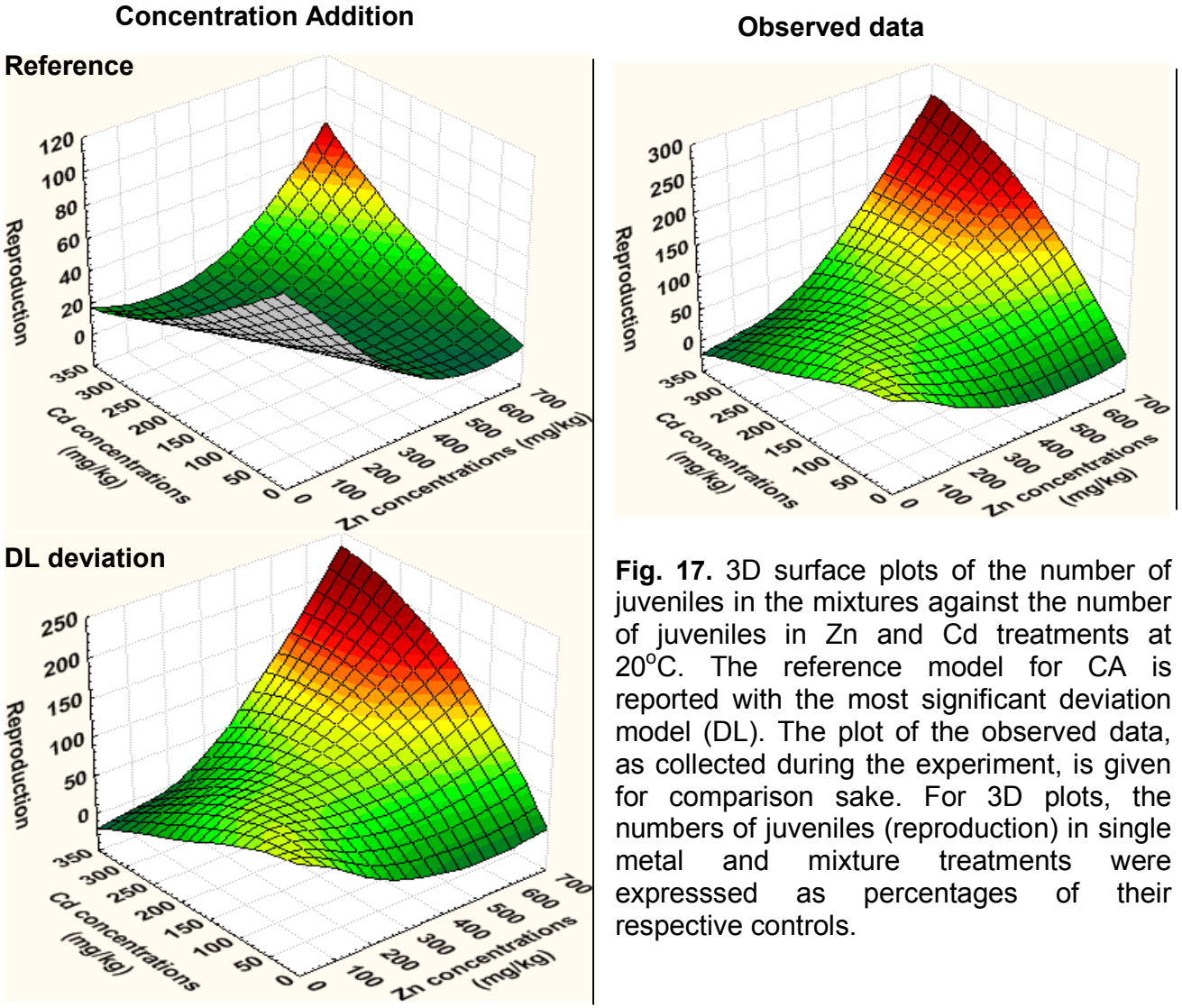


Fig. 17. 3D surface plots of the number of juveniles in the mixtures against the number of juveniles in Zn and Cd treatments at 20°C. The reference model for CA is reported with the most significant deviation model (DL). The plot of the observed data, as collected during the experiment, is given for comparison sake. For 3D plots, the numbers of juveniles (reproduction) in single metal and mixture treatments were expresssed as percentages of their respective controls.

4.3.3.3. Modeling antagonistic interactions at 25°C

When reproduction data from single metal and mixture exposures at 25°C were modeled using the MixToxModules, antagonism in the mixture treatments was confirmed under both the CA and the assumptions (parameter $a > 0$ when testing for S/A; Table 7).

Further tests investigating DR or DL dependency showed that DL was the most significant interaction under both the CA and the IA assumptions ($p[\chi^2] < 0.001$; Table 7).

The interpretation of the DL model parameters under the CA reference model (using Table 2 from Chapter 2; section 2.5) showed the following: a = 37.02 indicated antagonism at low mixture concentrations and synergism at high mixture concentrations. b_{DL} = 1 indicated that the change from antagonism to synergism occurred at EC_{50mix} level. Thus, this switch occurred at 11.454TU, which was the EC_{50mix} at 25°C (Fig. 14).

The interpretation of the DL model parameters under the IA reference model (using Table 2 from Chapter 2; section 2.5) showed the following: a = 1948.79 indicated antagonism at low mixture concentrations and synergism at high mixture concentrations. b_{DL} = 1.011 indicated that the change from antagonism to synergism occurred at mixture concentration equivalent to $\frac{1}{b_{DL}} EC_{50mix}$. EC_{50mix} at 25°C was 11.454 TU (Fig. 14). Thus, this switch occurred at 11.329TU.

Fig. 18 depicts 3D surface plots of the number of juveniles in mixture and single metal treatments. The reference models are reported with the most significant deviation models (DL for both concentration addition independent action). Model fitness, independently, in both cases was extremely significant ($p[\chi^2] < 0.001$; Table 7).

Table 7. Summary of the modeling of the effect of Cd and Zn on the reproduction of *E. doerjesi* after exposure in artificial soil for 4 weeks at 25°C. b is the slope of the individual dose response curve; EC_{50} (in mg/kg) is the median effect concentration; a, b_{DL}, and b_{Zn} are parameters in the deviation functions; SS is the sum of squares; χ^2 is the test statistic; Df is the degrees of freedom; and $p(\chi^2)$ indicates the outcome of the likelihood ratio test. S/A is synergism/antagonism, DL is dose level dependent deviation from the reference, and DR is dose ratio dependent deviation from the reference. The abbreviation na means that the quantity is not applicable.

	Concentration addition				Independent action			
	Reference	S/A	DR	DL	Reference	S/A	DR	DL
EC_{50Cd}	149.33	16.02	16.02	16.61	131.72	18.76	16.61	14.47
EC_{50Zn}	235.61	103.00	103.00	116.34	181.60	95.25	116.34	100.83
a	na	37.02	37.02	37.02	na	35.20	35.20	1948.79
b_{DL}	na	na	na	1	na	na	na	1.011
b_{Zn}	na	na	$6.59 \cdot 10^{-5}$	na	na	na	0	na
SS	22131.48	4630.56	4630.56	749.20	19263.88	6294.23	5402.45	2692.26
χ^2	na	32.85	$8.11 \cdot 10^{-10}$	38.24	na	23.49	3.20	17.83
Df	na	1	2	1	na	1	2	1
$p(\chi^2)$	na	<0.001	0.99	<0.001	na	<0.001	0.07	<0.001

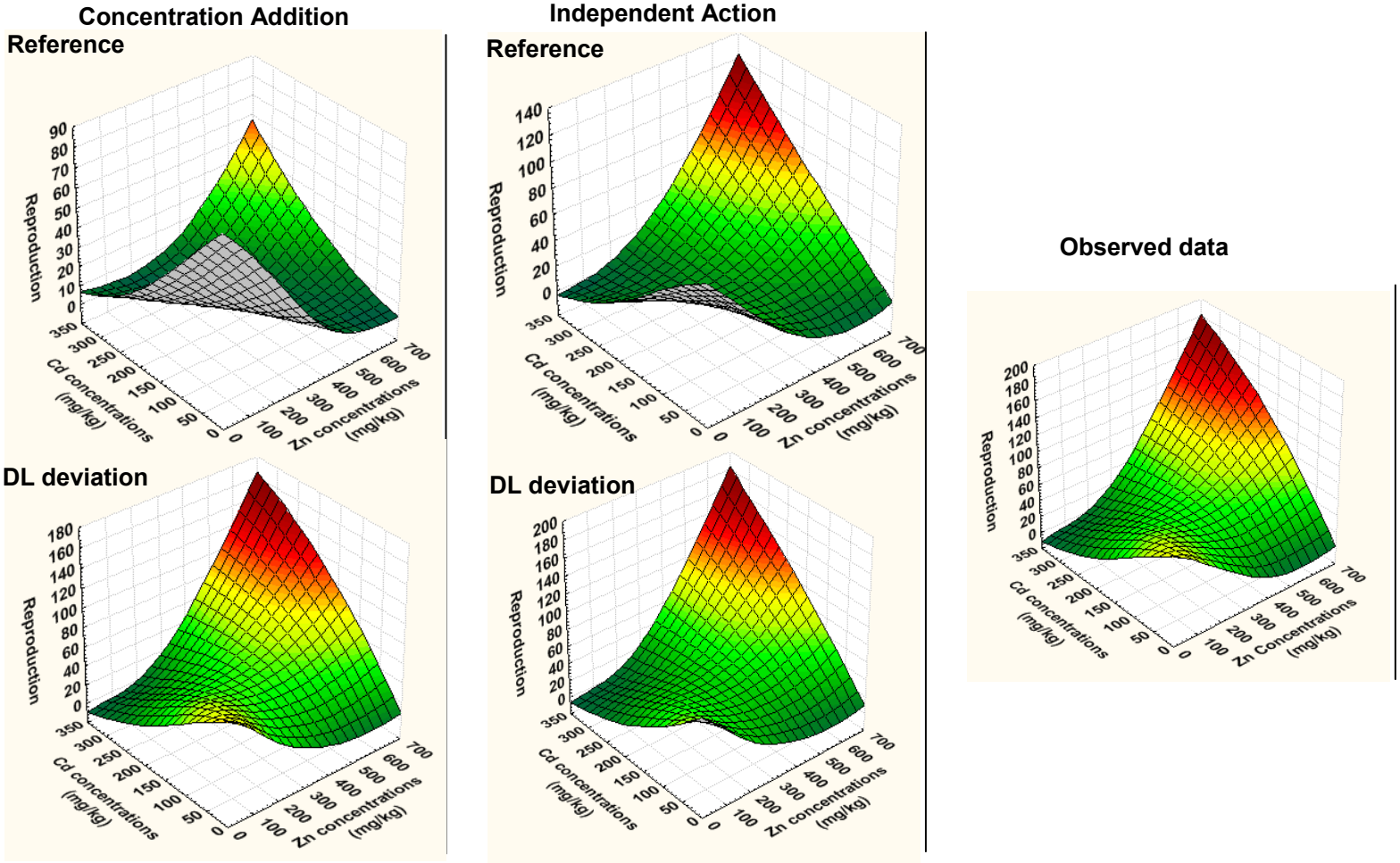


Fig. 18. 3D surface plots of the number of juveniles in the mixtures against the number of juveniles in Zn and Cd treatments at 25^{o}C. The reference models are reported with the most significant deviation models (DL for both CA and IA). The plot of the observed data, as collected during the experiment, is given for comparison sake. For 3D plots, the numbers of juveniles (reproduction) in single metal and mixture treatments were expresssed as percentages of their respective controls.

4.4. Discussion

4.4.1. Survival and reproduction in single metal exposures

The analysis of surviving adults revealed that Cd LC_{50s}, decreased consistently as temperature increased suggesting that this metal becomes increasingly lethal at elevated temperature. In the case of Zn, this pattern was reversed as Zn LC_{50s} were found to increase with temperature, suggesting a decrease of lethality at higher temperature.

The interaction between metal and temperature in explaining these results is significant for Zn (Table 2) but not for Cd (Table 1). Cd nonetheless was increasingly lethality at higher temperature.

Furthermore, single metal exposures indicated a dose response relationship between metal concentrations and reproduction in each metal (Fig. 7 & 10). For Cd, when we considered the range of incubation temperature used in this study, there was a general decrease of EC_{50} from 23.93 mg/kg at 15^{o}C to 16.84 mg/kg at 25^{o}C (Fig. 8) suggesting more deleterious Cd effects on reproduction at higher temperature. In the case of Zn the reverse was found as Zn EC_{50} increased from 41.47 mg/kg at 15^{o}C to 112.17 at 25^{o}C (Fig. 11). This further suggested that Zn becomes less toxic to this organism at higher temperature.

In the case of reproduction, the interaction between metal and temperature in explaining the number of juveniles was extremely significant in the case of both metals ($p < 0.0001$, Tables 3 & 4). This suggested that the effect of Cd on the reproduction of *E. doerjesi* is positively correlated to temperature while it is the contrary for Zn. Increasing Cd toxicity with increasing temperature has previously been reported in the case of earthworm species such as *Dendrobaena veneta*, *Lumbricus rubellus* (Olchawa *et al.* 2003; Spurgeon *et al.* 2005; Wieczorek-Olchawa *et al.* 2006).

From the EC_{50s} and the LC_{50s} generated in this study, it can also be concluded, as far as Cd toxicity is concerned, that 20°C is a more advantageous temperature for *E. doerjesi.* At this particular temperature, Cd had the least strain on reproduction (highest EC_{50}; Fig. 8) and was not as lethal as at 25°C. Without the pressure exerted by any toxicant, nonetheless (as represented by the control treatments), 25°C could significantly improve the reproduction of *E. doerjesi* which means that in a pristine environment this temperature would be more suitable than 15°C and 20°C for the reproduction of this species.

In the present study, Zn toxicity to *E. doerjesi* was found to decrease with increasing temperature. While no such information is available on enchytraeids, Zn toxicity has been shown to increase with temperature in *E. fetida* (Spurgeon *et al.* 1997). However, the present results found in *E. doerjesi* corroborate the results of Zn toxicity to *E. andrei* reported on chapter 3, sections 3.3.1.1 and 3.3.1.2. This implied that enchytraeids and lumbricids may not intrinsically differ in their capacity to deal with metal toxicity.

4.4.2. Survival and reproduction in mixture exposures

The analysis of surviving adults in mixture exposures showed that an LC_{50mix} could only be estimated at 25°C. It was shown to be 9.02 TU. A comparison of survival rates at temperature level (Fig. 6) suggested that mixture lethality increased significantly ($p < 0.01$) with temperature. The fact that mixture induced 50% mortality rates could not be recorded at 15 and 20°C, while such rates were caused at both these temperatures by single exposures of Cd and Zn suggests that mixtures were less lethal than the separate metals.

EC_{50mix} values, with regard to the range of incubation temperatures, increased from 9.77TU at 15°C to 11.45TU at 25°C (Fig 14). These values suggest a decrease in mixture toxicity on the reproduction of *E. doerjesi* with increasing temperatures. Thus, mixtures were more lethal with increasing temperature, yet less deleterious to reproduction at the highest temperatures. This could be

explained by the fact that as temperature increased the energy trade-off in these organisms was more in favor of reproduction than survival (Arrillo & Melodia 1991) as witnessed by higher juvenile numbers at elevated temperatures (Fig. 13). Reproducing adults would however become increasingly vulnerable to mixture toxicity as a result because of the high-energy cost involved in reproduction.

4.4.3. Metal interactions in mixture exposures

Although antagonism was already established in mixture exposures by considering EC_{50mix} values (EC_{50mix} >1), the modeling of single metal and mixture data using MixToxModules shed more light on the type of antagonistic interactions involved. At 15^{o}C, dose level dependent antagonism in both the CA and IA reference models was found (Fig. 16 & Table 5). At 20^{o}C, dose level dependent antagonism in the CA reference model only was found while the IA model could not explain the variation in the data (Fig. 17 & Table 6). At 25^{o}C, dose level dependent antagonism was found in both the CA and the IA reference models (Fig 18 & Table 7).

These results showed that the incubation temperature had no direct effect on the type of antagonistic interaction present as the main interaction (DL) was found at each temperature investigated in this study.

If it is considered as suggested by Jonker *et al*. (2005), that the CA and IA reference models are not mutually exclusive, the results of both assumptions have to be taken into account in validating the findings of this study. As already stated, the CA model can indicate whether the relative toxicity of a mixture correlates to the relative toxicity of its individual chemicals and the IA model whether the chemicals involved in a mixture could still cause independent responses.

Based on these two different assumptions, our results suggest that at 15^{o}C (Table 5), the relative toxicity of mixtures correlated to the relative toxicity of Cd

and Zn toxicities separately. At that temperature however, both metals interacted independently and in a dose level dependent manner (as suggested by both CA and IA reference models). Antagonism occurred mainly at low mixture concentrations while synergism was more prevalent in high mixture concentrations (a > 0 while testing for DL; Table 5). As previously established, the switch from antagonism to synergism at this temperature occurred at EC_{50mix} level.

At 20^{o}C, the relative toxicity of mixtures correlated to the relative toxicity of Cd and Zn separately and both metals interacted in a dose level dependent antagonistic manner (Table 6). At this temperature, there was no independent action from either of the metals. Nonetheless, parameter a > 0 (in the test for DL; Table 6), indicated that antagonism was dominant at low mixture concentrations and synergism at high mixture concentrations. In this case, b_{DL} = 1 (Table 6) indicated that the change from antagonism to synergism occurred at EC_{50mix} level.

At 25^{o}C, the relative toxicity of mixtures correlated to the relative toxicity of Cd and Zn separately Moreover, Cd and Zn at 25^{o}C acted independently and interacted in a dose level dependent antagonistic manner (as suggested by both CA and IA reference models). Moreover, $a > 0$ in both reference models implied the presence of antagonism at low mixture concentrations and synergism at high mixture concentrations. The switch from antagonism to synergism was found to occur between 11.32-11.45 TU.

The results of these analyses revealed that overall, the “switch points” from antagonism to synergism increased with increasing temperature between 15 and 25^{o}C. Although the AI reference model could not explain the variation of the data at 20^{o}C, in the CA model this switch occurred at a lower mixture concentration than at the other two temperatures. This indicated 20^{o}C to be the temperature with the earliest onset of synergistic interactions between Cd and Zn. Moreover, these switch points were the same for both reference models (CA and IA)

indicating that both models predicted the same scenario of mixture behaviour. Lock and Janssen (2002), who assessed the effects of binary mixtures of Zn, Cd, Cu and Pb in the potworm *E. albidus*, had, on the contrary, reported that the CA model represented the worse case scenario in that species. These authors, however, used other analytical approaches (central composite design and toxic units) and did not make use of MixToxModules.

Because of the fixed dose ratio used in the mixture experimental design (2:1, Cd: Zn) one would have expected that such a design would predominantly cause dose ratio interactions but it was not the case in this study.

The present findings regarding the main antagonistic nature of the interactions between Cd and Zn corroborate with previous findings. Such antagonistic interactions between Cd and Zn have been reported after mixture exposures of these metals to other soil-dwelling organisms such as the springtail *Folsomia candida* (Van Gestel & Hensbergen 1997) the earthworm *E. fetida* (Demuynck *et al.* 2007) and the isopod *Porcellio scaber* (Zidar *et al.* 2009).

4.5. Conclusion

The present findings show that an increase of 5°C above the considered laboratory optimal (20°C) does not alter the effects of Cd and Zn (single exposures) on the reproduction of *E. doerjesi* considerably. Nevertheless, Cd toxicity increases with increasing temperature and Zn toxicity consistently decreases with increasing temperature.

Mixture exposures, in the case of these two metals, are here shown to be less toxic than the metals separately. However, mixtures are dynamic processes with antagonism and synergism occurring interchangeably depending on the dose (rather than the ratio) of the toxicants involved. The effect of mixture toxicity on the survival of this species increases with temperature and could be exacerbated within such a temperature increase as the one predicted under the current global warming scenario (IPCC 2001).

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5. Cytotoxic and genotoxic effects of cadmium and zinc in single and mixture exposures at different temperatures on *Eisenia andrei*

5.1. Introduction

Heavy metal toxicity has the potential to affect the contaminated organisms at different levels. Besides impairments at life cycle level, heavy metal toxicity can induce an array of responses within the affected organisms. These responses can be used as biomarkers of exposure or effect. According to Dallinger *et al*. (2000), a biomarker can be defined as "*any molecular, biochemical, histological and physiological parameter at the sub-individual level that varies in response to an environmental toxicant"*.

Investigating effects on organisms using biomarkers can provide early warnings of potentially deleterious processes within the organism before the whole individual, and eventually the whole population (and perhaps community and ecosystem), are irremediably affected by exposure to a toxicant. Segner & Braunbeck (1997) have argued that changes occurring below the individual level can potentially evolve into ecological changes. In order to verify such an assumption, Maboeta *et al*. (2002) compared the biomarker Neutral Red Retention time (NRRT; testing cytotoxicity) between two experimental groups of an indigenous South African oligochaete species of the genus *Microchaetus* in a natural environment. One group was treated with an increasing concentration gradient of copper oxychloride while the other was kept as an uncontaminated control. The decrease in NRR time in the treated population, showing increased cellular damage within months of the start of copper oxychloride spraying, translated into lower earthworm biomass and abundance in these experimental plots a year later. These authors concluded that this particular biomarker (NRR test) could be useful in environmental risk assessment as it conclusively helped provide a warning of imminent ecological changes subsequent to a contamination event.

Many biomarkers have been used successfully in oligochaetes. They are, amongst several others, metallothioneins (Lukkari *et al*. 2004; Demuynck *et al.* 2006) cytochrome P4501A (Lukkari *et al*. 2004), glutathione-S-transferase (Saint-Denis *et al*. 2001; Lukkari *et al*. 2004; Laszczyca *et al.* 2004), NRRT (Maboeta *et al.* 2002; Maboeta *et al.* 2003, Maboeta *et al.* 2004; Reinecke & Reinecke 2003; Reinecke & Reinecke 2007), acetylcholinesterase inhibition (Saint-Denis *et al*. 2001; Reinecke & Reinecke 2007), sperm count/morphology (Cikutovic *et al*. 1993; Zheng & Li 2009), etc.

Two other biomarkers that have been used increasingly for the testing of effects of toxic chemicals on oligochaetes are the MTT assay that gives an indication of cytotoxicity at mitochondrial level after a stress event (Mosmann 1983) and the comet assay that allows assessing genotoxicity by measuring DNA strand breaks (Östling & Johanson 1984).

The MTT assay as developed by Mosmann (1983) measures cytotoxicity, cell proliferation and activity. It is a colorimetric assay in which isolated living cells incubated with the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cause the tetrazolium ring of this salt to cleave. The result is a blue coloured product (blue formazan) that is contained within the cell membrane and therefore accumulates in healthy cells (Fotakis & Timbrell 2006). The concentration of this blue formazan gives an estimation of cell activity and proliferation when measured by spectrophotometry. Because this process takes place when active, healthy mitochondria are present, it will only occur in living cells (Mosmann 1983), and can give an indication of cell survival (or the lack thereof) after treatment with a toxicant. This assay has been used successfully to assess the effect of the heavy metals Cu, Zn and Ag on the digestive system of the cuttlefish *Sepia officinalis* (Le Bihan *et al.* 2004). Similarly, Seth *et al.* (2004) conclusively assessed copper induced toxicity in the human hepatoma line, Hep G2 using the MTT assay amongst other methods.

Maleri *et al*. (2008) assessed the effects of Cd on earthworm coelomocytes using the MTT assay. Their results showed a clear dose response relationship between Cd concentrations and cellular activity. More recently, Voua Otomo and Reinecke (2010) used the MTT assay to assess the long-term cytotoxic effects of Cd in *E. fetida* and established that long-term exposure to Cd in the laboratory had conferred to *E. fetida* an increased tolerance to Cd at cellular level.

The comet assay originated from the work of Östling & Johanson (1984) who developed an electrophoretic technique for the visualization and quantification of DNA damage in individual cells in the form of single strand breaks. The technique was called the single cell gel (SCG) assay or microgel electrophoresis (MGE). Singh *et al*. (1988) improved the method of Östling & Johanson (1984) by allowing the visualization of DNA double strand breaks. Besides its usefulness in the detection of DNA strand breaks, the single cell gel electrophoresis assay (also known as the comet assay) has found relevance in the assessment of (1) excisable DNA damage (e.g. strand breaks produced by DNA repair mechanisms); (2) DNA interstrand crosslinks (lesions mainly caused by chemotherapeutic agents); (3) oxidative stress damage (from reactive oxygen species such as hydrogen peroxide); (4) cellular death by apoptosis and (5) genetic toxicology (Fairbairn *et al*. 1995; Collins 2004).

In oligochaetes, a number of investigations on metal genotoxicity have been undertaken using the comet assay (Reinecke & Reinecke 2004, Zhu *el al*. 2006; Fourie *et al*. 2007; Manerikar *et al*. 2008). Reinecke and Reinecke (2004) assessed the effect of Ni on the earthworm *E. fetida* and reported the genotoxic potential of this metal. Their results also suggested that earthworms might be useful organisms for the assessment of heavy metal genotoxicity using the comet assay. Zhu *el al*. (2006) assessed the separate and combined effects of Cd and phenanthrene on *E. andrei* using the comet assay. Their results indicated that these two substances alone were less genotoxic than their mixtures. Fourie *et al*. (2007) used the comet assay to compare the genotoxicity of Cd to five species of earthworms and to determine species sensitivity amongst them. *E. fetida* was

found to be the most sensitive amongst *Amynthas diffringens*, *Aporrectodea caliginosa*, *Dendrodrilus rubidus*, and *Microchaetus benhami*. Manerikar *et al*. (2008) established that Cr genotoxicity to the coelomocytes of the earthworm *Dichogaster curgensis* was different between *in vitro* and *in vivo* experiments by using the comet assay. More recently, the application of the comet assay in ecotoxicological studies using oligochaetes has increased (see Bigorgne *et al*. 2010; Bonnard *et al*. 2010; Button *et al.* 2010; Giovanetti *et al*. 2010; Hu *et al*. 2010 and Voua Otomo & Reinecke 2010).

Voua Otomo and Reinecke (2010) used the comet assay to investigate genotoxic effects developed after long-term exposure to Cd in *E. fetida*. The results indicated that long-term exposure to Cd in the laboratory had conferred increased genotoxic tolerance to Cd in *E. fetida.* A similar study by Button *et al*. (2010) makes use of the comet assay to establish resistance to arsenic in earthworms (such as *L. rubellus, D. rubidus and L. terrestris*) native to a former mine site in the UK.

Although it has been established that temperature is a key parameter for the survival and reproduction of earthworms (Viljoen *et al*. 1992; Viljoen & Reinecke 1992; Wever *et al.* 2001; Eriksen-Hamel & Whalen 2006), the effects of temperature on the below-individual level have not been investigated. Moreover, neither the MTT test nor the comet assay have been used to investigate the toxicity of metal mixtures.

The aim of this study was to investigate the separate and combined effect of Cd and Zn on mitochondrial activity using the MTT assay and DNA integrity using the comet assay in *E. andrei* at three different temperatures.

5.2 Materials & Methods

5.2.1 Acclimation and metal exposure of experimental animals

Acclimation to OECD soil and subsequent exposure of *E. andrei* to Cd and Zn are provided in sections 3.2.1 and 3.2.2 of this thesis.

5.2.2 Cell collection

In order to perform the MTT and comet assays, coelomic cells were collected by extrusion from five randomly selected *E. andrei* specimens per treatment. Cell extrusion was carried out by means of an extrusion solution (0.2 g EDTA in 76 ml PBS; 80 mg Guaiacol Gliserol Ether; 4 ml EtOH abs) as suggested by Eyambe *et al*. (1991). Each animal was immersed in 1 ml of the extrusion solution for 3 min in a 1.5 ml Eppendorf tube. Thereafter, the animals were removed and the contents of the tubes centrifuged at 2000 *g* (Biofuge fresco, Heraeus Instruments) for 15 min at 4^{o}C. After centrifugation, most of the supernatant in each tube was discarded and the pellet (made of cells) was re-suspended in PBS up to a volume of 0.5 ml. Cell density and viability in the cell suspensions was assessed using the trypan blue exclusion method.

5.2.3 Trypan blue exclusion method

Equal volumes of Trypan blue and cell suspensions (10 µl of each) were mixed in Eppendorf tubes and incubated for 2 minutes. Using a Neubauer hemocytometer, cell viability was assessed and cell concentration was quantified as follows:

Sample cell concentration =

$$\frac{number\ of\ living\ cells}{(proportion\ \ of\ chamber\ counted)\ X\ (volume\ of\ chamber)}\ X\ \frac{original\ volume\ of\ sample}{volume\ of\ sample\ stained}$$

5.2.4 The MTT assay

50µl of the extruded cell samples (2.5 – 3.5 x 10^6 cells/ml) were pipetted in duplicate onto a 96 well ELISA plate and 50µl of the MTT colouring solution (2.5mg MTT in 5ml PBS) were added to the wells. The plate was incubated in the dark, overnight, at room temperature, before the addition of the formazan extraction buffer (20µl 70% HCl in 18ml Isopropanol, 10% Triton X, pH 4.7). After another 2 hour incubation period, absorbance was measured at 570nm using a multiwell scanning spectrophotometer (Multiskan® Ex, Thermo Electron Corporation). The recorded absorbance values were an indication of the reduction of the yellow MTT to blue formazan. This reduction occurs in living mitochondria and is an indication of cell viability/proliferation (Mosmann 1983)

5.2.5 The comet assay

Microscope slides were coated beforehand with a layer of 1% normal melting point agarose (in PBS) and left to dry. Then 10µl of the cell suspensions mixed with 70µl of low melting point agarose (0.5% in PBS) were pipetted onto the first layer of hardened normal melting point agarose. The slides were covered with cover slips and kept on ice until the cell-containing layer of agarose had hardened. The cover slips were removed and a third layer of agarose (75µl of low melting point agarose) was added on top. The slides were covered again and kept on ice until the last layer of agarose had hardened. Then, the cover slips were removed and the slides with gels were immersed in a cold cell-membrane lysing solution at 4^{o}C overnight.

The lysing solution consisted of 37.2g of 100mM EDTA, 146.1g of 2.5M NaCl, 1.2g of 10mM Tris and 8g of 0.2M NaOH diluted in 890ml of distilled H_2O. The final pH was adjusted to 10 using either concentrated HCl or NaOH. Immediately before cell immersion, 10ml of Triton X-100 and 100ml of DMSO was added to make up a final volume of 1000ml. After removal from the lysing solution, the slides were washed in distilled water and immersed for 20min in the

electrophoresis buffer (3% NaOH, 0.5% of EDTA in distilled water, pH>13) to allow for DNA unwinding. Then, electrophoresis was carried out for 10 min at 25V (300mA).

After electrophoresis, the slides were washed with distilled water and immersed in a neutralization buffer (48.5g of 0.4M Tris in 1000ml of H_2O, pH 7.5) for 5min. Thereafter, slides were stored in the dark until DNA staining and comet scoring was carried out. A microscope slide was prepared for each earthworm cell extract and 50 cells were scored per slide.

The slides were stained with ± 100µL of ethidium bromide (20µg/ml) dripped on top of the gel before visualisation. They were visualised under a Leitz Diaplan fluorescent microscope with Ploemopak 2.3 (excitation filter 515–650 nm, barrier filter 580 nm). The comets were assessed using two separate software packages; IM50 V1.20 (Leica Microsystems AG, Heerbrugg, Switzerland), and CASP (Konca *et al*. 2003). The first served to take pictures of the cells on microscope slides and the latter was used to measure tail DNA percentage. Tail DNA percentage is a more effective parameter than comet tail length, as the quantity of DNA in the tail increases in proportion with DNA strand breaks (Collins 2004).

5.2.6. Statistical Analysis

Descriptive statistics were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Normally distributed data with homogenous variances (most of the MTT data sets) were analyzed using a parametric multiple test (One-way ANOVA, followed with Bonferroni posttest). Non-parametric data (all comet assay data sets) were analyzed using the Kruskal-Wallis ANOVA followed by Dunns' test.

Factorial ANOVA followed by Bonferroni posttest was performed to assess the interaction between temperature and metal on the two selected endpoints at all three temperatures and metal/mixture concentrations. These analyses were

performed using Statistica (Data analysis software system), version 9. (Statsoft, Inc, 2009, Tulsa, Oklahoma, www.statsoft.com). The mixture exposure range was converted into toxic unit following the findings at life-cycle parameters (see section 3.3.2.3.1.). The level of significance was $p < 0.05$. Because most the responses for both the MTT and comet assays in mixture treatments did not usually represent a 50% reduction of the responses observed in the controls, it was not possible to compute EC_{50} values. It was consequently not possible to model mixture interactions at cellular and molecular level using MixToxModules.

5.3. Results

5.3.1. MTT assay

5.3.1.1. Cd exposures

The spectrophotometer reading of the quantity of blue formazan in the Cd experiment revealed that at 15°C, the absorbance in cell suspensions from the worms exposed to 250 mg/kg Cd was the only value significantly lower than the value measured in the control ($p < 0.05$; Fig 1). In all the other treatments, there was no difference in absorbance with the control. At 20°C, the quantity of blue formazan decreased consistently with increasing Cd concentration, although statistically lower absorbance readings than the control were only recorded in 500, 750 and 1000 mg/kg ($p \leq 0.01$). At 25°C, absorbance readings were statistically lower than the value in the control in both 500 and 1000 mg/kg only ($p \leq 0.01$; Fig 1).

Factorial ANOVA revealed that temperature alone accounted for 54.19% ($p < 0.0001$) of the observed results and that the interaction between temperature and Cd was very significant ($p = 0.0097$; Table 1)

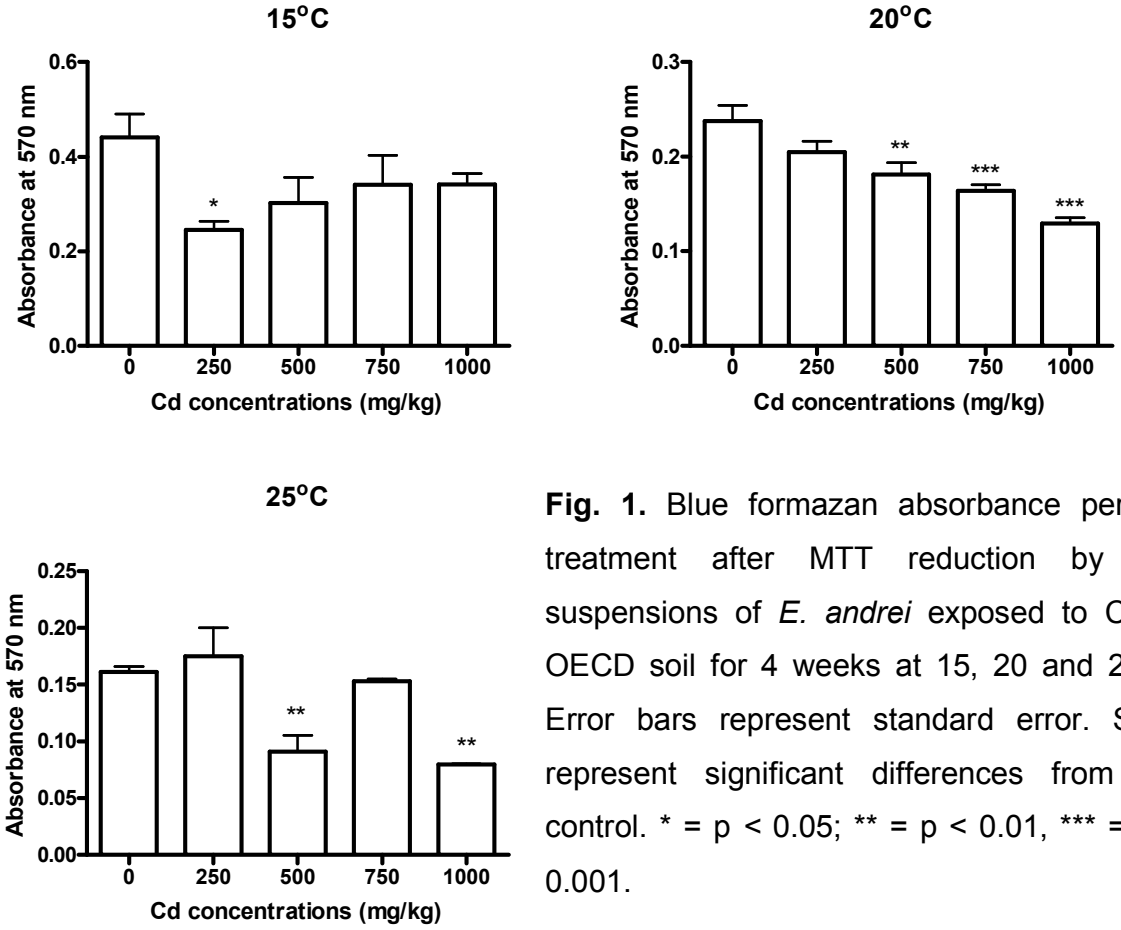

Fig. 1. Blue formazan absorbance per Cd treatment after MTT reduction by cell suspensions of *E. andrei* exposed to Cd in OECD soil for 4 weeks at 15, 20 and 25°C. Error bars represent standard error. Stars represent significant differences from the control. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

Table 1. Two-way ANOVA results of the analysis of the effects of temperature and Cd on the reduction of the MTT by cell suspensions of *E. andrei* after exposure of *E. andrei* to Cd in OECD artificial soil for four weeks at 15, 20 and 25°C.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	8.49	8	0.1035	0.01294	2.770	0.0097
Temperature	54.19	2	0.6602	0.3301	70.69	p<0.0001
Cd concentrations	8.57	4	0.1045	0.02611	5.592	0.0005
Residual	8.49	75	0.3502	0.004670		

The comparison of absorbance values between all three temperatures showed that in all treatments, formazan absorbance values were constantly high at 15°C ($p \leq 0.05$). Although between 20 and 25°C there were no statistical differences in absorbance, absorbance values tended to be the lowest at 25°C (Fig. 2)

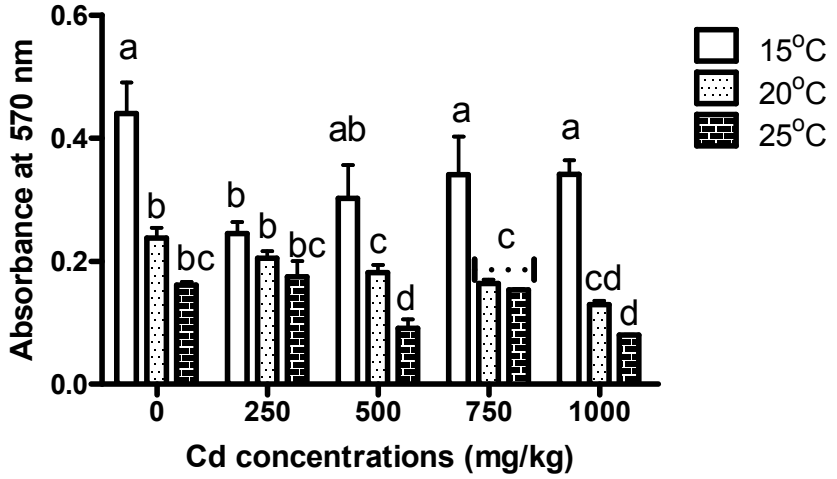

Fig. 2. Comparison of blue formazan absorbance at temperature level after MTT reduction by cell suspensions of *E. andrei* exposed to Cd in OECD soil for 4 weeks at 15, 20 and 25°C. Error bars represent standard error. Different letters above bars represent statistical differences.

5.3.1.2. Zn exposures

The spectrophotometer reading of the quantity of blue formazan in the Zn experiment revealed that at 15°C, absorbance values in both 750 and 1000 mg/kg Zn were statistically lower than in the control ($p \leq 0.05$). At 20°C, there were no differences in absorbance between the control and the Zn treatments. At 25°C, statistical differences with the control were recorded in 250, 750 and 1000 mg/kg Zn ($p < 0.001$; Fig. 3).

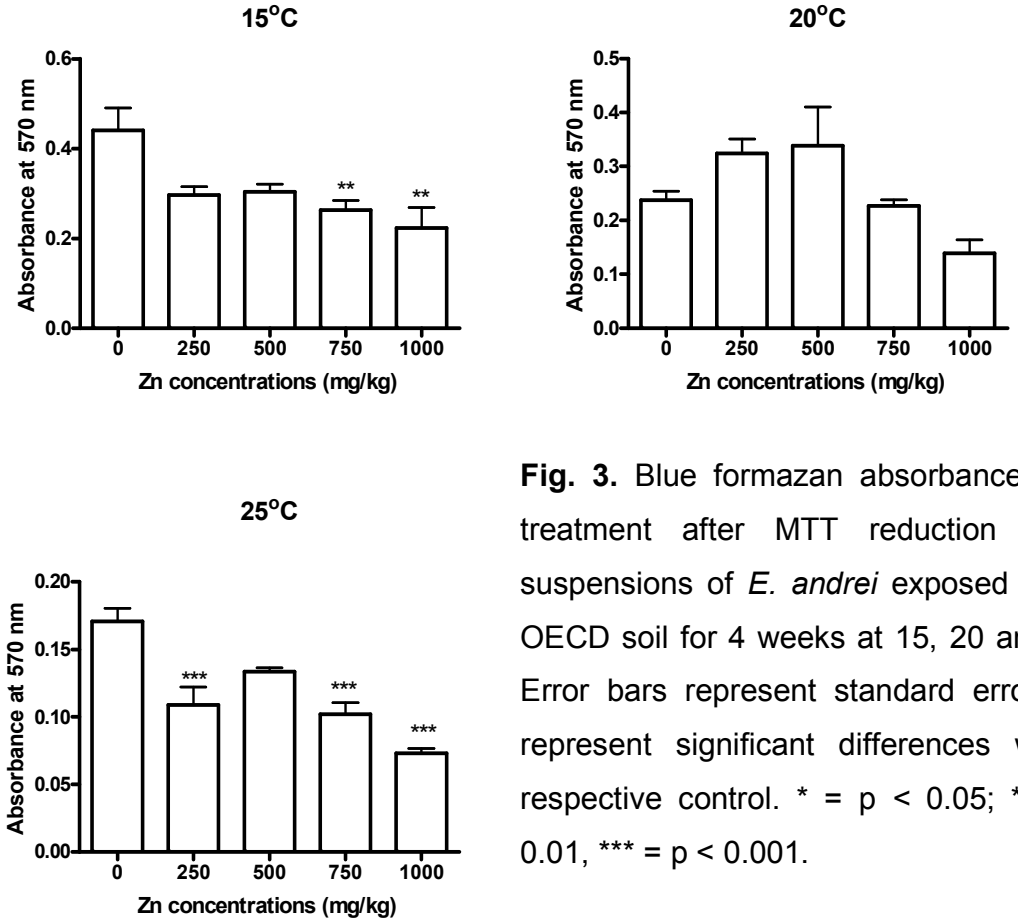

Fig. 3. Blue formazan absorbance per Zn treatment after MTT reduction by cell suspensions of *E. andrei* exposed to Zn in OECD soil for 4 weeks at 15, 20 and 25°C. Error bars represent standard error. Stars represent significant differences with the respective control. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

Factorial ANOVA revealed that temperature alone accounted for 41.65% ($p < 0.0001$) of the observed results and that there was no interaction between temperature and Zn (Table 2).

Table 2. Two-way ANOVA results of the analysis of the effects of temperature and Zn on the reduction of the MTT by cell suspensions of *E. andrei* after exposure of *E. andrei* to Cd in OECD artificial soil for four weeks at 15, 20 and 25^{o}C. The abbreviation ns means not significant.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	5.21	8	0.08126	0.01016	1.735	0.1040^{ns}
Temperature	41.65	2	0.6497	0.3248	55.50	P<0.0001
Zn concentrations	25.00	4	0.3901	0.09751	16.66	P<0.0001
Residual		75	0.4390	0.005853		

The comparison of absorbance values between all three temperatures showed that in all treatments, formazan absorbance values were not different between 15 and 20^{o}C. These values however were statistically higher than the absorbance values recorded at 25^{o}C ($p \leq 0.5$) except at 1000 mg/Kg Zn between 20 and 25^{o}C where there were no statistical differences in absorbance (Fig. 4)

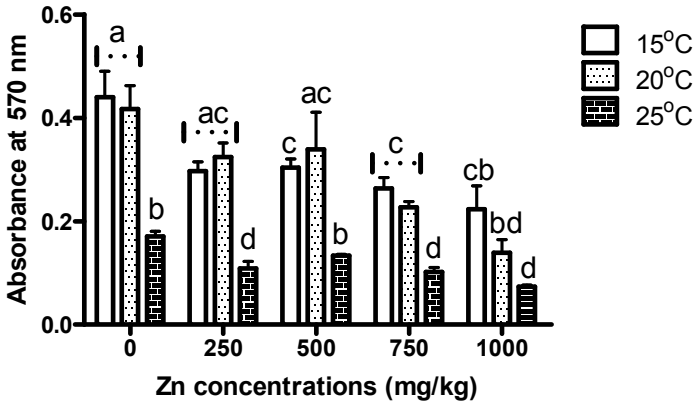

Fig. 4. Comparison of blue formazan absorbance at temperature level after MTT reduction by cell suspensions of *E. andrei* exposed to Zn in OECD soil for 4 weeks at 15, 20 and 25^{o}C. Error bars represent standard error. Different letters above bars represent statistical differences

5.3.1.3. Mixture exposures

In the mixture treatments at 15°C, the quantity of blue formazan in worms exposed to both the 1.26 and 12.61 TU was statistically lower than in worms exposed to the control ($p \leq 0.05$). Earthworms from the other treatments showed no statistical difference in absorbance with those exposed to the control. At 20 and 25°C, absorbance values in worms exposed to all the mixture treatments were not statistically different from the absorbance value measured in worms exposed to the control (Fig.5).

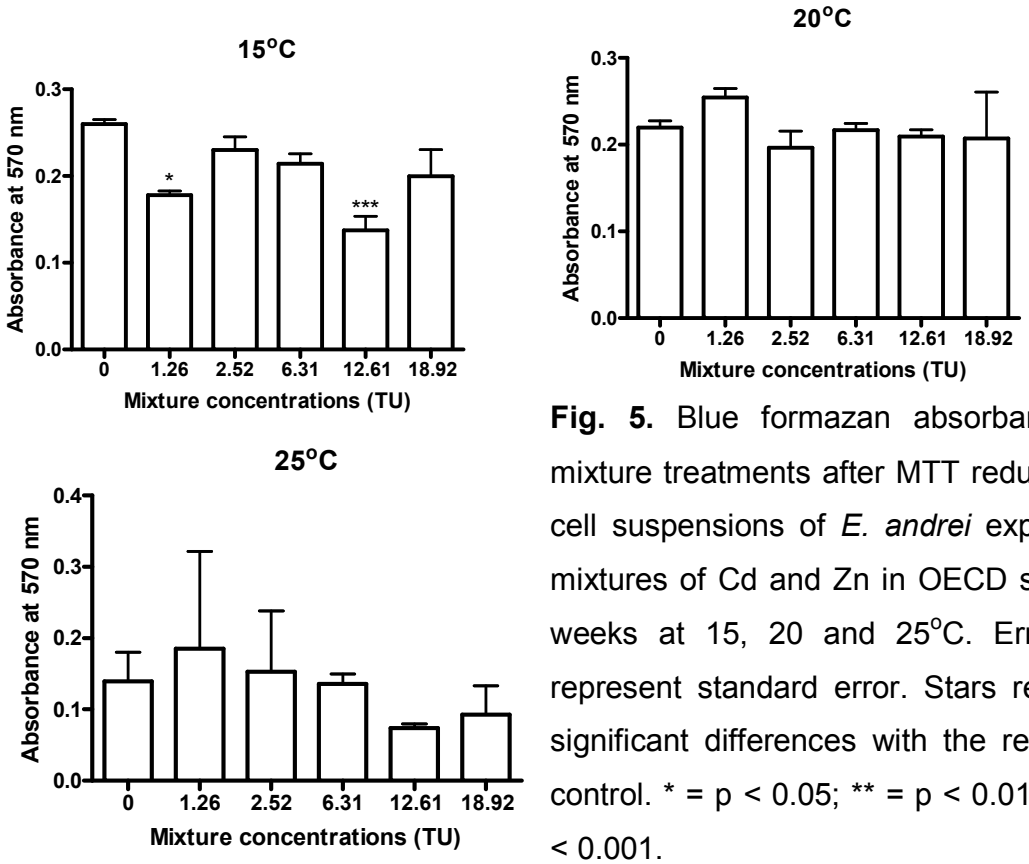

Fig. 5. Blue formazan absorbance per mixture treatments after MTT reduction by cell suspensions of *E. andrei* exposed to mixtures of Cd and Zn in OECD soil for 4 weeks at 15, 20 and 25°C. Error bars represent standard error. Stars represent significant differences with the respective control. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

Two-way ANOVA followed by Bonferroni posttests showed that temperature accounted for 25.76% of the observed results and that the interaction between mixture concentrations and temperature was very significant ($p < 0.0013$; Table 3).

Table 3. Two-way ANOVA results of the analysis of the effects of temperature and mixtures of Cd and Zn on the reduction of the MTT by cell suspensions of *E. andrei* after exposure of *E. andrei* to Cd in OECD artificial soil for four weeks at 15, 20 and 25°C.

Source of Variation	% of total variation	Df	Sum-of-squares	Mean square	F	p
Interaction	15.56	10	0.07450	0.007450	3.240	0.0013
Temperature	25.76	2	0.1233	0.06165	26.81	P<0.0001
Mixture concentrations	15.44	5	0.07392	0.01478	6.430	P<0.0001
Residual		90	0.2070	0.002299		

The comparison of absorbance values between all temperatures showed that except for 1.26 TU, there was no difference in formazan absorbance between 15 and 20°C. Moreover, except for 1.26 and 2.52 TU, absorbance values at 25°C were significantly lower than to ones measured at both 15 and 20°C ($p \leq 0.05$; Fig. 6).

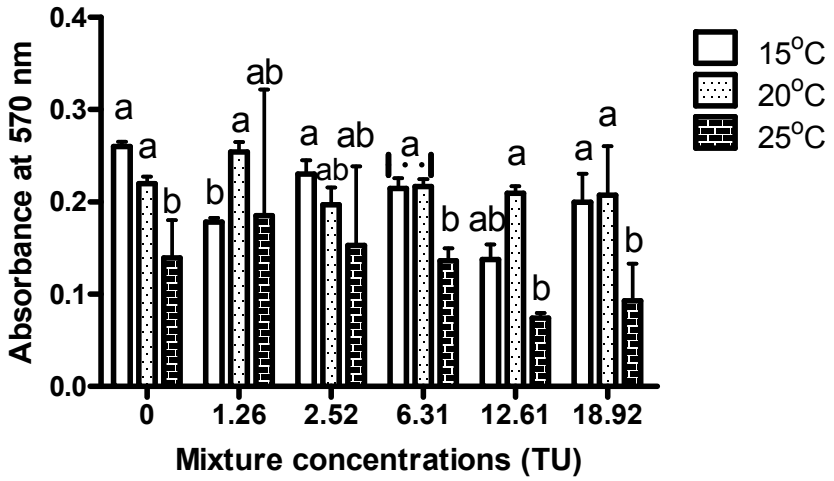

Fig. 6. Comparison of blue formazan absorbance at temperature level after MTT reduction by cell suspensions of *E. andrei* exposed to mixtures of Cd and Zn in OECD soil for 4 weeks at 15, 20 and 25°C. Error bars represent standard error. Different letters above bars represent statistical differences.

In order to compare the difference in responses between single metal and mixture exposures, selected absorbance measurements between the two exposure regimes were compared in a pair wise manner. Absorbance measurements in worms exposed to 0; 250; 500 & 750 mg/kg in the single metal exposures were compared to the values measured in worms exposed to 0; 6.31; 12.61 & 18.92 TU respectively. In these selected pairs of exposures treatments, *E. andrei* was exposed to the same nominal concentrations of either of the metals, alone (expressed in $mg.kg^{-1}$) or in mixtures (expressed in TU). These pairs of exposure concentrations were 0/0; 250/6.31; 500/12.61 and 750/18.9 $mg.kg^{-1}$/TU.

In the case of Cd, it was found that at 15^{o}C, the absorbance values in the mixture treatments were significantly lower than in their corresponding Cd treatments within the 500/12.61 and 750/18.92 $mg.kg^{-1}$/TU pairs of exposure concentrations ($p \leq 0.01$). In the other treatments, although there was a tendency for lower absorbance values in the mixture treatments, there were no statistical differences in absorbance between Cd and mixture exposures (Fig. 7).

At 20 and 25^{o}C, there were no significant differences in absorbance values between the two exposure regimes although at 25^{o}C for instance, consistently lower absorbance values were measured in the mixture treatments (Fig. 7).

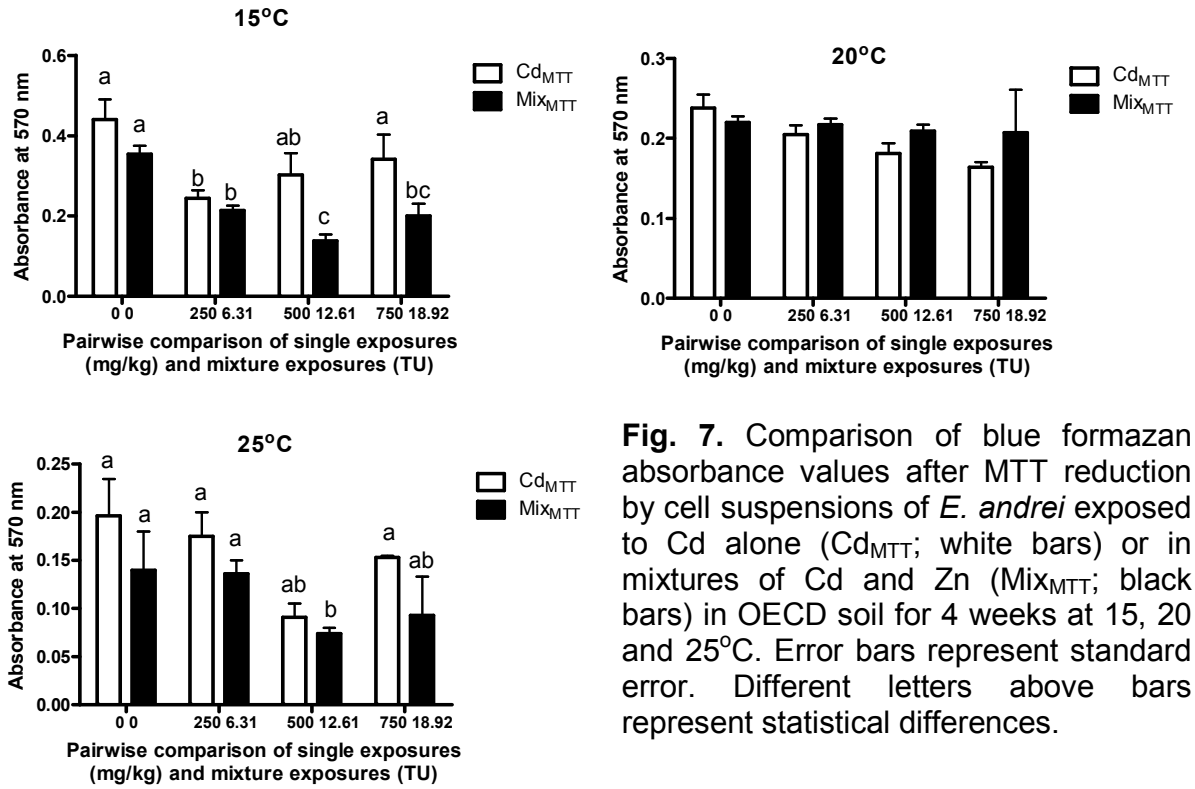

Fig. 7. Comparison of blue formazan absorbance values after MTT reduction by cell suspensions of *E. andrei* exposed to Cd alone (Cd_{MTT}; white bars) or in mixtures of Cd and Zn (Mix_{MTT}; black bars) in OECD soil for 4 weeks at 15, 20 and 25°C. Error bars represent standard error. Different letters above bars represent statistical differences.

In the case of Zn, similarly absorbance values were mostly lower in the mixture than in the single Zn exposures. At 15°C however, these values were significantly different only within the 500/12.61 mg.kg^{-1}/TU pair of exposure concentrations ($p < 0.001$; Fig. 8). At 20°C, similar significantly lower differences in the mixture treatments were found within both the 250/6.31 and the 500/12.61 mg.kg^{-1}/TU pair of exposure concentrations ($p \leq 0.05$). At 25°C, despite the prevalence of lower absorbance values in the mixture treatments, there were no statistical differences between the values recorded in the two exposure regimes.

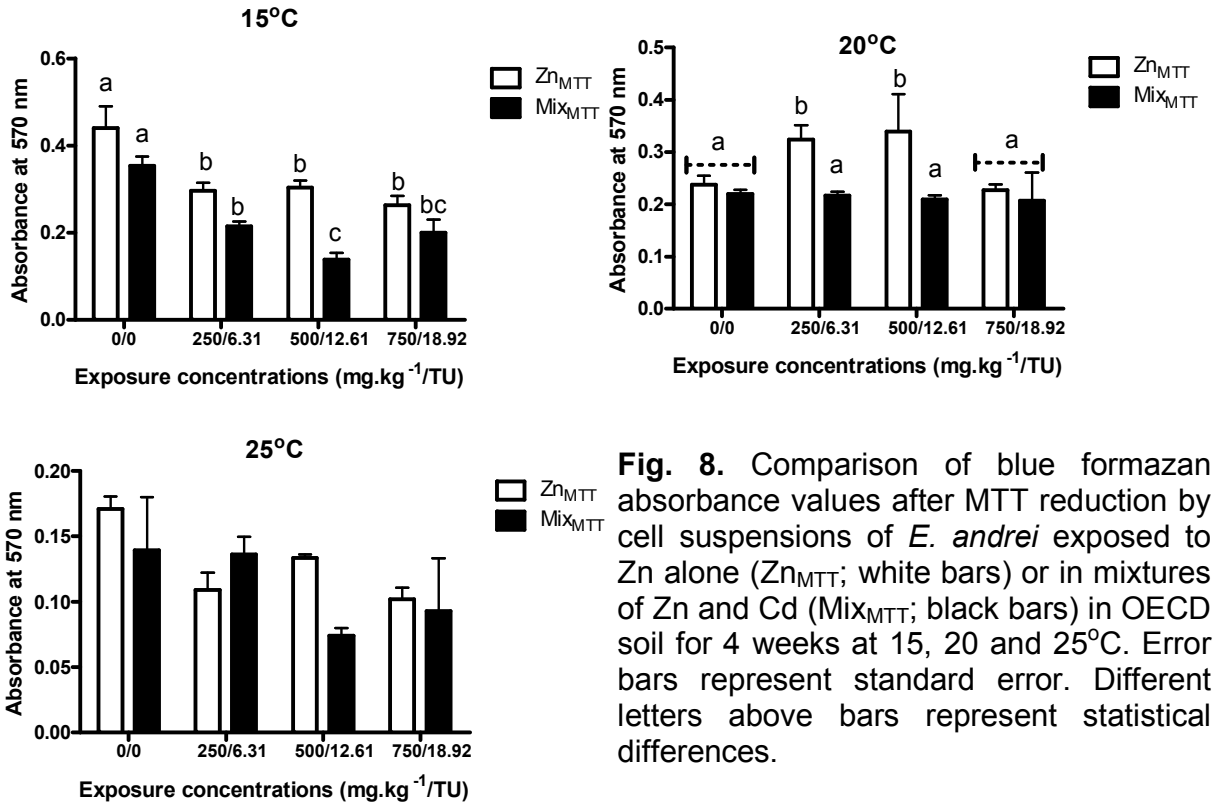

Fig. 8. Comparison of blue formazan absorbance values after MTT reduction by cell suspensions of *E. andrei* exposed to Zn alone (Zn_{MTT}; white bars) or in mixtures of Zn and Cd (Mix_{MTT}; black bars) in OECD soil for 4 weeks at 15, 20 and 25°C. Error bars represent standard error. Different letters above bars represent statistical differences.

5.3.2. Comet assay

5.3.2.1. Cd exposures

The assessment of Tail DNA percentage after *E. andrei* exposure to Cd for four weeks in OECD soil showed that at 15°C, Cd-induced DNA strand breaks in the worms exposed to 500, 750 and 1000 mg/kg were significantly lower than the "background level" in the worms exposed to the control treatment ($p \leq 0.01$). At 20 and 25°C, inversely, Cd genotoxicity in the worms exposed to 500, 750 and 1000 was significantly higher than in the worms exposed to the control ($p \leq 0.01$ Fig 9).

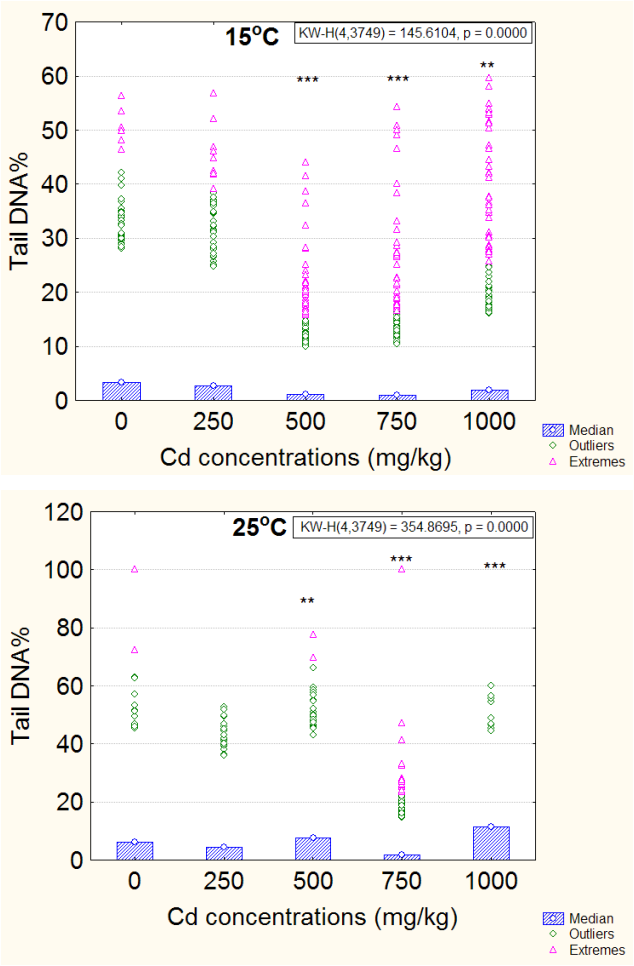

Fig. 9. Tail DNA % per Cd treatments after exposure to *E. andrei* in OECD soil for 4 weeks at 15, 20 and 25°C. Stars represent significant differences with the respective control. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

Factorial ANOVA showed that there was a highly significant interaction between Cd and temperature influencing the genotoxicity of Cd to *E. andrei* (Table 4).

Table 4. Two-way ANOVA results of the analysis of the effects of temperature and Cd on DNA strands of *E. andrei* after exposure in OECD artificial soil for four weeks at 15, 20 and 25°C.

Effect	Sum-of-squares	Df	Mean square	F	p
Intercept	656026	1	656026.0	6833.079	< 0.0001
Cd concentrations	26357	4	6589.1	68.632	< 0.0001
Temperature	40210	2	20105.1	209.412	< 0.0001
Interaction	38313	8	4789.2	49.883	< 0.0001
Error	1078355	11232	96.0		

A comparison of Tail DNA % between the three temperatures revealed prevalently higher Cd genotoxicity occurring at 25°C, although significant differences supporting this observation were only measured in the worms incubated at 0, 250, and 1000 mg/kg ($p \leq 0.05$; Fig. 10).

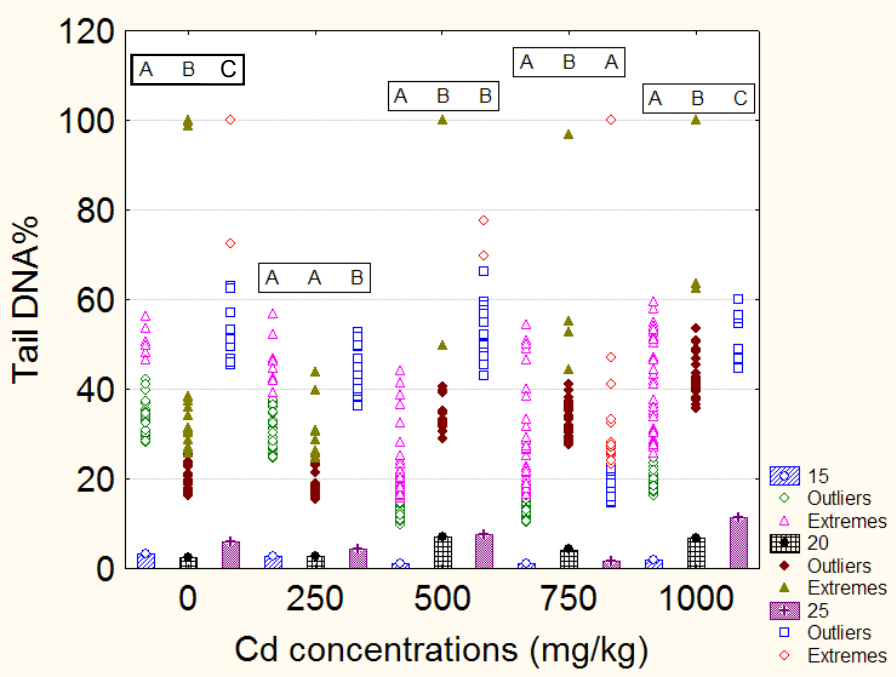

Fig. 10. Comparison of Tail DNA % per Cd treatments at temperature level after exposure to *E. andrei* in OECD soil for 4 weeks at 15, 20 and 25°C. Different letters above bars represent significant differences between the three temperatures within the indicated treatments.

5.3.2.2. Zn exposures

The assessment of Tail DNA % after *E. andrei* exposure to Zn for four weeks in OECD soil showed that at 15°C, Zn-induced DNA strand breaks were significantly higher in the worms exposed to 500, 750 and 1000 mg/kg Zn when compared to worms exposed to the control ($p < 0.001$). At 20°C, similarly results were observed in the worms exposed to 250, 500, and 1000 mg/kg Zn when compared to worms exposed to the control ($p < 0.001$). At 25°C however, Zn-induced DNA strand breaks were all lower than the "background level" in the control treatment, with statistically lower Tail DNA % observed in the worms exposed to 250, 500, 750 mg/kg ($p \leq 0.05$; Fig 11).

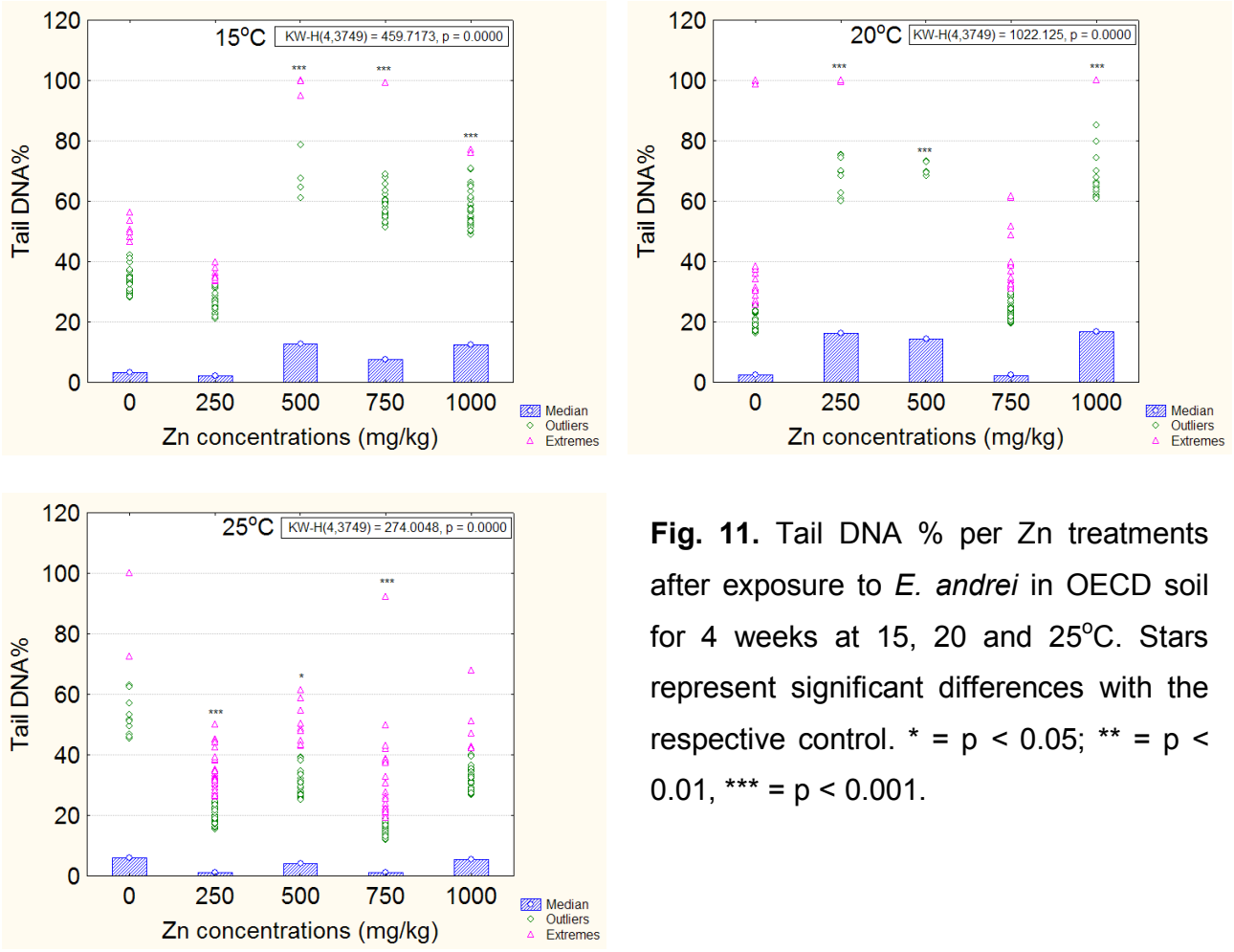

Fig. 11. Tail DNA % per Zn treatments after exposure to *E. andrei* in OECD soil for 4 weeks at 15, 20 and 25°C. Stars represent significant differences with the respective control. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

Factorial ANOVA showed that there was a highly significant interaction between Zn and temperature influencing the genotoxicity of Zn to *E. andrei* (Table 5).

Table 5. Two-way ANOVA results of the analysis of the effects of temperature and Zn on DNA strands of *E. andrei* after exposure in OECD artificial soil for four weeks at 15, 20 and 25°C.

Effect	Sum-of-squares	Df	Mean square	F	p
Intercept	1347385	1	1347385	8891.210	<0.0001
Zn concentrations	105844	4	26461	174.613	<0.0001
Temperature	88713	2	44356	292.702	<0.0001
Interaction	166655	8	20832	137.466	<0.0001
Error	1702111	11232	152		

A comparison of Tail DNA % between the three temperatures revealed prevalently lower Zn genotoxicity occurring at 25°C. Significant differences supporting this observation were measured in the worms incubated at 250, 500, 750, and 1000 mg/kg ($p \leq 0.01$; Fig. 12).

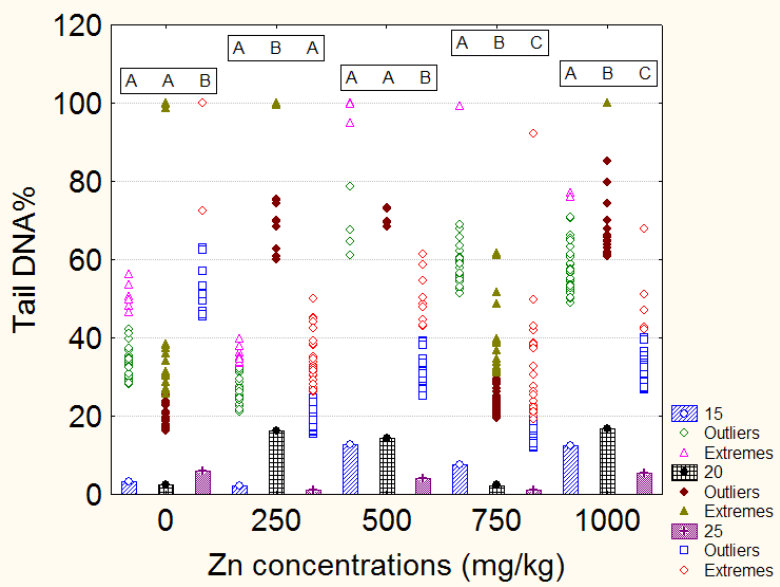

Fig. 12. Comparison of Tail DNA % per Zn treatments at temperature level after exposure to *E. andrei* in OECD soil for 4 weeks at 15, 20 and 25°C. Different letters above bars represent significant differences between the three temperatures within the indicated treatments.

5.3.2.3. Mixture exposures

The assessment of Tail DNA % after *E. andrei* exposure to mixtures of Cd and Zn for four weeks in OECD soil showed that at 15°C, mixture-induced DNA strand breaks in the worms exposed in 12.61 and 18.92 TU were significantly lower than the "background level" observed in the worms from the control treatment ($p \leq 0.05$). At 20°C however, the worms exposed in the mixture treatments showed significantly higher DNA strand breaks than the ones exposed to the control ($p < 0.001$). At 25°C, such significantly higher DNA strand breaks were measured in worms exposed to 1.26, 6.31 and 18.92 TU when compared to the worms exposed in the control ($p \leq 0.05$; Fig. 13).

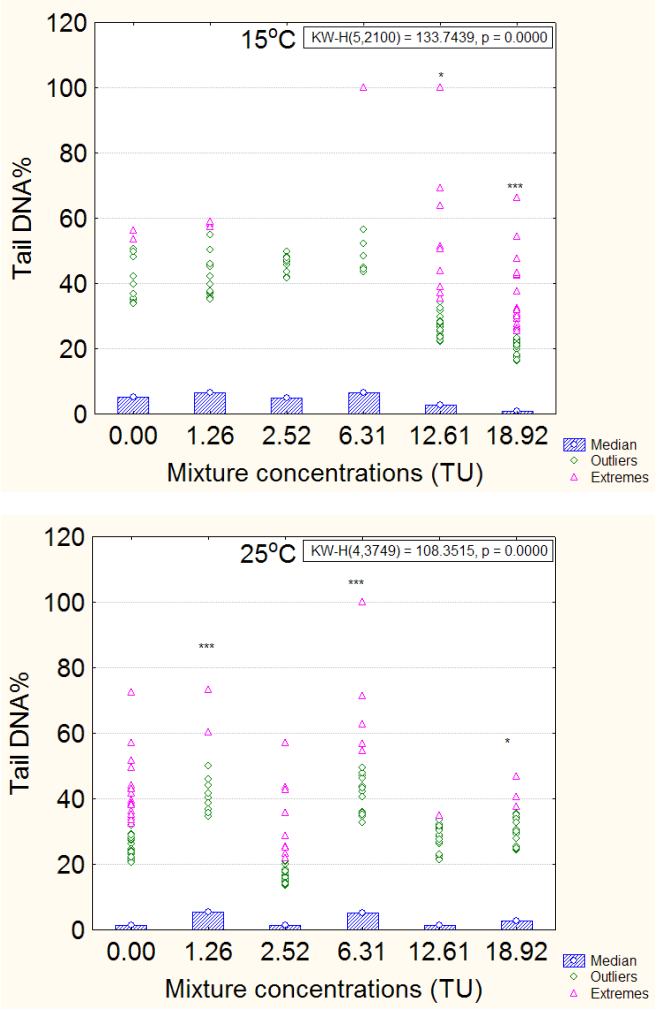

Fig. 13. Tail DNA % per mixture treatments after exposure to *E. andrei* in OECD soil for 4 weeks at 15, 20 and 25°C. Stars represent significant differences from the respective control. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$.

Factorial ANOVA showed that there was a highly significant interaction between mixtures and temperature influencing mixture genotoxicity to *E. andrei* (Table 6).

Table 6. Two-way ANOVA results of the analysis of the effects of temperature and mixtures of Cd and Zn on DNA strands of *E. andrei* after exposure in OECD artificial soil for four weeks at 15, 20 and 25°C.

Effect	Sum-of-squares	Df	Mean square	F	p
Intercept	472706.3	1	472706.3	4152.881	<0.0001
Mixture concentrations	15305.7	5	3061.1	26.893	<0.0001
Temperature	10714.5	2	5357.2	47.065	<0.0001
Interaction	17735.8	10	1773.6	15.581	<0.0001
Error	715055.8	6282	113.8		

A comparison of Tail DNA % between the three temperature revealed prevalently lower mixtures genotoxicity occurring at 25°C, although significant differences supporting this observation were only recorded in the worms incubated at 2.52 12.61 and 18.92 TU ($p \leq 0.01$; Fig. 14).

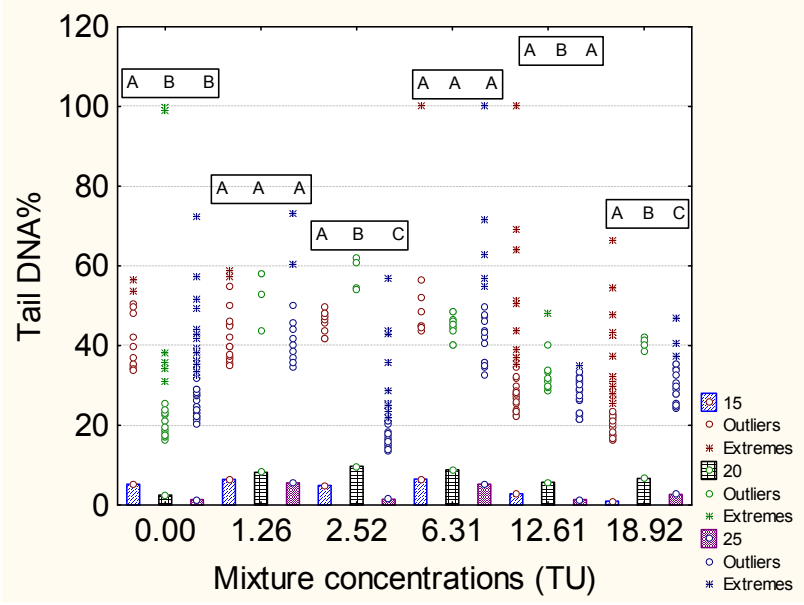

Fig. 14. Comparison of Tail DNA % per mixture treatments at temperature level after exposure to *E. andrei* in OECD soil for 4 weeks at 15, 20 and 25°C. Different letters above bars represent significant differences between the three temperatures within the indicated treatments.

In order to compare the difference in responses between single metal and mixture exposures, selected tail DNA% measurements between the two exposure regimes were compared in a pair wise manner. Tail DNA % measurements in worms exposed to 0; 250; 500 & 750 mg/kg in the single metal exposures were compared to the values measured in worms exposed to 0; 6.31; 12.61 & 18.92 TU respectively. In these selected pairs of exposures treatments, *E. andrei* was exposed to the same nominal concentrations of either of the metals, alone (expressed in $mg.kg^{-1}$) or in mixtures (expressed in TU). These pairs of exposure concentrations were 0/0; 250/6.31; 500/12.61 and 750/18.9 $mg.kg^{-1}$/TU.

When the level of genotoxicity was compared between Cd and Mixture exposures, it was found that at 15^{o}C, there was a tendency for higher Tail DNA% in worms exposed to the mixtures than in those exposed to Cd alone. However, significant differences were only observed within both the 250/6.31 $mg.kg^{-1}$/TU and the 500/12.61 $mg.kg^{-1}$/TU exposure pairs ($p \leq 0.05$; Fig. 15). At 20^{o}C, similar significantly higher mixture-induced DNA strand breaks were also measured within both the 250/6.31 $mg.kg^{-1}$/TU and the 750/18.92 $mg.kg^{-1}$/TU exposure pairs ($p \leq 0.001$). At 25^{o}C, there was no difference in genotoxicity between the exposure regimes.

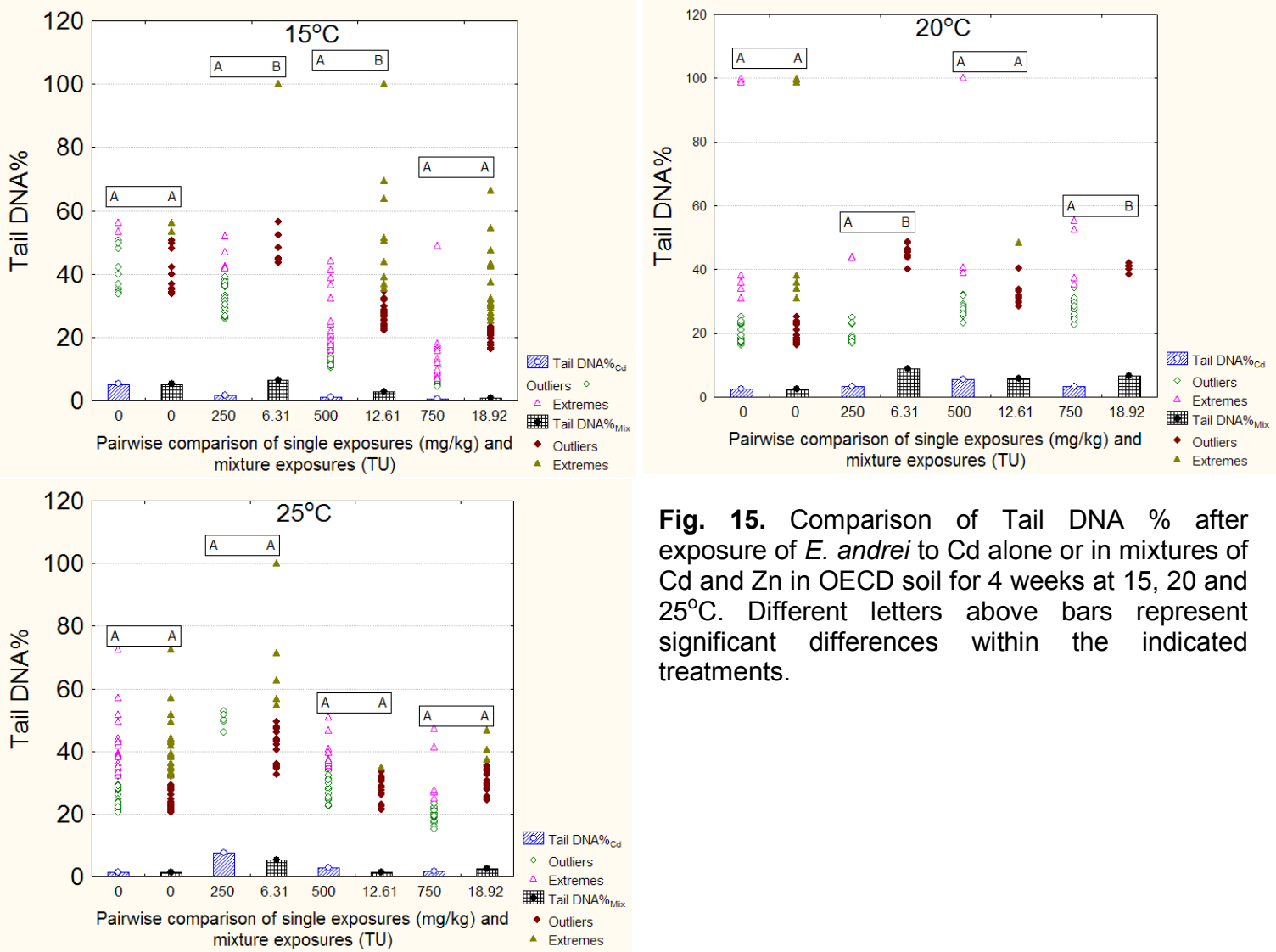

Fig. 15. Comparison of Tail DNA % after exposure of *E. andrei* to Cd alone or in mixtures of Cd and Zn in OECD soil for 4 weeks at 15, 20 and 25°C. Different letters above bars represent significant differences within the indicated treatments.

In the case of Zinc, the comparison of genotoxicity between Zn and mixture exposures showed that at 15°C, there was a prevailing tendency for higher Tail DNA% in worms exposed to Zn alone than in those exposed to the mixtures. However, significant differences supporting this observation were only found within both the 500/12.61 and 750/18.92 $mg.kg^{-1}$/TU exposure pairs ($p \leq 0.001$; Fig. 16). At 20°C, similar significantly higher Zn-induced DNA strand breaks were also measured within both the 250/6.31 and the 500/12.61 $mg.kg^{-1}$/TU exposure pairs ($p \leq 0.01$). At 25°C inversely, prevailing mixture-induced DNA strand breaks were observed within both the 250/6.31 and the 750/18.92 $mg.kg^{-1}$/TU exposure pairs ($p \leq 0.05$).

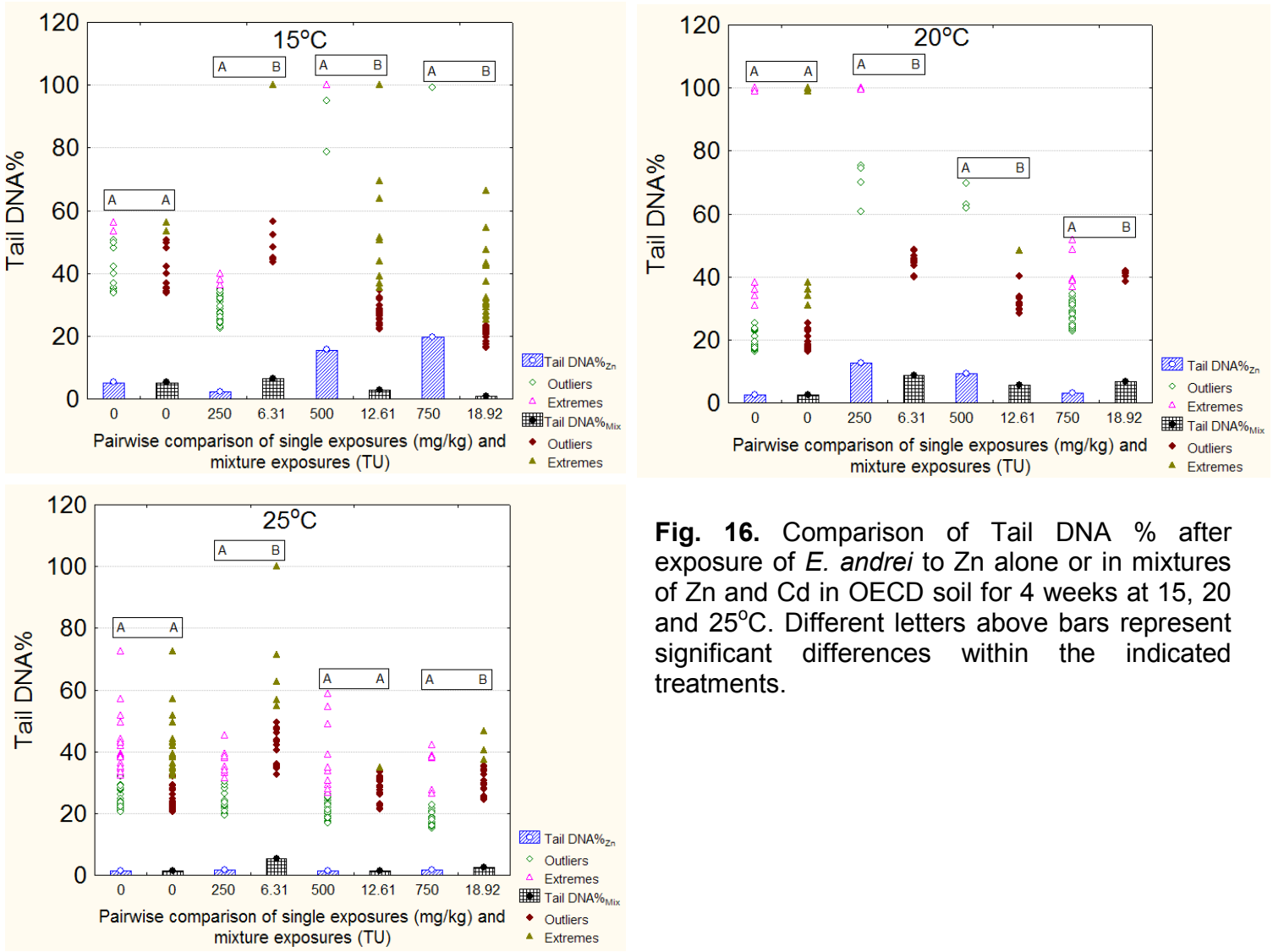

Fig. 16. Comparison of Tail DNA % after exposure of *E. andrei* to Zn alone or in mixtures of Zn and Cd in OECD soil for 4 weeks at 15, 20 and 25°C. Different letters above bars represent significant differences within the indicated treatments.

5.4. Discussion

5.4.1. MTT reduction in single exposures

In the case of Cd, the present results showed a decrease in absorbance values (i.e. an increase in Cd cytotoxicity) with increasing temperature (Figs. 1 & 2). Fig. 2 especially revealed that this decrease in absorbance values also occurred in the control treatment (in the absence of Cd). This was also noticeable in Zn exposures where the same temperature-driven decrease in absorbance in worms from the control treatments was recorded (Fig. 4). This observation pointed to temperature rather than Cd or Zn as the main factor controlling the reduction of MTT in *E. andrei*. This was confirmed by factorial ANOVA analyses in Cd and Zn exposures that indicated that temperature alone accounted respectively for 54.19% and 41.65% of the observed results (Table 1 & 2). Both metals nonetheless were found to be increasingly cytotoxic to *E. andrei* at higher temperature as significantly lower absorbance values than the respective controls were measured at higher temperatures and metal concentrations (Figs 1 & 3). Consequently, the temperature-dependent decrease of absorbance values in the controls was not an indication of [metal] cytotoxicity but rather a sign of decreased mitochondrial activity perhaps cause by high reproduction.

As documented in chapter 3 section 3.3.2., reproduction primarily occurred in the controls and increased noticeable with temperature. This certainly came at a high-energy cost to the worms concerned. According to Arrillo & Melodia (1991) a temperature-induced mitochondrial cycle helps improve reproduction in *E. fetida* during moderate increase of ambient temperature. Since the reduction of MTT is also carried out by mitochondria (Mosmann 1983); it is probable that low MTT reduction in the controls at higher temperature was a consequence of energy redistribution in an attempt to increase reproduction. The decrease in MTT reduction in worms from the metal-spiked treatments was, however, a combination of temperature and metal cytotoxicity.

Research has shown that in the mitochondrion, Cd causes altered transmembrane electrical potential, consequently inducing premature depression of mitochondrial ATP levels (Dorta *et al.* 2003). This subsequently inhibits the electron transfer chain

(ETC), triggering the formation of reactive oxygen species (ROS) and causing the opening of membrane permeability pores which could cause apoptosis (Li *et al*. 2003). Cell death by apoptosis could perhaps explain why at 20 and 25^{o}C in Cd treatments, increasingly lower absorbance values from the control were measured. However, it may be argued that in the case of Zn these lower absorbance values from the control were caused rather by lower levels of energy in the mitochondria caused by the effort to regulate Zn at higher concentrations and temperatures.

The MTT assay is based on the reduction of the MTT salt by mitochondrial dehydrogenases from live cells (Carmichael *et al.* 1987). Although the effects of Zn on such process have never been investigated, Ivanina *et* al (2008) have shown that Cd impairs mitochondrial dehydrogenases (in the oyster *Crassostrea virginica*) and that such Cd effects are enhanced by temperature. Lannig *et al* (2006) and Sokolova & Lannig (2008) have also reported an increased toxicity of Cd in the mitochondria of *C. virginica* at an elevated temperature. These previous studies may explain why in the present study Cd exposure caused less MTT reduction at higher temperatures (Fig. 2). No similar studies on the effects of Zn could be found in the literature, thus the interpretation of the present results for Zn could only be speculative.

5.4.2. MTT reduction in mixture exposures

Temperature–dependent decrease in absorbance values was also observed in the control treatments during the mixture experiments. Mixture exposures however, did not cause significant decrease in cytotoxicity with increased temperature and concentrations, as cytotoxic levels in higher concentrations and temperatures did not differ from the respective controls (Figs. 5 & 6). This observation also suggested that mixture treatments were less cytotoxic than Cd and Zn separately. In single exposures of Cd and Zn it was found that these metals significantly impaired MTT reduction when compared to their respective controls. This was to some extent supported by the comparison of absorbance values measured in worms from single and mixture exposures (Figs 7 & 8). Figs 7 & 8 indeed showed that MTT reduction in worms from the single metal exposures was often significantly higher than that in worms from mixture exposures. This observation pointed toward possible antagonistic interactions between Cd and Zn in the mitochondria. However, this could not be formally tested using MixToxModules due to the lack of suitable data as

stated above. Mixture exposures also suggested that the cytotoxicity of mixtures tended to decrease with increasing temperature, as no significantly low absorbance values than those recorded in the respective controls were recorded in mixture treatments at both 20 and 25^{o}C (Figure 5).

5.4.3. Comet assay in single exposures

The assessment of Tail DNA % revealed that while Cd genotoxicity to *E. andrei* increased with increasing temperature it was the opposite for Zn (Figs 9 & 11). In the case of Cd, Tail DNA % values in the worms exposed at 15^{o}C, were even significantly lower than the "background" values recorded in the worms from the control (Fig. 9).

Mayer *et al*. (1987) reported that mild hypothermia improves DNA repair mechanism by slowing down processes such as ROS formation that ultimately lead to DNA damage. Cd especially has been linked to the formation of ROS and has been found to interfere with antioxidant defense mechanisms (thus promoting the production of ROS), which subsequently alters gene expression and induces apoptosis (Risso-de Faverney *et al.* 2001). In the present study, such Cd induced impairments may have been slowed down at 15^{o}C, which is a sub-optimal temperature to *E. andrei*.

Moreover, a recent study by Connelly *et al*. (2009) has found evidence of enhanced rates of DNA repair at 10^{o}C compared to 20^{o}C, in four UV radiated freshwater daphniid species. If a comparable scenario were possible in oligochaetes, it would particularly benefit specimens exposed to genotoxic substances at lower temperature such as 15^{o}C.

Furthermore, as established in chapter 3 section 3.3.4., at 15^{o}C, metal accumulation was lower than at the other two temperatures. Limited metal uptake would subsequently prevent higher metal-induced genotoxicity. This coupled with enhanced DNA repair at low temperature (Mayer *et al*. 1987; Connelly *et al*. 2009) may justify why at 15^{o}C, in the case of Cd exposures DNA Tail % were very low. Following this rationale, however, would render it hard to explain the significantly high Tail DNA % observed at 15^{o}C in Zn exposures. These high Tail DNA % observed at 15^{o}C were perhaps caused by higher Zn body burden at 15^{o}C than at

the other two temperatures (as previously observed in chapter 3, Fig. 18). However, the Zn body burdens at 15^{o}C were even lower than the levels measured at 15^{o}C in the Cd exposures (see chapter 3, Fig. 17). The present results suggest that Zn was more cytotoxic than Cd at 15^{o}C temperature while Cd was more cytotoxic than Zn at the higher temperatures.

The results of the comet assay also indicated non-significant increase in Tail DNA % with temperature in the control treatments (in the absance of Cd and Zn; Fig. 10 & 12). A temperature of 34^{o}C has been found, in the absence of a genotoxicant, to cause DNA strand breaks in gold fish (Anitha *et al*. 2000). Buschini *et al.* (2003) similarly have found increasing DNA damage with increasing temperature between four groups of the freshwater mussel *Dreissena polymorpha* kept at 4, 18, 28 and 37^{o}C. The non-significantly higher Tail DNA% at 25^{o}C in the controls (Figs. 10 & 12) was perhaps an onset of increased DNA damage solely caused by higher temperature in *E. andrei*. A further increase in incubation temperature could have caused significant temperature induced DNA damage in the control treatments.

5.4.4. Comet assay in mixture exposures

In mixture treatments, Tail DNA % were the lowest in worms exposed at 25^{o}C (Figs. 13 &14) suggesting decreasing genotoxicity with increasing temperature. When Tail DNA % were compared between single and mixture exposures, it was found that mixtures showed the tendency of higher genotoxic potential than Cd at 15 and 20^{o}C (Fig. 15). At higher temperature, however, there was no difference in genotoxicty between the two exposure regimes. Similar comparison between Zn and mixture induced genotoxicities showed that Zn was more genotoxic at lower temperature whereas mixture seemed more genotoxic at higher temperatures (Fig. 16). These observations between single and mixture exposures pointed toward complex metal interactions between Cd and Zn at molecular level. These, however, could not be formally assessed using MixToxModules due to the lack of suitable data as stated above.

5.5. Conclusion

Cadmium and Zinc cytotoxicity and genotoxicity in single and mixture exposures are here evidenced and measured. The assessment of metal cytotoxicity suggests that both Cd and Zn have cytotoxic potential in the earthworm *E. andrei*. Mixture exposures indicate decreasing cytotoxicity with increasing temperature and possible antagonism between Cd and Zn at cellular level. The assessment of metal genotoxicity suggests that Cd and Zn genotoxicity profiles are inversely correlated. Cd tends to be less genotoxic at low temperature and becomes increasingly deleterious at higher temperature while it is the exact opposite for Zn. Mixture exposures indicate decreasing genotoxicity with increasing temperature and suggest that the interactions between Cd and Zn at molecular level are probably not simply antagonistic and could more complex.

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6. General discussion & concluding remarks

6.1. *E. andrei* & *E. doerjesi*

The rare studies that have investigated the combined effects of temperature and metal toxicity in oligochaetes have exclusively done so using lumbricid species (Janssen *et al.* 1996; Spurgeon *et al.* 1997; Wieczorek-Olchawa *et al.* 2003; Spurgeon *et al.* 2005; Olchawa *et al.* 2006). Such studies also have never looked at how both temperature and metal toxicity would in combination affect biomarker responses at cellular and molecular levels. In the present study, these shortcomings were addressed by looking at the coupled effects of metal contamination (Cd and Zn) and temperature in both a lumbricid (*Eisenia andrei)* and an enchytraeid (*Enchytraeus doerjesi)* species. These effects were measured using whole organismal endpoints (in both experimental species) and cellular and molecular effects (in *E. andrei*) using selected biomarker responses; the MTT reduction and the comet assay.

Although only two similar endpoints (survival and reproduction) were assessed in both *E. andrei* and *E. doerjesi*, results indicated conclusively that *E. doerjesi* was the most sensitive of the two species. Exposures to Cd singly showed the Cd LC_{50} to be 725.46 mg/kg at 25^{o}C in *E. andrei* while at the same temperature the Cd LC_{50} was 231.79 mg/kg in *E. doerjesi*. At the lower temperatures (15 and 20^{o}C) where Cd LC_{50}s could not be computed in the case of *E. andrei* for lack of sufficient mortality, the LC_{50} for Cd in *E. doerjesi* was 293.01 and 261.62 mg/kg Cd respectively. Similar results for Zn and mixture exposures supported the conclusion that *E. doerjesi* was more sensitive than *E. andrei* in both single and mixture exposures. This observation corroborates the findings of Kuperman *et al*. (2004) who have reported higher manganese (Mn) sensitivity in *Enchytraeus crypticus* (enchytraeidae) compared to *E. fetida* (lumbricidae). The difference in sensitivity observed between *E. doerjesi* (smaller species) and *E. andrei* (larger species) might be explained by the respectvie sizes of the worms. However, this could not be stated as a rule as experiments assessing the effects of oil residues on these same species have shown that *E. andrei* is more sensitive than *E. doerjesi* (Van Wyk, personal communication). More reliable explanations could lie in metal detoxification mechanisms. Indeed, metals such as Zn, Cu, Hg and Cd have been associated with the encoding of a cysteine

rich non-metallothionein protein, whose role is to alleviate the effects of metal toxicity in *Enchytraeus buchholzi* (Willuhn *et al.* 1994, Willuhn *et al.* 1996a & b). It may be possible that the cysteine rich non-metallothionein protein reported in *E. buchholzi* is also found in *E. doerjesi*. Such a protein, to our knowledge, has not been reported in lumbricids whose analogous means of defense are metallothioneins. The fundamental difference between metallothioneins and the cysteine rich non-metallothionein protein (especially their capacity to withstand temperature changes) could perhaps explain the difference in sensitivity between *E. andrei* and *E. doerjesi*.

6.2. Life cycle parameters and biomarkers

The present study showed that the deleterious effects of Cd on all the endpoints investigated in both *E. andrei* and *E. doerjesi* were aggravated by an increasing temperature gradient of 10^{o}C. Because all physiological and biochemical processes in ectotherms depend on temperature it is not surprising that temperature would be one of the main factors controlling the susceptibility of these organisms to metal toxicity (Katsikatsou *et al* 2011). Results showed a decrease in Zn toxicity with increasing temperature at all the endpoints assessed in both experimental species. Zinc, as opposed to Cd, is an essential metal that is regulated in oligochaetes (Morgan & Morgan 1991; Lock & Janssen 2001; Demuynck *et al*. 2007). The present study, moreover, documents increasing Zn regulation with increasing temperature (in *E. andrei*) probably caused by faster temperature-driven metabolic rates. Zinc regulation however was seemingly prevented in the presence of Cd, as metal analysis showed that in mixtures, Zn uptake increased at higher temperature. The reverse was observed with Cd, although an increase in uptake was observed with increasing temperature, less Cd was accumulated in mixture exposures than in single Cd exposures. The antagonistic relationship between Cd and Zn, based on their tendency to bind to the same proteins in the organism (Sunderman & Barber 1988; Brzóska & Moniuszko-Jakoniuk 2001), may explain why their respective uptakes were considerably altered in mixtures.

Mixture exposures in both experimental species indicated that temperature increased mixture toxicity for some endpoints (biomass and survival) and decreased mixture toxicity for others (reproduction and biomarkers). These results show that during a stress event, an oligochaete would be prone to safeguard reproduction and

physiological-biochemical functioning at the expense of growth and adult survival. This particular finding is a typical illustration of r-selected species for which the ability to reproduce is essential (especially in unstable environments) even though reproduction takes place at the cost of parental investment (MacArthur & Wilson 1967).

The findings for mixture exposures highlight the importance of investigating the effects of toxicants on life cycle parameters and biomarkers concomitantly. In the present study, the assessment of the effect of mixtures on reproduction or genotoxicity alone would have indicated that increasing temperature would lessen the deleterious effects of the metals. In contrast, the other endpoints showed that, this apparent decrease in mixture toxicity (as measured by reproduction and biomarkers) was an attempt to mitigate metal toxicity to these vital processes. Perhaps this was to ensure the survival of the species in an unstable environment comparable to the one created by current climatic trends.

Temperature however did not have an influence on the type of interaction that took place between Cd and Zn in mixtures. Mixture exposures using *E. doerjesi* had a 2:1 fixed ratio where the nominal concentration of Cd was double that of Zn. Mixture exposures using *E. andrei* had a 1:1 fixed ratio where the nominal concentration for both metals was the same. It was nevertheless found in both experimental species and at the three temperatures investigated that the interactions between Cd and Zn were dose level (DL) dependent rather than dose ratio (DR) dependent. This is perhaps because the most toxic metal (Cd) in both cases contributed the most to the toxicity of the mixtures. Had the proportion of Zn been higher than that of Cd the nature of the interactions would perhaps have been different. Another approach that could have caused different Cd and Zn interactions would have been to use equitoxic mixtures based on the respective toxic units of the metals (adding the same concentrations in TU) rather than their nominal concentrations (adding the same concentrations in mg/kg).

Novel comet and MTT assay findings from the present study have indicated that temperature has the potential to exacerbate Cd and Zn toxicity at both cellular and molecular levels. Similar abiotic factors such as pH (van Gestel & Hoogerwerf 2001; Sivakumar & Subbhuraam 2005; Ownby *et al*. 2005) and drought (Sørensen &

Holmstrup 2005) are known to influence metal toxicity to soil organisms. Temperature, moisture and pH are particularly important in the investigation of the influence of environmental factors in metal toxicity to soil organism. While temperature and precipitation (moisture) are the main parameters controlling the partitioning of chemical toxicants (Noyes *et al*. (2009), pH is the key factor influencing the distribution and bioavailability of metals in soils (Anderson & Christensen 1998; Crommentuijn *et al*. 1997). Future studies looking at the effect of combinations of these factors and metal contamination in enchytraeids species would be valuable contributions to the current state of our knowledge.

6.3. Climate change

The findings reported in this study show that a temperature increase within the range predicted by the Intergovernmental Panel on Climate Change (IPCC 2001) is likely to play a crucial role in metal toxicity to oligochaetes. Although these results show that a moderate increase in temperature in the absence of toxicants could improve reproduction and growth in both experimental species, the combination of increasing temperature and pollution is more likely to cause adverse effects.

Pörtner & Farrell (2008) list the following current temperature-driven ecosystem changes caused by climate change: (1) poleward or altitudinal shifts in geographical distribution, (2) population collapses or local extinctions, (3) failure of large-scale animal migrations, (4) changes in the seasonal timing of biological events and (5) changes in food availability and food web structure. Van Jaarsveld & Chown (2001) had already predicted that a temperature increase of 1 to 3°C in the South African context could cause an alteration of the geographical ranges of up to 44% of plant and 80% of animal species. Oligochaetes that have poor dispersal ability (Van der Werff *et al*. 1998) could already be affected by global warming. In polluted areas, the selecting pressure could even be higher.

Somero (2010) has for instance reported that a single amino acid replacement is sufficient to adapt a protein to a new thermal range. Such a replacement at protein level, however, would be unachievable if DNA integrity is compromised by exposure to genotoxic substances such as Cd (that was increasingly genotoxic at high temperature in this study). The threat posed by anthropogenic pollution in a warming

environment is being investigated increasingly in various organisms (see recent review by Holmstrup *et al*. 2010) and most studies agree that heat stress enhances toxicant effects. In other words, the interaction between increased temperature and toxicants is usually synergistic as found in the present study. Many of the studies investigating the combined effects of heat stress and toxicants however focus on aquatic species. Such studies on soil organisms such as enchytraeids and earthworms are lacking.

The findings of the present study finally show the importance of assessing mixture exposures in studies investigating the combined effects of heat stress and toxicants in organisms. Cadmium being a byproduct of Zn production (Plachy 1997) means that both these metals have a greater likelihood of occurring together in nature. Each of these metals separately was more toxic than the mixtures of the two at all the endpoints assessed in the present study. Since mixture assessments provide data that are more comparable to the reality in the field, mixture screening should become a common procedure in studies investigating the effects of global warming and pollution will have on terrestrial ecosystems. A relevant approach would perhaps be to make an inventory of the prevalent toxicants in the areas of interest in parallel with a study of past and present environmental trends followed by an attempt to recreate these conditions in ecotoxicological studies in the laboratory.

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