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Studies with the freeze tolerant earthworm *Dendrobaena octaedra*

PhD Thesis, 2008
Anne-Mette Bindesbøl

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Abstract: In traditional ecotoxicological studies, test organisms are usually exposed to a single chemical at increasing concentrations, performed under otherwise optimal conditions. However, in natural environments it is very likely that these organisms will also be exposed to other stressful factors, including climatic stressors as frost and drought. This means that traditional laboratory tests may underestimate the toxic effects of chemicals in natural environments.

This PhD thesis examined the interaction between climatic and toxic stress, using the globally distributed freeze tolerant earthworm *Dendrobaena octaedra* as test organism.

The physiological mechanisms known to affect the freeze tolerance of *D. octaedra*, including glucose production and adjustments in membrane phospholipid composition, was examined in worms exposed to copper, which is shown to interact synergistically with freezing temperatures. To test how general this phenomenon was, worms were furthermore exposed to a number of other chemicals with different modes of action in combination with freezing temperatures.

In general, synergistic interactions seemed to occur mostly at high levels of climatic stress in combination with high concentrations of the chemical. It is suggested that it is important to include natural stressors as frost and drought in risk assessment, especially taken into consideration the predictions of future climate change.

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Preface

This PhD thesis is submitted to the Faculty of Science, Aarhus University, Denmark. Most of the work has been conducted at the Department of Terrestrial Ecology at NERI in Silkeborg, Denmark.
The objective of this PhD was to examine the interactions between toxic and climatic stress, using the freeze tolerant earthworm *Dendrobaena octaedra* as test organism. The introduction of the thesis has the structure of a review paper on the interactions of environmental contaminants with freezing temperatures and drought in terrestrial invertebrates. Following this are five papers, all concerning the freeze tolerant earthworm *D. octaedra*. Three of these have been published, one has been submitted, and one is ready for submission.

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Finally, I would like to thank my friends and family, especially my husband Asser for support and critical review, and my daughter Ida for taking my mind off science.

Randers, November 2008

Anne-Mette Bindesbøl

Review

Photo: Brian Rasmussen

The interactions of environmental contaminants with freezing temperatures and drought in terrestrial invertebrates:

Effects, mechanisms and consequences for ecotoxicology

Abstract

Although the numbers of studies concerned with the effects of environmental contaminants on the ability of organisms to tolerate climatic stress, such as winter frost or summer drought, are still limited, the issue is receiving increasing attention in the ecotoxicological literature. This paper reviews the interactions of environmental contaminants with survival of sub-zero temperatures and drought stress in terrestrial invertebrates. A comprihensive database search was used to acquire relevant literature, and a total of 48 papers were found dealing with this subject, the majority of these focusing on interactions with drought. Further, studies regarding adaptation strategies to drought and freezing temperatures, as well as studies concerning toxicants' mode of action were included. Together, these studies were used to assess the occurrence and known types of interactions, as well as discussing the mechanisms underlying such interactions. Some general patterns seem to exist; synergistic interactions were commonly seen between heavy metals and frost survival, whereas polycyclic aromatic hydrocarbons (PAHs) interacted antagonistically with frost survival. Further, surfactants and to a great extent PAHs interacted synergistically with drought causing elevated mortality. On the other hand, interaction between heavy metals and drought and between surfactants and frost survival seemed less clear. The impact of surfactants on frost survival seems to depend on which cold hardiness strategy is employed by the organism. Pesticides did not seem to interact with frost or drought tolerance. In general it can be concluded that traditional laboratory studies, where the organisms are exposed to increasing concentrations of a single compound under otherwise optimal conditions, may underestimate the toxicity of some compounds in the field.

Introduction

Ecotoxicological risk assessments of environmental contaminants are primarily based on results of laboratory studies, where test organisms are exposed to increasing concentrations of a single compound. In such laboratory experiments, the test organisms often have optimal conditions (temperature, moisture, food etc.) to optimize control survival and isolate the effects of the chemical in question. The risk assessment of such contaminants is usually based on results from acute lethality tests or chronic reproduction tests, but also effects on population growth rate have gained increased focus (Forbes and Calow, 1999; Hansen et al., 1999; Bindesbøl et al., 2007). These are all useful methods for assessment of the effects of toxic compounds, and the methods have been greatly improved and standardised during the last decades. However, organisms in their natural environments will at most times be simultaneously exposed to several stressful factors, and among these are sub-optimal and occasionally stressful environmental conditions. These stressful environmental conditions may significantly alter an organisms tolerance towards a given contaminant (and vice versa), an alteration that is not taken into consideration under traditional, well-controlled laboratory conditions. To reduce the chance of underestimating risk, an uncertainty factor is used to extrapolate the results from laboratory tests to field situations. The validity of these uncertainty factors has, however, frequently been questioned (e.g. Chapman et al., 1998), as the choice for these factors are arbitrary and often not based on science. To be able to improve risk assessment, it therefore seems appropriate to supplement traditional ecotoxicological studies with investigations on how these natural stressors interact with chemical stressors.

Photo: Brian Rasmussen

The purpose of the present review is to analyse interactions between chemicals and freezing temperatures, as well as the interactions between chemicals and drought in terrestrial invertebrates. I have chosen to focus on these two forms of climatic stress, since considerable evidence suggests that there are many common physiological adaptations in response to both drought and freezing temperatures (Ring and Danks, 1994; Bayley et al., 2001, Holmstrup et al., 2002a) and thus, that the nature of chemicals' interference with these adaptations may be comparable.

It is important to keep in mind that toxicants and freezing temperatures, and toxicants and drought, can interact at different levels. The bioavailability and the toxicokinetics of the toxicant will inevitable change during the changing temperatures and moisture conditions of soil or habitat in general (Jannsen and Bergema, 1991; Bruus Pedersen et al., 1997; van Gestel, 1997). Toxicants may also interfere with several physiological mechanisms important for cold and drought tolerance. The present review puts most emphasis on the latter issue.

This review is structured by first presenting an overview of the different adaptations involved in cold and drought tolerance. I then continue to discuss relevant studies addressing interactions between toxicants and climatic stressors, as well as discussing the documented and theoretical mechanisms behind possible interactions. As various toxicants have different effects on organisms, chemicals are distinguished into four classes of chemicals: heavy metals, polycyclic aromatic hydrocarbons (PAHs), surfactants and pesticides. The term synergistic interaction is used when the combined effect of the two stressors is greater than expected, and the term antagonistic interaction is used if the combined effect is less than expected from the combination of the effects from each stressor alone.

Mechanisms for tolerating freezing temperatures

Cold hardy invertebrates are traditionally divided into freeze avoiding or freeze tolerant species (Zachariassen, 1985; Ramløv, 2000). Freeze avoiding species die if their body fluids freeze. They survive sub-zero temperatures by supercooling their body fluids or dehydrating until the melting point of their body fluids is lowered to the ambient temperature (cryoprotective dehydration) (Holmstrup and Westh, 1994). Freeze tolerant species, on the other hand, can survive freezing of their extracellular body fluids. Intracellular ice formation is, with few exceptions (Wharton and Ferns, 1995), considered to be lethal (Zachariassen, 1985).

Freeze avoidance

Freeze avoiding species depending on supercooling are faced with the risk of spontaneous or inoculative freezing (Zachariassen, 1985). The supercooling point (SCP) is the temperature where crystallisation occurs, and in cold tolerant invertebrates is thereby equal to their

lower lethal temperature (Ramløv, 2000). The task of these organisms is to lower the SCP well below the ambient temperature and to stabilize the supercooled state (Storey and Storey, 1992). They do so by removal or inactivation of ice nucleating agents (INAs) (Zachariassen, 1980; Wu and Duman, 1991). Furthermore, they accumulate high concentrations of sugars and polyols (SPs), which further depress the SCP as well as stabilizing membranes and cellular proteins (Ramløv, 2000). In addition, many freeze avoiding species (mostly insects) usually produce highly active antifreeze proteins (AFPs), which are known for their ability to prevent the growth of seeding ice crystals upon cooling (Wu et al., 1991).

Supercooling can however be a problematic solution for permeable invertebrates living in frozen soils. They are at great risk of extensive dehydration because the water vapour pressure of their supercooled body fluids may often be higher than that of ice in their frozen habitat. These organisms depend on cryoprotective dehydration (Holmstrup et al., 2002b). This strategy is effective in organisms that can lower their melting point of their body fluid at the same rate as the decline in environmental temperature and thereby avoid the need for supercooling (Holmstrup, 2003). As temperature decreases they start to dehydrate, which induces accumulation of SPs, which further depresses the melting point and thereby reduces the amount of water lost by the organism (Holmstrup et al., 2002b) as well as stabilising membranes. To be successful, however, these organisms should be able to tolerate extensive dehydration.

Photo: Brian Rasmussen

Freeze tolerance

Freeze tolerant species are faced with several problems when extracellular body fluid freezes. They are faced with the risk of mechanical damage by ice crystals, which may penetrate tissues and cell membranes (Grout and Morris, 1987). The cells can also experience extensive dehydration (Zachariassen, 1985). The ice crystals consist of pure water, and as these grow, the solute concentrations in the extracellular fluid increases, which leads to an osmotic outflow of water from the cells. This may decrease the cell volume below the so called minimum volume (Lee, 1991), where the membranes begin to rest on the extracellular structures. This may eventually rupture the cell membranes. The increases in solute concentrations, both extra- and intracellular, may lead to changes in enzyme activity and denaturation of proteins (Ramløv, 2000). Finally, the extensive dehydration may cause phase changes in the membranes, due to removal of the forces keeping the membrane in its bilayer conformation (Hazel, 1995).

Freeze tolerant organisms usually induce the formation of ice crystals at relatively high sub-zero temperatures to ensure a controlled development of ice in the extracellular body fluids (Block, 1990; Storey and Storey, 1992). This early crystallisation is achieved by INAs (Zachariassen, 1985). Further, freeze tolerant organisms often accumulate SPs, which reduce the amount of ice formed and controls the minimum volume and stabilizes membranes and proteins (Storey and Storey, 1992). Antifreeze proteins are sometimes present, and act as recrystallisation inhibitors, preventing growth and redistribution of ice crystals once these have formed (Knight and Duman, 1986; Duman, 2001).

Mechanisms for tolerating drought (summer temperatures)

During drought conditions, there will be a water activity gradient favouring a net flux of water from an organism to its surroundings, causing a reduction of the body water content which may become critical. Animals have developed a variety of mechanisms to control their water content, from the cellular to the behavioural level. One way of coping with drought is by reducing the water permeability of the integument, a strategy frequently used by insects (Hadley, 1994). However, many organisms such as soil invertebrates have a highly permeable integument to allow gas exchange, and have thus evolved the ability to tolerate extensive water loss, which is comparable to cryoprotective dehydration (Petersen et al., 2008). Other soil invertebrates such as Collembola are able to absorb water from unsaturated soil pore air. When exposed to drought, these organisms initially lose a substantial amount of water to the surrounding soil. This dehydration initiates the accumulation of SPs to a degree that makes them hyperosmotic to their surroundings, enabling them to passively absorb water vapour from soil pore air (Bayley and Holmstrup, 1999). The accumulation of SPs also stabilizes membranes during dehydration (Crowe et al., 1992).

Some earthworms enter diapause during summer if the soil water potential gets too low (Gerard, 1967; Nordström, 1975; Holmstrup, 2001). During this process, several worm species excavate a spherical mucus-lined chamber in the soil. By coiling itself into a ball in the soil cell, water loss is minimized during drought. However, earthworms can generally tolerate extensive water loss (Grant, 1955). They do not, however, accumulate protective SPs during this dehydration, and must therefore be able to tolerate high concentrations of inorganic ions as chloride, potassium and sodium.

Shared mechanisms in cold and drought tolerance

Accumulation in SPs seems to be a central mechanism in both tolerance of freezing temperatures and drought. Thus, SPs may slow down dehydration rates in freeze avoiding species during winter, and have the same effect during summer drought. Accumulation of SPs can also reduce cellular dehydration in freeze tolerant organisms during winter and reduce the equilibrium dehydration level in summer drought exposed organisms. Also, the specific protection of membranes and proteins by SPs is important during winter dehydration of both freeze avoiding and freeze tolerant organisms, as well as during summer drought dehydration.

An additional common mechanism between cold and drought strategies seems to be the adjustment in phospholipid fatty acids (PLFA) of membranes (Bayley et al., 2001; Holmstrup et al., 2002a). Fully functional membranes exist in a liquid-crystalline phase, but when biological membranes are cooled below a certain temperature, T_m (temperature of phase transition), they change from the liquid-crystalline phase to the more ordered gel phase, whereby they become non-functional and lose their selective properties (Hazel, 1995). Characteristically, lowering of environmental temperatures induces homeoviscous adaptation, during which the chemical composition of biological membranes are modified to maintain an appropriate degree of fluidity (Sinensky, 1974; Hazel, 1995). Also drought, followed by substantial dehydration, may induce an increased proportion of unsaturated PLFAs. This is explained by the lower water activity during dehydration, which causes a tighter packing of the membranes and with that a lower fluidity (Hazel and Williams, 1990). Unsaturation of the PLFAs can maintain appropriate fluidity during these drought conditions.

It should, however, be kept in mind that the study of PLFA adjustment under drought conditions has been neglected compared to adjustment during cold acclimation.

Interactions between freezing temperatures and chemicals

Since both freeze tolerance and freeze avoidance depends on the accumulation of SPs and also on membrane adjustments, it is expected that toxicants interfering with these processes will significantly reduce survival at low temperatures. Also, toxicants interfering with INAs as well as AFPs may reduce freeze tolerance and freeze avoidance, respectively. As pointed out by Aarset and Zachariassen (1982), freezing is likely to potentiate the effect of toxicants by concentrating them in the fluid fraction of the frozen body fluids.

Interactions with heavy metals

Heavy metals are naturally occurring elements, but due to increasing anthropogenic activities, like mining, they can become locally concentrated and are becoming widespread environmental contaminants all over the world (Kozlov and Zvereva, 2007). As elements, heavy metals are non-degradable and will have permanent effect on organisms in contaminated areas. Studies have shown that heavy metals such as copper, cadmium, lead, mercury and nickel exhibit the ability to produce reactive oxygen species, induce lipid peroxidation, DNA damage, deplete sulfhydryls, denaturate proteins, inhibit enzymes and alter cell homeostasis (Stohs and Bagchi, 1995; Valko et al., 2005).

A number of studies concerning heavy metals have shown synergistic interaction with freezing temperatures, except for lead, where no interaction has been observed (Table 1).

Bindesbøl et al. (2005; 2008a) showed that a synergistic interaction occurred between freezing temperatures and environmentally realistic copper concentrations in the earthworm *Dendrobaena octaedra*. These interactions were investigated using a full factorial design with six sub-lethal copper concentrations between 0 and 300 mg Cu/kg dry weight (dw) and five temperatures from +2 to –8 °C (Bindesbøl et al., 2008a). The synergistic interaction between copper and freezing is illustrated in Figure 1, showing that the temperature where 50 % of the worms died was higher when exposed to high soil copper concentrations. For example, if the worms were exposed to 60 mg/kg dw, the lethal temperature where 50 % died was –7 °C compared to –3 °C at 240 mg/kg dw. The synergistic interaction became

Table 1. Overview of interactions between the toxicant and freezing temperatures. The term "synergistic" is used when the combined effect of the two stressors is greater than expected, and the term "antagonistic" is used if the combined effect is less than expected from the combination of the effects from each stressor alone. No interaction is indicated as "none". *Freeze tolerant, † Freeze avoidant – supercooling, ‡ Freeze avoidant – cryoprotective dehydration, #Chill tolerant.

Toxicant	Test organism	Species	Life stage	Interaction	Reference
Heavy metals					
Copper	*Earthworm	*Dendrobaena octaedra*	Adult	Synergistic	Bindesbøl et al. (2005)
Copper	*Earthworm	*Dendrobaena octaedra*	Adult	Synergistic	Bindesbøl et al. (2008a)
Copper	‡Earthworm	*Dendrobaena octaedra*	Cocoon	Synergistic	Holmstrup et al. (1998)
Copper	‡Earthworm	*Aporrectodea calliginosa*	Cocoon	Synergistic	Holmstrup et al. (1998)
Copper	‡Springtail	*Protaphorura armata*	Adults	Synergistic	Bossen (2001)
Nickel	*Earthworm	*Dendrobaena octaedra*	Adult	Synergistic	Bindesbøl et al. (2008b)
Mercury	*Earthworm	*Dendrobaena octaedra*	Adult	Synergistic	Bindesbøl et al. (2008b)
Mercury	#Springtail	*Folsomia candida*	Adult	Synergistic	Holmstrup et al. (2008)
Mercury	*Insect	*Chilo Suppressalis*	Larvae tissue	Synergistic	Izumi et al. (2006)
Mercury	*Insect	*Eurosta solidaginis*	Larvae tissue	Synergistic	Philip et al. (2008)
Lead	*Earthworm	*Dendrobaena octaedra*	Adult	None	Bindesbøl et al. (2008b)
Polycyclic aromatic hydrocarbons (PAHs)					
Pyrene	‡Springtail	*Protaphorura armata*	Adult	Antagonistic	Sjursen & Holmstrup (2004)
Pyrene	*Earthworm	*Dendrobaena octaedra*	Adult	None	Bindesbøl et al. (2008b)
Phenanthrene	*Earthworm	*Dendrobaena octaedra*	Adult	Antagonistic	Bindesbøl et al. (2008b)
Surfactants					
Nonylphenol	*Earthworm	*Dendrobaena octaedra*	Adult	None	Jensen et al. (2008)
Surfactants	†Insect	*Cacopsylla pyricola*	Adult	Synergistic	Horton et al. (1996)
Pesticides					
Abamectin	*Earthworm	*Dendrobaena octaedra*	Adult	None	Bindesbøl et al. (2008b)
Carbendazim	*Earthworm	*Dendrobaena octaedra*	Adult	None	Bindesbøl et al. (2008b)

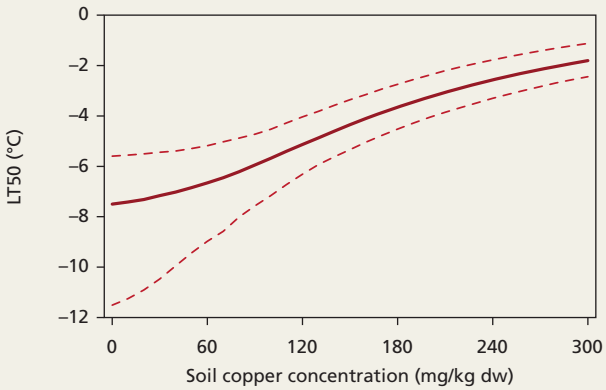

Figure 1. The estimated temperature causing 50% mortality (LT50) as a function of soil copper concentrations in *Dendrobaena octaedra*. The dashed lines indicate 95% credibility interval. (From Bindesbøl et al., 2008a).

most apparent at copper concentrations above 120 mg/kg dw, as well as at temperatures below –2 °C. Synergistic interaction between copper and freezing temperatures was observed across species as in cocoons of *D. octaedra* and *Aporrectodea caliginosa* (Holmstrup et al., 1998), as well as the collembolan *Protaphorura armata* (Bossen, 2001).

Synergistic interactions between mercury and freezing temperatures were also observed across species. Mercury significantly reduced the ability of *D. octaedra* to survive at –6 °C (Bindesbøl et al., 2008b). The LC50 decreased from approximately 40 mg/kg dw at the control temperature of 2 °C to less that 10 mg/kg dw at –6 °C. Likewise, mercury reduced the cold shock tolerance of the springtail *Folsomia candida* as well as reducing the beneficial effect of rapid cold hardening (Holmstrup et al., 2008). Reduced freeze tolerance was also observed when different tissues of the larvae *Eurosta solidaginis* and *Chilo suppressalis* were exposed to mercury (Philip et al., 2008; Izumi et al., 2006). These authors explained the reduced freeze tolerance by the ability of mercury to block aquaporins, which are integral proteins channelling trans-membranous transport of water and osmolytes such as glycerol (Borgnia et al., 1999). Blocking of aquaporins may increase the risk of intracellular freezing in freeze tolerant organisms, and perhaps increasing the risk of inoculative crystallisation in organisms depending on cryoprotective dehydration. By using radiotracer techniques, Izumi et al. (2006) showed that the transport of both water and glycerol was almost eliminated by mercury, suggesting that this could explain the reduced freeze tolerance. Copper ions have been shown to inhibit the water and glycerol permeability of aquaporins in human erythrocytes (Zelenia et al., 2004) suggesting that also this metals ability to block aquaporins could be a reasonable explanation for the observed synergistic interactions with freezing temperatures. However, blocking of aquaporins can not be the mechanistic explanation for the reduced cold shock tolerance of *F. candida* after rapid cold hardening (Holmstrup et al., 2008), where water transport across cell membranes is not an issue, because they are exposed to temperatures between their melting point and super cooling point, and do not dehydrate during the relatively short exposure period. Bindesbøl et al. (2008a) proposed changes in membrane PLFAs as the mechanistic explanation for the decreased freeze tolerance in *D. octaedra* when exposed to copper. These changes in PLFAs can probably be explained by coppers ability to induce lipid peroxidation, where especially PLFAs containing two or more double bonds are particularly susceptible to oxidation by free radicals and other highly reactive species (Valko et al., 2005). The study by

Bindesbøl et al. (2008a) showed that copper had an especially significant negative effect on the PLFA 18:2ω6,9 (Figure 2), which has previously been reported to correlate positively (R^2 = 0.92) with freeze tolerance in *D. octaedra* (Holmstrup et al., 2007). This supports the proposal that the observed synergistic interactions between copper and freezing temperatures are due to membrane changes. Mercury may also change the PLFA composition in membranes due to its ability to induce lipid peroxidation, and this could explain the mercury-induced reduction of cold shock tolerance after rapid cold hardening in *F. candida*, especially because rapid cold hardening in *F. candida* is likely to involve changes in membrane PLFA composition, including an increase in PLFA 18:2ω6,9 (Overgaard et al., 2005).

Bindesbøl et al. (2005) tested the hypothesis that the reduced freeze tolerance in *D. octaedra* could be explained by copper interacting with glucose accumulation, which is believed to be the major component of the cryoprotectant system in this earthworm species (Rasmussen and Holmstrup, 2002). This was, however, not the case, as shown in figure 3. Contrary to this, Zachariassen and Lundheim (1995) found that exposure to copper and cadmium caused a reduction in the rate of the removal of the cryoprotectant glycerol in the cold-exposed beetle *Rhagium inquisitor* after ten days of warm acclimation. The enzymes involved in the breakdown are the same as those involved in the production of SPs, and may suggest that production of SPs during cold acclimation could be reduced during exposure to heavy metals and thereby reduce the cold-hardiness of this insect (Zachariassen and Lundheim, 1995). The authors did not test this suggestion or the effect of heavy metals on cold tolerance, which makes it hard to conclude if this could be a mechanistic explanation for the reduced cold tolerance of insects exposed to heavy metals in general.

Zachariassen et al. (2004) proposed that metals may affect the cryoprotective mechanism of AFPs. Both AFPs and metallothioneins contain high amounts of cysteine, which means that exposure to metals may deplete the cysteine pool and thereby reduce the exposed organism's ability to produce AFPs. Pedersen et al. (2006) explored this idea and found that AFP production was reduced in the freeze avoidant meal worm (*Tenebrio molitor*) when exposed to metals (copper, zinc and cadmium) at summer temperatures (20 °C), but not at winter temperatures (4 °C). Unfortunately, they did not measure these organisms ability to survive freezing temperatures during these different exposure regimes, and it is therefore hard to conclude anything, but the results suggests that metals do not affect AFP production during cold acclimation, and as far as we know neither of the organ-

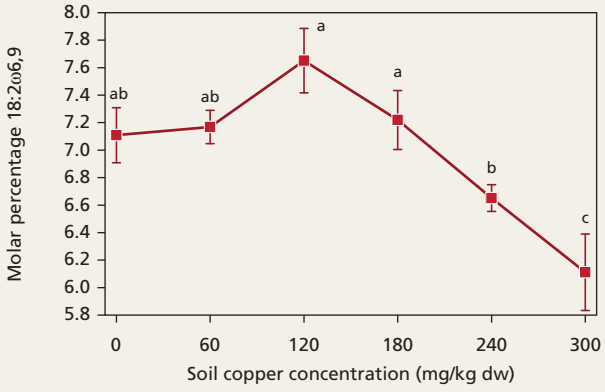

Figure 2. The molar percentage (mean ± SE) of the phospholipid fatty acid, 18:2ω6,9, in *Dendrobaena octaedra* exposed to increasing soil copper concentrations during 6 weeks of cold acclimation. (From Bindesbøl et al., 2008a).

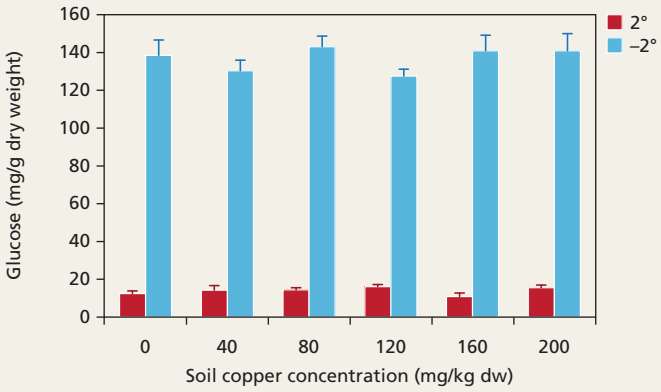

Figure 3. Mean glucose content in *Dendrobaena octaedra* after exposure to increasing soil copper concentrations for four weeks at 2 °C, followed by exposure to either 2 °C or –2 °C for another 2 days.

isms dealt with in the previous section produce AFPs (M. Holmstrup, personal communication). It therefore seems reasonable to suggest that the mechanistic explanation behind the observed synergistic interactions between heavy metals and freezing temperatures (across species) is at least partly due to membrane damage. This is supported by Taulovuori et al. (2005), who hypothesised that heavy metals increase the risk of frost injury due to membrane alterations in plants at northern high latitudes. Further, Pukacki (2004) found a reduction in the PLFAs 18:2 and 18:3 of cell membranes in needles of Scots pine (*Pinus sylvestris*) near a copper smelter in Glogow, Poland, which very well could lead to a reduced freeze tolerance in those tissues. Thus, such reduction in freeze tolerance was recorded in a study by Sutinen et al. (1996), who observed that needles of *P. sylvestris* were more susceptible to frost near a copper-nickel smelter in Russia than those further away from the smelter.

Interactions with polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants, composed of fused aromatic carbon rings. They are produced by a number of natural and anthropogenic activities, mainly from the incomplete combustion of fossil fuels and the pyrolysis of a wide range of plastics (Chun-The et al., 2001). Contamination by PAHs is widespread. Based on their structure with no functional groups, the mode of toxic action is likely to be interference with membrane function and fluidity, a phenomenon called nonpolar narcosis (Chaisuksant et al., 1999). This mode of toxic action is directly associated with the quantity, rather than the chemical structure of the toxicant (Mullins, 1954). Some PAHs are, however, known to have mutagenic properties as well as photoinduced toxicity has been found for some of them (Weinstein et al., 1997).

Only two studies concerning the interactions between freezing temperatures and PAHs exist (Table 1; Bindesbøl et al., 2008b; Sjursen and Holmstrup, 2004). In the study by Bindesbøl et al. (2008b), the earthworm *D. octaedra* were exposed to increasing concentrations of phenanthrene and pyrene, respectively, in soil for one week at 10 °C, followed by one week

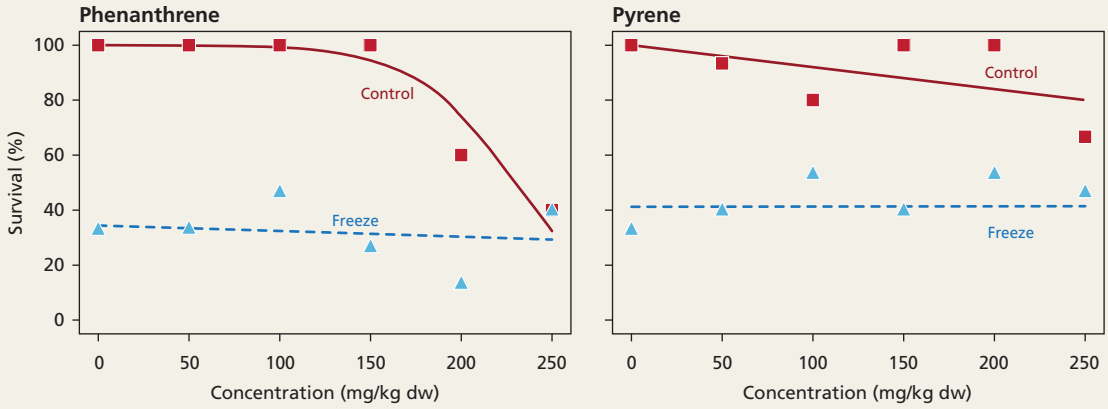

Figure 4. Dose-response relationship for the effects of phenanthrene and pyrene on control survival (red squares) and freeze survival (blue triangles). Lines between points represent estimated curves. (From Bindesbøl et al., 2008b).

at 5 °C, and finally for 4 weeks at 2 °C prior to exposure to the freezing temperature of –6 °C. The control worms were exposed to the same treatment, except that they were exposed at 2 °C for one more week, as long as the freezing lasted. The worms were significantly more susceptible to phenanthrene at control temperature than at the freezing temperature. This tendency, though not significant, was also observed with pyrene (Figure 4). It is possible that the worms in the control group had higher internal PAH concentrations at the end of the experiment than those exposed to –6 °C, where no further accumulation would be expected because the water surrounding them was frozen. However, no increased mortality in the control groups was observed after the time of freezing, and we therefore do not expect this to be the reason for increased mortality at control conditions. PAHs are likely to accumulate in membranes because of their lipophilic characteristics and structure with no functional groups (Moriarty, 1983), and may thereby increase fluidity (Chaisuksant et al., 1999). Such an increase in fluidity may be an advantage during freezing, thereby counteracting the expected mortality with increasing exposure concentrations, as observed at control conditions.

Sjursen and Holmstrup (2004) reported a higher survival in pyrene exposed *P. armata* at –3 °C compared to 5 and 20 °C. The collembolans exposed to –3 °C were pre-exposed to increasing pyrene concentrations at 5 °C for two weeks before being exposed to –3 °C for another two weeks. The other collembolans were exposed to increasing pyrene concentrations for four weeks at 5 and 20 °C, respectively. The increased survival at –3 °C was statistically significant only at the highest tested concentration of 300 mg/kg dw. Sjursen and Holmstrup (2004) explained this increase in survival at –3 °C by an increased accumulation of pyrene at 5 °C and 20 °C, because they assumed that the uptake of the toxicant was probably reduced due to a lowered metabolism, as well as because a larger fraction of the toxicant is bound to the soil particles at decreasing temperatures (Piatt et al., 1996). If the higher survival at –3 °C compared to 5 and 20 °C had been due to a lower accumulation of pyrene at lower temperatures, the difference in survival would have been evident at the lower pyrene concentrations as well, and not only at the highest tested concentration. Further, survival at 5 °C would be expected to be higher than at 20 °C, but this was not the case.

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This might support the above suggested mechanistic explanation, that a PAH increased fluidity counteracts the expected mortality observed at non freezing temperatures. The highest tested concentration of 300 mg/kg dw might actually have been high enough to give rise to an increased fluidity of the membranes and thereby counteract the expected mortality observed at higher temperatures (Chaisuksant et al., 1999). I therefore suggest that the mechanistic explanation behind the observed antagonistic interaction between the tested PAHs and freezing temperatures compared to control temperatures could be due to PAH increased membrane fluidity rather than decreased PAH uptake at freezing temperatures. Measurements of internal PAH concentrations are, however, necessary to obtain further insight into this question.

Interactions with surfactants

Surfactants are widely used in household and industrial detergents and reach the terrestrial environment as sludge produced by sewage treatment facilities. Surfactants are organic compounds which have both polar and non-polar characteristics (Walker et al., 1996). Surfactants lower the surface tension of water and because of their amphiphilic nature and the consequent ability to be adsorbed at interfaces, surfactants may interact with biological membranes and may cause permeability changes as well as denaturate proteins (Schwuger and Bartnik, 1980).

Horton et al. (1996) found that spraying pear psylla (*Cacopsylla pyricola*; Hemiptera; Psyllidae) with four different surfactants individually, caused a dramatic decrease in survival of frost. Spraying with water also decreased freeze survival, however, though not to the same extent as spraying with surfactants. Temperatures causing 50 % mortality increased from below –18 °C in control and water sprayed animals to between –2.6 to –12.7 °C in surfactant treated animals. *C. pyricola* is a cold-hardy, freeze avoiding species, able to supercool to temperatures well below –20 °C during winter, and freezing of their body fluids is lethal. The increased mortality in water and surfactant exposed animals is probably

a result of inoculative freezing. Salt (1963) showed that detergents caused increased inoculative freezing in the blowfly larvae, which might be explained by the detergents ability to increase water permeability of the integument of the larvae and thereby increasing the risk of inoculative freezing. Spraying with surfactant might also increase the water permeability of the cuticle of *C. pyricola* increasing the risk of inoculative freezing.

Jensen et al. (2009) found that the freeze tolerance of *D. octaedra* was not reduced after exposure to the surfactant nonylphenol. This observation was based on results from a full factorial design using concentrations as high as 900 mg/kg dw nonylphenol and freezing temperatures down to –6.4 °C. A 100 % mortality was obvious already at –3.5 °C, which is much higher than normally observed. Usually, *D. octaedra* have a 50 % survival at –8 °C in a Danish population (Bindesbøl et al., 2008a). This failure to survive rather high freezing temperatures makes it quite hard to conclude anything from these results. Whereas a possible inoculative effect of a surfactant, like nonylphenol, will greatly affect the survival of a freeze avoiding species, as the above mentioned *C. pyricola,* the survival of *D. octaedra*, a freeze tolerant species, would not be expected to be reduced on this account.

Interactions with pesticides

Pesticides are the most important pollutant in agricultural soils and are applied as sprays, granules or dusts. Modern pesticides are mostly readily biodegradable and thereby not strongly persistent in the environment. Pesticides exercise a very specific mode of action, such as acetylcholine esterase inhibition in organophosphorus insecticides (Walker et al., 1996), whereas others like abamectin and lindane, inhibits the gamma-aminobutyric acid induced neurotransmission and causes paralysis in parasites (Shoop et al., 1995; Walker et al., 1996). Many pesticides are very hydrophobic, and could be expected to interfere with membrane lipids (Gabbianelli et al., 2002).

Two pesticides, abamectin and carbendazim, have been tested but none of them had any effect on freeze tolerance of *D. octaedra* at –6 °C (Bindesbøl et al., 2008b). This is probably due to their quite specific mode of action. Abamectin inhibits the gamma-aminobutyric acid induced neurotransmission and causes paralysis in parasites (Campell et al., 1983; Shoop et al., 1995) and carbendazim works by inhibiting the development of fungi, probably by interfering with spindle formation at mitosis. These modes of action are presumably the same in *D. octaedra*, and both chemicals were inherently toxic, but had no effect on freeze tolerance.

Interactions between drought and chemicals

As with cold hardy organisms, drought tolerance often also depends on the accumulation of SPs as well as membrane adjustments. Therefore it is expected that toxicants interfering these processes will significantly reduce drought survival. Further, dehydration will reduce the volume of liquid water in the organism thereby increasing the concentration of chemicals and the risk of toxic damage.

Table 2. Overview of interactions between toxicant and drought. The term "synergistic" is used when the combined effect of the two stressors is greater than expected, and the term "antagonistic" interaction is used if the combined effect is less than expected from the combination of the effects from each stressor alone. No interaction is indicated as "none".

Toxicant	Test organism	Species	Life stage	Interaction	Reference
Heavy metals					
Copper	Earthworm	*Dendrobaena octaedra*	Juvenile	None	Bindesbøl (unpl. data)
Copper	Earthworm	*Dendrobaena octaedra*	Cocoon	Synergistic	Holmstrup et al. (1998)
Copper	Earthworm	*Aporrectodea calliginosa*	Cocoon	Synergistic	Holmstrup et al. (1998)
Copper	Earthworm	*Aporrectodea calliginosa*	Adult	Synergistic	Friis et al. (2004)
Copper	Springtail	*Folsomia candida*	Adult	Synergistic	Holmstrup (1997)
Copper	Springtail	*Folsomia candida*	Adult	None	Sørensen and Holmstrup (2005)
Cadmium	Springtail	*Folsomia candida*	Adult	None	Sørensen and Holmstrup (2005)
Polycyclic aromatic hydrocarbons (PAHs)					
Pyrene	Springtail	*Protaphorura armata*	Adult	Synergistic	Sjursen and Holmstrup (2004)
Pyrene	Springtail	*Folsomia candida*	Adult	Synergistic	Skovlund et al. (2006)
Pyrene	Springtail	*Folsomia candida*	Adult	Synergistic	Sørensen and Holmstrup (2005)
Pyrene	Springtail	*Folsomia fimetaria*	Adult	Synergistic	Sjursen et al. (2001)
Flourene	Springtail	*Folsomia fimetaria*	Adult	Synergistic	Sjursen et al. (2001)
Flourene	Springtail	*Folsomia candida*	Adult	Synergistic	Sørensen and Holmstrup (2005)
Flouranthene	Springtail	*Folsomia fimetaria*	Adult	Synergistic	Sjursen et al. (2001)
Flouranthene	Earthworm	*Lumbricus rubellus*	Adult	None	Long et al. (2008)
Dibenzothiophene	Springtail	*Folsomia fimetaria*	Adult	Synergistic	Sjursen et al. (2001)
Acridine	Springtail	*Folsomia fimetaria*	Adult	None	Sjursen et al. (2001)
Dibenzofuran	Springtail	*Folsomia fimetaria*	Adult	None	Sjursen et al. (2001)
Carbazole	Springtail	*Folsomia fimetaria*	Adult	None	Sjursen et al. (2001)
Surfactants					
Nonylphenol	Springtail	*Folsomia candida*	Adult	Synergistic	Skovlund et al. (2006)
Nonylphenol	Springtail	*Folsomia candida*	Adult	Synergistic	Sørensen and Holmstrup (2005)
Nonylphenol	Springtail	*Folsomia candida*	Adult	Synergistic	Holmstrup (1997)
Nonylphenol	Springtail	*Folsomia candida*	Adult	Synergistic	Højer et al. (2001)
LAS	Springtail	*Folsomia candida*	Adult	Synergistic	Sørensen and Holmstrup (2005)
LAS	Springtail	*Folsomia candida*	Adult	Synergistic	Holmstrup (1997)
Pesticides					
DDT	Springtail	*Folsomia candida*	Adult	None	Skovlund et al. (2006)
Cypermethrin	Springtail	*Folsomia candida*	Adult	None	Sørensen and Holmstrup (2005)
Abamectin	Potworm	*Enchytraeus doerjesi*	Adult	None	Kramarz (unpl. data)
Dimethoate	Springtail	*Folsomia candida*	Adult	None	Sørensen and Holmstrup (2005)
Lindane	Springtail	*Onychiurus quadriocellatus*	Adult	Synergistic	Demon and Eijsackers (1985)

Interactions with heavy metals

Interaction between drought and heavy metals showed both synergistic interaction and no interaction (Table 2). Most studies so far have investigated the effect of copper on drought tolerance and no clear trend is found. For example, copper did not reduce the drought tolerance of juveniles of the earthworm *D. octaedra* (Bindesbøl, unpublished), whereas the drought tolerance was reduced by copper in the earthworm *A. caliginosa* (Friis et al., 2004) as well as in cocoons of both *D. octaedra* and *A. caliginosa* (Holmstrup et al., 1998). Furthermore, copper significantly reduced the drought tolerance of the collembolan *F. candida* in a study by Holmstrup (1997), whereas no interaction was observed between copper and drought in the same species in a study by Sørensen and Holmstrup (2005). This difference in interactions in *F. candida* may be explained by the drought exposure levels. Holmstrup (1997) exposed *F. candida* to 300 mg Cu/kg dw for one week at 20 °C, followed by exposure to different drought stresses ranging from 99.6 % relative humidity (RH) to 96.8 % RH for seven days. The synergistic interaction became apparent at drought levels lower than 97.8 % RH, whereas no interaction with copper occurred at higher drought exposures. In the study by Sørensen and Holmstrup (2005) *F. candida* were exposed to increasing concentrations of copper and cadmium, followed by drought stress at 97.8 % RH for seven days. This drought level is less severe than those showing synergistic interactions with copper in the study by Holmstrup (1997), and might be the explanation why no synergistic interaction was observed. This may also explain why no reduced drought tolerance was observed in copper exposed *D. octaedra's* (Bindesbøl, unpublished) However, as sensitivity to humidity may differ greatly between species, such a comparison may be difficult, and there is currently no sound explanation for this difference in interaction between copper and drought.

Friis et al. (2004) exposed the earthworm *A. caliginosa* to a sublethal copper concentration (150 mg/kg dw) at different drought levels, obtaining water potentials from pF 1.5 (wet) to pF 5 (very dry). They found that drought tolerance decreased in copper exposed worms (Figure 5). At drought levels where mortality is starting to occur in controls (pF 4.0–4.5), copper increased the mortality rate 2 to 3-fold. *A. caliginosa* enters diapause during summer if the water potential gets too low (Gerard, 1967; Nordström, 1975), during which process the worm enclose itself into an estivation cell, which minimize water loss during drought. Friis et al. (2004) found that the development of estivation cells was significantly depressed in copper exposed worms, and that worms without estivation cells were more prone to drought induced mortality compared to worms with intact estivation cells. However, the water

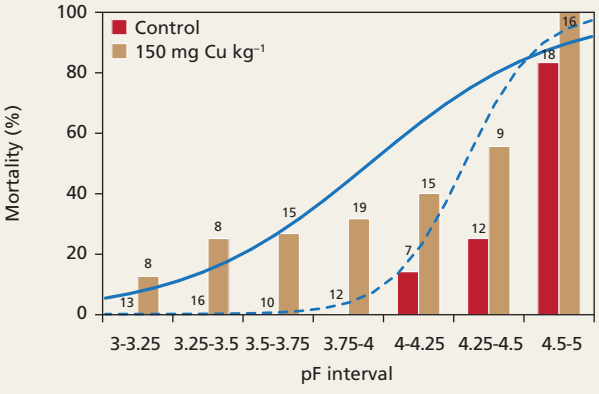

Figure 5. Percentage mortality of control and copper exposed *Aporrectodea caliginosa* in different classes of drought levels. The number of replicates is indicated above bars. The lines represent estimated curves. (From Friis et al., 2004).

potential where 50 % of the worms died (LWP50) for worms not exposed to copper, all having estivation cells, was higher than LWP50 for copper exposed worms with estivations cells (pF 4.48 and 4.31 respectively), which suggest that the lack of estivation cells is not the only explanation for the observed synergistic interaction. An additional explanation for the observed synergy could be that the internal copper concentration increased at increased drought stress and might have reached a lethal level, as found by Friis et al. (2004). However, as copper concentrations were only determined in surviving worms, it was not possible to determine whether dead worms contained lethal concentrations. The authors suggested that the observed increase in copper burden at high drought stress could be due to dehydrated worms losing the ability to regulate internal copper levels. Furthermore, Friis et al. (2004) found that the body fluid osmolality of copper exposed worms was consistently higher than in control worms, even if they had the same water content. Since *A. caliginosa* do not accumulate SPs in high concentrations, this increased osmolality must predominantly have been due to original solutes (e.g. Na^+, K^+, Ca^{++}, Cl^-), that might have reached toxic levels. Nevertheless, the mechanistic explanation behind the observed synergy was probably based on both behavioural and physiological responses.

Interactions with polycyclic aromatic hydrocarbons

Sjursen et al. (2001) tested the effects of seven PAHs on the drought tolerance of the springtail *F. fimetaria*. The springtails were exposed to a drought level of 98.2 % RH or 100 % RH (control) after exposure to increasing concentrations of the different PAHs. Synergistic effects between the PAH and drought could be seen for flourene, pyrene, flouranthene, dibenzothiophene and carbazole, whereas dibenzofuran and acridine did not reduce the drought tolerance. Synergistic interaction between pyrene and drought was also observed in three other studies with springtails (Sørensen and Holmstrup, 2005; Skovlund et al., 2006; Sjursen et al., 2001). Skovlund et al. (2006) tested the effect of drought and pyrene using a full factorial design with six sub-lethal pyrene concentrations between 0–150 mg/kg dw and six drought levels from 100 % RH to 97 % RH. The synergistic interaction became apparent at drought levels lower than 98.2 % RH and at the highest tested pyrene concentration of 150 mg/kg. At a drought level of 97.8 % RH, used in the study by Sørensen and Holmstrup (2005), the synergistic interaction with pyrene also became clear at a concentration of 150 mg/kg dw and higher. They suggested that the reduced drought tolerance was due to disrupted membrane functionality, because PAHs are known to interact with mem-

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branes. Functional cell membranes are crucial for the osmoregulatory changes associated with dehydration, and membrane disturbance might detrimentally influence these processes, which in turn would increase mortality.

Long et al. (2008) tested the effects of flouranthene on survival and reproduction during drought exposure in the earthworm *Lumbricus rubellus*. This was tested using a full factorial design with five flouranthene concentrations and four drought treatments, including controls. Survival was not significantly affected by any of the exposure treatments. Cocoon production, however, was significantly reduced by both drought and flouranthene, but no synergistic interaction was observed between the two stressors that seemed to work in concert by simple additive effects. These results are in contradiction to the results with springtails, were synergistic interactions was observed with almost all tested PAHs and drought. This may be explained by the rather low degree of drought stress used in the study by Long et al. (2008), which in itself will probably not cause the worms to dehydrate, as is the case with the collembolans discussed above. At drought levels comparable to those in the study by Long et al. (2008), no dehydration of the earthworm *A. caliginosa* was observed (Holmstrup, 2001; Friis et al., 2004), and it is assumed that the same will be the case in *L. rubellus*. It is likely that flouranthene, as observed for other PAHs, would interact synergistically at higher degrees of drought stress, where functional membranes are important for the osmoregulatory changes associated with dehydration.

Interactions with surfactants

Højer et al. (2001) showed that exposure to nonylphenol caused a reduction in the drought tolerance of *F. candida*. The two stressors were varied in a full factorial design with six nonylphenol concentrations (0–62.5 mg/kg dw) and seven drought levels (99.7–97.0 % RH). The synergistic interaction became most pronounced at a drought level of 97.9 % RH and below. The relative humidity causing 50 % mortality (LRH50) increased from approximately 98 % RH with no nonylphenol exposure to 98.6 % RH in animals exposed to a nonylphenol concentration of 60 mg/kg dw. Furthermore, it was shown that nonylphenol caused a small but significant increase in water permeability across the integument, as well as inhibiting the synthesis of both glucose and myo-inisitol (SPs). The springtail *F. candida* has a highly permeable integument and depends on its ability to regulate body fluid osmolality through the synthesis of SPs during drought (water vapour absorption strategy) (Bayley and Holmstrup, 1999). When exposed to drought, *F. candida* initially loses a substantial

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amount of water to the surrounding soil. Following this dehydration, they start to accumulate SPs during the first 24–48 hours, which makes them hyperosmotic to the surroundings. This enables them to passively absorb water vapour from the unsaturated air around them (Bayley and Holmstrup, 1999). However, during the first 24 hours, before SP accumulation occurs, water loss is prominent and the observed increase in water permeability across the integument can make them even more vulnerable to desiccation as suggested by Højer et al. (2001). Furthermore, the reduced SP synthesis in nonylphenol exposed animals will make them even more susceptible to drought. It is not known whether the reduced production of SPs is due to a reduction in glycogen supplies because of a metabolically expensive detoxification mechanism or due to reduced expression and/or activity of the enzyme system responsible for glycogen breakdown. A full factorial design was also used by Skovlund et al. (2006) to test the interaction between nonylphenol and drought in the springtail *F. candida*. As in Højer et al. (2001), the observed synergistic interaction became apparent at relatively high drought stress – below 98 % RH – and became very severe at the highest nonylphenol concentrations in both studies. Also, Sørensen and Holmstrup (2005) as well as Holmstrup (1997), recorded a highly synergistic interaction between nonylphenol and a drought level below 98 % RH in *F. candida*. Synergistic interaction was also recorded between drought and the surfactant linear alkylbenzene sulphonate (LAS) in *F. candida* (Holmstrup, 1997; Sørensen and Holmstrup, 2005), though not to the same extent as observed with nonylphenol. This difference between the abilities of nonylphenol and LAS to create synergistic interactions with drought within the same species may be explained by a difference in lipophilicity, with nonylphenol being more lipophile than LAS. Furthermore, the molecular structure of nonylphenol is similar to membrane phospholipids in the sense that both have a nonpolar carbon chain and a polar head-region which supposedly could become embedded in and likely interfere with cellular membranes.

Interactions with pesticides

Skovlund et al. (2006) tested the effect of the pesticide residue, DDE, on drought tolerance of *F. candida* applying a full factorial design. No interaction between the two stressors was observed, even at the highest drought level exposure of 97 % RH. This was also evident in a study with the same species by Sørensen and Holmstrup (2005), where no interaction was observed between two insecticides, dimethoate and cypermethrin, and drought even though a severe effect on reproduction was observed. Further, no synergistically increased mortality was evident in the enchytraeid, *Enchytraeus doerjesi*, exposed to different concentrations of abamectin in combination with different drought levels (P. Kramarz, unpublished results). However, Demon and Eijsackers (1985) found that the pesticide lindane

reduced the drought tolerance of the springtail *Onychiurus quadriocellatus*. This reduced drought tolerance may be explained by lindanes high lipophilicity (Walker et al., 1996), which makes it able to interfere with membranes. However, many of the other tested pesticides have high lipophilicities as well (Gülden et al., 2002). Gabbianelli et al. (2002) found that cypermethrin disturbed membrane structure as well as inducing oxidative stress in erythrocytes from rats. These findings would suggest that this pesticide could create synergistic interactions with drought as well. The lack of such findings may be explained by the fact that many of the pesticides are so toxic because of their very specific mode of action, resulting in the organisms dying before the concentrations reach a level that can affect general membrane stability and function.

Conclusion

The present review clearly shows that synergistic interactions between environmental contaminants and natural stressors, like cold and drought, are not uncommon, as illustrated in Figure 6. This means that traditional laboratory studies, where the organisms are exposed to increasing concentrations of a single compound under otherwise optimal conditions, might underestimate the toxicity of the chemical in the field. Although the available literature is still limited, some general patterns seem to emerge. Thus, in nearly all studies, synergistic interactions are observed between heavy metals and freezing temperatures. It also appears that surfactants and to a great extent PAHs interfere with drought tolerance. On the other hand, no clear relationship is observed between heavy metals and drought, or between surfactants and freezing. In the case of surfactants, it seems likely that the outcome depends on the cold hardiness strategy employed by the organism. With the exception of one study, pesticides do not seem to interact with either of the two natural stressors. One peculiar observation gained from the present review is that PAHs have different interactions depending on which natural stressor it is combined with. PAHs seems to interact synergistically with drought, whereas they seem to interact antagonistically with freezing temperatures, i.e. the freeze mortality at increasing PAH concentrations is lower than expected from control treatments. This observation is rather unexpected as there are many

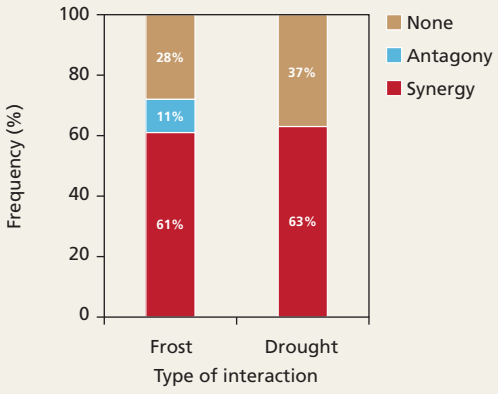

Figure 6. Frequency of occurrence of interactions between toxicants and the two natural stressors; freezing temperatures and drought, with respect to mortality. The term "synergy" is used when the combined effect of the two stressors is greater than expected, and the term "antagony" is used if the combined effect is less than expected from the combination of the effects from each stressor alone. No interaction is indicated as "none".

common physiological adaptations in response to both drought and freezing temperatures. A straight forward explanation for this difference would be that the uptake of the toxicant is reduced during acclimation in the freezing experiments compared to the drought experiments. This does not seem to be the reason, however, as discussed in previous sections. Another possible explanation can be that a PAH increased fluidity counteracts the expected mortality observed at non freezing temperatures. However more experiments are needed to support the observed antagonistic interaction between freezing temperatures and PAHs.

In general, synergistic interactions seem to occur mostly at high drought stress and rather low temperatures in combination with quite high concentrations of the toxicant. This suggests that the impact of pollution becomes most significant in situations of extreme climatic stress. Chemicals pollute the local areas where they are released, but due to airborne transport they also cause global pollution. Many chemicals enter the arctic and cold temperate areas, which are sinks for a large number of environmental contaminants because of cold distillation of windborne pollutants from industrialised countries (AMAP 2002). This may change the geographical distribution of organisms living in these areas. Additionally, as one of the most commonly predicted consequences of future climate warming is an increase in the frequency and severity of drought periods (Good et al., 2006), it is likely that the soil drought levels will become more extreme. An overall warming of northern regions may, although it seems paradoxical, result in occasional but extreme soil freezing, since thinner snowpacks have a less insulating effect (Isard et al., 2007). The synergistic interactions reviewed in the present study may thus represent a widespread exposure scenario making it even more important to include natural stressors, like cold and drought, in future risk assessment.

Photo: Brian Rasmussen

References

Aarset, A.V. and Zachariassen, K.E. (1982). Effects of oil pollutopn on the freezing tolerance and solute concentration of the blue mussel *Mytilus edulis*. – Marine Biology 72: 45–51.

AMAP (2002). AMAP Assessment Report: Heavy Metals in the Arctic. – Artic Monitoring and Assessment Programme (AMAP). Oslo, Norway.

Bayley, M. and Holmstrup, M. (1999).Water vapour absorption in arthropods by accumulation of myoinositol and glucose. – Science 285: 1909–1911.

Bayley, M., Petersen, S.O., Knigge, T., Köhler, H.R. and Holmstrup, M. (2001). Drought acclimation confers cold tolerance in the soil collembolan *Folsomia candida*. – Journal of Insect Physiology 47: 1197–1204.

Bindesbøl, A., Holmstrup, M., Damgaard, C. and Bayley, M. (2005). Stress synergy between environmentally realistic levels of copper and frost in the earthworm *Dendrobaena octaedra*. – Environmental Toxicology and Chemistry 24: 1462–1467.

Bindesbøl, A., Bayley, M., Damgaard, C. and Holmstrup, M. (2007). Life–history traits and population growth rate in the laboratory of the earthworm *Dendrobaena octaedra* cultured in copper–contaminated soil. – Applied Soil Ecology 35: 46–56.

Bindesbøl, A., Bayley, M., Damgaard, C., Hedlund, K. and Holmstrup, M. (2008a). Changes in membrane phospholipids as a mechanistic explanation for decreased freeze tolerance in earthworms exposed to sub–lethal copper concentrations. – Submitted to ESandT.

Bindesbøl, A., Bayley, M., Damgaard, C. and Holmstrup, M. (2008b). Metals reduce freeze tolerance of the earthworm *Dendrobaena octaedra*. – In Preparation.

Block, W. (1990). Cold tolerance of insects and other arthropods. – Philosophical Transactions of the Royal Society of London series B–biology sciences 326: 513–633.

Borgnia, M., Nielsen, S., Engel, A. and Agre, P. (1999). Cellular and molecular biology of the aquaporin water channels. – Annual Reviews of Biochemistry 68: 425–458.

Bossen, S. (2001). The effects of combined cold and copper exposure in *Protaphorura armata* (Collembola). – Master's Thesis. University of Aarhus, Denmark (In Danish).

Bruus Pedersen, M., Temminghoff, E., Marinaussen, M., Elmegaard, N. and Van Gestel, C.A.M. (1997). Copper accumulation and fitness of *Folsomia candida* Willem in a copper contaminated sandy soil as affected by pH and soil moisture. – Applied Soil Ecology 6: 135–146.

Campell, W.C., Fisher, M.H., Stapley, E.O., Albers–Schönberg, G. and Jacob, T.A. (1983). Ivermectin: a potent new parasitic agent. – Science 221: 823–828.

Chaisuksant, Y., Yu, Q.M. and Connell, D.W. (1999). The internal critical level concept of nonspecific toxicity. – Reviews of Environmental Contamination and Toxicology 162: 1–41.

Chapman, P.M., Fairbrothers, A. and Browns, D. (1998). A critical evaluation of safety (uncertainty) factors for ecological risk assessment. – Environmental Toxicology and Chemistry 17: 99–108.

Chun–The, L., Huan–Kai, Z., Lien–Te, H., Wen–Jhy, L. and Meng–Chun, T. (2001). PAH emission from the incineration of three plastic wastes. – Environment International 27: 61–67.

Crowe, J.H., Oliver, A.E. and Tablin, F. (1992). Anhydrobiosis. – Annual Reviews of Physiology 54: 579–599.

Demon, A. and Eijsackers, H. (1985). The effect of lindane and azinphosmethyl on survival time of soil animals, under extreme or fluctuating temperature and moisture conditions. – Zeitschrift für angewandter Entomologie 100: 504–510.

Duman, J.G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. – Annual Review of Physiology 61: 243–282.

Forbes, V.E. and Calow, P. (1999). Is the per capita rate of increase a good measure of population-level effects in ecotoxicolgy ? – Environmental Toxicology and Chemistry 18: 1544–1556.

Friis, K., Damgaard, C. and Holmstrup, M. (2004). Sublethal soil copper concentrations increase mortality in the earthworm *Aporrectodea calignosa* during drought. – Ecotoxicology and Environmental Safety 57: 65–73.

Gabbianelli, R., Falciono, G., Nasuti, C. and Cantalamessa, F. (2002). Cypermethrin–induced plasma membrane perturbation on erythrocytes from rats: reduction of fluidity in the hydrophobic core and in glutathione peroxidise activity. – Toxicology 175: 91–101.

Gerard, B. (1967). Factors affecting earthworms in pastures. – Journal of Animal Ecology 36: 235–252.

Grant, W. (1955). Studies on moisture relationships in earthworms. – Ecology 36: 400–407.

Grout and Morris (1987). The Effect of Low Temperature on Biological Systems. Edward Arnold, London, UK.

Gülden, M., Mörchelm, S., Tahan, S. and Seibert, H. (2002). Impact of protein binding on the availability and cytotoxic potency of organochlorine pesticides and chlorophenols. – Toxicology 175: 201–213.

Hadley, N.F. (1994). Water Relations of Terrestrial Arthropods. – Academic Press. San Diego, USA.

Hansen, F.T., Forbes, V.E. and Forbes, T.L. (1999). Effects of 4–n–nonylphenol on life–history traits and population dynamics of a polychaete. – Ecological Applications 9: 482–495.

Hazel, J.R. and Williams, E.E. (1990). The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. – Progress in Lipid Research 29: 167–227.

Hazel, J.R. (1995). Thermal adaptation in biological membranes: Is homeoviscous adaptations the explanation? – Annual Reviews of Physiology 57: 19–42.

Holmstrup, M. and Westh, P. (1994). Dehydration of earthworm cocoons exposed to cold: a novel hardiness mechanism. – Journal of Comparative Physiology B 164: 312–315.

Holmstrup, M. (1997). Drought tolerance in *Folsomia candida* Willem (Collembola) after exposure to sublethal concentrations of three soil polluting chemicals. – Pedobiologia 41: 354–361.

Holmstrup, M., Petersen, B.F. and Larsen, M.M. (1998). Combined effects of copper, desiccation, and frost on the viability of earthworm cocoons. – Environmental Toxicology and Chemistry 17: 897–901.

Holmstrup, M. (2001). Sensitivity of life history parameters in the earthworm *Aporrectodea caliganosa* to small changes in water potential. – Soil Biology and Biochemistry 33: 1217–1223.

Holmstrup, M., Hedlund, K. and Boriss, H. (2002a). Drought acclimation and lipid composition in *Folsomia candida*: implications for cold shock, heat shock and acute desiccation stress. – Journal of Insect Physiology 48: 961– 970.

Holmstrup, M., Bayley, M. and Ramløv, H. (2002b). Supercool or dehydrate? A experimental analysis of overwintering strategies in small permeable arctic invertebrates. – Proceedings of the National Academy of Sciences of the United States of America 99: 5716–5720.

Holmstrup, M. (2003). Overwintering adaptations in earthworms. – Pedobiologia 47: 504–510.

Holmstrup, M., Sørensen, L.I., Bindesbøl, A. and Hedlund, K. (2007). Cold acclimation and lipid composition in the earthworm *Dendrobaena octaedra*. – Comparative Biochemistry and Physiology, Part A. 147: 911–919.

Holmstrup, M., Aubail, A. and Damgaard, C. (2008). Exposure to mercury reduces cold tolerance in the springtale *Folsomia candida*. – Comparative Biochemistry and Physiology Part C: 148: 172–177.

Horton, D.R., Lewism, T.M. and Neven, L.G. (1996). Reduced cold–hardiness of pear psylla (Homoptera: Psyllidae) caused by exposure to external water and surfactants. – The Canadian Entomologist 128: 825–830.

Højer, R., Bayley, M., Damgaard, C. and Holmstrup, M. (2001). Stress synergy between drought and a common environmental contaminant: studies with the collembolan *Folsomia candida*. – Global Change Biology 7: 485–494.

Isard, S.A., Schaetzl, R.J. and Andresen, J.A. (2007). Soils cool as climate warms in the Great Lakes region: 1951–2000. – Annals of the Association of American Geographers 97: 467–476.

Izumi, Y., Sonoda, S., Uoshida, H., Danks, H.V. and Tsumuki, H. (2006). Role of membrane transport of water and glycerol in the freeze tolerance of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). – Journal of Insect Physiology 52: 215–220.

Janssen, M.P.M. and Bergema, W.F. (1991). The effects of temperature on cadmium kinetics and oxygen–consumption in soil arthropods. – Environmental Toxicology and Chemistry 10: 1493–1501.

Jensen, D., Bayley, M. and Holmstrup, M. (2009). Synergistic interactions between 4–nonylphenol and high but not low temperatures in *Dendrobaena octaedra*. – Ecotoxicology and Environmental Safety 72: 10–16

Knight, C.A. and Duman, J.G. (1986). Inhibition of recrystallization of ice by insect thermal hysteresis proteins: a possible cryoprotective role. – Cryobiology 23: 256–262.

Kozlow, M.V. and Zvereva, E.L. (2007). Industrial barrens: extreme habitats created by non–ferrous metallurgy. – Reviews of Environmental Science and Biotechnology 6: 231–259.

Lee, R.E. (1991). Principles of insects at low temperature tolerance. In: Insects at Low Temperature (Ed. Lee, R.E. and Denlinger, D.). – Chapman and Hall, New York. pp. 17–46.

Long, S.M., Reichenberg, F., Lister, L.J., Hankard, P.K., Townsend, J., Mayer, P., Wright, J., Holmstrup, M., Svendsen, C. and Spurgeon, D.J. (2008). Combined chemical (fluoranthene) and drought effects on *Lumbricus rubellus* demonstrates the applicability of the independent action model for multiple stressor assessment. – In Press.

Moriarty, F. (1983). Ecotoxicology: The study of pollutants in ecosystems. – Academic Press, London, UK.

Mullins, L.J. (1954). Some physical mechanisms in narcosis. – Chemical Reviews 54: 289–323.

Nordström, S. (1975). Seasonal activity of lumbricids in southern Sweden. – Oikos 26: 307–315.

Overgaard, J., Sørensen, J.G., Petersen, S.O., Loeschcke, V. and Holmstrup, M. (2005). Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. – Journal of Insect Physiology 51: 1173–1182.

Pedersen, S.A., Kristiansen, E., Hansen, B.H., Andersen, R.A. and Zachariassen, K.E. (2006). Cold hardiness in relation to trace metal stress in the freeze–avoiding beetle *Tenebrio molitor*. – Journal of Insect Physiology 52: 846–853.

Petersen, C.R., Holmstrup, M., Malmendal, A., Bayley, M. and Overgaard, J. (2008). Slow dessication improves dehydration tolerance and accumulation of compatible osmolytes in earthworm cocoons (*Dendrobaena octaedra* Savigny). – The Journal of Experimental Biology 211: 1903–1910.

Philip, B.N., Yi, S.X., Elnitsky, M.A. and Lee, R.E. (2008). Aquaporins play a role in desiccation and freeze tolerance in larvae of the goldenrod gall fly, *Eurosta solidagnis*. – The Journal of Experimental Biology 211: 1114–1119.

Piatt, J.J., Backhus, D.A., Capel, P.D. and Eisenreich, S.J. (1996). Temperature–dependent sorption of naphthalene, phenanthere and pyrene to low organic carbon aquifer sediments. – Environmental Science and Technology 30: 751–760.

Pukacki, P.M. (2004). The effects of industrial air pollution on the membrane lipid composition of scots pine (*Pinus sylvestris L.*) needles. – Acta Societatis Botanicorum Polonia 73: 187–191.

Ramløv, H. (2000). Aspects of natural cold tolerance in ectothermic animals. – Human Reproduction 15: 26–46.

Rasmussen, L.M. and Holmstrup, M. (2002). Geographic variation of freeze–tolerance in the earthworm *Dendrobaena octaedra*. – Journal of Comparative Physiology B 172: 691–698.

Ring, A. and Danks, H.V. (1994). Desiccation and cryoprotection: overlapping adaptations. – Cryoletters 15: 181–190.

Salt, R.W. (1963). Delayed inoculative freezing of insects. – The Canadian Entomologist 95: 1190–1202.

Schwuger, M.J. and Bartnik, F.G. (1980). Interactions of anionic surfactants with proteins, enzymes and membranes. In: Anionic Surfactants – Biochemistry, Toxicology, Dermatology. (Ed. Dekker, M.). New York, USA. pp. 1–49.

Shoop, W.L., Morzik, H. and Fisher, M.H. 1995. Structure and activity of avermectina and milbemycins in animal health. – Veterinary Parasitology 59: 139–156.

Sinensky, M. (1974). Homeoviscous adaptation – a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. – Proceedings of the National Academy of Sciences of the United States of America 71: 522–525.

Sjursen, H., Sverdrup, L.E. and Krogh, P.H. (2001). Effects of polycyclic aromatic compounds on the drought tolerance of *Folsomia fimeteria* (Collembola, Isotomidae). – Environmental Toxicology and Chemistry 20: 2899–2902.

Sjursen, H. and Holmstrup, M. (2004). Cold and drought stress in combination with pyrene exposure: studies wit *Protaphorura armata* (Collembola: Onychiuridae). – Ecotoxicology and Environmental Safety 57: 145–152.

Skovlund, G., Damggard, C., Bayley, M. and Holmstrup, M. (2006). Does lipophilicity of toxic compounds determine effects on drought tolerance of the soil collembolan *Folsomia candida*? – Environmental Pollution 144: 808–815.

Stohs, S.J and Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. – Free Radical Biology and Medicine 18: 321–336.

Storey, K.B. and Storey, J.M. (1992). Biochemical adaptations for winter survival in insects. In: Advances in Low temperature Biology. (Ed. Steponkus, P.L.). – JAI Press, London, UK. pp. 101–140.

Sutinen, M.J., Raitio, H., Nivala, V., Ollikainen, R. and Ritari, A. (1996). Effects of emissions from copper–nickel smelters on the frost hardiness of *Pinus sylvetris* needles in the subarctic region. – New Phytologist 132: 503–512.

Sørensen, T.S. and Holmstrup, M. (2005). A comparative analysis of the toxicity of eight common soil contaminants and their effects on drought tolerance in the collembolan *Folsomia candida*. – Ecotoxicologi and Environmental Safety 60: 132–139.

Taulavuori, K., Prasad, M.N.V., Taulavuori, E. and Laine, K. (2005). Metal stress consequences of frost hardiness of plants at northern high latitudes: a review and hypothesis. – Environmental Pollution 135: 209–220.

Valko, M., Morris, H. and Cronin, M.T.D. (2005). Metals, toxicity and oxidative stress. – Current Medical Chemistry: 12: 1161–1208.

Van Gestel, C.A.M. (1997). Scientific basis for extrapolating results from soil ecotoxicity tests to field conditions and the use of bioassays. In: Ecological Risk Assessment of Contaminants in Soil. (Ed. van Straalen, N.M. and Løkke, H.). – Chapman and Hall, London, UK. pp. 25–50.

Walker, C.H., Hopkin, S.P., Sibly, R.M. and Peakall, D.B. (1996). Principles of Ecotoxicology. – Taylor and Francis, London, UK.

Weinstein, J.E., Oris, J.T. and Taylor, D.H. (1997). An ultrastructural examination of the mode of UV–induced toxic action of flouranthene in the fathead minnow, *Pimephales promelas*. – Aquatic Toxicology 39: 1–22.

Wharton, D.A. and Ferns, D.J. (1995). Survival of intracellular freezing by the Antarctic nematode *Panagrolaimus davidi*. – Journal of Experimental Biology 198: 1381–1387.

Wu, D.W. and Duman, J.G. (1991). Activation of antifreeze proteins from larvae of the beetle *Dendroides Canadensis*. – Journal of Comparative Physiology 161B: 279–281.

Wu, D.W., Duman, J.G., Cheng, C.H.C. and Castellino, F.J. (1991). Purification and charatization of antifreeze proteins from larvae of the beetle *Dendroides Canadensis*. – Journal of Comparative Physiology 161B: 271–278.

Zachariassen, K.E. (1980). The role of polyols and nucleating agents in cold-hardy beetles. – Journal of Comparative Physiology 140: 227–234.

Zachariassen, K.E. (1985). Physiology of cold tolerance in insects. – Physiological Review 65: 799–832.

Zachariassen, K.E and Lundheim, R. (1995). Effects of environmental pollutants on the cold–hardiness of Arctic and boreal ectothermic animals. In: The Contamination in the Nordic Ecosystem: Dynamics, Processes and Fate. (Ed. Munawar, M. and Luotola, M.). – SPS Academic Publishing, Amsterdam, The Netherlands. pp. 71–83.

Zachariassen, K.E., Kristiansen, E. and Pedersen, S.A. (2004). Inorganic ions in cold–hardiness. – Cryobiology 48: 309–321.

Zelenia, M., Tritto, S., Bondar, A.A., Zeleni, S. and Aperia, A. (2004). Copper inhibits the water and glycerol permeability of aquaporin–3. – Journal of Biological Chemistry 279: 51939–51943.

Paper 1

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Life-history traits and population growth rate in the laboratory of the earthworm *Dendrobaena octaedra* cultured in copper-contaminated soil

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Life-history traits and population growth rate in the laboratory of the earthworm *Dendrobaena octaedra* cultured in copper-contaminated soil

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Abstract

A study on the widespread earthworm *Dendrobaena octaedra* was conducted to determine which individual life history traits were the most sensitive to copper and to determine the contribution of changes in individual traits to changes in the population growth rate (λ). The study showed that the effect of copper on population growth rate mirrored the effects seen on growth, maturation and reproductive output, with stimulation at the lowest concentrations and inhibition at the highest concentration. A decomposition analysis showed that the mean change in λ was mainly driven by time between consecutive cocoon productions, except at the highest copper concentration (200 mg/kg dry soil) where decreased production of fertile cocoons also contributed to the reductions in λ. The highest population growth rate ($\lambda = 1.18\ \text{week}^{-1}$) occurred at 80 mg Cu/kg dry soil. At higher concentrations λ became gradually smaller, and was almost $1\ \text{week}^{-1}$ (where no population increase or decrease occurs) at the highest exposure concentration of 200 mg Cu/kg dry soil suggesting that extinction would occur if a population of *D. octaedra* were to be exposed to copper concentrations only slightly higher than this level.

Keywords: Ecotoxicology; Copper; Population growth rate; Elasticity analysis; Decomposition analysis

1. Introduction

Earthworms are important members of the soil fauna because of their ability to improve soil structure, their contribution to the breakdown of organic matter and release of plant nutrients (Edwards and Bohlen, 1996). The earthworm *Dendrobaena octaedra* is widely distributed in the northern boreal zone including Europe, Siberia, North America and Greenland (Stöp-Bowitz, 1969; Hendrix, 1995; Dymond et al., 1997; Berman et al., 2001). It lives and deposits its cocoons in litter, between plant roots, under moss and in decaying tree stumps (Stöp-Bowitz, 1969). As a result of its wide geographical distribution and since it is surface dwelling, populations of this species are frequently exposed to chemicals of anthropogenic origin. With respect to metals, surface litter and humus are the principal metal sinks in the forest floor (Bengtsson et al., 1983). Copper is one of the most common metal contaminants in terrestrial surface ecosystems. It can

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originate from smelters (Rozen, 2003), brass mills (Bengtsson et al., 1992), from the use of copper fungicides (Helling et al., 2000) or from the use of pig slurry as fertilizer (Kerr and McGavin, 1991).

Most available information on the effects of toxic compounds on earthworms has been biased toward measures of individual survival during short-term exposure to high concentrations or individual growth and reproduction during long-term exposure to lower concentrations (Van Gestel et al., 1991; Helling et al., 2000; Spurgeon et al., 2004). Recently, however, it has been emphasised that ecotoxicological investigations should preferably include the complete set of life-history parameters of an organism in order to evaluate more precisely how chemicals influence population growth dynamics and risk of extinction. The relationship between individual and population responses is not necessarily linear or simple. The application of life-history models could therefore increase our knowledge of how earthworm populations respond to toxic chemicals in the environment (Hansen et al., 1999; Spurgeon et al., 2003; Widarto et al., 2004). The importance of various life-history traits for the performance of species exposed to stress will depend on the life history strategy of the particular species tested (Kammenga and Riksen, 1996).

In the present study, we investigated the life-history traits of *D. octaedra* when exposed to sublethal concentrations of copper under laboratory conditions. Survival, growth, maturation time, time to first reproduction, cocoon production, time between production of cocoons and cocoon viability were investigated by following newly hatched juveniles kept in copper contaminated soil.

Thus, the main aims of this study were to determine which individual traits were the most/least sensitive to copper; to interpret the effects on individual traits in relation to effects on population growth rate (λ) and to identify which traits were mainly responsible for the effects on λ.

2. Materials and methods

2.1. Animals

D. octaedra were collected in a coniferous forest near Silkeborg, Jutland in 2003. The earthworms were kept in culture at 15 ± 1 °C in moist soil and fed on a diet of cow dung (50% cow dung and 50% soil). See the detailed description of soil type below. Cocoons collected from the culture were incubated in Petri dishes layered with wet filter paper. Undeveloped cocoons (yellow colour) were incubated at 20 °C, whereas developed cocoons (red colour) were incubated at 15 °C in order to slow down development and synchronize hatching within a short time interval. The newly hatched juveniles were kept in the Petri dishes for a maximum of 4 days without food at 15 °C until the start of the experiment.

2.2. Soil

Topsoil from an ecologically farmed Danish pea field (Foulum, Viborg) was used in all experiments. The soil was a loamy sand consisting of 35% coarse sand, 45% fine sand, 9.4% silt, 8.9% clay, 1.7% organic matter and a pH-H_2O of approximately 6.8. The total copper content of the soil was 11 mg/kg dry soil. Prior to use, the soil was dried for 24 h at 80 °C, sieved through a 2 mm mesh and stored at room temperature until use. In the toxicity experiments, the soil was spiked with $CuCl_2 \cdot 2H_2O$ (>99% purity, Merck, Darmstadt, Germany), and the water content adjusted to 20% of dry weight (pF approximately 2, corresponding to approximately 50% of water holding capacity). The soil was stored for one day before further use.

2.3. Life–history traits

A total of 70 newly hatched worms (2–4 days old) were exposed to copper. Ten randomly chosen individuals were exposed at the 4 lowest concentrations (0, 40, 80 and 120 mg Cu/kg soil) and 15 individuals at the two highest concentrations (160 and 200 mg Cu/kg soil). The average weights of the worms in each treatment were equal. Each individual was placed separately in a 200 ml plastic beaker (diameter 7 cm; height 4.2 cm) containing 50 ± 1 g moist soil (wet weight) with the required concentration of copper and 4.0 ± 0.1 g earthworm food (wet weight). The cow-dung feed was produced by adding 400 ml demineralised water to 150 g dried and finely ground cow-dung. Every 4 weeks the substrate and food was renewed using freshly prepared soil as described. From 4 weeks onwards, the amount of substrate was increased to 75 ± 1 g containing 6 ± 0.1 g food. All containers were covered with lids with ventilation holes, and kept in cardboard boxes at 15 ± 1 °C.

Every second week, mortality, fresh weight and developmental stage of the worms were determined up to the 20th week. Mortality was confirmed if remains were found, but if this was not the case the soil was hand sorted again at the following sampling date, and if the worm was still not found it was assumed dead at the earlier inspection date.

The surviving worms were rinsed in tap water, gently dried on filter paper, and weighed to the nearest 0.1 mg before being put back into the container again. Sexual development was recorded according to the scheme of Van Gestel et al. (1991). Worms with a full clitellum were recorded as adults, those with fully developed tubercula pubertatis but no clitellum as subadults, and individuals without either of these reproductive structures as juveniles. Cocoons were sampled by wet sieving the soil through a 1-mm mesh every fourth week. Cocoons to be used for analysis of internal copper concentrations were kept in the original copper spiked soil at 5 °C to halt development (Holmstrup et al., 1991). To determine the viability of cocoons produced in the contaminated soil, 26–38 cocoons from each copper concentration were incubated in Petri dishes on wet filter paper at 20 °C, and their hatching recorded.

2.4. Population growth rate (λ)

We calculated λ for each treatment by fitting the life-history data to a two-stage model (Calow and Sibly, 1990):

$$1 = nS_j\lambda^{-t_j} + S_a\lambda^{-t_a}$$

where n denotes the number of hatched cocoons per worm per week (in *D. octaedra* only one juvenile hatches from each cocoon), S_j represents juvenile survival (the probability that a juvenile survives from birth to first reproduction), S_a represents adult survival (the probability that an adult survives between census dates), and t_j is the time to first reproduction measured as the time from a cocoon being produced until the time where that individual produced its first cocoon. Since cocoons were collected by wet sieving at regular intervals, the time to first reproduction was estimated as the time elapsed to the first observation of cocoons minus half of the time interval between census dates (the cocoons are assigned an average production time exactly between the census dates). At the highest copper concentration some of the worms had still not produced any cocoons at the end of the experiment (week 20). In this case we made the assumption that such a worm would have produced the first cocoon two weeks after the last census date. According to Holmstrup et al. (1991) hatching time is 92 days at 15 °C. Finally, t_a is the time between the production of cocoons, which was estimated from the period between census dates divided by the number of cocoons produced during that census period.

The effect of copper on population growth rate was analysed by calculating 95% confidence intervals around mean values of λ. The confidence intervals were calculated from the total variance in λ, which is the sum of the variance contributions from each of the five life-history traits (n, t_j, t_a, S_j, S_a) as described by Sibly et al. (2000). Differences between population growth rates at differing copper concentrations were judged to be statistically significant if there was no overlap in the corresponding 95% confidence intervals.

2.5. Elasticity and decomposition analysis

An elasticity analysis was carried out to determine the relative sensitivity of population growth rate (λ) to changes in each of the five life history traits, n, t_j, t_a, S_j and S_a. Elasticity was measured using the equation $e_a = (a/\lambda)(d\lambda/da)$, where a is a life-history trait and $d\lambda/da$ is the sensitivity of λ to changes in the particular life-history trait a (Caswell, 2001). The elasticity was calculated by implicit differentiation as described in Forbes et al. (2001), i.e.,

$$\frac{t_j}{\lambda}\frac{\partial\lambda}{\partial t_j} = -\frac{nS_jt_j\ln(\lambda)}{T},$$

$$\frac{t_a}{\lambda}\frac{\partial\lambda}{\partial t_a} = -\frac{S_at_a\lambda^{t_j-t_a}\ln(\lambda)}{T}, \quad \frac{n}{\lambda}\frac{\partial\lambda}{\partial n} = \frac{nS_j}{T},$$

$$\frac{S_j}{\lambda}\frac{\partial\lambda}{\partial S_j} = \frac{nS_j}{T}, \quad \frac{S_a}{\lambda}\frac{\partial\lambda}{\partial S_a} = \frac{S_at_a\lambda^{t_j-t_a}}{T}$$

where the generation time T is defined as $T = nS_jt_j + S_at_a\lambda^{t_j-t_a}$.

Decomposition analysis was conducted to determine how much each life-history trait contributed to the mean changes in λ. The effect of copper on the contribution of each life-history trait to λ was calculated following Caswell (1989) and Levin et al. (1996).

2.6. Determination of copper concentration in adults

At the end of the experiment, i.e., after 20 weeks, 4–6 worms from each concentration were individually placed in Petri dishes on moist filter paper for 48 h at 15 °C to allow the worms to empty their gut. The animals were then frozen at −80 °C and freeze-dried before copper analysis. The dried samples were acid digested using concentrated nitric acid at increasing temperature (80–129 °C) until the samples were dry. When all fluid had evaporated 1 ml 14 M nitric acid was added and again heated until dryness. The samples were redissolved in 1.5 ml 0.1 M nitric acid and analysed using flame atomic absorption spectrometry (AAS; Perkin-Elmer 4100, Ueberlingen, Germany). Certified

reference material (oyster tissue material from the NBS, US National Institute of Standards and Technology, Department of Commerce, United States, and lobster hepatopancreas from the National Research Council Canada) was analysed to verify the efficiency of the digestion and AAS procedure, resulting in a measured concentration of approximately 95% of the certified values. All samples were analysed in one run.

2.7. Determination of copper concentration in cocoons

Previous observations (M. Holmstrup, unpublished) have shown that a significant amount of copper is adsorbed onto the cocoon wall. This copper is probably not available to the developing embryo, and it is therefore more sensible to estimate the copper concentration of cocoon contents rather than of the whole cocoon. The cocoons were sampled by wet sieving, gently dried and the fresh weight determined to the nearest 0.01 mg. After weighing, the cocoon contents were squeezed out into the bottom of an acid-rinsed glass test tube (3 ml) sealed with Parafilm. The cocoon wall was weighed and the mass of the cocoon contents (albuminous fluid and embryo) calculated. The cocoon contents were acid digested as described above. The samples originating from the 40 and 80 mg Cu/kg soil treatments were re-dissolved in 1.5 ml supra pure 0.1 M HNO_3, those from 120 mg Cu/kg soil in 2.5 ml, and those from the 160 mg Cu/kg soils in 3 ml. The supra pure 0.1 M HNO_3 was prepared by mixing 6 ml concentrated (16 M) supra pure HNO_3 (J.T. Baker) in 1 l ELGA-purified water (ELGA Maxima, ELGA Ldt, UK). The re-dissolved samples were transferred to disposable polyethylene cups (3 ml, Sarstedt, no. 73.646) and analyzed using a Perkin-Elmer 5100pc graphite furnace AAS (Norwalk, CT, USA). The copper concentration was calculated on the basis of estimated tissue dry weight. This was calculated from the wet mass data and the relative water content of embryo tissue, which was assumed to be 75.7% (Holmstrup, 1994). Certified reference material (see previous section) was analysed to verify the efficiency of the digestion and graphite furnace procedure, resulting in an average 94% of the certified values.

2.8. Statistical analysis

One-way ANOVA was used to test for differences in maturation time, time between production of cocoons (t_a), time to first reproduction (t_j) and total cocoon production. Growth data were analysed with repeated measures ANOVA. Data were transformed appropriately to achieve homogeneity of variance and normal distribution. In cases where the F-test was significant Tukey's multiple comparisons test was used to determine differences between specific treatments.

3. Results

3.1. Survival

Juvenile survival was affected only at the highest copper concentration (200 mg/kg dry soil), where 5 out of 15 worms died within the first 12 weeks, not reaching a weight above 10 mg. Copper in concentrations up to 200 mg/kg soil had no apparent effect on adult survival. This was also evident in an earlier range finding experiment where copper concentrations up to 300 mg/kg soil caused no mortality in adult worms (data not shown).

3.2. Growth

The worms in all groups gained weight until week 16 after which most groups ceased to grow (Fig. 1). There was a significant effect of time ($p < 0.0001$), copper ($p < 0.0001$), and a significant interaction between time and copper ($p < 0.0001$). The results indicate that growth was stimulated at all copper concentrations except for the worms exposed to 200 mg Cu/kg. A post hoc Tukey test showed that the worms exposed to 200 mg Cu/kg had a significantly lower weight than the worms at the other exposure concentrations at week 10, 14 and 18 ($p < 0.05$). At week 10, 14 and 18 the fresh weight of worms exposed to 80 mg Cu/kg was

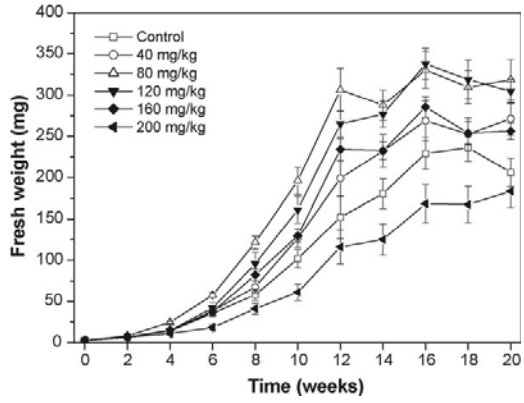

Fig. 1. Growth of *Dendrobaena octaedra* in a loamy sand soil with different levels of copper spiking. Error bars indicate standard error of the mean.

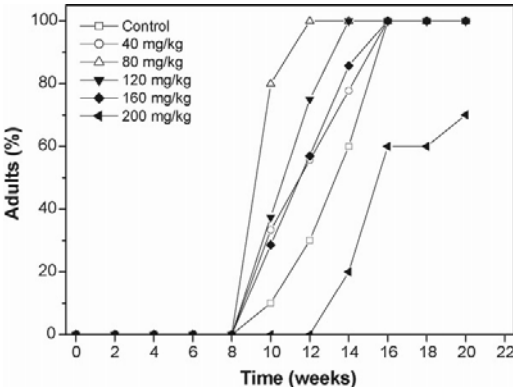

Fig. 2. Maturation time of newly hatched *D. octaedra* in a loamy sand soil with different copper treatments shown as the percentage of the individuals that have reached the adult stage (n = 10–15). The values for 200 mg/kg include only the worms that survived the juvenile stage.

significantly higher than all other groups except those exposed to 120 mg Cu/kg (Tukey, $p < 0.05$).

3.3. Maturation time

Maturation time followed the same pattern as growth; the faster worms grew the faster they reached maturity. Maturation time was shortest for the worms exposed to 80 mg Cu/kg where 100% reached maturity after 12 weeks (Fig. 2). At week 14 all the worms exposed to 120 mg Cu/kg reached maturity and at week 16 all in control, 40 and 160 mg Cu also reached maturity. At the end of the experiment (i.e., after 20 weeks) 70% of the worms exposed to the highest concentration had reached maturity (7 out of 10 worms). To calculate mean maturation time at 200 mg/kg where not all individuals were mature at the end of the experiment, we assumed that those still subadult would be fully mature within 2 weeks, and that those still juvenile needed four more weeks to reach maturity. This assumption was based on observations of growth and maturity during the experiment (data not shown). The estimated mean maturation time of 18 weeks at the highest concentration was significantly longer than the mean maturation time of controls, which was 14 weeks (Tukey, $p < 0.0001$). The shortest maturation time was found at 80 mg Cu/kg (10.4 weeks).

3.4. Time to first reproduction

The worms exposed to the highest concentration needed significantly longer time to reach the reproductive stage. On average these worms produced the first cocoon after 31.4 weeks, whereas the worms in the control produced cocoons after 27.2 weeks (Table 1). The worms exposed to 80 mg Cu/kg produced cocoons as early as after 24.3 weeks, but this was not significantly different from the worms exposed to 40, 120 and 160 mg/kg.

3.5. Cocoon production and time between production of individual cocoons

Cocoons were observed for the first time at week 12 (Fig. 3). The worms exposed to 80 mg Cu/kg had the highest production of cocoons and reached a total of 33.4 cocoons per worm during the whole period of 20 weeks which was significantly higher than the mean cumulated production of 16.9 cocoons per worm in controls in the same period (Tukey, $p < 0.0001$). The average total production was 3.6 cocoons per worm for the worms exposed to 200 mg/kg, which was significantly lower than the production at all other exposures.

The interval between consecutive cocoon productions was almost three weeks for the worms exposed to 200 mg/kg and this was significantly longer than for the other exposure groups (Table 1). The worms exposed to

Table 1
Time to first reproduction (t_j), time between production of cocoons (t_a), and hatchability of cocoons of *Dendrobaena octaedra* exposed to copper contaminated loamy sand soil

Copper concentration (mg/kg dry soil)	t_j (weeks) (mean ± S.E.M.)	t_a (weeks) (mean ± S.E.M.)	Hatchability (%)
0 (control)	27.2 ± 0.74 a	0.89 ± 0.25 ac	90
40	26.0 ± 0.5 ab	0.5 ± 0.06 abc	91
80	24.3 ± 0.3 b	0.34 0.02 b	74
120	25.1 ± 0.54 ab	0.37 ± 0.029 abc	79
160	25.5 ± 0.42 ab	0.52 ± 0.05 ac	74
200	31.4 ± 1.26 c	2.99 ± 0.16 d	31

Mean values followed by the same letters are not statistically different (Tukey, $P > 0.05$).

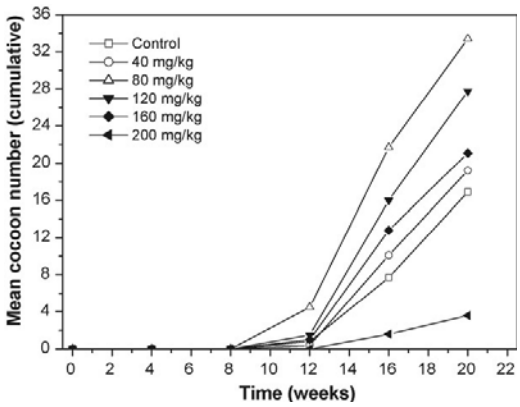

Fig. 3. Mean cumulative cocoon production of *D. octaedra* in a loamy sand soil spiked with different copper concentrations (n = 10–15).

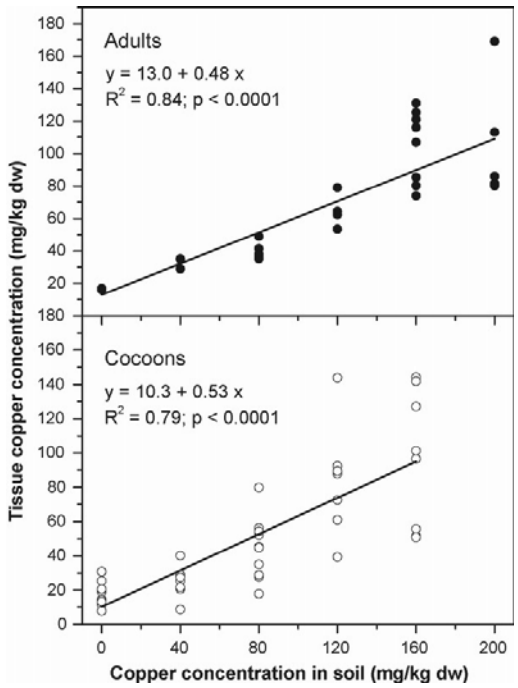

Fig. 4. Copper concentrations in worm tissues of 20 weeks old adult *D. octaedra* that had been exposed to copper in a loamy sand soil throughout their lives (Upper panel), and copper concentration in cocoon contents (excluding walls) produced in copper contaminated soil (lower panel). Note that copper in cocoon contents may originate both from parents and from soil. Linear regression lines describing the relationship between soil and tissue concentrations are fitted.

80 mg/kg showed the shortest time between consecutive cocoon productions of 0.34 weeks.

3.6. Copper content of cocoons and adult worms

A significant positive correlation was found between the copper concentrations in cocoons and substrate and between those in adults and substrate (Fig. 4). The adult control worms contained approximately 16 mg Cu/kg dry tissue weight and this increased to approximately 100 mg/kg at the two highest exposure concentrations. The same result was observed for cocoon contents (excluding the cocoon wall) with a concentration of about 18 mg/kg in controls increasing up to about 96 mg/kg at the highest exposure concentration where enough cocoons were available for analysis (160 mg/kg soil).

3.7. Hatching success

The viability of the cocoons declined with exposure to increasing copper concentrations (Table 1). Approximately 90% hatched in controls, whereas only 31% hatched at the highest copper concentration of 200 mg/kg.

3.8. Population growth rate (λ)

The estimated population growth rate increased from control to 80 mg/kg and then declined again and reached the lowest value at 200 mg/kg (Fig. 5). Mean λ at 80 mg Cu/kg was significantly higher than at all other concentrations except for 120 mg Cu/kg. There was no significant difference between λ in control, 40, 120 and

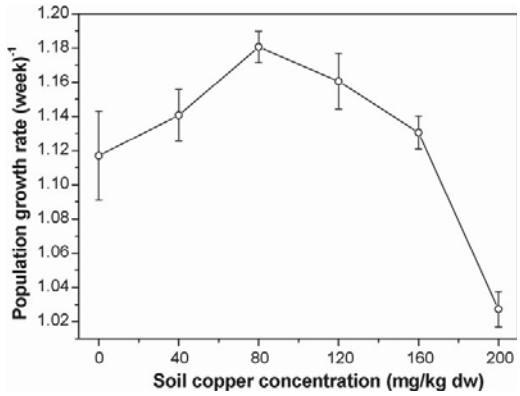

Fig. 5. Population growth rate (λ) of *D. octaedra* exposed to copper in a loamy sand soil. Error bars represent 95% confidence intervals.

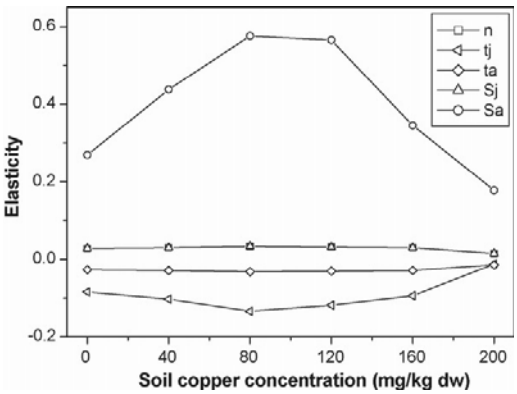

Fig. 6. Elasticity of hatched cocoons per worm per week (n), time to first reproduction (t_j), time between production of cocoons (t_a), juvenile survival (S_j) and adult survival (S_a) of *D. octaedra* as a function of copper exposure in a loamy sand soil.

160 mg/kg copper exposure. The population growth rate of 1.02 week^{-1} at 200 mg/kg was much lower than at the other concentrations and was very close to unity, i.e., where no population growth occurs.

3.9. Elasticity and decomposition analysis

The elasticity analysis showed that λ was most sensitive to changes in adult survival (S_a), which in the present study was not affected by any of the copper concentrations assessed. The sensitivity of λ to this trait was several times higher than to the other traits and was least sensitive at the lowest and highest copper concentrations (Fig. 6). A negative elasticity value means that as the value of the trait becomes larger (e.g., t_a, t_j), the value of λ declines. For other traits (such as n, S_j, S_a) the larger the value of the trait, the greater λ becomes. λ was moderately sensitive to changes in the time to first reproduction (t_j) except at the highest concentration where λ was relatively insensitive. This was also the case for juvenile survival (S_j), time between production of cocoons (t_a) and number of hatched cocoons per worm per week (n). The elasticity for S_j, t_a and n was largely unchanged as a function of copper exposure.

In a decomposition analysis the copper-influenced change in λ is distributed among the different life history stages in proportion to its importance, i.e., a life stage which is highly affected by copper and has a large effect on λ will show up in the decomposition analysis with a high relative proportion. The decomposition analysis showed that, in all but the highest copper treatment, time between productions of cocoons (t_a) was the trait causing most of the changes in λ with an average contribution to change in λ of 54.5% (Table 2). The average contribution of time to first reproduction and fecundity to changes in λ were approximately the same (20.2% and 23.7%, respectively). At the highest copper concentration λ was affected most by a reduction in fecundity (n) which caused 51% of the reduction in λ followed by (t_a) which caused a further 34% of the change.

Table 2
Decomposition analysis of life history traits in *D. octaedra* exposed to copper contaminated loamy sand soil

Copper concentration (mg/kg dry soil)	Proportional contribution of individual traits (%)			
	S_j	t_j	t_a	N
0 (control)				
40	0	16.9	67.6	15.5
80	0	20.0	56.3	23.6
120	0	16.7	60.6	22.8
160	0	40.6	53.8	5.6
200	7.7	6.9	34.3	51.1
Mean	1.5	20.2	54.5	23.7

4. Discussion

4.1. Stimulation of population growth rate

In the present study we have shown that copper stimulated growth, maturation time and reproduction in *D. octaedra* at moderate copper concentrations, but inhibited these same parameters at higher exposure concentrations. Adult survival was unaffected at any of the concentrations tested and juvenile survival was only affected at the highest concentration of 200 mg Cu/kg soil. The viability of the cocoons, on the other hand, showed a monotone decrease with increasing copper exposure (Table 1). As a result, the effects of copper on population growth rate mirrored the effects seen on growth, maturation and reproductive output, with stimulation at the lowest concentrations and inhibition at the highest concentration.

Stimulation of for example body growth and reproduction by a toxicant is not an unusual phenomenon in ecotoxicological studies. This has been observed in several experiments including ones with earthworms (Calabrese and Baldwin, 1998; Spurgeon et al., 1994, 2003). Spurgeon et al. (2004) exposed the earthworm *Lumbricus rubellus* to clay loam spiked with copper, and also observed stimulation of growth at lower copper concentrations (10 and 40 mg/kg dry soil), no effect at 160 mg/kg, and a reduction only at the

highest exposure concentration of 640 mg/kg. Since this soil was a clay loam soil it is likely that the bioavailability was lower than in the loamy sand soil used in the present study. Spurgeon et al. (2004) interpreted the stimulation at lower concentrations as hormesis—the stimulatory effect caused by low levels of potentially toxic agents (Stebbing, 1982). However, this stimulation in growth could also be explained by copper functioning as an antibiotic, hence reducing the presence of possible earthworm parasites and pathogens in the test soil. According to Gunnarsson and Rundgren (1986) copper significantly reduced the infestation of *Dendrodrilus rubidus* cocoons by parasitic nematodes. In their control group approximately 50% of cocoons were infested, causing death of the embryos, whereas only 15% were infested when exposed to 100 mg Cu/kg in a mixture of sand and dried cow dung.

4.2. Internal copper concentrations

Copper is an essential element and it is therefore important to view the results of this study in terms of the body accumulation since toxic effects can only be expected when copper levels exceed a threshold value within the organism (Bogomolov et al., 1996). The internal concentrations of copper observed in the present study were very similar to body concentrations found in a previous study under identical soil conditions but where worms were exposed for only 4 weeks at 2 °C (Bindesbøl et al., 2005). The internal concentrations measured in the present study were slightly higher, probably because of a longer exposure time at a higher temperature. The concentrations measured were also very similar to those measured by Weeks and Svendsen (1996) in *L. rubellus* under similar experimental conditions. It is interesting to note that Weeks and Svendsen (1996) observed negative effects on neutral red retention time (i.e., effects at the cellular level) even at body concentrations as low as 20 mg/kg dry weight, whereas negative effects at the level of individuals in our study only occurred at body concentrations in the range 80–170 mg/kg dry weight. Bengtsson et al. (1983) measured internal copper concentrations in *D. octaedra* collected in the field ranging from 100 to 300 mg/kg dry weight suggesting that internal concentrations in the present study are environmentally realistic.

The internal copper concentrations in the cocoons (Fig. 4) were very similar to the concentrations measured in the parent adult worms. Bengtsson et al. (1986) found 77–240 mg/kg (average 134 mg/kg) in *D. rubidus* cocoons but did not specify whether copper measurements were made on whole cocoons or only on cocoon contents. A large proportion of the copper entering the cocoons from soil is probably adsorbed onto the cocoon wall. The copper concentration in cocoon contents is biologically more relevant than the total cocoon concentration (Holmstrup et al., 1998) and the higher concentrations reported by Bengtsson et al. (1986) could be a result of including the cocoon wall in the measurements. Holmstrup et al. (1998) found copper concentrations up to 200 mg/kg dry weight in embryos of cocoons from *D. octaedra* exposed to aqueous copper solutions. The aqueous copper concentrations used were, however, much higher than would normally be found in the pore water of polluted soils (Marinussen et al., 1997).

According to Streit and Jäggy (1982) the earthworm *Octolasium cyaneum* regulates tissue copper concentrations from approximately 40 to 100 mg/kg when exposed in soil having copper concentrations up to 400 mg/kg. The increased variation in the tissue copper concentrations with increasing soil concentrations observed in the present study (Fig. 4) is likely to be caused by differences in individual efficiencies of the copper regulatory mechanisms in the adult worms, which is also reflected in the cocoons.

4.3. Population growth rate (λ)

Population growth rate is a better measure of responses to toxicants, than are effects on single life-history traits, because it integrates potentially complex interactions among the life-history traits and provides a more relevant measure of ecological impact (Forbes and Calow, 1999). The present study showed the same trend in population growth as in (individual) growth, maturation time and reproduction, i.e., an increase at moderate copper concentrations and significant reduction at 200 mg/kg. The control population growth rate had a mean value of 1.12 $week^{-1}$ compared to a value of approximately 1.19 $week^{-1}$ in a study by Widarto et al. (2004) using the same species in a similar study of toxicity of nonylphenol. The discrepancy between these two studies can be explained by a difference in the definition of time to first reproduction used in the two studies. In the present study we included the cocoon development time as part of the time to first reproduction whereas in Widarto et al. (2004) cocoon development time was excluded. Reanalysis of our data using their definition results in exactly the same value of 1.19 $week^{-1}$, which shows that this is a robust estimate of λ.

The population growth rate was declining at concentrations above 80 mg Cu/kg and very close to 1 week^{-1} at the highest exposure concentration of 200 mg Cu/kg dry soil, suggesting that extinction would occur if a population of *D. octaedra* were exposed to copper concentrations only slightly higher than 200 mg/kg. These concentrations are not unusual in the field, but it should be noted that our study may not be extrapolated uncritically to field conditions. Factors like density, competition, predation and food availability can probably modify the effects of copper when considering natural populations in contaminated areas. The copper concentrations used in this experiment are within the range that can be found in natural soils, which can vary from 2 to 250 mg/kg, depending on soil type and parent rock (Adriano, 1986). Near a brass mill in Southeast Sweden high copper concentrations are found in the litter layer in areas where populations of *D. octaedra* exist (Bengtsson et al., 1983). At the most contaminated sites closest to the mill copper concentrations exceeded more than 1000 mg/kg dry soil, and at these high concentrations no individuals of *D. octaedra* were found.

Elasticity measures the proportional change in λ resulting from a proportional change in a given life history trait, holding all other traits constant. If a trait has a high elasticity, then relatively small proportional changes in the trait will have a relatively large influence on λ. The life cycle of *D. octaedra* under the laboratory conditions used in the present study was such that the elasticity of adult survival (S_a) was much higher than the other traits. That is, a given impact on adult survival (especially at the moderate copper concentrations) would be expected to have much greater consequences for population dynamics than would a similar proportional impact on juvenile survival, time to first reproduction, time between production of cocoons and number of hatched cocoons per week. In the present study, adult survival was unaffected by copper, which means that even though this trait had a high elasticity it did not contribute to the change in population growth rate (λ). This phenomenon is not unusual and it seems to be a general observation that the most elastic traits are not those that make the largest contribution to the observed effects on population growth rate (Caswell, 1996). In the study by Widarto et al. (2004) juvenile survival and adult survival had the highest elasticities, but neither of them contributed to the changes in population growth rate because no mortality was observed in the toxicant range (4-nonylphenol) they used. It was therefore necessary to perform a decomposition analysis to determine the contribution of treatment on the individual life-history traits to the observed effects on λ (Caswell, 1989). Since no adult mortality was observed within the copper range used in this experiment, the contribution of adult survival was zero. By contrast the decomposition analysis showed that the mean change in λ was mainly explained by time between production of cocoons, which had an average contribution of 54.5% to the change in λ, except at the highest concentration where both fecundity (51%) and the time between production of individual cocoons (34%) both contributed to the reduction in λ.

The present study has shown that the effect of copper on population growth rate (Fig. 5) mirrored the effects seen on growth, maturation and reproductive output (Figs. 1–3), with a stimulation at the lowest concentrations and an inhibition at the highest concentration. If we had based our assessment of copper toxicity on adult survival alone we would not have seen any effect at the exposure range used in this study. Likewise, juvenile *D. octaedra* did develop to adulthood and produced cocoons, albeit at a slow rate, even at the highest tested concentration, which might lead one to believe that populations would not be critically affected under these circumstances. However, the observed population growth rate at this concentration suggests that the population would be at risk if other stress factors are also influencing population growth, e.g., competition or limited food availability. Thus, the trends for a single trait cannot necessarily be directly related to the effects at the population level, and it seems that the full analysis of life history traits including the calculation of λ, provides a more sensitive measure of the sustainability of a population than the individual life history traits by themselves. The fact that exposure to copper and other metals can result in local extinction of sensitive earthworm communities (Abdul and Bouché, 1995; Bengtsson et al., 1983; Spurgeon and Hopkin, 1999), emphasizes the need in risk assessment of contaminated soils to be able to predict the potential effects of chemical exposure on the stability of earthworm populations.

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References

Abdul, A.M.M., Bouché, M.B., 1995. The eradication of an earthworm genus by heavy metals in southern France. Appl. Soil Ecol. 2, 45–52.

Adriano, D.C., 1986. Trace Elements in The Terrestrial Environment. Springer-Verlag, New York, pp. 867.

Bengtsson, G., Nordström, S., Rundgren, S., 1983. Population density and tissue metal concentration of lumbricids in forest soils near a brass mill. Environ. Pollut. 30, 87–108.

Bengtsson, G., Gunnarsson, T., Rundgren, S., 1986. Effects of metal pollution on the earthworm *Dendrobaena rubida* (sav) in acidified soils. Water Air Soil Pollut. 28, 361–383.

Bengtsson, G., Ek, H., Rundgren, S., 1992. Evolutionary response of earthworms to long-term metal exposure. Oikos 63, 289–297.

Berman, D.I., Meshcheryakova, E.N., Alfimov, A.V., Leirikh, A.N., 2001. Spread of the earthworm *Dendrobaena octaedra* (Lumbricidae: Oligochaeta) from Europe to Northern Asia is restricted by its insufficient freeze tolerance. Dokl. Biol. Sci. 377, 145–148.

Bindesbøl, A., Holmstrup, M., Damgaard, C., Bayley, M., 2005. Stress synergy between environmentally realistic levels of copper and frost in the earthworm *Dendrobaena octaedra*. Environ. Toxicol. Chem. 24, 1462–1467.

Bogomolov, D.M., Chen, S.-K., Parmelee, R.W., Subler, S., Edwards, C.A., 1996. An ecosystem approach to soil toxicity testing: a study of copper contamination in laboratory soil microcosms. Appl. Soil Ecol. 4, 95–105.

Calabrese, E.J., Baldwin, L.A., 1998. Hormesis as a biological hypothesis. Environ. Health Persp. 106 (Suppl. 1), 357–362.

Calow, P., Sibly, R.M., 1990. A physiological-basis of population processes – ecotoxicological implications. Funct. Ecol. 4, 283–288.

Caswell, H., 1989. Analysis of life table response experiments I. Decomposition of effects on population growth rate. Ecol. Model. 46, 221–237.

Caswell, H., 1996. Analysis of life history table response experiments II. Alternative parameterization for size- and stage-structured models. Ecol. Model. 88, 73–82.

Caswell, H., 2001. Matrix Population Models. Construction, Analysis and Interpretation, 2nd ed. Sinauer, Sunderland, pp. 727.

Dymond, P., Scheu, S., Parkinson, D., 1997. Density and distribution of *Dendrobaena octaedra* (Lumbricidae) in a and pine forests in the Canadian Rocky Mountains (Alberta). Soil Biol. Biochem. 29, 265–273.

Edwards, C., Bohlen, P., 1996. Biology and Ecology of Earthworms, 3rd ed. Chapman and Hall, London, pp. 426.

Forbes, V.E., Calow, P., 1999. Is the per capita rate of increase a good measure of population-level effects in ecotoxicology? Environ. Toxicol. Chem. 18, 1544–1556.

Forbes, V.E., Calow, P., Sibly, R.M., 2001. Are current species extrapolation models a good basis for ecological risk assessment? Environ. Toxicol. Chem. 20, 442–447.

Gunnarsson, T., Rundgren, S., 1986. Nematode infestation and hatching failure of lumbricid cocoons in acidified and polluted soils. Pedobiologia 29, 165–173.

Hansen, F.T., Forbes, V.E., Forbes, T.L., 1999. Effects of 4-*n*-nonylphenol on life-history traits and population dynamics of a polychaete. Ecol. Appl. 9, 482–495.

Helling, B., Reinecke, S.A., Reinecke, A.J., 2000. Effects of the fungicide copper oxychloride on the growth and reproduction of *Eisenia fetida* (oligochaeta). Ecotoxicol. Environ. Safety 46, 108–116.

Hendrix, P.F., 1995. Earthworm Ecology and Biogeography in North America. Lewis Publishers, Boca Raton, p. 256.

Holmstrup, M., Østergaard, I.K., Nielsen, A., Hansen, B., 1991. The relationship between temperature and cocoon incubation time for some lumbricid earthworm species. Pedobiologia 35, 179–184.

Holmstrup, M., 1994. Physiology of cold hardiness in cocoons of five earthworm taxa (Lumbricidae: Oligochaeta). J. Comp. Physiol. B 164, 222–228.

Holmstrup, M., Petersen, B.F., Larsen, M.M., 1998. Combined effects of copper, desiccation and frost on the viability of earthworm cocoons. Environ. Toxicol. Chem. 14, 897–901.

Kammenga, J.E., Riksen, J.A.G., 1996. Comparing differences in species sensitivity to toxicants: phenotypic plasticity versus concentration–response relationships. Environ. Toxicol. Chem. 15, 1649–1653.

Kerr, L.A., McGavin, H.D., 1991. Chronic copper poisoning in sheep grazing pastures fertilized with swine manure. J. Am. Vet. Med. Assoc. 198, 99–101.

Levin, L., Caswell, H., Bridges, T., DiBacco, C., Cabrera, D., Plai, G., 1996. Demographic responses of estuarine polychaetes to pollutants: life table response experiments. Ecol. Appl. 6, 1295–1313.

Marinussen, M.P.J.C., Van der Zee, S.E.A.T.M., de Haan, F.A.M., Bouwman, L.M., Hefting, M.M., 1997. Heavy metal (copper, lead and zinc) accumulation and excretion by the earthworm *Dendrobaena veneta*. J. Environ. Qual. 26, 278–284.

Rozen, A., 2003. Do earthworms (*Dendrobaena octaedra*) from differently polluted forests vary in life-history parameters? Eur. J. Soil Biol. 39, 73–77.

Sibly, R.M., Hansen, F.T., Forbes, V.E., 2000. Confidence intervals for population growth rate of organisms with two-stage life histories. Oikos 88, 335–340.

Spurgeon, D.J., Hopkin, S.P., Jones, D.T., 1994. Effects of cadmium, copper, lead and zinc on growth, reproduction and survival of the earthworm *Eisenia fetida* (Savigny): assessing the environmental impact of point-source metal contamination in terrestrial ecosystems. Environ. Pollut. 84, 123–130.

Spurgeon, D.J., Hopkin, S.P., 1999. Seasonal variation in the abundance, biomass and biodiversity of earthworms in soils contaminated with metal emissions from a primary smelting works. J. Appl. Ecol. 36, 173–183.

Spurgeon, D.J., Svendsen, C., Weeks, J.M., Hankard, P.K., Stubberud, H.E., Kammenga, J.E., 2003. Quantifying copper and cadmium impacts on intrinsic rate of population increase in the terrestrial oligochaete *Lumbricus rubellus*. Environ. Toxicol. Chem. 22, 1465–1472.

Spurgeon, D.J., Svendsen, C., Kille, P., Morgan, A.J., Weeks, J.M., 2004. Responses of earthworms (*Lumbricus rubellus*) to copper and cadmium as determined as measurements of juvenile traits in a specifically designed test system. Ecotoxicol. Environ. Saf. 57, 54–64.

Stebbing, A.R.D., 1982. Hormesis—the stimulation of growth by low levels of inhibitors. Sci. Total Environ. 22, 213–234.

Stöp-Bowitz, C., 1969. A contribution to our knowledge of the systematics and zoogeography of Norwegian earthworms (Annelida Oligochaeta: Lumbricidae). Nytt. Mag. Zool. 17, 169–280.

Streit, B., Jäggy, A., 1982. Effect of soil type on copper toxicity and copper uptake in *Octolasium cyaneum* (Lumbricidae). In: Lebrun, Ph., André, H.M., Medts, A., Grégoire-Wibo, C., Wauthy, G. (Eds.), New Trends in Soil Biology. Dieu-Brichart, Ottignies-Louvain-la-Neuve, Belgium, pp. 569–575.

Van Gestel, C.A.M., Van Dis, W.A., Van Breemen, A.M.D., Sparenburg, P.M., Baerselmann, R., 1991. Influence of cadmium, copper and pentachlorophenol on the growth and sexual development of *Eisenia andrei* (Oligochaeta Annelida). Biol. Fertil. Soils 12, 117–121.

Weeks, J.M., Svendsen, C., 1996. Neutral red retention by lysosomes from earthworm (*Lumbricus rubellus*) coelomocytes: a simple biomarker of exposure to soil copper. Environ. Toxicol. Chem. 15, 1801–1805.

Widarto, T.H., Holmstrup, M., Forbes, V.E., 2004. The influence of nonylphenol on the life-history traits of earthworm *Dendrobaena octaedra* Savigny: linking effects from individual- to the population-level. Ecotoxicol. Environ. Saf. 58, 147–159.

Paper 2

Photo: Brian Rasmussen

Stress synergy between environmentally realistic levels of copper and frost in the earthworm *Dendrobaena octaedra*

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STRESS SYNERGY BETWEEN ENVIRONMENTALLY REALISTIC LEVELS OF COPPER AND FROST IN THE EARTHWORM *DENDROBAENA OCTAEDRA*

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Abstract—In their natural habitat, animals are exposed to a variety of stress factors, including extreme temperatures, low water availability, and toxic stress from chemical pollutants. In this study we examined the interaction between realistic environmental levels of soil–copper contamination and realistic winter temperatures on survival of the cosmopolitan freeze-tolerant earthworm *Dendrobaena octaedra*. These interactions were tested using a full factorial design with six copper concentrations between 0 and 200 mg Cu/kg dry soil and five temperatures from +2 to −8°C. A highly significant synergistic interaction existed that demonstrates that exposure to subzero temperatures significantly reduced copper tolerance and, conversely, that copper exposure significantly reduced freeze tolerance. Copper had no effect on glucose production, which is believed to be a major component of the cryoprotective system and the only known cryoprotectant in *D. octaedra*. This points to other mechanisms behind the observed synergy, possibly impaired osmoregulatory function of the cell membrane. The results support the working hypothesis that interactions between toxicants and dominant natural stress factors can alter the organisms' tolerance to these individual stressors.

Keywords—Frost tolerance Copper *Dendrobaena octaedra* Synergy Risk assessment

INTRODUCTION

In natural environments it is not unusual for an organism to be exposed to several stressful factors, both physical and chemical, at the same time. These could include climatic stress or the exposure to chemicals of anthropogenic origin. In traditional ecotoxicological studies, organisms usually are exposed to a single chemical at increasing concentrations, while factors such as temperature and moisture content are held at a constant optimum. These traditional laboratory tests, therefore, can lead to an underestimate of the toxicity of the chemical in natural environments where the organism periodically will encounter several physical and chemical factors simultaneously. In the context of the current concerns over global climate changes, it is important to study the interactions between the toxic chemicals present in the environment and the climatic events likely to alter species biogeography.

Earthworms are important members of the soil fauna due to their ability to improve soil structure and their contribution to the breakdown of organic matter and release of plant nutrients [1]. *Dendrobaena octaedra* has a worldwide distribution. It is present in almost the entire European forest zone and tundra, and also is found in Russia (Siberia), North America, Canada, and Greenland [2–5]. Because of its wide geographical distribution, populations of this species frequently are exposed to chemicals of anthropogenic origin. Examples include heavy-metal pollution of surface soils from smelters [6] and brass mills [7] or from the use of copper fungicides [8]. Surface litter and humus are the principal metal sinks in the forest floor [9]. This means that *D. octaedra*, which is a litter-dwelling species, is likely to be more exposed to metals than burrowing species.

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One of the climatic factors important in defining the geographical limits of ectotherm species is the winter temperature regime and many ectotherm species at some time in their life span will be exposed to subzero temperatures that may cause freezing of their body fluids. These cold-hardy animals have developed one of two strategies: Freeze-avoidance or freeze-tolerance [10]. Freeze-avoiding species die if frozen. They survive subzero temperatures by supercooling their body fluids or dehydrating until the melting point of their body fluids is lowered to the ambient temperature [11,12]. Freeze-tolerant species, on the other hand, can survive freezing of their extracellular body fluids. The earthworm *D. octaedra* is one of the only two known earthworm species that can survive this extracellular ice formation [13]. Although most other earthworm species migrate to deeper soil layers to avoid subzero temperatures, *D. octaedra* overwinters in the litter layer [14]. To facilitate this winter survival, *D. octaedra* accumulates glucose to high concentrations at the onset of the body fluid–freezing process [15]. This accumulation of glucose contributes to a slowing down of the freezing process, and to a reduction in the amount of ice formed. Furthermore, glucose is known to aid in the preservation of the structure and function of membranes and proteins during freezing-induced dehydration of tissues [16,17].

The aim of the present study was to investigate the possible existence of a synergistic interaction between a common metal contaminant at realistic and sublethal soil concentrations and realistic winter temperatures in *D. octaedra*. Further, because of the importance of glucose production to winter survival in this species, the effects of copper on glucose synthesis were investigated.

MATERIALS AND METHODS

Animals

Animals used in this study were subadult and adult individuals of *D. octaedra* collected in the year 2000 around the

Arctic Station, Godhavn, Disko West Greenland, and since kept in culture at 15°C in the soil described below on a diet of cow dung. Animals used for experimentation were approximately three months old and had a fresh weight of between 100 and 320 mg.

Soil

Topsoil from an ecologically farmed Danish pea field (Foulum, Viborg) was used for the experiment. The soil was a loamy sand consisting of 35% coarse sand, 45% fine sand, 9.4% silt, 8.9% clay, and 1.7% organic matter. The pH–H_2O was approximately 6.8. Prior to use, the soil was dried for 24 h at 80°C and sieved through a 2-mm mesh. The soil was copper-spiked with $CuCl_2 \cdot 2H_2O$ (>99%, Merck, Damstadt, Germany), and the water content adjusted to 20% of dry weight ($\sim pF$ = 2 and 50% water-holding capacity) and stored for 2 d before further use.

Experimental design

Copper and temperature were varied in a full factorial design with six Cu concentrations and five temperatures, giving a total of 30 treatments, including the control groups.

Animals were exposed to nominal soil concentrations of 0, 40, 80, 120, 160, and 200 mg Cu/kg dry weight for four weeks at 2°C before exposure to the experimental temperatures. Each group had three to 10 worms, depending on the treatment. Each worm was weighed and kept individually in a small container with 75 g of soil (wet wt) and 4 g of cow dung (wet wt) mixed into the soil. The cow-dung feed was produced by adding 400 ml of demineralized water to 150 g of dried and finely ground cow-dung. Animals in each treatment had the same average fresh weight. All containers were covered with lids having the same number of holes so that ventilation was equal between treatments. The pH was found to be equal (6.8) in all copper treatments after the addition of cow-dung. After four weeks at 2°C, 14 individuals from each Cu concentration were allowed to empty their gut for 48 h on filter paper kept at 5°C, rinsed, and then frozen at −80°C. Ten animals were used for copper analysis and four for glucose analysis. With the exception of the controls (+2°C), the remaining worms were placed in 8-ml tubes along with a few grams of the appropriate substrate. In each lid, two small needle holes were made for ventilation. The worms were exposed to −2, −4, −6, and −8°C in a freezer cabinet (WTB Binder Labortechnik, Tuttlingen, Germany). The freezer cabinet was programmed to lower the temperature from 0°C to −8°C at 0.042°C/h. When the temperature reached approximately −1.5°C, a small ice crystal was added to each tube to initiate freezing. As the temperature fell, the animals were removed to separate freezer cabinets at their intended experimental temperature. The animals remained at their experimental temperature for different periods such that each group remained at subzero temperatures for equal time.

Eight worms from each copper concentration were removed from the −2°C group and stored at −80°C for glucose analysis. Because Rasmussen and Holmstrup [13] showed that glucose production reached a maximum at −2°C, glucose was not measured at the lower temperatures.

When the final groups had reached their intended temperature (−8°C), the temperature was raised from −8 to 0°C within a 24-h period so that all groups reached 0°C at the same time. Prior to the assessment of survival rate, the temperature was raised from 0 to 2°C and the worms allowed 24 h to thaw. The earthworms were considered to have survived if there was a reaction to tactile stimuli, normal locomotor activity, and no visible signs of freezing damage.

Glucose analysis

Worms were removed from the −80°C freezer and freeze-dried immediately for 24 h. Glucose was measured using high-performance liquid chromatography (HPLC). The freeze-dried worms were pulverized individually in an Eppendorf tube using a glass rod. Approximately 3 mg of the pulverized tissue was added to a 600-μl Eppendorf tube. Cryoprotectants were extracted according to Bayley and Holmstrup [18] in 100-μl 40% ethanol with a rotating glass rod. Following homogenization, the samples were placed in an ultrasonic bath for 30 min. After warming to 80°C for 5 min in a heat block, the tubes were centrifuged for 10 min and the supernatant was removed to a 1,500-μl Eppendorf tube. The pellet then was rinsed again with 40% ethanol and centrifuged. The combined supernatants were left in the heat block at 60°C until dryness. This sample was redissolved in 1,000 μl. Five hundred μl of this solution was diluted to 1,500 μl with HPLC-grade water and filtered through a 0.45-μl filter before HPLC analysis. Twenty-five–μl samples were run in duplicate on a Shimadzu HPLC (Shimadzu Corporation, Kyoto, Japan) system described in detail by Holmstrup et al. [19]. Glucose concentrations were calculated using a D(+) glucose standard curve (Supelco-standard 4–7249 [Sigma-Aldrich, St. Louis, MO, USA]). In the present study, only an external standard was used, but recovery of internal standards using this methodology at our laboratory average 75% with a low variance (standard deviation 5%). Corrections for this were not applied because recovery rates may vary between individual samples. Concentrations reported in this paper, therefore, are slightly conservative.

Copper analysis

The worms were freeze-dried for 24 h and the whole worm was acid-digested using 3 ml of 14 M nitric acid at increasing temperatures (80–135°C). When all fluid had evaporated, 1 ml 14 M of nitric acid was added and again heated until dryness. The samples were redissolved in 0.1 M of nitric acid and analyzed using flame atomic absorption spectrometry (Perkin-Elmer 4100, Ueberlingen, Germany). Certified reference material (oyster tissue material from the National Institute of Standards and Technology, U.S. Department of Commerce and lobster hepatopancreas from National Research Council Canada) was analyzed to verify the efficiency of the digestion and atomic absorption spectrometry procedure, resulting in a measured concentration of approximately 95% of the certified values. All samples were analyzed in one run.

Statistical analysis

Traditionally, probability of mortality or survival has been modeled by constructing linear (additive) or log-linear (multiplicative) models after an appropriate transformation of the mortality data (e.g., the probit- or logit transformations). However, because mortality data are binary, it is natural to model the probability of dying as a function of the investigated factor(s) [20]. When two or more factors are considered, it is easier to model the inverse of the probability of mortality (i.e., the probability of survival) due to the rule of combined events.

The effect of subzero temperatures and copper and any

possible interaction were modeled using a modified sigmoid dose-response function.

$$f(x; b, x_0) = \frac{1 + \exp(-bx_0)}{1 + \exp[b(x - x_0)]} \quad x \geq 0, \quad b \geq 0 \quad (1)$$

where $f(x)$ is the expected probability of survival at a level of a single stress factor x, x_0 is the point of inflection, and b is the shape parameter of the function. For a more detailed description of the model, the reader is referred to Damgaard et al. [21].

It is very difficult to predict the target for the toxicity of both copper and subzero temperatures; therefore, the effects of subzero temperatures and copper on *D. octaedra* are assumed to be independent physically (they affect different cellular processes), and the effect of subzero temperatures, copper, and the interaction effect of combining the two factors are assumed to be multiplicative [22]. Consequently, the expected probability of survival $p(st, [\mathrm{Cu}])$, when subjected to subzero temperatures and copper can be modeled as

$$p(st, [\mathrm{Cu}]) = (1 - \lambda) f(st; b_{st}, x_{0,st}) f([\mathrm{Cu}]; b_{[\mathrm{cu}]}, x_{0,[\mathrm{cu}]}) \times f(st \cdot [\mathrm{Cu}]; b_{st\cdot[\mathrm{cu}]}, x_{0,st\cdot[\mathrm{cu}]}) \quad (2)$$

where st is the subzero temperature, [Cu] is the concentration of copper, and $\lambda \in [0, 1]$ the residual or control mortality [21]. Note that the interaction effect of combining subzero temperatures and copper is assumed to be a dose-response function of the level of subzero temperatures multiplied by the concentration of copper.

Equation 2 was fitted to the mortality data of *D. octaedra* exposed to various levels of subzero-temperature stress and concentrations of the heavy-metal copper by the maximum likelihood approach. The number of survivors X, out of n individuals exposed to a given degree of subzero temperatures and copper concentrations was assumed to be binomial-distributed with a probability calculated by Equation 2. The likelihood function is

$$L = \prod_R p(st, [\mathrm{Cu}])^X [1 - p(st, [\mathrm{Cu}])]^{n-X} \quad (3)$$

where R is the number of trials. The maximum likelihood estimates were found using the NMaximize routine in Mathematica [23], but any software that optimizes nonlinear functions might be used instead.

The fit of the model was tested by checking whether a null-model, where each of 30 treatment combinations was assigned a specific probability, fitted the mortality data significantly better than Equation 2.

A linear regression model was used to test the correlation between copper in the soil and the subsequent concentration in earthworm tissue. A visual inspection of the residuals suggested that the data should be log transformed. After this transformation, the residuals approximately were distributed normally and the variation homogeneous.

Differences in glucose concentrations between treatments were analyzed using analysis of variance after analysis for variance heterogeneity.

RESULTS

Effect of subzero temperatures and copper on survival

A highly significant interaction occurred between copper toxicity and the severity of frost exposure ($p = 0.0004$). This means that there is a significant increase in copper toxicity with decreasing temperatures and, conversely, a significant reduction in frost tolerance with copper exposure. This synergistic interaction between subzero temperatures and copper is illustrated in Figure 1 by the probability isoclines of mortality when both stresses are present. Survival of *D. octaedra* exposed to 70 mg Cu/kg soil dry weight will be reduced by 5% when the worms are exposed to −2°C and by 20% when exposed to −6°C. At a higher but still realistic copper concentration of 170 mg Cu/kg soil dry weight, 10% of the worms will die after exposure to −1.5°C and this increases to 50% when exposed to −7°C. If the results are considered in terms of the concentrations of copper resulting in a 10% mortality (Fig. 2), it becomes evident that exposure even to mild subzero temperatures radically changes the toxicity estimate of this metal and the Cu-concentration that is lethal to 10% of the worms estimate drops from 200 mg Cu/kg dry soil at −1°C to only 100 mg Cu/kg dry soil at −3°C.

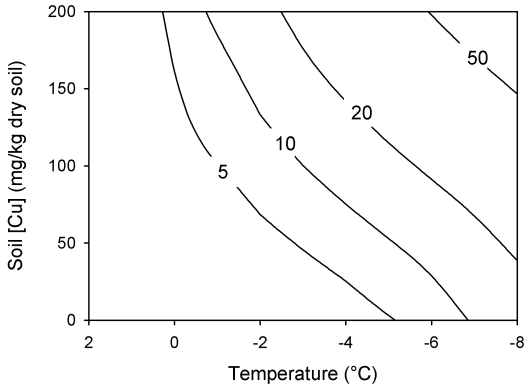

Fig. 1. Mortality probability isoclines for stress combinations of copper and subzero temperatures, fitted to the data shown in Table 2 by the maximum likelihood approach.

The mathematical model used to predict the effect of subzero temperatures and copper on mortality is sufficient to give an adequate description of the data. A null-model, where each of 30 treatment combinations were assigned a specific probability, did not fit the mortality data significantly better ($p =$

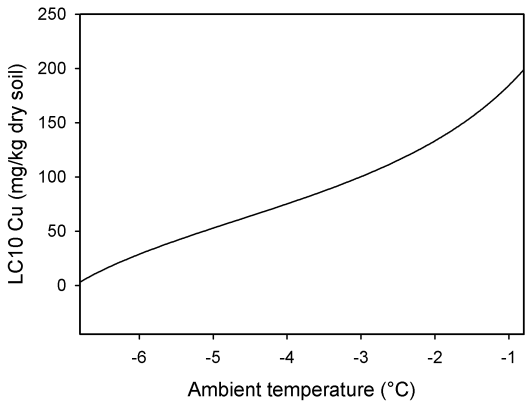

Fig. 2. The estimated concentrations of copper causing 10% mortality (LC10) as a function of subzero temperatures.

Table 1. Survival (%) of *Dendrobaena octaedra* after four weeks of exposure to copper at 2°C followed by 10 d at subzero temperatures in the same soil. The numbers in parentheses are the values calculated from the 0-model

	Soil copper concn. mg Cu/kg dry wt soil					
Temperature	Control	40	80	120	160	200
2°C	100% (99.9%)	100% (99.9%)	100% (99.9%)	100% (99.9%)	100% (99.9%)	100% (99.9%)
−2°C	100% (97.5%)	100% (95.9%)	100% (93.8%)	87.5% (91.1%)	75.0% (87.9%)	87.5% (84.0%)
−4°C	87.5% (94.6%)	100% (92.1%)	90.0% (88.4%)	80.0% (83.5%)	50.0% (76.9%)	80.0% (68.8%)
−6°C	87.5% (89.5%)	100% (86.1%)	100% (80.1%)	66.7% (72.8%)	77.8% (62.2%)	33.3% (49.6%)
−8°C	87.5% (81.1%)	66.7% (76.9%)	66.7% (69.8%)	50.0% (58.9%)	66.7% (44.9%)	33.3% (30.4%)

0.38) (Table 1). The maximum likelihood estimates are listed in Table 2. The shape parameter of copper (b_{cu}) is zero because we have assessed only sublethal copper concentrations. This means that the inflection point (x_{0cu}) of copper cannot be estimated and that a full dose-response for copper alone cannot be obtained from this data.

The model in Equation 2 was expanded with a covariance function of size to test whether the size of the animal interacted with the observed mortality. We could find no evidence of any effects of size ($p > 0.9$), possibly due to the rather limited size range of animals used in this study.

Copper content in worms

Earthworm internal body copper burden increased linearly with exposure to elevated soil–copper concentrations. A significant positive correlation existed between copper of earthworm tissue and copper content in substrate ($r^2 = 0.80$, $p < 0.0001$; Fig. 3). All 10 worms exposed to control soil had approximately the same Cu content in their tissue (11 mg/kg). At the highest Cu–soil concentration, the mean tissue Cu concentration was 94 mg/kg. The variation in the internal copper concentrations in the earthworm increased with increasing copper exposure.

Glucose content in worms

Copper had no effect on glucose production ($p > 0.3$), but there was a highly significant temperature effect in all copper concentrations ($p < 0.001$). The mean glucose concentration of the control worms (2°C), independent of copper treatments, was 14.1 ± 3.2 mg/g worm dry weight (mean ± standard deviation). Worms frozen at −2°C synthesized, on average, 137 ± 18.2 mg glucose/g worm dry weight.

DISCUSSION

This study has revealed a highly significant interaction between environmentally realistic levels of soil–copper contamination and winter temperatures that are encountered frequently by this earthworm species in the climatic zones it inhabits. The implications of this result are twofold. First, that the biogeography of this species, in all probability, will be affected by the presence of copper in its environment. Second, that traditional laboratory estimation of copper toxicity, which is performed at constant benign temperatures, will underestimate the toxicity of this metal in the field because earthworms inevitably will encounter subzero temperatures during the winter [13,14]. Holmstrup et al. [24] previously have observed a trend indicating a synergistic interaction between copper and subzero temperatures on the viability of cocoons from *D. octaedra*. However, this trend was only significant at −8°C. Comparison with the present study is difficult because the cocoons were exposed to copper in aqueous solution and cocoons of *D. octaedra* are not freeze-tolerant like adult worms, but dehydrate at subzero temperatures to an extent that their melting point equilibrates with the environmental temperature [15]. Considerable evidence suggests that there are many common physiological adaptations in response to drought and subzero temperatures because both involve dehydration [12,25,26]. Indeed, copper previously has been shown to affect summer drought tolerance in the euedaphic collembolan *Folsomia candida* [27] and in the earthworm *Aporrectodea calignosa* [28]. However, the design in these studies only allowed the inclusion of a single contamination level at 300 and 150 mg Cu/kg dry soil, respectively.

In the present study, copper had no influence on glucose

Table 2. Maximum likelihood estimates of the survival data model

Model parameter[a]		Max. likelihood estimate
λ	(residual mortality)	0
b_{st}	(shape parameter at $x_{0,st}$)	0.408
$x_{0,st}$	(inflexion point of subzero temperature dose-response curve)	−12.19
$b_{[Cu]}$	(shape parameter at $X_{0,[Cu]}$)	0
$X_{0,[Cu]}$	(inflexion point of copper dose-response curve)	−NE[b]
$b_{st\cdot[Cu]}$	(shape parameter at $x_{0,st\cdot[Cu]}$)	0.00163
$x_{0,st[Cu]}$	(inflexion point at interaction dose-response curve)	1,582.65[c]

[a] *st* is subzero temperatures and [Cu] is copper.
[b] Not estimable.
[c] Transformed value to fit the model: $(-t + 2)\cdot[Cu](mg/kg) = 1{,}582.65$.

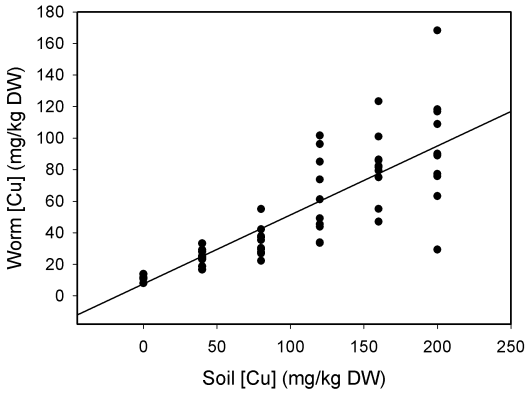

Fig. 3. Copper content in *Dendrobaena octaedra* exposed to different soil copper concentrations at 2°C for four weeks. Individual measurements are shown as closed circles. DW = dry weight.

synthesis, which was chosen for measurement due to its significance in frost tolerance [13,29]. Hence, copper must have had other effects that underlie the interaction between copper and subzero temperatures on worm survival seen in this study. One of the alternative targets for copper toxicity that might explain this interaction between toxic and climatic stress is the cell membrane. Copper concentrations in the range used in the present study have been shown to deteriorate the membrane stability of lysosomes from coelomocytes of the earthworm *Lumbricus rubellus*, measured as the ability of these cell components to retain a neutral red dye [30]. The phase behavior and physical properties of membrane phospholipids fatty acids are extremely sensitive to temperature changes [31]. As the temperature falls, ectothermic organisms increasingly will introduce unsaturated phospholipids into membranes, thus maintaining optimal membrane fluidity at low temperatures [32]. Interaction between copper and the membrane enzymes involved in the control of membrane fluidity possibly could have a significant impact on the ability of the organism to survive changes in temperature. Alternatively, copper may interfere with the ability of the earthworm cells to regulate their volume, which has been shown in the marine flagellates [33] and which would be of particular importance during the rehydration that occurs during thaw [34]. In many of the worms that failed to survive subzero temperatures at higher copper concentrations, there was evidence of edema and internal bleeding that could point to such effects.

The copper concentrations used in this experiment are within the range that can be found in natural soils, which can vary from 2 to 250 mg/kg, depending on the soil type and the mother rock [35]. Near a brass mill in southeast Sweden, *D. octaedra* were found in a coniferous forest contaminated with copper. The earthworm density increased with increasing distance to the mill [9]. At the most-contaminated sites closest to the mill, no individuals were found, which may indicate that synergistic interactions like those seen in this study exist in the field. The internal copper concentrations in this experiment range from a mean of 11 to 94 mg/kg dry weight in worms exposed to control soil and soil containing 200 mg Cu/kg dry weight, respectively. Bengtsson et al. [9] measured internal copper concentrations in *D. octaedra* collected in the field ranging from approximately 100 to 300 mg/kg dry weight, which would suggest, in the light of our data, that these field animals would be vulnerable to subzero winter temperatures. The concentrations in the worms in the present experiment were very similar to those measured by Weeks and Svendsen [30] in *Lumbricus rubellus* under similar experimental conditions. Also, in a laboratory experiment performed by Bengtsson et al. [36], the earthworm *Dendrobaena rubida* (=*Dendrodrilus rubidus*) had internal copper concentrations in the muscle of approximately 57 mg/kg dry weight when exposed to 100 mg Cu/kg soil at pH 6.5 for 21 d. These observations are in good agreement with our results. According to Spurgeon and Hopkin [37], the worm *Aporrectodea calignosa* excretes copper at a fast rate after transfer to clean soil with a half-life of less than 1 d. This suggests that the internal copper concentrations measured in this study are lower than they would be in field populations confronting a similar contamination, because the worms in the present study were depurated for 48 h on wet filter paper before freeze drying and analysis to avoid copper-contaminated gut contents affecting the measured concentrations. Soil pH also has an effect on copper uptake. The uptake is higher at lower pH [36] and, because *D. octaedra* often is found in acidic soils [38], the copper uptake in the present study may well be lower than the uptake seen in natural populations.

According to Streit and Jäggy [39], the earthworm *Octolasium cyaneum* regulates tissue copper concentrations from approximately 40 mg/kg to 100 mg/kg. The increased variation in the tissue copper concentrations with increasing copper exposure seen in the present study is likely to be caused by differences in individual efficiencies of the copper regulatory mechanisms.

Earthworms living in copper-contaminated soil obviously will be exposed periodically to subzero temperatures and copper at the same time. The experimental design in the present study was chosen to simulate natural conditions as far as possible. The worms were first acclimated in copper-contaminated soil and then exposed to subzero temperatures and copper in the soil at the same time. It could be speculated that the exposure to the subzero temperatures and copper should be separated to enable the study of the synergistic interaction between stressors, without the confusing interaction between bioavailability and temperature [40]. However, the earthworms exposed to subzero temperatures do not move and, because the water surrounding them at subzero temperatures is frozen, they probably would not accumulate copper during the exposure to frost.

CONCLUSION

In conclusion, our data indicate that the assessment of the toxicity copper by the traditional laboratory studies where test organisms are exposed to only one stress factor and otherwise optimal conditions (e.g., temperature and humidity), will underestimate the impact of the pollutant on the survival of field populations, which regularly will encounter stressful climatic conditions. Our data also indicate that the presence of environmental contaminants such as copper will alter the climatic tolerance limits of *D. octaedra* and, thereby, its geographical distribution.

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REFERENCES

1. Edwards C, Bohlen P. 1996. *Biology and Ecology of Earthworms*, 3rd ed. Chapman & Hall, London, UK.
2. Stöp-Bowitz C. 1969. A contribution to our knowledge of the systematics and zoogeography of Norwegian earthworms (Annelida Oligochaeta: Lumbricidae). *Nytt Mag Zool* 17:169–280.
3. Hendrix PF. 1995. *Earthworm Ecology and Biography in North America.* Lewis, Boca Raton, FL, USA.
4. Dymond P, Scheu S, Parkinson D. 1997. Density and distribution of *Dendrobaena octaedra* (Lumbricidae) in aspen and pine forests in the Canadian Rocky mountains (Alberta). *Soil Biol Biochem* 29:265–273.
5. Berman DI, Meshcheryakova EN, Alfimov AV, Leirikh AN. 2001. Spread of the earthworm *Dendrobaena octaedra* (Lumbricidae: Oligochaeta) from Europe to Northern Asia is restricted by its insufficient freeze tolerance. *Dokl Biol Sci* 377:145–148.
6. Rozen A. 2003. Do earthworms (*Dendrobaena octaedra*) from differently polluted forests vary in life-history parameters? *Eur J Soil Biol* 39:73–77.
7. Bengtsson G, Ek H, Rundgren S. 1992. Evolutionary response of earthworms to long-term metal exposure. *Oikos* 63:289–297.
8. Helling B, Reinecke SA, Reinecke AJ. 2000. Effects of the fungicide copper oxychloride on the growth and reproduction of *Eisenia fetida* (oligochaeta). *Ecotoxicol Environ Saf* 46:108–116.
9. Bengtsson G, Nordström S, Rundgren S. 1983. Population density and tissue metal concentration of lumbricids in forest soils near a brass mill. *Environ Pollut A* 30:87–108.
10. Zachariassen KE. 1985. Physiology of cold tolerance in insects. *Physiol Rev* 65:799–832.
11. Holmstrup M, Westh P. 1994. Dehydration of earthworm cocoons exposed to cold: A novel cold hardiness mechanism. *J Comp Physiol B* 164:312–315.
12. Holmstrup M, Bayley M, Ramløv H. 2002. Supercool or dehydrate? An experimental analysis of overwintering strategies in small permeable arctic invertebrates. *Proc Natl Acad Sci USA* 99:5716–5720.
13. Rasmussen LM, Holmstrup M. 2002. Geographic variation of freeze-tolerance in the earthworm *Dendrobaena octaedra*. *J Comp Physiol B* 172:691–698.
14. Rundgren S. 1975. Vertical distribution of lumbricids in southern Sweden. *Oikos* 26:299–306.
15. Holmstrup M. 2003. Overwintering adaptations in earthworms. *Pedobiologia* 47:504–510.
16. Storey KB, Storey JM. 1996. Natural freezing survival in animals. *Annu Rev Ecol Syst* 27:365–386.
17. Holmstrup M, Costanzo JP, Lee RE. 1999. Cryoprotective and osmotic responses to cold acclimation and freezing in freeze-tolerant and freeze-intolerant earthworms. *J Comp Physiol B* 169: 207–214.
18. Bayley M, Holmstrup M. 1999. Water vapor absorption in arthropods by accumulation of myoinositol and glucose. *Science* 285:1909–1911.
19. Holmstrup M, Sjursen H, Ravn H, Bayley M. 2001. Dehydration tolerance and water vapor absorption in two species of soil-dwelling collembolan by accumulation of sugars and polyols. *Funct Ecol* 15:647–653.
20. Kooijman SALM, Bedaux JJM. 1996. *The Analysis of Aquatic Toxicity Data.* VU University, Amsterdam, The Netherlands.
21. Damgaard CF, Hojer R, Bayley M, Scott-Fordsmand JJ, Holmstrup M. 2002. Dose-response curve modeling of excess mortality caused by two forms of stress. *Environ Ecol Stat* 9:195–200.
22. Greco W, Unkelbach H-D, Pöch G, Sühnel J, Kundi M, Bödeker W. 1992. Consensus on concepts and terminology for combined-action assessment: The Saariselkä agreement. *Archives of Complex Environmental Studies* 4:65–69.
23. Wolfram S. 1999. *The Mathematica Book.* Cambridge University, Cambridge, UK.
24. Holmstrup M, Petersen BF, Larsen MM. 1998. Combined effects of copper, desiccation, and frost on the viability of earthworm cocoons. *Environ Toxicol Chem* 17:897–901.
25. Ring A, Danks HV. 1994. Dessication and cryoprotection: Overlapping adaptations. *Cryoletters* 15:181–190.
26. Bayley M, Petersen SO, Knigge T, Köhler H-R, Holmstrup M. 2001. Drought acclimation confers cold tolerance in the soil collembolan *Folsomia candida*. *J Insect Physiol* 47:1197–1204.
27. Holmstrup M. 1997. Drought tolerance in *Folsomia candida* Willem (Collembola) after exposure to sublethal concentrations of three soil polluting chemicals. *Pedobiologia* 41:354–361.
28. Friis K, Damgaard CF, Holmstrup M. 2004. Sublethal soil copper concentrations increase mortality in the earthworm *Aporrectodea calignosa* during drought. *Ecotoxicol Environ Saf* 57:65–73.
29. Costanzo JP, Lee RE, Lortz PH. 1993. Glucose concentration regulates freeze tolerance in the wood frog *Rana sylvatica*. *J Exp Biol* 181:245–255.
30. Weeks JM, Svendsen C. 1996. Neutral red retention by lysosomes from earthworm (*Lumbricus rubellus*) coelomocytes: A simple biomarker of exposure to soil copper. *Environ Toxicol Chem* 15: 1801–1805.
31. Hazel JR, Williams EE. 1990. The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Prog Lipid Res* 29:167–227.
32. Hazel JR. 1995. Thermal adaptation in biological membranes: Is homeoviscous adaptations the explanation? *Annu Rev Physiol* 57: 19–42.
33. Riisgaard HU. 1979. Effect of copper on volume regulation in the marine flagellate *Dunaliella marina*. *Mar Biol* 50:189–193.
34. Holmstrup M, Zachariassen KE. 1996. Physiology of cold hardiness in earthworms. *Comp Biochem Phys A* 115:91–101.
35. Adriano DC. 1986. *Trace Elements in the Terrestrial Environment.* Springer-Verlag, New York, NY, USA.
36. Bengtsson G, Gunnarsson T, Rundgren S. 1986. Effects of metal pollution on the earthworm *Dendrobaena rubida* (sav.) in acified soils. *Water Air Soil Pollut* 28:361–383.
37. Spurgeon DJ, Hopkin SP. 1999. Comparisons of metal accumulation and excretion kinetics in earthworms (*Eisenia fetida*) exposed to contaminated field and laboratory soils. *Appl Soil Ecol* 11:227–243.
38. Satchell JE. 1955. Some aspects of earthworm ecology. In Kevan K McE, ed, *Soil Zoology*. Butterworth, London, UK, pp 259–322.
39. Streit B, Jäggy A. 1982. Effect of soil type on copper toxicity and copper uptake in *Octolasium cyaneum* (Lumbricidae). In Lerbun Ph, André HM, Medts A, Grégoire-Wibo C, Wauthy G, eds, *New Trends in Soil Biology.* Dieu-Brichart, Ottignies-Louvain-la-Neuve, Belgium, pp 569–575.
40. Højer R, Bayley M, Damgaard CF, Holmstrup M. 2001. Stress synergy between drought and a common environmental contaminant: Studies with the collembolan *Folsomia candida*. *Global Change Biology* 7:485–494.

Paper 3


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Cold acclimation and lipid composition in the earthworm *Dendrobaena octaedra*

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Abstract

We have investigated the lipid chemistry during cold acclimation in the freeze tolerant earthworm *Dendrobaena octaedra*. The dominant phospholipid fatty acids (PLFA) of *D. octaedra* were 20:4, 20:5 and 20:1 (50% of total PLFA) followed by 18:0, 18:1 and 18:2ω6,9 (25% of total PLFA). The ability to tolerate freezing in this species was acquired after acclimation at low temperature for 2–4 weeks. During this period one particular membrane PLFA, 18:2ω6,9, increased significantly and there was a good correlation between the proportion of this PLFA and the survival of freezing. The composition of neutral lipid fatty acids (NLFA), most likely representing storage lipids (triacylglycerides), also changed during cold acclimation so that the overall degree of unsaturation increased. Using a common-garden experiment approach, we compared lipid composition of three genetically different populations (Denmark, Finland and Greenland) that differed in their freeze tolerance. Inter-populational differences and differences due to cold acclimation in overall fatty acid composition were evident in both PLFAs and NLFAs. Specifically, the PLFAs, 20:4 and 20:5, were considerably more represented in worms from Greenland, and this contributed to a higher UI of PLFAs in this population.

Keywords: Cold acclimation; Earthworms; Freeze tolerance; Homeoviscous adaptation; Membrane phospholipids

1. Introduction

Changes in the composition of cellular lipids that aid to preserve appropriate fluidity of the cell membrane under changing temperatures, is a common phenomenon in ectothermic animals, known as "homeoviscous adaptation" (Hazel, 1995; Sinensky, 1974). Cell membranes function as selective barriers between the intra- and extracellular compartments. In fully functioning cells they are in a liquid–crystalline phase, but when biological membranes are cooled sufficiently they gradually go from the liquid–crystalline phase to the gel phase, whereby the membranes partly lose their selective properties (Cossins and Raynard, 1987; Hazel, 1995). Such phase transition can lead to loss of intracellular metabolites and ions, as well as to a damaging uptake of sodium and calcium to the intracellular compartment (Watson and Morris, 1987). Living organisms must therefore adapt to low temperature by modifying the physical conditions of the cell membrane.

The physical structure of biological membranes is governed by several properties including phospholipid head-groups, cholesterol content and the composition of the phospholipid fatty acyl side-chains (PLFA) (Cossins and Raynard, 1987; Hazel, 1995). In terms of cold adaptation in ectothermic animals the changes in PLFAs are typically associated with an increase in the degree of unsaturation and, in particular, an increased proportion of polyunsaturated long-chain fatty acids leading to a more disordered membrane which is less likely to undergo phase transition at low temperature (Cossins et al., 2002; Hazel, 1995; Lee and Chapman, 1987; Lee et al., 2006). Thus, several studies have shown that seasonal changes in cold tolerance of terrestrial invertebrates are typically associated with alterations of the PLFA composition (Bennett et al., 1997; Kostal et al., 2003; Kostal and Simek, 1998; Ohtsu et al., 1998; Slachta et al., 2002). Moreover, storage lipids (primarily triacylglycerols) are also modified during cold acclimation in order to preserve

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fluidity. This is probably a necessary modification in order to secure the use of triacylglycerols as fuel for metabolism during winter (Bennett et al., 1997; Joanisse and Storey, 1996; Ohtsu et al., 1993).

Most studies of membrane adaptations in cold tolerant invertebrates have been concerned with freeze avoiding species whereas only few studies have investigated membrane adaptations in freeze tolerant species (Bennett et al., 1997; Kostal et al., 2003; Tooke and Holland, 1985). Freeze tolerant species are in particular interesting in this regard because membrane fluidity is not only affected by low temperature but also from freeze-induced dehydration of the cells when extracellular ice is formed (Steponkus, 1984; Webb et al., 1994).

Recently, we have investigated the physiology of freeze tolerance in the earthworm *Dendrobaena octaedra*. The ability to tolerate freezing in this species is acquired after acclimation at low temperature for 2–4 weeks and is probably closely linked to the freeze-induced accumulation of glucose that can act as a cryoprotectant (Rasmussen and Holmstrup, 2002). *D. octaedra* mobilise high concentrations of glucose (10–15% of dry weight) during freezing, which is probably one of the reasons why it tolerates freezing at low temperatures, probably even down to −15 °C but depending on the geographic origin (Bindesbøl et al., 2005; Holmstrup et al., submitted for publication). Other physiological adaptations to low temperature such as membrane lipid composition are likely to be important for freeze tolerance. Studies on other earthworm species show that adjustments of membrane lipids do occur during cold acclimation (Petersen and Holmstrup, 2000). In the present study we wanted to investigate the changes in membrane PLFA composition and neutral lipid fatty acids (NLFA) during cold acclimation by simulating the natural temperature decline during autumn. By assessing the ability to tolerate freezing during a progressing cold acclimation there was a possibility to link putative changes in the composition of PLFA and NLFA to the acquisition of freeze tolerance. A second aim was to compare, in a common-garden experiment, three geographically and genetically distinct populations of *D. octaedra* that differed considerably in their ability to tolerate freezing. This was done in order to evaluate if membrane PLFA composition was related to their degree of freeze tolerance.

2. Materials and methods

2.1. Animals

Specimens of *D. octaedra* (Lumbricidae: Oligochaeta) were obtained from Denmark, Finland and Greenland. The worms from Denmark were collected in a coniferous forest in the vicinity of Silkeborg. Specimens from Greenland were collected around the Arctic station near Godhavn, Disko (West Greenland), and the specimens from Finland were collected in forests surrounding Jyväskylä, Central Finland. The three populations were kept in laboratory cultures at 15 °C using moist loamy sand soil as substrate and fed a mixture of soil and cow manure for cocoon production (Holmstrup et al., 1991). The cocoons were hatched in Petri dishes with moist filter paper, and a F1 generation of each population was raised. Worms used in experiments were adults and large juveniles sampled at random from the F1 cultures. The average fresh weight was approximately 120 mg. The freeze tolerance of the three populations was determined in a parallel study with results shown in Table 1.

2.2. Thermal acclimation protocol

Worms were placed individually in 200 mL plastic beakers containing 75 g moist soil and 6 g cow dung as food. The beakers were closed with perforated lids allowing ventilation. The worms were placed at 15±0.2 °C for one week followed by one week at 10 °C, one week at 5 °C, three weeks at 0±0.2 °C, and finally one week at −2±0.2 °C as described below. For the Greenlandic population, six randomly chosen worms were sampled at the end of each week for lipid analysis. At the same time points, fifteen worms were used for a freeze tolerance assay to estimate the acquisition of freeze tolerance in relation to the cold acclimation regime. The worms from Finland and Denmark were subjected to the same acclimation protocol but only sampled for lipid analysis at the end of the 7-week acclimation period.

A control group of worms were kept at constant 15 °C for the entire 7-week period. These worms were sampled every 2 weeks (Greenlandic worms) or at the end of the 7-week acclimation period (Finland and Denmark). The animals collected for lipid analysis were frozen at −80 °C, freeze dried, and stored at −80 °C until analysis.

2.3. Freeze tolerance assay

The worms collected for freeze tolerance assay were transferred to a small Petri dish with a small amount of soil at −1 °C for 24 h. A small piece of ice was added to each Petri dish to initiate freezing and the worms were left at −1 °C for another 24 h. The Petri dishes were then placed at −2 °C for 5 days. After the freeze period the Petri dishes were placed at 2 °C for 1 day to allow thawing. Worms responding to tactile stimuli with normal movements were scored as survivors.

2.4. Fatty acid analysis

Single, freeze-dried worms were homogenised in 500 µl buffer (50 mM K_2HPO_4, pH 7.4) in 1.5 mL microcentrifuge

Table 1
The estimated lower temperature causing 50% mortality (Lethal Temperature; LT50) and 90% mortality (LT90), respectively

Population	LT50 (°C)	LT90 (°C)	Average minimum air temperature of coldest month (°C)
Denmark (Silkeborg)	-1.3^{a}	-4.3^{a}	−3.1
Finland (Jyväskylä)	-3.1^{b}	-10.3^{b}	−12.9
Greenland (Disko)	-4.4^{b}	-14.6^{b}	−24.7

LT50 and LT90 estimates (non-linear regression) followed by different superscript letters are significantly different at the 5% level. Briefly, groups of earthworms were frozen by inoculation via frozen soil at −1.5 °C and then gradually cooled (1 °C/day) to a series of target temperatures down to −14.5 °C. Data from Holmstrup et al. (submitted for publication).

tubes. Homogenates were transferred to glass test tubes and extracted with a one-phase mixture of chloroform, methanol and buffer (1:2:0.8 v/v/v) (Bayley et al., 2001). Lipid extract was dissolved in 100 µl chloroform and fractionated on prepacked columns with 100 mg silic acid (Bond Elut Extraction Cartridges, Varian US). Neutral lipids fatty acids (NLFA) were extracted with 1.5 mL chloroform and phospholipid fatty acids (PLFA) with 1.5 mL methanol. The phospholipid and neutral lipid fractions were collected and methyl nonadecanoate added as an internal standard (2.4 mg/sample), and then transesterified by a mild alkaline methanolysis (Dowling et al., 1986). The resulting fatty acid methyl esters were separated on a Hewlett Packard 6890 gas chromatograph equipped with a flame ionization detector and a HP5 capillary column (inner diameter 0.25 mm, length 25 m). Hydrogen was used as carrier gas and injections were made in splitless mode. The temperature programme for the column was 80 °C for 1 min, 20 °C min^{-1} up to 160 °C and 5 °C min^{-1} up to 270 °C. Relative retention times of the fatty acid methyl esters were compared to those of a set of known fatty acid methyl esters. Fatty acids were designated as *X*:*Y*ω*Z*, where *X* is the number of carbon atoms, *Y* the number of double bonds, and *Z* indicates the position of the first double bond from the methyl end of the molecule, if known. Identification was verified by GC-MS and to standard compounds. In compounds with no match to standards the number of double bonds could be verified with MS, though not the position of double bonds. In cases with unknown positions of the double bounds, the *Z* indications were not used. The prefix, Me, indicates a methyl group on the fatty acid. The mol percentage distribution of identified compounds was calculated by dividing integrated areas by that of the internal standard methyl nonadecanoate (with a known concentration in each sample) and converting the amount to nmol. The mol percentage of each sample was determined by dividing the amount of each peak with the total amount of PLFAs or NLFAs that were determined in each sample. Overlapping peaks of 20:1 and 20:2 were identified in the GC-MS analysis, and called 20:1&2. The degree of unsaturation (UI) was calculated as: Σ (% monoenes + 2 × % dienes + 3 × % trienes ...) / 100 (Kates, 1986).

2.5. Statistical analysis

The effects of population and acclimation treatment on the degree of unsaturation and selected fatty acids were tested by two-way analysis of variance (ANOVA) with population and treatment as fixed factors using the statistical package SAS (SAS Institute, Cary, NC, USA). Similarly, the effects of treatment and acclimation time (Greenlandic worms) were tested using a two-way ANOVA. A *T*-test was used to make pairwise comparisons in some cases, and hence corrected for the total variation within the ANOVA. Appropriate transformations of data were performed as proposed by SAS to improve normality of data and homogeneity of variance. Comparison of fatty acid composition as influenced by population origin and acclimation regime was done using Principal Component Analysis (PCA) of the mol percentage distributions of individual fatty acids. Differences in the first and second Principal Component (PC1 and PC2) between populations and acclimation treatments were tested using two-way ANOVA.

Table 2
Molar percentage distribution (mean ± SD, *N* = 6) of phospholipid fatty acids in *Dendrobaena octaedra* held constantly at 15 °C or gradually acclimated to low temperature

PLFA	Control				Cold acclimation					
	Time at 15 °C (weeks)				Time (weeks) with temperature in previous week shown [°C]					
	1	3	5	7	2 [10 °C]	3 [5 °C]	4 [0 °C]	5 [0 °C]	6 [0 °C]	7 [−2 °C][a]
14:0	0.69±0.13	0.97±0.09	0.84±0.06	0.87±0.09	0.64±0.053	0.67±0.09	0.65±0.08	0.81±0.11	0.66±0.07	0.64±0.07
Me14:0	1.18±0.22	1.64±0.17	1.45±0.11	1.44±0.10	1.37±0.10	1.56±0.18	1.68±0.14	2.09±0.29	1.82±0.18	1.81±0.21
15:0	3.88±0.43	4.91±0.44	3.98±0.31	4.30±0.39	3.45±0.31	3.70±0.50	3.58±0.29	4.13±0.45	3.77±0.45	3.71±0.35
16:0	1.99±0.74	1.74±0.15	1.74±0.11	1.64±0.16	1.53±0.21	1.52±0.25	1.69±0.20	2.11±0.28	1.65±0.19	1.69±0.14
Me16:0	1.03±0.12	1.08±0.05	1.07±0.04	1.24±0.16	0.97±0.06	1.04±0.14	1.22±0.12	1.50±0.17	1.26±0.13	1.32±0.12
16:1ω7	0.80±0.09	1.00±0.05	0.86±0.07	0.89±0.10	0.84±0.05	0.90±0.12	0.92±0.10	1.10±0.12	0.99±0.08	0.82±0.17
17:0	2.74±0.56	2.12±0.16	2.40±0.12	2.59±0.21	2.11±0.19	2.05±0.39	2.45±0.26	3.04±0.30	2.51±0.33	2.73±0.23
17:1	3.30±0.30	3.75±0.33	3.16±0.35	3.39±0.25	3.90±0.35	3.88±0.55	3.30±0.33	3.24±0.37	3.55±0.56	2.88±0.23
18:0	8.81±1.74	6.75±0.51	8.03±0.66	8.83±1.15	6.72±0.65	6.54±1.24	7.41±0.90	8.64±0.70	7.06±0.89	7.74±0.51
Me18:0	1.12±0.06	1.28±0.05	1.11±0.07	1.10±0.10	1.13±0.04	1.19±0.07	1.04±0.04	0.86±0.04	0.96±0.06	0.83±0.03
18:1ω7	9.38±0.62	10.43±0.74	9.47±0.22	10.98±0.80	8.57±0.61	9.14±0.89	9.63±0.40	10.36±0.89	9.37±0.47	8.95±0.75
18:1ω9	2.59±0.32	2.92±0.33	2.28±0.14	2.37±0.19	2.73±0.23	2.71±0.30	2.79±0.32	3.05±0.42	2.86±1.25	3.04±0.27
18:2ω6,9	5.92±0.35	6.72±0.25	7.03±0.30	7.04±0.39	6.28±0.37	6.72±0.46	8.06±0.57	9.52±0.62	8.80±0.55	8.72±0.38
20:0	0.79±0.16	0.73±0.04	0.62±0.08	0.59±0.02	0.65±0.08	0.68±0.06	0.62±0.10	0.64±0.23	0.68±0.05	0.62±0.04
20:1&2	15.95±1.02	17.14±0.92	14.72±0.77	15.01±1.70	14.98±0.53	16.24±1.48	13.90±0.96	9.59±4.05	13.55±1.19	11.61±0.61
20:2	1.76±0.18	1.95±0.11	1.73±0.12	1.77±0.12	1.76±0.28	2.12±0.20	1.83±0.33	1.62±0.12	1.88±0.16	1.72±0.04
20:3	2.35±0.29	2.66±0.21	3.09±0.22	2.83±0.25	2.68±0.57	3.07±0.15	3.02±0.66	3.22±0.18	3.17±0.23	3.18±0.25
20:4	17.13±1.21	15.33±1.02	18.23±0.94	16.08±3.13	19.0±0.99	17.24±0.90	16.57±0.50	16.26±1.47	16.53±0.52	17.69±0.53
20:5	18.59±0.79	16.89±0.83	18.17±0.43	17.02±2.69	20.68±0.91	19.02±1.35	19.64±0.82	18.20±1.91	18.93±0.78	20.27±0.95
UI	2.24±0.06	2.15±0.06	2.27±0.04	2.16±0.17	2.41±0.08	2.31±0.08	2.27±0.09	2.18±0.15	2.25±0.06	2.36±0.06

Degree of unsaturation (UI; mean ± SD) is indicated in the bottom of the table.
[a] Worms were frozen at −2 °C for 5 days prior to sampling.

3. Results

3.1. Phospholipid and neutral fatty acids

A total of 19 fatty acids were identified in the PLFA fraction of all three populations (Table 2; data only shown for Greenlandic worms). Six fatty acids; 18:0, 18:1ω7, 18:2ω6,9, 20:1, 20:4 and 20:5, accounted for more than 75% of the total amount of PLFA. Especially the long-chain unsaturated fatty acids, 20:*n* and 18:*n*, were abundant whereas shorter chain fatty acids were much less abundant.

Twenty-three fatty acids were identified in the NLFA fraction of all three populations (Table 3; data only shown for Greenlandic worms). Most of the fatty acids found in the PLFA fraction were also found in the NLFA fraction. However, in the NLFA fraction a much more even distribution of the various fatty acids were found. Thus, no single fatty acid accounted for more than 10% of the total amount as was seen in the PLFA fraction. In particular, the 20:*n* fatty acids, were only found in relatively low amounts contrary to what was observed for the PLFA fraction. Moreover, 20:5, which was prominent in the PLFA fraction, was completely missing in the NLFA fraction (Table 2 and 3). In the PLFA pool no fatty acids with chain length shorter than 14-C were found, whereas 12-and 13-C fatty acids were found in the NLFA pool.

3.2. Temporal changes during cold acclimation (Greenlandic worms)

Cold acclimation had no significant influence on the short-chain PLFA (14 to 17 Carbon chains). A clear increase in linoleic acid (18:2ω6,9) was seen as a response to cold acclimation (Table 2; ANOVA, $P<0.0001$). Several significant changes were observed in the NLFA pool as a response to cold acclimation (Table 3). For example, many of the short-chain NLFAs were down regulated, whereas increased proportions of 18:0, 18:1ω7 and 18:2ω6,9 were observed.

The degree of unsaturation (UI) of PLFAs increased slightly over time in the cold acclimated group of worms (Table 2). Similarly, UI of NLFAs increased significantly over time in the cold acclimated worms (Table 3). UI of both PLFAs and NLFAs of control worms kept at 15 °C was largely constant during the experiment.

Worms had no freeze tolerance when acclimated at 15 °C or 10 °C, but began to increase their freeze tolerance after 1 week at 5 °C. All worms tolerated freezing at −2 °C after being acclimated for 3 weeks at 0 °C (Fig. 1). The temporal development of freeze tolerance was positively correlated ($R^2=0.92$; $P<0.0001$) with the relative proportion of the PLFA, 18:2ω6,9 (Figs. 2 and 3). Freeze tolerance did not correlate significantly with any other single PLFA or UI.

Table 3
Molar percentage distribution (mean±SD, $N=6$) of neutral lipid fatty acids in *Dendrobaena octaedra* held constantly at 15 °C or gradually acclimated to low temperature

NLFA	Control				Cold acclimation					
	Time at 15 °C (weeks)				Time (weeks) with temperature in previous week shown [°C]					
	1	3	5	7	2 [10 °C]	3 [5 °C]	4 [0 °C]	5 [0 °C]	6 [0 °C]	7 [−2 °C][a]
12:0	3.89±1.58	9.65±2.81	10.07±4.51	7.50±3.00	7.49±3.50	8.74±1.50	5.38±0.95	5.30±2.20	1.98±0.52	1.28±0.47
Me12:0a	5.34±1.56	9.76±2.56	10.27±3.26	8.55±2.30	8.44±1.22	9.49±1.20	7.16±0.78	8.00±2.42	4.04±0.69	5.68±0.92
Me12:0b	2.70±0.65	5.12±2.56	5.30±2.56	3.71±1.02	4.64±2.15	3.93±0.34	3.45±0.38	3.91±0.82	2.45±0.38	3.79±0.64
13:0	3.44±0.82	6.10±1.63	6.58±1.46	5.64±1.98	4.19±0.28	4.93±0.43	3.99±0.41	4.13±0.89	2.99±0.40	2.36±0.36
Me13:0a	2.60±0.74	4.17±1.15	4.37±1.41	3.26±1.55	3.62±0.66	3.53±0.56	2.77±0.52	2.75±0.61	1.83±0.43	1.01±0.17
Me13:0b	1.89±0.48	3.27±0.99	3.76±1.09	3.15±1.33	2.20±0.28	2.38±0.35	1.64±0.24	1.79±0.46	1.03±0.13	0.78±0.21
14:0	4.20±0.88	6.28±0.28	6.35±0.99	5.22±1.33	5.17±0.61	5.41±0.56	4.18±0.33	3.98±0.76	2.99±0.36	1.95±0.40
Me14:0	6.73±1.46	7.54±0.69	7.17±0.60	6.05±1.04	7.89±0.73	8.62±0.57	8.42±0.66	8.91±0.66	9.69±0.96	6.89±0.71
15:0	9.85±2.07	8.84±2.18	7.46±2.35	9.28±1.67	10.08±0.70	10.05±0.75	9.63±0.68	9.97±0.74	9.17±1.05	7.18±0.67
16:0	3.94±0.60	2.73±0.18	2.36±0.40	2.62±0.39	2.87±0.47	2.74±0.26	2.69±0.34	2.42±0.25	3.09±0.27	2.91±0.36
Me16:0	3.07±0.44	2.61±0.51	2.21±0.53	2.78±0.50	2.42±0.35	2.59±0.27	3.05±0.26	2.89±0.31	3.34±0.14	3.20±0.15
16:1ω7	1.44±0.24	1.01±0.14	0.87±0.16	1.05±0.34	1.18±0.27	1.14±0.17	1.23±0.24	1.25±0.42	1.43±0.12	1.28±0.21
17:0	2.91±0.55	2.17±0.65	2.04±0.74	2.90±1.02	2.27±2.01	1.74±0.23	2.53±0.77	2.03±0.40	2.36±0.43	3.62±0.50
17:1	2.64±0.46	2.16±0.36	1.48±0.42	1.81±0.53	2.00±0.41	2.29±0.49	2.47±0.23	2.20±0.40	2.87±0.48	2.64±0.13
18:0	8.27±2.37	4.48±0.52	5.50±2.20	7.88±4.91	3.99±0.50	4.76±1.09	5.73±1.49	5.01±1.13	6.36±0.98	10.53±1.31
Me18:0	1.11±0.67	1.15±0.60	1.00±0.49	1.15±0.58	0.89±0.43	0.87±0.25	1.26±0.35	0.97±0.41	1.55±0.35	1.37±0.20
18:1ω7	9.18±0.88	5.90±0.93	6.06±1.14	7.83±3.36	8.40±1.34	8.90±1.50	10.43±1.42	9.66±2.19	11.50±1.62	10.57±0.91
18:1ω9	4.68±0.47	2.79±0.42	2.35±0.85	2.74±0.54	3.75±0.67	3.37±0.48	3.52±0.35	4.08±1.84	4.50±0.36	3.96±0.13
18:2ω6	6.45±1.04	5.34±0.66	4.55±1.48	4.95±0.72	5.91±1.25	5.72±0.94	7.72±0.46	6.97±1.94	9.91±1.11	9.32±1.04
20:0	4.31±1.82	3.43±1.06	2.88±1.43	3.97±1.45	2.90±1.61	2.72±0.20	3.83±0.27	3.97±0.61	4.88±0.62	4.97±0.17
20:1&2	1.54±0.23	1.17±0.29	1.80±0.40	1.94±0.38	1.58±1.17	1.10±0.06	1.51±0.08	1.64±0.14	1.93±0.22	2.50±0.21
20:3	2.54±0.28	1.58±0.50	2.22±0.52	2.02±0.14	2.64±0.43	1.89±0.17	2.75±0.40	2.99±0.51	3.44±0.59	3.64±0.25
20:4	7.26±2.93	2.75±0.59	3.80±1.29	3.95±0.98	5.46±0.83	3.08±0.58	4.68±0.85	5.18±1.71	6.67±1.88	8.59±1.04
UI	0.67±0.13	0.38±0.04	0.43±0.09	0.46±0.09	0.57±0.07	0.45±0.03	0.60±0.04	0.61±0.13	0.77±0.08	0.83±0.05

Degree of unsaturation (UI; mean±SD) is indicated in the bottom of the table.
[a] Worms were frozen at −2 °C for 5 days prior to sampling.

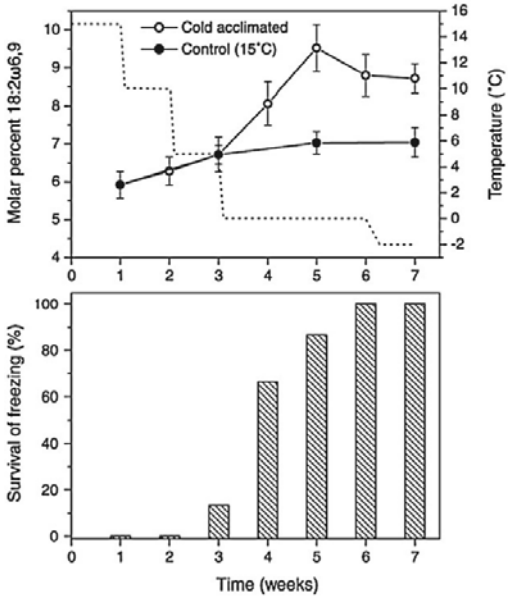

Fig. 1. Temporal development in the proportion of the phospholipid fatty acid, 18:2ω6,9 (mean±SD, $N=6$), and parallel acquisition of freeze tolerance in *Dendrobaena octaedra* from Greenland during cold acclimation. The temperature during the 7-week cold acclimation is shown as the dotted line.

3.3. Responses to cold acclimation in different populations

After 7 weeks of acclimation a significantly higher proportion of the PLFA, 18:2ω6,9 was observed in cold acclimated worms of all three populations as compared to control worms (Fig. 3; 2-way ANOVA, $P<0.0001$). However, the effect of population or population*treatment was not significant (2-way ANOVA, $P=0.51$ and 0.09, respectively).

The degree of unsaturation in PLFAs at week 7 was significantly influenced by both acclimation treatment (Fig. 4;

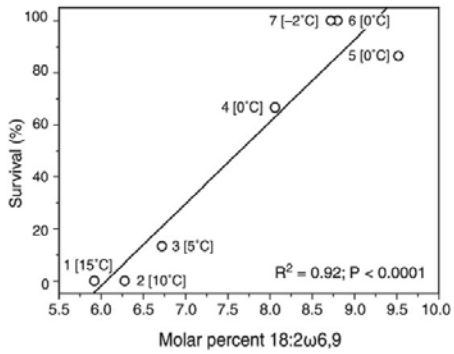

Fig. 2. The relationship between molar percentage of the phospholipid fatty acid, 18:2ω6,9 (mean values, $N=6$), and survival (%, $N=10$–15) of freezing at −2 °C for 5 days in *Dendrobaena octaedra* from Greenland. Numbers next to symbols indicate the sampling time (week number in the acclimation experiment) and the temperature during the previous week.

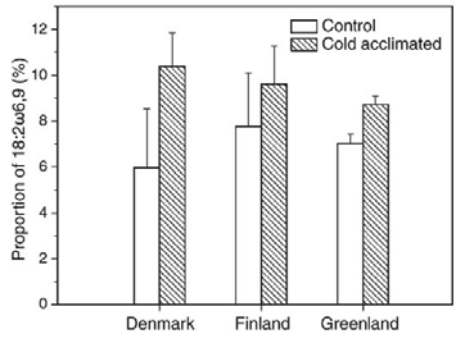

Fig. 3. The proportion of the phospholipid fatty acid, 18:2ω6,9 (mean±SD, $N=6$), of *Dendrobaena octaedra* that were kept at constant 15 °C (Control) or gradually cooled to −2 °C over 7 weeks (Cold acclimated).

2-way ANOVA, $P<0.0002$) and population (2-way ANOVA, $P<0.0003$). Thus, cold acclimation resulted in a significant increase of UI in worms from Greenland and Finland, but not in worms from Denmark. There was a significant interaction between treatment and population (2-way ANOVA, $P<0.033$). For NLFAs, there was a significant effect of treatment on UI (2-way ANOVA, $P<0.0042$), a significant interaction between treatment and population (2-way ANOVA, $P<0.0053$), but not a significant effect of population (2-way ANOVA, $P=0.78$). Pairwise comparisons showed that cold acclimation significantly

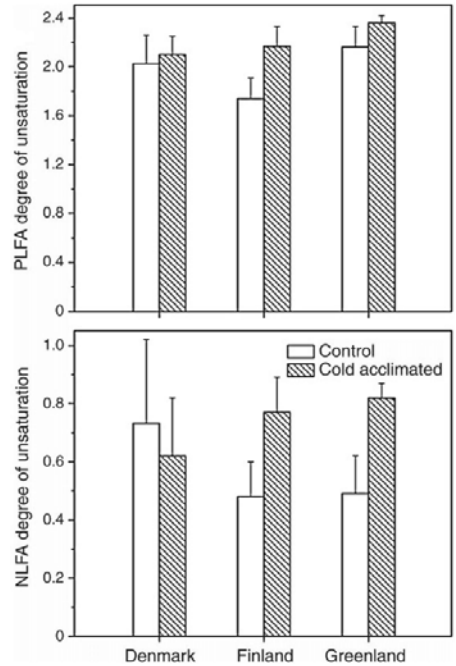

Fig. 4. Degree of unsaturation for Phospholipid Fatty Acids (PLFA, upper panel) and Neutral Lipid Fatty Acids (NLFA, lower panel) of *Dendrobaena octaedra* that were kept at constant 15 °C (Control) or gradually cooled to −2 °C over 7 weeks (Cold acclimated). Data are shown as mean±SD ($N=6$).

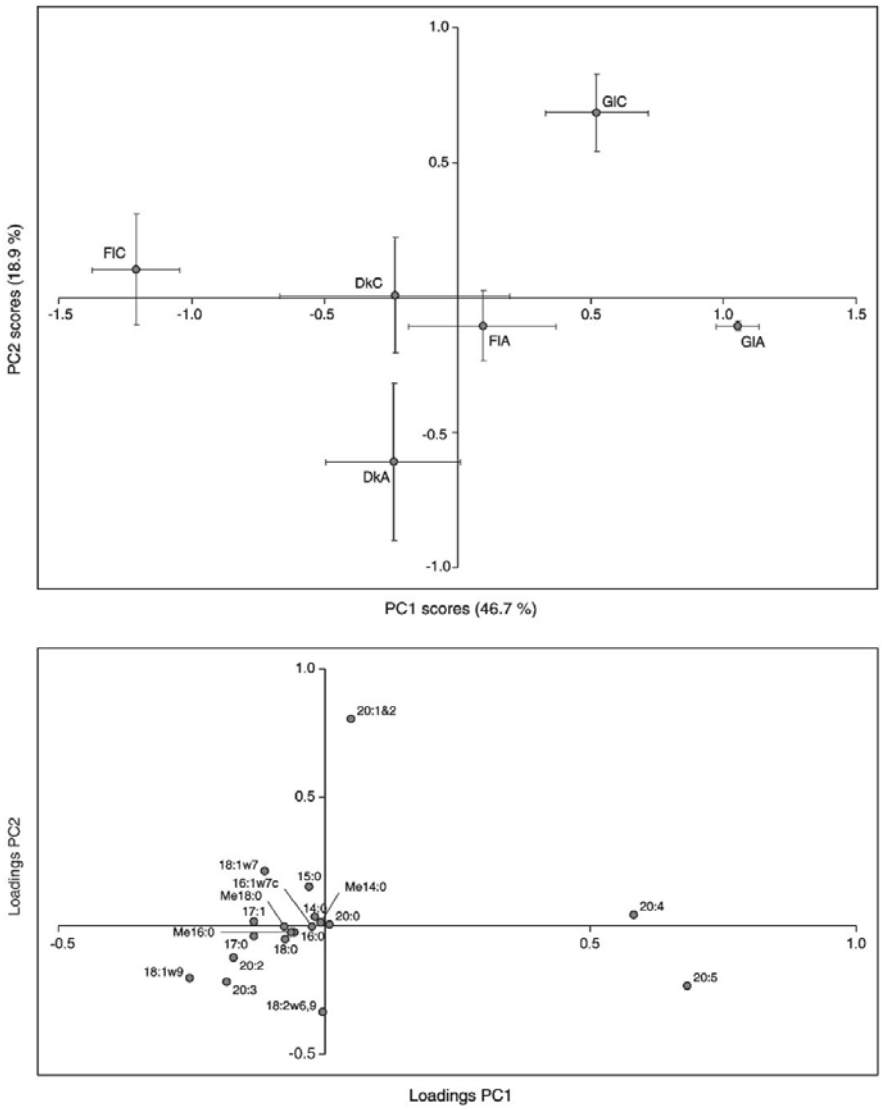

Fig. 5. Mol percentages of the phospholipid fatty acids (PLFA) extracted from *Dendrobaena octaedra* were used for principal component analysis. Upper panel: Case scores for PC1 and PC2, with the percentage variation explained by each component in parentheses. DkC: Control worms (15 °C) from Denmark; DkA: Cold acclimated worms (−2 °C) from Denmark; FlC: Control worms (15 °C) from Finland; FlA: Cold acclimated worms (−2 °C) from Finland; GlC: Control worms (15 °C) from Greenland; GlA: Cold acclimated worms (−2 °C) from Greenland. Lower panel: Component loading values.

increased UI of NLFAs in worms from Finland (*T*-test; $P=0.012$) and Greenland (*T*-test; $P=0.001$), but in worms from Denmark no significant change of UI was observed (*T*-test; $P=0.45$).

Inter-populational differences in PLFA composition were evident and control individuals from Denmark, Finland and Greenland were separated primarily along the PC1 axis (Fig. 5). The effect of thermal treatment was seen for all three populations along both PC1 and PC2. These two first components together explained 65% of the variation in the data. A two-way ANOVA showed that population, thermal treatment and population * treatment all had a significant effect on PC1 ($P<0.05$). For PC2, population and treatment had a significant effect ($P<0.01$), but no significant interaction between the two was observed. The co-occurrence of the loading values (the position of the PLFAs) (Fig. 5) and the position of the cases (the mean values of the treatments; data not shown) showed that polyunsaturated PLFAs, 20:4 and 20:5, tended to be present at higher concentrations in worms from Greenland, especially in cold acclimated worms. Thus, a two-way ANOVA revealed a

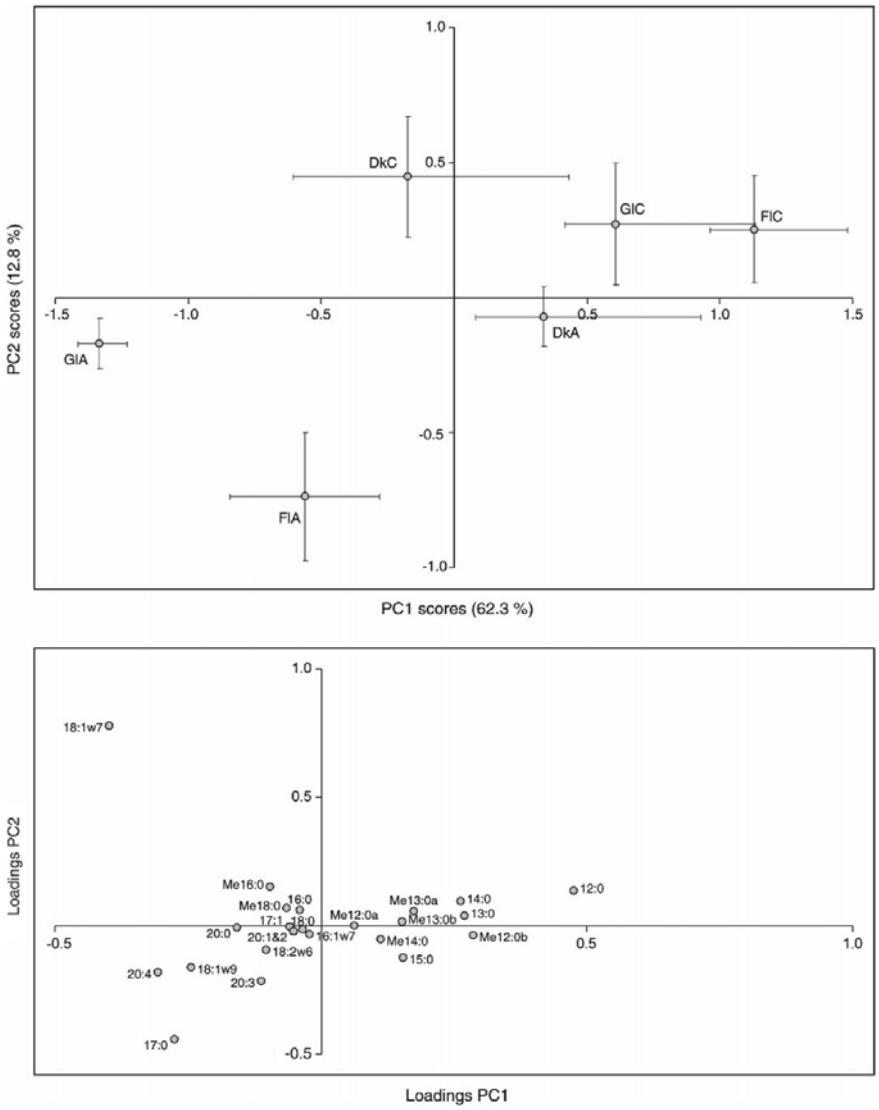

Fig. 6. Mol percentages of the neutral lipid fatty acids (NLFA) extracted from *Dendrobaena octaedra* were used for principal component analysis. Upper panel: Scores for PC1 and PC2, with the percentage variation explained by each component in parentheses. DkC: Control worms (15 °C) from Denmark; DkA: Cold acclimated worms (−2 °C) from Denmark; FlC: Control worms (15 °C) from Finland; FlA: Cold acclimated worms (−2 °C) from Finland; GlC: Control worms (15 °C) from Greenland; GlA: Cold acclimated worms (−2 °C) from Greenland. Lower panel: Component loading values.

significant effect of population ($P<0.0001$) but not of acclimation ($P=0.15$) in the PLFA, 20:4. This PLFA contributed about 16% of total PLFAs in worms from Greenland, but only about 12% in worms from Denmark and Finland. For the PLFA, 20:5, both population and acclimation treatment had significant effects ($P=0.002$ and $P=0.009$, respectively). Greenlandic worms had the highest percentage of 20:5 (data not shown).

Also for NLFAs, distinct inter-populational differences were found with populations separated primarily along the PC1 axis, which explained 65% of the variation in the data (Fig. 6). PC2 explained 13% of variation. Population and treatment both had a significant effect on PC1 (two-way ANOVA, $P=0.005$ and 0.01, respectively). For PC2, only treatment had a significant effect (two-way ANOVA, $P=0.0001$).

4. Discussion

The dominant phospholipid fatty acids of *D. octaedra* were 20:4, 20:5 and 20:1 (50% of total PLFA) followed by 18:0, 18:1

and 18:2 (25% of total PLFA). This is largely the same distribution as observed in other earthworm species such as *Lumbricus terrestris* (Albro et al., 1992), *L. rubellus* and *Eisenia nordenskioldi* (Petersen and Holmstrup, 2000) even though fatty acids reported for the latter two species also included neutral lipid fatty acids. Several of the fatty acids listed in Tables 1 and 2 are known as biomarkers of bacteria (e.g. 15:0, 16:1ω7, 17:0, 18:1ω7 and methylated fatty acids) and have previously been associated with the gut microflora of earthworms (Sampedro and Whalen, 2007). These fatty acids have probably been derived from the food and incorporated in earthworm tissues. In the present study we did not attempt to quantify the absolute amounts of different lipid classes of *D. octaedra* tissues. However, previous studies of other earthworm species (*L. terrestris*) report that total lipids may contain 30–35% neutral lipids (mainly triglycerides and cholesterol), 10–40% glycolipids and 25–60% phospholipids (Albro et al., 1992; Lee et al., 1988). Of the phospholipids, phosphatidylethanolamine and phosphatidylcholine were the most abundant accounting for 33% and 40% of the phospholipids, respectively (Albro et al., 1992). Unpublished studies (Overgaard et al., unpublished) have shown that total lipid contents of *D. octaedra* collected in the field (forest soil, Denmark) remain constant during all seasons at around 10% of dry weight, which is about the same lipid content found for *L. terrestris* (Albro et al., 1992; Lee et al., 1988).

Inter-populational differences and differences due to cold acclimation in overall fatty acid composition were evident in both PLFAs and NLFAs (Figs. 5 and 6). Specifically, the PLFAs, 20:4 and 20:5, were considerably more represented in worms from Greenland, and this contributed to a higher UI of PLFAs in this population (Fig. 4). Since this study was designed as a common-garden experiment where individuals were cultured under nearly identical conditions, these results suggest that the differences in membrane composition as well as in the storage lipids must, at least partly, have a genetic component. An earlier reported phylogenetic analysis using isozymes of the populations studied here shows that worms from Greenland form a distinct cluster separated from European populations (Hansen et al., 2006). This is supporting results of the present study where the lipid composition of especially cold acclimated worms from Greenland was clearly separated from other populations.

Cold acclimation was expected to increase UI of storage lipids (NLFA). The maintenance of fluidity in storage lipids may be a necessary requirement in order to make lipids available for energy production during winter (Bennett et al., 1997). Consistent with this notion, an overall increase in the degree of unsaturation of NLFAs was found in cold acclimated worms from Finland and Greenland but, surprisingly, not in worms from Denmark. A possible reason for this difference could be that worms from Denmark seldom experience very low temperatures (Table 1), whereas this is probably the case on a regular basis for the other populations.

Changes in the PLFA, 18:2ω6,9 seemed to be an important element of cold acclimation and correlated with development of freeze tolerance in *D. octaedra* from Greenland. Although this is no formal proof of a causal relationship, it is in accordance with results reported previously for the Drosophilid, *Chymomyza costata*, where it was observed that an increased proportion of 18:2ω6,9 was also highly correlated with the freeze tolerance of various life stages differing in their freeze tolerance (Kostal et al., 2003). An increased proportion of this particular PLFA was observed in all three populations and no effects of population, or population * treatment interaction was observed. Altogether it shows that the upregulation of 18:2ω6,9 is a robust observation, which indeed could be important for acquisition of freeze tolerance. However, when comparing the cold-induced relative increase in 18:2ω6,9 of the three populations with the general cold hardiness of these populations (see Fig. 3 and Table 1) there was no similar pattern. The highest proportion of 18:2ω6,9 was found in worms from Denmark, yet this population had the poorest freeze tolerance. Therefore, even though an increased proportion of 18:2ω6,9 seems important for the ability to tolerate freezing, other physiological traits must also be important, for example the ability to mobilise high concentrations of glucose (Holmstrup et al., 1999; Holmstrup et al., submitted for publication; Rasmussen and Holmstrup, 2002).

As expected, the degree of unsaturation of PLFAs increased in all three populations as a response to cold acclimation, although not statistically significant in worms from Denmark (Fig. 4). This result is consistent with earlier reports of ectotherms in general (Hazel and Williams, 1990) and earthworms in particular (Petersen and Holmstrup, 2000). There seemed to be a positive correlation between UI and freeze tolerance (Table 1 and Fig. 4). In opposition to this, other authors have observed inverse temperature compensation in terms of fluidity, with no change in phospholipid fatty acid profiles (Crockett et al., 2001). It has also been demonstrated, using fish models, that a very efficient means of effecting homeoviscous adaptation is the insertion of 18:1ω9 into the sn-1 position of membrane phospholipids (Buda et al., 1994). Such changes in molecular species structure would not necessarily be reflected in changes in the UI or other measures of unsaturation. More studies are therefore needed to substantiate this relationship in *D. octaedra* as no firm conclusions can be drawn from observations of only 3 populations. Moreover, UI of PLFAs is not the only membrane property that can alter the fluidity of membranes and result in homeoviscous adaptation. For example, membrane cholesterol concentrations and the distribution among different phospholipid head-groups (e.g. phosphatidylethanolamine or phosphatidylcholine) may also influence membrane fluidity (Crockett, 1998; Hazel, 1995). However, cholesterol levels in the freeze tolerant earthworm, *E. nordenskioldi*, and the freeze susceptible *L. rubellus*, were equal in worms acclimated at either 0 °C or 20 °C (Petersen and Holmstrup, 2000) suggesting that unsaturation of phospholipid fatty acids play a significant role in homeoviscous adaptation in earthworms.

Phospholipid fatty acid distribution may be organ or tissue specific. For example, it has been reported that mitochondrial membranes of *L. terrestris* were dominated by 18:0, 18:1 and 20:1 (65% of total PLFA) whereas 20:4 and 20:5 were rare (3% of total PLFA) (Crockett et al., 2001). This composition is very different from what is seen for whole worm extracts, and

stresses that attempts to correlate PLFA composition with tolerance of low temperature are complicated. Such analyses using data from whole-body extracts suffer from low resolution because lipids of many different tissues are mixed, which may mask membrane adjustments in particular cell types (Kostal et al., 2003). Nevertheless, the results of the present study show that overall changes in PLFA composition during cold acclimation were consistent with the current understanding of adaptations of invertebrates to low temperature.

Acknowledgement

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References

Albro, P.W., Schroeder, J.L., Corbett, J.T., 1992. Lipids of the earthworm *Lumbricus terrestris*. Lipids 27, 136–143.

Bayley, M., Petersen, S.O., Knigge, T., Kohler, H.R., Holmstrup, M., 2001. Drought acclimation confers cold tolerance in the soil collembolan *Folsomia candida*. J. Insect Physiol. 47, 1197–1204.

Bennett, V., Pruitt, N., Lee, R., 1997. Seasonal changes in fatty acid composition associated with cold-hardening in third instar larvae of *Eurosta solidaginis*. J. Comp. Physiol. B 167, 249–255.

Bindesbøl, A.M., Holmstrup, M., Damgaard, C., Bayley, M., 2005. Stress synergy between environmentally realistic levels of copper and frost in the earthworm *Dendrobaena octaedra*. Environ. Toxicol. Chem. 24, 1462–1467.

Buda, C., Dey, I., Balogh, N., Horvath, L.I., Maderspach, K., Juhasz, M., Yeo, Y.K., Farkas, T., 1994. Structural order of membranes and composition of phospholipids in fish brain-cells during thermal acclimatization. Proc. Natl. Acad. Sci. U. S. A. 91, 8234–8238.

Cossins, A.R., Raynard, R.S., 1987. Adaptive responses of animal cell membranes to temperature. In: Bowler, K., Fuller, B.J. (Eds.), Temperature and Animal Cells. The Company of Biologists Limited, Cambridge, pp. 91–112.

Cossins, A.R., Murray, P.A., Gracey, A.Y., Logue, J., Polley, S., Caddick, M., Brooks, S., Postle, T., Maclean, N., 2002. The role of desaturases in cold-induced lipid restructuring. Biochem. Soc. Trans. 30, 1082–1086.

Crockett, E.L., 1998. Cholesterol function in plasma membranes from ectotherms: membrane-specific roles in adaptation to temperature. Am. Zool. 38, 291–304.

Crockett, E.L., Dougherty, B.E., McNamer, A.N., 2001. Effects of acclimation temperature on enzymatic capacities and mitochondrial membranes from the body wall of the earthworm *Lumbricus terrestris*. Comp. Biochem. Physiol. B 130, 419–426.

Dowling, N., Widdel, F., White, D., 1986. Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulphate-reducers and other sulphide-forming bacteria. J. Gen. Microbiol. 132, 1815–1825.

Hansen, P., Holmstrup, M., Bayley, M., Simonsen, V., 2006. Low genetic variation for *Dendrobaena octaedra* from Greenland compared to populations from Europe and North America: refuge or selection? Pedobiologia 50, 225–234.

Hazel, J., 1995. Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation. Annu. Rev. Physiol. 57, 19–42.

Hazel, J.R., Williams, E.E., 1990. The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. Prog. Lipid Res. 29, 167–227.

Holmstrup, M., Costanzo, J.P., Lee, R.E., 1999. Cryoprotective and osmotic responses to cold acclimation and freezing in freeze-tolerant and freeze-intolerant earthworms. J. Comp. Physiol. B 169, 207–214.

Holmstrup, M., Østergaard, I.K., Nielsen, A., Hansen, B.T., 1991. The relationship between temperature and cocoon incubation time for some Lumbricid earthworm species. Pedobiologia 35, 179–184.

Holmstrup, M., Overgaard, J., Bindesbol, A.-M., Pertoldi, C., Bayley, M., submitted for publication. Adaptations to overwintering in the earthworm Dendrobaena octaedra: Genetic differences in glucose mobilisation and freeze tolerance.

Joanisse, D., Storey, K., 1996. Fatty acid content and enzymes of fatty acid metabolism in overwintering cold hardy gall insects. Physiol. Zool. 69, 1079–1095.

Kates, M., 1986. Techniques of lipidology. Elsevier, Amsterdam.

Kostal, V., Simek, P., 1998. Changes in fatty acid composition of phospholipids and triacylglycerols after cold-acclimation of an aestivating insect prepupa. J. Comp. Physiol. B 168, 453–460.

Kostal, V., Berkova, P., Simek, P., 2003. Remodelling of membrane phospholipids during transition to diapause and cold-acclimation in the larvae of *Chymomyza costata* (Drosophilidae). Comp. Biochem. Physiol. B 135, 407–419.

Lee, D.C., Chapman, D., 1987. The effects of temperature on biological membranes and their models. In: Bowler, K., Fuller, B.J. (Eds.), Temperature and Animal Cells. The Company of Biologists Limited, Cambridge, pp. 35–52.

Lee, H.K., Kim, H.S., Adachi, S., Ito, S., Okamoto, K., Kametaka, M., 1988. Occurrence of unusual fatty acids in the earthworm, *Lumbricus terrestris*. Agric. Biol. Chem. 52, 2379–2380.

Lee, R.E., Damodaran, K., Yi, S.X., Lorigan, G.A., 2006. Rapid cold-hardening increases membrane fluidity and cold tolerance of insect cells. Cryobiology 52, 459–463.

Ohtsu, T., Katagiri, C., Kimura, T., Hori, S., 1993. Cold adaptations in *Drosophila*. Qualitative changes of triacylglycerols with relation to overwintering. J. Biol. Chem. 268, 1830–1834.

Ohtsu, T., Kimura, M., Katagiri, C., 1998. How *Drosophila* species acquire cold tolerance. Qualitative changes of phospholipids. Eur. J. Biochem. 252, 608–611.

Petersen, S., Holmstrup, M., 2000. Temperature effects on lipid composition of the earthworms *Lumbricus rubellus* and *Eisenia nordenskioeldi*. Soil Biol. Biochem. 32, 1787–1791.

Rasmussen, L., Holmstrup, M., 2002. Geographic variation of freeze-tolerance in the earthworm *Dendrobaena octaedra*. J. Comp. Physiol. B 172, 691–698.

Sampedro, L., Whalen, J.K., 2007. Changes in the fatty acid profiles through the digestive tract of the earthworm *Lumbricus terrestris* L. Appl. Soil Ecol. 35, 226–236.

Sinensky, M., 1974. Homeoviscous adaptation — a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. 71, 522–525.

Slachta, M., Berkova, P., Vambera, J., Kostal, V., 2002. Physiology of cold-acclimation in non-diapausing adults of *Pyrrhocoris apterus* (Heteroptera). Eur. J. Entomol. 99, 181–187.

Steponkus, P.L., 1984. Role of the plasma-membrane in freezing-injury and cold-acclimation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 35, 543–584.

Tooke, N.E., Holland, D.L., 1985. Phospholipid fatty-acid composition and cold tolerance in 2 species of barnacle, *Balanus balanoides* (L) and *Elminius modestus* Darwin. 1. Summer versus winter variations in phospholipid fatty-acid composition of whole animals. J. Exp. Mar. Biol. Ecol. 87, 241–253.

Watson, P.F., Morris, G.J., 1987. Cold shock injury in animal cells. In: Bowler, K., Fuller, B.J. (Eds.), Temperature and Animal Cells. The Company of Biologists Limited, Cambridge, pp. 311–340.

Webb, M.S., Uemura, M., Steponkus, P.L., 1994. A comparison of freezing-injury in oat and rye- 2 cereals at the extremes of freezing tolerance. Plant Physiol. 104, 467–478.

Paper 4

Photo: Brian Rasmussen

Changes in membrane phospholipids as a mechanistic explanation for decreased freeze tolerance in earthworms exposed to sub-lethal copper concentrations

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Changes in membrane phospholipids as a mechanistic explanation for decreased freeze tolerance in earthworms exposed to sub-lethal copper concentrations

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Abstract

At low temperature, cell membrane functionality depends on adjustments of membrane phospholipid fatty acid (PLFA) composition. We here test the hypothesis that synergistic interactions between copper (Cu) and freezing are due to Cu-induced changes of PLFA composition of cell membranes in the freeze tolerant earthworm *Dendrobaena octaedra*. Cu levels and freezing temperatures were varied in a full factorial design. We measured PLFA composition and lipid peroxidation. A highly synergistic interaction was observed between subzero temperatures and Cu concentrations above 120 mg/kg dry soil. Lipid peroxidation increased slightly in worms exposed to Cu. In particular, the analysis showed that Cu had a significant negative effect on the PLFA 18:2ω6,9, which has previously been reported to correlate positively (R^2 = 0.92) with freeze tolerance in *D. octaedra.* This supports our hypothesis that synergistic interaction between Cu and frost may be due to membrane damage.

Keywords: Copper; *Dendrobaena octaedra*; Freeze tolerance; Membrane phospholipids; Lipid peroxidation

Introduction

In natural environments, it is not unusual for an organism to be exposed to several stressful factors, both physical and chemical, at the same time. Several studies have shown that the interactions between chemicals and climatic stressors can lead to synergistically increased mortality, and it is therefore important to include such aspects in risk assessment of chemicals (*1, 2*). The freeze tolerant earthworm *Dendrobaena octaedra* is likely to be exposed to several environmental stressors at the same time. These could include both climatic stress and the exposure to chemicals of anthropogenic origin. Examples include heavy metal pollution of surface soils from smelters (*3*), and brass mills (*4*) or from the use of copper (Cu) fungicides, e.g. in vineyards (*5*). Surface litter and humus are the principal metal sinks in the forest floor (*6*). This means that *D. octaedra,* which is a litter-dwelling species, is likely to be more exposed to metals than deep-burrowing earthworm species (*7*). The fact that *D. octaedra* is a litter-dwelling species, and does not migrate to deeper soil layers, means that it will be exposed to sub-zero temperatures during winter. To ensure winter survival, *D. octaedra* accumulates glucose as a cryoprotectant to high concentrations at the onset of the body fluid freezing process (*8*) and changes the lipid chemistry during cold acclimation (*9*). Especially one particular membrane phospholipid fatty acid (PLFA), 18:2ω6,9, has been shown to increase during acclimation to cold and correlate positively with freeze tolerance (*9*).

We have previously shown that Cu can significantly reduce the freeze tolerance of *D. octaedra.* However, Cu had no effect on cryoprotectant production, which points to the need for investigation of other mechanisms to explain the observed synergistic interaction between Cu and frost survival (*10*).

Cell membranes function as selective barriers between intra- and extra-cellular compartments and their proper function is essential for the survival of ectothermic organisms at all temperatures. Characteristically, lowering of environmental temperatures induces homeoviscous adaptation, during which the chemical composition of biological membranes are modified to maintain an appropriate degree of fluidity (*11, 12*). Fully functional membranes exist in a liquid-crystalline phase, but when biological membranes are cooled below a certain temperature, T_m (temperature of phase transition), they change from the liquid-crystalline phase to the more ordered gel phase, whereby they become non-functional and lose their selective properties (*12*).

Cu has been shown to deteriorate the membrane stability of lysosomes from coelomocytes of the earthworm *Lumbricus rubellus* (*13*). A possible mechanism behind this effect may be that Cu causes lipid peroxidation, which in turn changes the PLFA composition of the membrane (*14*). Indeed, it has been shown that Cu exposure alters the PLFA composition in several plant species (*15–17*).

In the present study we test the hypothesis that the synergistic interactions between Cu and freezing temperatures are due to detrimental Cu-induced changes of the membrane phospholipids, causing *D. octaedra* to be less tolerant to freezing. This problem was investigated by determining abundance of lipid peroxidation products and analysis of the PLFA composition of Cu-exposed *D. octaedra*.

Materials and methods

Animals

D. octaedra were collected in a coniferous forest near Silkeborg, Denmark, in spring 2007. The earthworms were kept in culture at 15 °C in moist soil and fed on a diet of cow dung. Cocoons collected from the culture were incubated at 20 °C in Petri dishes layered with wet filter paper. Newly hatched juveniles of approximately same age were transferred to moist soil and kept in the same way as the adults until they had reached a suitable size for the experiment (65-340 mg).

Soil

Topsoil from an ecologically farmed Danish pea field (Foulum, Viborg) was used for the experiment. The soil was loamy sand consisting of: 35 % coarse sand, 45 % fine sand, 9.4 % silt, 8.9 % clay and 1.7 % organic matter. The pH-H_2O was 6.8. Prior to use, the soil was dried for 24 hours at 80 °C and sieved through a 2 mm mesh. The soil was Cu spiked with $CuCl_2 \cdot 2H_2O$ (> 99%, Merck, Darmstadt, Germany), and the water content adjusted to 20 % of dry weight (dw) (approximately pF = 2 and 50% water holding capacity) and stored for one day before further use.

Experimental design

Cu and temperature were varied in a full factorial design with six Cu concentrations and five temperatures giving a total of 30 treatments, including the control-groups. Animals were exposed to nominal soil concentrations of 0, 60, 120, 180, 240 and 300 mg Cu/kg dry weight (dw) for one week at 10 °C followed by one week at 5 °C and finally for four weeks at 2 °C prior to exposure to the freezing temperatures. There were 10 to 15 worms in each group, depending on the treatment. Each worm was kept individually in a small container with 75 g of soil (fresh weight) and 6 g of cow dung (fresh weight) mixed into the soil. The cow-dung feed was produced by adding 400 ml demineralised water to 150 g dried and finely ground cow-dung. The soil pH was equal (pH 6.8) in all copper treatments after the addition of cow-dung. All containers were covered with lids having the same number of holes so that ventilation was equal between treatments. After six weeks of acclimation eight individuals from each Cu concentration were allowed to empty their gut for 48 hours on wet filter paper kept at 2 °C, rinsed and then frozen at –80 °C for Cu analysis. Furthermore, 20 worms from each Cu concentration were rinsed, snap-frozen in liquid nitrogen and stored at –80 °C until further analysis; 10 animals from each concentration were used for PLFA analysis and 10 for the analysis of lipid peroxidation.

With the exception of the controls (+2 °C), the remaining worms were placed singly in 8 ml tubes along with a few grams of the appropriate substrate. In each lid, 2 small needle holes were made for ventilation. These worms were placed at –1 °C and after 24 hours a small ice crystal were added to each tube to ensure inoculative freezing of the worm (*8*). After another 24 hours the worms were transferred to four different freezer cabinets programmed to gradually lower the temperature from –1 °C (0.042 °C/h) to the experimental temperatures (–2, –4, –6 and –8 °C). The worms remained at their experimental temperature for different periods such that each group were exposed to subzero temperatures for equal time (10 days).

Prior to the assessment of survival rate all the worms were transferred to 2°C and allowed to thaw. The earthworms were considered to have survived if there was a reaction to tactile stimuli, normal locomotor activity, and if no signs of freezing damage were visible after 24 hours.

PLFA and Cu analysis

Two to 3 mg of freeze-dried and crushed earthworm tissue was homogenized in 50 µl buffer (50 mM K_2HPO_4, pH 7.4) in 1.5 ml Eppendorf tubes and used for analysis of PLFA composition. Homogenates were transferred to glass test tubes and washed with 2x100 µl of the buffer and extracted with a one-phase mixture of chloroform, methanol and buffer (1:2:0.8 v/v/v) (*18*). Lipid extracts were dissolved in 100 µl chloroform and fractionated on prepacked columns with 100 mg silic acid (Bond Elut Extraction Cartridges, Varian US). The PLFA fraction was extracted from the columns with 1.5 ml methanol. After collection of the PLFAs an internal standard (19:0) was added (2.3 mg/sample) and the sample transesterified by a mild alkaline methanolysis (*19*). The resulting fatty acid methyl esters were separated on a Hewlett Packard 6890 gas chromatograph and identified through GC-MS analysis as described previously (*9*).

Fatty acids were designated as *X*:*Y*ω*Z*, where *X* is the number of carbon atoms, *Y* is the number of double bonds and *Z* indicates the position of the first double bond from the methyl end of the molecule, if known.

The molar percentage of each PLFA in each sample was estimated by dividing the amount of each peak with the total amount of PLFAs that were determined in each sample. Overlapping peaks of 20:1 and 20:2 were identified in the GC-MS analysis, and called "20:1&2" (*9*). The degree of unsaturation (UI) was calculated as: Σ (% monoenes + 2 x % dienes + 3 x % trienes….)/100 (*20*). In addition, the unsaturation ratio, UFA/SFA, and the average chain length at all copper concentrations were calculated.

The internal Cu concentration of the earthworms was analyzed using AAS as described by Bindesbøl et al. (*10*).

Lipid peroxidation

Approximately 100 mg (fresh weight) of earthworm tissue in total was taken from the head, middle and tail region and homogenized on ice for one minute in a phosphate buffer, with EDTA and pH 7.0 in a 1:10 ratio, using a Ultra-Turrax T8 homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). The homogenate was centrifuged at 9000*g* for 30 min at 4 °C to yield the post-mitochondrial fraction (supernatant: S9). This fraction was purified using 10,000 NMWL spin filter column (UFC3LGC00, Millipore, Denmark) centrifuged at 5000 *g* for two hours at 4 °C. Aliquots were frozen at –80 °C until further use. Lipid peroxidation was measured using a commercial reagent kit (Northwest Life Science Specialities LLC, Vancouver, WA), which is based on the formation of thiobarbituric acid reactive substances (TBARS) and quantified in terms of malondialdehyde (MDA).

Statistical analysis

One-way analysis of variance (ANOVA) was used to test for differences in PLFA, UI, UFA/SFA and average number of C-atoms in the PLFAs (chain length). Data were transformed appropriately to achieve homogeneity of variance and normal distribution. In cases were the F-test was significant, Fishers PLSD was used to determine differences between spe-

cific treatments. The sequential Bonferroni correction for multiple testing test, with an initial α level of $p<0.05/k$ (k = number of tests), was performed to avoid the confounding effects of multiple testing (*21*).

The effect of the proportion of PLFA 18:2ω6,9 on survival was investigated in a generalized linear model (SAS 9.1 PROC GENMOD) with thermal treatment as a class variable and the proportion of 18:2ω6,9 as a quantitative variable, and assuming the survival data to be binomially distributed (*22*).

The effects on survival of Cu, temperature and possible interaction effect of combining the two factors were modelled by a modified dose-response model as described in Bindesbøl et al. (*10*). Credibility intervals (95 %) of the modelled LT50 (temperature causing 50 % mortality) were estimated as described in Holmstrup et al. (*23*).

A linear regression model was used to test the relationship between Cu in the soil and the subsequent concentrations in earthworm tissue, and the relationship between Cu and lipid peroxidation (MDA). A visual inspection of the residuals suggested that the data should be log transformed. After this transformation the residuals were found to be normally distributed and with variance homogeneity in all cases.

Results

Effects of subzero temperatures and Cu on survival

A highly significant synergistic interaction occurred between Cu and frost ($p<0.0001$; Table 1) indicating that Cu significantly reduced the freeze tolerance of *D. octaedra*. Without Cu, survival declined from 100 % in worms at +2 °C and –2 °C to approximately 50 % in worms exposed to –4, –6 and –8 °C, whereas Cu exposed earthworms were significantly less freeze tolerant (Table 1). The synergistic interaction between Cu and frost is illustrated in Figure 1

Table 1. Observed and expected survival (%) of *Dendrobaena octaedra* after 6 weeks of thermal acclimation in copper contaminated soil followed by freezing for 10 days at various temperatures. The expected values, number in brackets, were calculated using a modified dose-response model as described in Bindesbøl et al. (*10*). A highly significant synergistic interaction existed between Cu and frost ($p<0.0001$)

Temperature (°C)	Copper concentration (mg / kg dry soil)					
	0	**60**	**120**	**180**	**240**	**300**
2	100 (100)	100 (99.5)	100 (99.1)	100 (98.6)	100 (98.1)	93 (97.7)
–2	100 (83.5)	90 (81.0)	100 (76.6)	10 (69.3)	30 (58.3)	80 (44.1)
–4	53 (71.2)	40 (67.6)	40 (59.4)	50 (44.2)	10 (25.4)	0 (10.3)
–6	47 (57.2)	60 (52.8)	20 (40.3)	11 (18.5)	10 (6.79)	0 (1.63)
–8	47 (43.1)	70 (38.2)	20 (23.1)	11 (6.27)	10 (1.37)	0 (0.24)

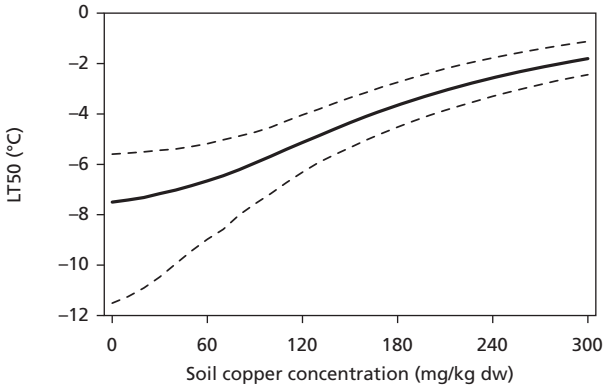

Figure 1. The estimated temperature causing 50 % mortality (LT50) as a function of soil copper concentrations. The dashed lines indicate 95% credibility interval.

showing that the temperature where 50 % of the worms died was higher when exposed to high soil Cu concentrations. For example, the temperature where 50 % of the worms died increased from –7 °C at 60 mg/kg dw to –3 °C at 240 mg/kg dw.

Cu content in worms

The internal Cu concentration increased linearly, except at the highest concentration, with exposure to increasing soil Cu concentrations (Figure 2). A significant positive correlation existed between Cu in soil and Cu in tissue ($R^2 = 0.76$, $p < 0.0001$). The highest internal concentration was found in the worms exposed to Cu soil concentrations of 240 mg/kg dw, where mean tissue concentration was 113 mg/kg DW.

Lipid peroxidation

Lipid peroxidation measured as MDA increased slightly at the highest Cu concentrations (Figure 3). A significant positive correlation existed between Cu in soil and lipid peroxidation in the tissue although variation between individuals was high ($R^2 = 0.10$, $p < 0.009$).

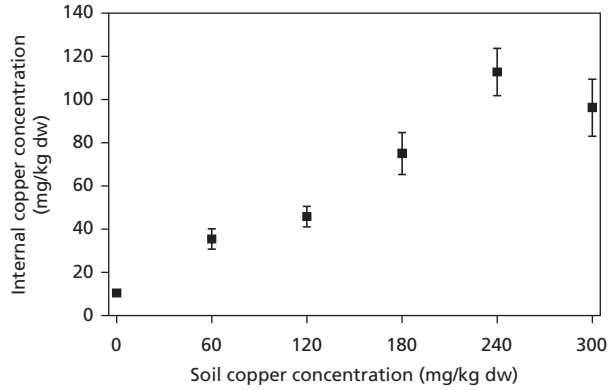

Figure 2. Copper content in *Dendrobaena octaedra* exposed to different soil copper concentrations for one week at 10 °C followed by one week at 5 °C and finally for 4 weeks at 2 °C (mean ± S.E., $N = 8$).

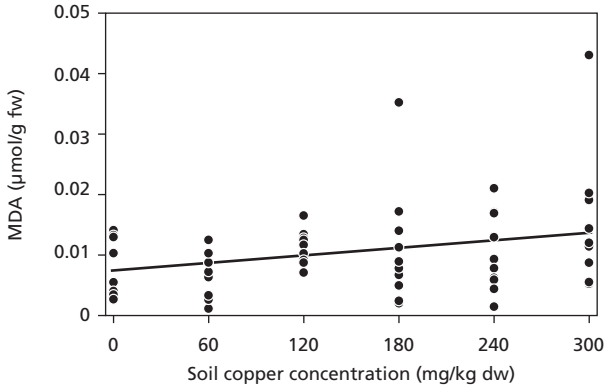

Figure 3. Lipid peroxidation, measured as malondialdehyd (MDA), in *Dendrobaena octaedra* exposed to different copper concentrations for 6 weeks. Individual measurements are shown as closed circles. A linear regression is indicated ($R^2 = 0.10$, $p < 0.009$).

Cu's effect on PLFA

Nineteen different PLFAs were identified in total. The observed changes in PLFA due to Cu were within an order of 2 mol% or less (Table 2). Cu had significant effects on three PLFAs through highly significant reductions in 16:0 (p = 0.0003), 20:3 and 18:2ω6,9 (p < 0.0001). Changes also pointed to an increase in the PLFA 20:5 and a decrease in 20:0. Cu had no significant effect on the degree of unsaturation UI (p = 0.09). However, we observed a significant effect on the unsaturation ratio UFA/SFA and chain length (p = 0.0024 and p = 0.0009, respectively) even though no directional trends due to Cu were obvious (Table 2). Fisher's PLSD test showed that the mol% of 18:2ω6,9 in worms exposed to the highest Cu concentration was significantly lower than in the worms from the other exposure concentrations (Figure 4). Further, a significant positive relationship existed between the proportion of 18:2ω6,9 and survival (p < 0.0001). It was tested whether the slopes of the assumed linear relationships between survival and 18:2ω6,9 varied with temperature, but such an interaction effect between 18:2ω6,9 and temperature on survival was not significant.Thus, data shows that poor freeze survival coincided with high Cu concentrations where relatively low proportions of 18:2ω6,9 were found.

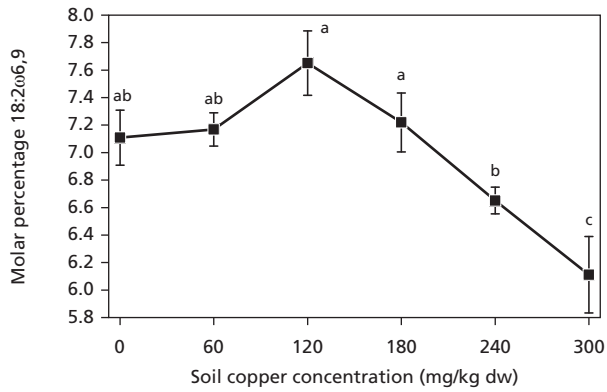

Figure 4. The molar percentage (mean ± SE) of the phospholipid fatty acid, 18:2ω6,9, in worms exposed to increasing copper concentrations during 6 weeks of cold acclimation.

Table 2. Molar percentage distribution (mean ± S.E., N = 10) of phospholipid fatty acids (PLFA) in *Dendrobaena octaedra*. Worms were exposed for 6 weeks to nominal soil concentrations of 0, 60, 120, 180, 240 and 300 mg Cu/kg dry soil.

PLFA	Copper concentration mg/kg dry soil					
	0	60	120	180	240	300
14:0	0.47 ± 0.12	0.71 ± 0.09	0.41 ± 0.02	0.43 ± 0.02	0.57 ± 0.17	0.36 ± 0.02
i15:0	1.12 ± 0.13	1.59 ± 0.11	1.30 ± 0.04	1.34 ± 0.06	1.37 ± 0.09	1.35 ± 0.09
Me14	0.95 ± 0.13	1.32 ± 0.06	1.15 ± 0.07	1.10 ± 0.07	0.92 ± 0.06	0.71 ± 0.08
15:0	0.27 ± 0.03	0.38 ± 0.03	0.29 ± 0.01	0.26 ± 0.01	0.22 ± 0.02	0.21 ± 0.01
16:1ω9	0.60 ± 0.03	0.74 ± 0.04	0.67 ± 0.02	0.65 ± 0.02	0.60 ± 0.02	0.59 ± 0.02
16:1ω7c	0.52 ± 0.06	0.58 ± 0.05	0.76 ± 0.09	0.56 ± 0.04	0.51 ± 0.03	0.49 ± 0.03
16:1ω5	0.18 ± 0.01	0.26 ± 0.01	0.23 ± 0.01	0.24 ± 0.02	0.24 ± 0.03	0.24 ± 0.02
16:0	2.11 ± 0.31	2.69 ± 0.26	2.05 ± 0.09	1.83 ± 0.16	1.60 ± 0.12	1.43 ± 0.10
Me16	1.70 ± 0.08	1.94 ± 0.09	1.98 ± 0.12	1.83 ± 0.09	1.64 ± 0.07	1.40 ± 0.08
17:0	3.06 ± 0.06	3.28 ± 0.15	3.40 ± 0.07	3.17 ± 0.05	3.18 ± 0.08	3.04 ± 0.08
18:2ω6	7.11 ± 0.20	7.17 ± 0.12	7.65 ± 0.23	7.22 ± 0.21	6.65 ± 0.10	6.11 ± 0.28
18:1ω9	4.10 ± 0.14	3.87 ± 0.14	4.18 ± 0.15	4.09 ± 0.16	4.43 ± 0.23	4.56 ± 0.13
18:1ω7	6.94 ± 0.11	7.49 ± 0.30	7.17 ± 0.22	6.78 ± 0.18	6.80 ± 0.16	6.55 ± 0.26
18:0	10.46 ± 0.22	10.71 ± 0.39	11.47 ± 0.45	10.66 ± 0.22	11.16 ± 0.37	10.30 ± 0.14
20:4	20.20 ± 1.08	19.55 ± 0.82	17.29 ± 0.54	18.92 ± 0.69	17.80 ± 0.40	19.90 ± 1.16
20:5	19.20 ± 1.07	17.46 ± 0.81	18.05 ± 0.98	19.45 ± 0.36	20.41 ± 0.74	21.19 ± 0.35
20:3	5.33 ± 0.37	4.99 ± 0.21	4.88 ± 0.10	4.38 ± 0.22	3.19 ± 0.14	3.41± 0.26
20:1&2	3.48 ± 0.18	2.95 ± 0.19	3.39 ± 0.09	3.36 ± 0.12	3.23 ± 0.16	3.27 ± 0.09
20:0	12.19 ± 0.70	12.35 ± 1.03	13.68 ± 0.43	13.72 ± 0.68	15.48 ± 0.67	14.90 ± 0.48
UI	2.25 ± 0.04	2.12 ± 0.07	2.12 ± 0.07	2.18 ± 0.03	2.14 ± 0.05	2.25 ± 0.04
UFA/SFA	3.44 ± 0.14	2.92 ± 0.13	2.95 ± 0.12	3.19 ± 0.07	3.11 ± 0.12	3.54 ± 0.11
Chain length	18.99 ± 0.04	18.86 ± 0.03	18.87 ± 0.03	18.97 ± 0.02	18.94 ± 0.03	19.05 ± 0.03

Discussion

This study clearly demonstrates that Cu exposure altered the PLFA composition in cellular membranes of *D. octaedra* despite the fact that we analysed data from whole-body extracts which may suffer from low resolution since lipids of many different tissues are mixed. This may mask membrane adjustment in particular tissues (*24*) but nevertheless a clear influence of Cu was detected in our study. In particular, the analysis showed that Cu significantly decreased the proportion of the PLFA, 18:2ω6,9, which previously has been reported to correlate positively ($R^2 = 0.92$) with freeze tolerance in *D. octaedra* (*9*) and other invertebrates (*24*). This result supports our hypothesis that synergistic interactions between Cu and freezing may be due to membrane damage. Taulovuori et al. (*25*) hypothesised that heavy metals increase the risk of frost injury due to membrane alterations in plants at northern high latitudes, but as far as we know this has never previously been explicitly detected in any organism.

Cu has previously been shown to change fatty acid (FA) composition of membranes in vascular plants. Thus, in maize seedlings a significant decrease of 18:2 (based on total FAs) occurred in the roots exposed to 100 μM Cu and a slight decrease in unsaturation occurred (*17*). Similar trends were observed in roots of wheat seedlings and tomatos exposed to an aqueous Cu concentration of 50 μM (*15, 16*). In these studies 18:2 and 18:3 decreased and a reduction in overall unsaturation was observed. These previous results are strikingly similar to those of the present study, especially for 18:2ω6,9, strongly suggesting that this effect of Cu is a general one occurring in a wide range of organisms.

The changes in PLFAs can probably be explained by Cu's ability to induce lipid peroxidation. Polyunsaturated fatty acids that contain two or more double bonds are particularly susceptible to oxidation by free radicals and other highly reactive species. Cu ions (Cu^{++}), in excess of cellular needs, mediate free radical production and direct oxidation of lipids (*14*). The lipid peroxidation products in our study (MDA) were only slightly increased at the end of the six week exposure period, but may have been higher at the beginning of the Cu exposure before free radical- protective mechanisms had been induced. Correia et al. (*26*) showed that lipid peroxidation in *Gammarus locusta* increased significantly after four days of Cu exposure but returned to control levels again after six days. This might also have been the case in our study.

The PLFA changes occurring in ectothermic animals under naturally decreasing temperatures (e. g. seasonal acclimatization during autumn) are typically associated with an increase in the degree of unsaturation leading to a more disordered membrane which is less likely to undergo phase transition at cold temperatures (*11, 12, 27*). These changes are an important part of the complement of physiological changes enabling ectothermic organisms to withstand low winter temperatures. However, we did not see any significant change in the overall unsaturation index in Cu exposed worms even though the freeze tolerance of these worms was significantly reduced. In the present study the potential decrease in unsaturation associated with the significant reduction in 18:2ω6,9 was counteracted by a concomitant increase in 20:5. Several other studies have shown that unsaturation does not necessarily correlate with tolerance to sub-zero temperatures (*9, 18, 28*). Regulation of membrane T_m via changes in PLFAs is, however, not only a matter of increasing the overall degree of unsaturation (*29-32*). The position and the number of double bonds seem to have a significant effect on T_m. Double bonds positioned in the middle of the FA chain leads to a lower T_m compared to double bonds positioned at the end of the FA (*32*). Studies have also shown that increasing the number of double bonds above two or three, depending on length of FA chain, actually can increase T_m (*29, 32*). Thus, the decrease in 18:2ω6,9 in our study which have a double bond positioned in the middle of the FA chain, and possibly the increase in 20:5, can give rise to an increased T_m even though unsaturation did not decrease. The lowered freeze tolerance we observed after Cu exposure could therefore be due to a phase transition in the membrane from the liquid-crystalline phase to the more ordered gel phase during freezing in the Cu exposed worms.

The observed changes in the composition of PLFAs in *D. octaedra* may seem rather small, but many other studies have shown that similarly slight changes in PFLA composition are associated with significant changes in freeze tolerance or cold tolerance in general (*18, 24, 33*). Therefore, we find it reasonable to conclude that the changes in PLFAs observed in the present study after Cu exposure could provide a mechanistic explanation for the reduced freeze tolerance. Having said this, it is clear that other molecular mechanisms of importance to freeze tolerance may also be influenced by Cu which is one of many metals known to cause protein denaturation as a result of its affinity for sulfhydryl groups (*14*).

The reduced freeze tolerance in the present study was especially apparent at soil Cu concentrations above 120 mg/kg dw. These Cu concentrations are within the range that can be found at many metal contaminated field sites where *D. octaedra* is often found (*6, 34, 35*). The internal concentrations of Cu observed in this experiment are also within the concentration that has been found in *D. octaedra* collected in the field ranging from approximately 100 to 300 mg Cu/kg dw at the metal contaminated site, Gusum, Sweden (*6*). This suggests that natural populations of *D. octaedra* in this area could be less tolerant to subzero temperatures that inevitably will occur during winter months. No published studies of freeze tolerance of invertebrates collected directly from polluted areas exist, but a few examples may be found in investigations of freeze tolerance of pine trees growing in metal polluted areas. For example, a reduction in the PLFAs, 18:2 and 18:3, of cell membranes in needles of Scots pine (*Pinus sylvestris*) has been observed in the field near a Cu smelter in Glogow, Poland (*36*). These changes could very well lead to a reduced freeze tolerance in those needles, and indeed, Sutinen et al. (*37*) observed that needles of *P. sylvestris* were more susceptible to frost near a Cu-nickel smelter in Russia than those further away from the smelter.

In conclusion, the present study and several studies of vascular plants suggest a causal link between perturbation of membrane PLFA composition as a consequence of Cu exposure and a reduced freeze tolerance. The fact that such effects of Cu occur across different species both in the laboratory and in the field strongly supports our conclusion.

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References

(1) Heugens, E. H. W.; Hendriks, A.J.; Dekker, T., van Straalen, N.M.; Admiraal, W. A. Review of the effects of multiple stressors on aquatic organisms and anlysis of uncertanty factors for use in risk assessment. *Crit. Rev. Toxicol*. **2001**, 31, 247-284.

(2) Friis, K.; Damgaard, C. F.; Holmstrup, M. Sublethal soil copper concentrations increase mortality in the earthworm *Aporrectodea calignosa* during drought. *Ecotox. Environ. Saf.* **2004**, 57, 65-73.

(3) Rozen, A. Do earthworms (*Dendrobaena octaedra*) from differently polluted forests vary in life-history parameters? *Eur. J. Soil. Biol.* **2003**, 39, 73-77.

(4) Bengtsson, G.; Ek, H.; Rundgren, S. Evolutionary response of earthworms to long-term metal exposure. *Oikos.* **1992**, 63, 289-297.

(5) Helling, B.; Reinecke, S. A.; Reinecke, A. J.. Effects of the fungicide copper oxychloride on the growth and reproduction of *Eisenia fetida* (oligochaeta). *Ecotox. Environ. Saf.* **2000**, 46, 108-116.

(6) Bengtsson, G.; Nordström, S.; Rundgren, S. Population density and tissue metal concentration of lumbricids in forest soils near a brass mill. *Environ Pollut A*. **1983**, 30, 87-108

(7) Rundgren, S. Vertical distribution of lumbricids in southern Sweden. *Oikos*. **1975**, 26, 299-306.

(8) Overgaard, J.; Slotsbo, S.; Holmstrup, M.; Bayley, M. Determining factors for cryoprotectant accumulation in the freeze-tolerant earthworm, *Dendrobaena octaedra*. *J. Exp. Zool. Part A*. **2007**, 307, 578-589.

(9) Holmstrup, M.; Sørensen, L. I.; Bindesbøl, A.; Hedlund, K. Cold acclimation and lipid composition in the earthworm *Dendrobaena octaedra*. *Comp. Biochem. Physiol. A*. **2007**. 147, 911-919.

(10) Bindesbøl, A.; Holmstrup, M.; Damgaard C. F.; Bayley, M.. Stress synergy between environmentally realistic levels of copper and frost in the earthworm *Dendrobaena octaedra*. *Environ. Toxicol. Chem*. **2005**, 24, 1462-1467.

(11) Sinensky, M.. Homeoviscous adaptation – a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A*. **1974**, 71, 522-525.

(12) Hazel, J. R.. Thermal adaptation in biological membranes: Is homeoviscous adaptations the explanation? *Annu. Rev. Physiol*. **1995**, 57, 19-42.

(13) Weeks, J. M.; Svendsen, C. Neutral red retention by lysosomes from earthworm (*Lumbricus rubellus*) coelomocytes: a simple biomarker of exposure to soil copper. *Environ. Toxicol. Chem*. **1996**, 15, 1801-1805.

(14) Valko, M.; Morris, H.; Cronin, M. T. D. Metals, toxicity and oxidative stress. *Curr. Med. Chem*. **2005**, 12, 1161-1208.

(15) Ouariti, O.; Boussama, N.; Zarrouk, M.; Cherif, A.; Ghorbal, M. H. Cadmium- and copper-induced changes in tomato membrane lipids. *Phytochemistry*. **1997**, 45, 1343-1350.

(16) Calucci, L.; Pinzino, C.; Quartacci, M. F.; Navari-Izzo, F. Copper excess reduces the fluidity of plama membrane lipids of wheat: a spin probe epr study. *J. Physical Bioch B*. **2003**, 107, 12021-12028.

(17) Chaffai, R.; Elhammadi, M. A.; Seybou, T. N.; Tekitek, A.; Marzouk, B.; El Ferjani, E.. Altered fatty acid profile of polar lipids in maize seedlings in response to excess copper. *J. Agron. Crop Sci*. **2007**, 193, 207-217.

(18) Bayley, M.; Petersen, S. O.; Knigge, T.; Köhler H. R.; Holmstrup, M. Drought acclimation confers cold tolerance in the soil collembolan *Folsomia candida*. *J. Insect physiol*. **2001**, 47, 1197-1204.

(19) Dowling, N.; Widdel, F.; White, D. Phospholipid ester-linked fatty acids biomarkers of acetate-oxidizing sulphate-reducers and other sulphide-forming bacteria. *J. Gen. Microbiol.* **1986**, 132, 1815-1825.

(20) Kates, M. *Techniques of Lipodology*. Elsevier, Amsterdam, Netherlands. 1986.

(21) Rice, W. R. Analyzing tables of statistical tests. *Evolution*. **1989**, 43, 223-225.

(22) McCullagh, P.; Nelder, J. A. *Generalized linear models*, 2 edn. CRC, Boca Raton. 1999.

(23) Holmstrup, M.; Aubail, A.; Damgaard, C.F. Exposure to mercury reduces cold tolerance in the springtail *Folsomia candida*. *Comp. Biochem. Physiol. B*. **2008**, 148, 172-177.

(24) Kostal, V.; Berkova, P.; Simek, P. Remodelling of membrane phospholipids during transition to diapause and cold-acclimation in the larvae of *Chymomyza costata* (Drosophilida). *Comp. Biochem. Physiol. B*. **2003**, 135, 407-419.

(25) Taulavuori, K.; Prasad, M.N.V.; Taulavuori, E.; Laine, K. Metal stress consequences of frost hardiness of plants at northern high latitudes: a review and hypothesis. *Environ. Pollut.* **2005**, 135, 209-220.

(26) Correia, A. D.; Livingstone, D. R.; Costa, M. H. Effects of water-borne copper on metallothionein and lipid peroxidation in the marine amphipod *Gammarus locusta*. *Mar. Environ. Res*. **2002**, 54, 357-360.

(27) Lee, Jr R. E.; Damodaran, K.; Yi, S.; Lorigan, G. A. Rapid cold-hardening increases membrane fluidity and cold tolerance of insect cells. *Cryobiology.* **2006**, 52, 459-463.

(28) Overgaard, J.; Tomacala, A.; Sørensen, J. G.; Holmstrup, M.; Krogh, P. H.; Simek, P.; Kostal, V. Effects of acclimation temperature on thermal tolerance and membrane phospholipid composition in the fruit fly *Drosophila melanogaster*. *J. Insect Physiol*. **2008**, 54, 619-629.

(29) Coolbear, K.P.; Berde, C.B.; Keough, K.M.W. Gel to liquid-crystalline phase transitions of aqueous dispersion of polyunsaturated mixed-acid phosphatidylcholines. *Biochemistry.* **1983**, 22, 1466-1473.

(30) Hazel, J. R.; Williams, E. E. The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Prog. Lipid Res*. **1990**, 29, 167-227.

(31) Wang, G.; Li, S.; Lin, H.; Brumbaugh, E. E.; Huang, C. Effects of various numbers and positions of *cis* double bonds in the *sn*-2 acyl chain of phosphatidylethanolamine on the chain-melting temperature. *J. Biol. Chem*. **1999**, 274, 12289-12299.

(32) Baenziger, J. E.; Jarrel, H. C.; Smith, I.C. P. Molecular motions and dynamics of a diunsaturated acyl chain in a lipid bilayer: implacations for the role of polyunsaturation in biological membranes. *Biochemistry*. **1992**, 31, 3377-3385.

(33) Overgaard, J.; Sørensen, J. G.; Petersen, S. O.; Loeschcke, V.; Holmstrup, M. Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. *J. Insect Physiol*. **2005**, 51, 1173-1182.

(34) Lukkari, T.; Taavitsainen, M.; Väisänen, A.; Haimi, J. Effects of heavy metals on earthworms along a contamination gradient in organic rich soils. *Ecotox. Environ. Saf*. **2004**, 59, 340-348.

(35) Koslow, M.V.; Zvereva, E.L. Industrial barrens: extreme habitats created by non-ferrous metallurgy. *Rev. Environ. Sci. Biotechnol*. **2007**, 6, 231-259.

(36) Pukacki, P. M. The effects of industrial air pollution on the membrane lipid composition of scots pine (*Pinus sylvestris* L.) needles. *Acta Soc. Bot. Pol*. **2004**, 73, 187-191.

(37) Sutinen, M-J.; Raitio, H.; Nivala, V.; Ollikainen, R.; Ritari, A. Effects of emissions from copper-nickel smelters on the frost hardiness of *Pinus sylvetris* needles in the subarctic region. *New. Phytol.* **1996**, 132, 503-512.

Paper 5

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Exposure to heavy metals reduces freeze tolerance of the earthworm *Dendrobaena octaedra*

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Exposure to heavy metals reduces freeze tolerance of the earthworm *Dendrobaena octaedra*

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Abstract

Previous studies have shown that the interactions between chemicals and climatic stressors can lead to synergistically increased mortality. In the present study we investigated the effect of seven common environmental contaminants on survival at –6 and 15 °C as well as reproduction at 15 °C in the earthworm *Dendrobaena octaedra*.

Three classes of chemicals were considered: heavy metals (nickel and mercury), polycyclic aromatic hydrocarbons (pyrene and phenanthrene) and pesticides (abamectin and carbendazim). Phenanthrene interacted antagonistically with freezing temperatures whereas no interaction was observed with any of the tested pesticides. Two out of the three tested metals reduced the freeze tolerance synergistically and especially mercury was very potent. This suggests that traditional laboratory studies, where the organisms are exposed to increasing concentrations of a single compound under otherwise optimal conditions, may underestimate the toxicity of some metals in field populations living in cold areas.

Keywords: *Dendrobaena octaedra*; Freeze tolerance; Environmental contaminants; Reproduction; Risk assessment

Introduction

In traditional ecotoxicological studies, organisms are usually exposed to a single chemical at increasing concentrations, performed under otherwise optimal conditions. The risk assessment of chemicals is usually based on such results from acute lethality tests or chronic reproduction tests. However, in natural environments it is very likely that these organisms will be exposed to other stressful factors in combination with chemicals of anthropogenic origin, including climatic stressors such as cold or drought. This suggests that traditional laboratory tests may underestimate the toxic effects of chemicals in natural environments. Several studies have shown that the interactions between chemicals and climatic stressors can lead to synergistically increased mortality, and it is therefore important to include such aspects in chemical risk assessment [1,2].

The freeze tolerant earthworm, *Dendrobaena octaedra*, is widely distributed in the northern hemisphere, including Europe, Siberia, North America and Greenland [3-6]. It lives in the litter layer, between plant roots and in decaying tree stumps [3], and is often exposed to sub-zero temperatures, including areas polluted by toxic chemicals. To ensure winter survival, *D. octaedra* accumulates glucose to high concentrations as a cryoprotectant at the onset of the body fluid freezing process [7] and changes the lipid chemistry during cold acclimation [8].

Earthworms play a major role in the breakdown of organic matter and the release of nutrients in many terrestrial ecosystems [9], and have therefore been widely used in ecotoxicological assessment of soil pollutants [10-12]. In these standard ecotoxicological bioassays, the aspects of multiple stressors including freeze tolerance are not considered, e.g. chemical influences on freeze tolerance. The chemical threshold level exerting such an effect on ecologically important tolerance mechanisms is interesting because it could play an important role in the difficult task of extrapolating from laboratory tests to field conditions.

Comparative studies of the toxicity of a variety of chemicals and their effects on tolerance towards another important climatic stressor, drought, have shown in Collembola that reproduction was the most sensitive parameter for most chemicals [13-15]. However, a few compounds did reduce drought tolerance at concentrations similar to or below the concentrations that had an effect on reproduction. It is possible that these chemicals specifically affect physiological mechanisms involved in drought tolerance, such as membrane fluidity adaptations.

It has been shown that the copper-induced reduction in freeze tolerance of *D. octaedra* could be explained by membrane perturbations [16]. The effects of other common pollutants on freeze tolerance have received limited attention and only a few studies exist [17,18] although the problem has been raised at several occasions during the last decades [e.g. 19,20]. In the present study we therefore examine the effects of several chemicals, chosen in order to represent different modes of action possibly enabling us to identify classes of toxicants with particular relevance for interactions with freeze tolerance. The compounds tested include heavy metals (nickel, mercury and lead), PAHs (pyrene and phenanthrene), and pesticides (abamectin and carbendazim). Heavy metals are able to change the lipid composition of membranes due to their ability to induce lipid peroxidation [21,16]. PAHs are likely to accumulate in membranes because of their lipophilic characteristics and their structure with no functional groups [22], which may affect the fluidity and function of membranes [23]. PAHs have been shown to reduce the drought tolerance of Collembola [13-15] and since freeze tolerance and drought tolerance have many physiological adaptations in common [24,25] we expect these chemicals to reduce freeze tolerance as well. We

hypothesize, that previous exposure to compounds affecting membrane function (metals and PAHs) reduces the freeze tolerance, whereas pesticides with more specific mode of action do not.

In addition to testing for effect on freeze tolerance, we tested the effect of the same chemicals on survival and reproduction (cocoon production) at 15 °C, enabling us to compare the concentration of each chemical causing a 50% decrease in survival and reproduction at optimal temperature (15 °C) with the concentration causing 50% decrease in freeze tolerance (measured as survival at –6 °C). These comparisons allow us to identify the groups of chemicals that may be particularly potent in reducing freeze tolerance.

Materials and Methods

Animals

D. octaedra were collected in a coniferous forest near Silkeborg, Denmark, in the spring of 2007. The earthworms were kept in culture at 15 °C in moist soil and fed on a diet of cow-dung. Cocoons collected from the culture were incubated at 20 °C in Petri dishes layered with wet filter paper. Newly hatched juveniles were transferred to moist soil and kept in the same way as the adults until they had reached a suitable size for the freeze experiment (above 50 mg) or reached adulthood for the survival and reproduction experiment.

Test substances

Pyrene, Phenanthrene, $NiCl_2$ and Abamectin were provided from Sigma-Aldrich. Acetone (J.T. Barker, Rødovre, Denmark) was used as a solvent. $PbCl_2$ and $NiCl_2$ were both provided from Merck, Germany, and Bavistin (carbendazim 50%) from BASF Agro, Ecully Cedex, France.

Soil and food

Topsoil from an ecologically farmed Danish pea field (Foulum, Viborg) was used for the experiment. The soil was a loamy sand consisting of 35 % coarse sand, 45 % fine sand, 9.4 % silt, 8.9 % clay and 1.7 % organic matter. The pH-H_2O was approximately 6.8. Prior to use, the soil was dried for 24 hours at 80 °C and sieved through a 2 mm mesh. The soil was contaminated by adding a solution of the chemicals dissolved in either deionised water or acetone to obtain ranges of nominal concentrations, as shown in the figures.

Acetone was used as a solvent for pyrene, phenanthrene and abamectin. After being mixed, these soils were placed in a fume cupboard overnight to let the acetone evaporate. The water content was then adjusted to the desired level of 20% of dry weight (dw) (approximately pF = 2 and 50% water holding capacity). Water was used as a solvent for the remaining chemicals. All test soils were left overnight in closed containers to equilibrate.

The cow-dung food was produced by adding 400 ml demineralised water to 150 g dried and finely ground cow-dung.

Survival and reproduction of adults

A range of 6-8 concentrations were used for each test chemical. Ten worms were exposed at each concentration. The age of the worms in each treatment was the same. Each individual was placed separately in a 200 ml plastic beaker (diameter 7 cm; height 4.2 cm) containing 75 ± 1 g moist soil (wet weight) with the required concentration of the tested

chemical. Six mg (wet weight) cow-dung was added as earthworm food. The soil pH was equal (pH 6.8) in all treatments after the addition of cow-dung. All containers were covered with perforated lids allowing ventilation. The worms were kept at 15 °C during the 4 week exposure period. At the end of this test period, survival was determined and the cocoons were sampled by wet sieving the soil through a 1 mm mesh and counted.

Frost and chemical exposure

Animals were exposed to the required concentrations of chemicals in soil for one week at 10 °C followed by one week at 5 °C, and finally for 4 weeks at 2 °C prior to exposure to the freezing temperature of –6 °C. This temperature was selected to ensure moderate effects of freezing [16]. For each concentration of the various chemicals 30 worms were exposed singly as described, of which 15 were used as temperature control without freezing (2 °C) and 15 were frozen as described below.

Unfrozen control worms were kept in the original beakers at 2 °C, whereas worms destined for freezing were placed in 8 ml tubes along with a few grams of the appropriate substrate. In each lid, two small needle holes were made for ventilation. These worms were placed at –1 ± 0.2 °C in a walk-in freezer and after 24 hours a small ice crystal was placed on the soil to ensure inoculative freezing of the worm [26]. After another 24 hours the worms were transferred to a programmable freezing cabinet precise to ± 0.2 °C and programmed to gradually lower the temperature from –1 °C to –6 °C (0.042 °C/h). Prior to the assessment of survival, the worms were transferred to 5 °C and allowed to thaw. The earthworms were considered to have survived if there was a reaction to tactile stimuli, normal locomotor activity, and if no signs of freezing damage were visible after 24 hours.

Statistics

The effect of the chemicals at different temperatures was compared using a modified dose-response function,

$$f(x;b,x_0)=\frac{1+\exp(-b\,x_0)}{1+\exp(b(x-x_0)} \qquad x\geq 0, b\geq 0, \tag{1}$$

where $f(x)$ is the expected effect of a single stress factor x, x_0 is the point of inflection (roughly indicating the 50% effect level) and b is the shape parameter of the function describing how steep the slope of the dose-response curve is at the point of inflexion. If b = 0, then $f(x)$ = 1 [27].

The effect of a chemical on the probability of survival is modelled by:

$$p(c)=(1-\lambda)\,f(c;b_c,x_{0,c}) \tag{2}$$

where c is the concentration of the chemical and $\lambda \in [0,1]$ is the estimated residual or control mortality. The mode of action of subzero temperature and the tested chemicals are assumed to be independent (i.e. the effects they inflict are mediated via different cellular processes), and therefore the combined effects of subzero temperature and chemicals should be estimated using a multiplicative rather than an additive model [28]. Our approach was to fit the survival data to the model (equation 2) under two circumstances:

where temperature was non-stressing (i.e. at 2 °C), and where worms were stressed by subzero temperature (i.e. at –6 °C). Synergistic or antagonistic interactions between temperature and the chemicals may be determined from possible differences of the dose response curves of the survival of *D. octaedra*. If there is synergy between temperature and the chemical, the point of inflection occurs at a lower concentration of the chemical (decrease in x_0) at the subzero temperature treatment compared to the control temperature treatment. Opposite, if there is antagony between temperature and the chemical, the point of inflection occurs at a higher concentration of the chemical (increase in x_0) in the subzero temperature treatment compared to the control temperature treatment. Further, interactions between temperature and chemicals may also appear as differences in the slope at the point of inflection, i.e. b of the dose-response curves. The occurrence of statistically significant differences of estimated values of b and x_0 in the two situations were tested using a likelihood-ratio test. Similarly, the effect of a chemical on the mean number of cocoons by a single adult is modelled by:

$$y(c) = a_c \; f(c;, b_c, x_{0,c}) \tag{3}$$

where c is the concentration of the chemical and a_c is the estimated mean number of offspring in the control treatment. The LC50 and the EC50 values of the chemicals are calculated by: $\log(2+\exp(b\, x_0))/b$

Eqs. (2) was fitted to the survival data of *D. octaedra* assuming the number of survivors were binomial distributed [27]. Eq. (3) was fitted to the reproduction data of *D. octaedra* assuming the number of offspring from each adult was Poisson distributed.

The log-likelihood functions of the regression models were maximized using the NMaximize routine in Mathematica (http://integrals.wolfram.com/index.jsp) with the described constraints on the parameters. The fit of the model was checked by plotting the residuals or by comparing the observed and predicted values in graphs and tables.

In order to calculate the 95% credibility intervals of the LC50 and EC50, the joint Bayesian posterior distribution of the parameters were calculated using MCMC methology (Metropolis-Hastings algorithm) with a multinomial candidate distribution (100,000 iterations with a burn-in period of 1000), and where the prior distribution of the parameters was assumed to be uniformly distributed of their domain [29].

Results

Adult survival and reproduction after exposure to chemicals

The survival of adults at 15 °C in all control treatments was 100%, but increasing concentrations of pyrene, phenanthrene, carbendazim, nickel and mercury caused a significant decline in survival compared to controls, whereas survival was unaffected at all tested concentrations of lead and abamectin (Fig 1).

A significant decline in reproduction was observed at increasing concentrations of all tested chemicals (Fig 1). Based on the results presented in Figure 1 the LC50 and EC50 were estimated when possible and otherwise indicated as larger than the maximum test concentration (Table 1).

Table 1. Estimated concentrations causing 50% reduction of cocoon production (EC50), 50% reduction of adult survival (LC50) and 50% reduction of freeze-survival (LC50F). Numbers in brackets indicate 95% credibility intervals.

	Lethality at 15 °C	Reproduction at 15 °C	Survival of freezing at –6 °C
Chemical	**LC50 (mg/kg)**	**EC50 (mg/kg)**	**LC50F (mg/kg)**
Pyrene	368 [335, 429]	148 [133, 164]	> 250
Phenanthrene	81 [71, 93]	30 [21, 47]	> 250
Carbendazim	1.9 [1.2, 2.8]	0.9 [0.7, 1.3]	> 2.5
Abamectin	> 9	6.6 [5.4, 7.6]	> 9
Lead	> 1200	501 [453, 568]	> 1000
Nickel	297 [247, 311]	121 [110, 133]	207 [169, 246]
Mercury	38 [29, 52]	7.7 [6, 10]	< 10
Copper*	> 300	160 < EC50 < 200	159 [129, 192]

*Results are reproduced from Bindesbøl et al. [16,58].

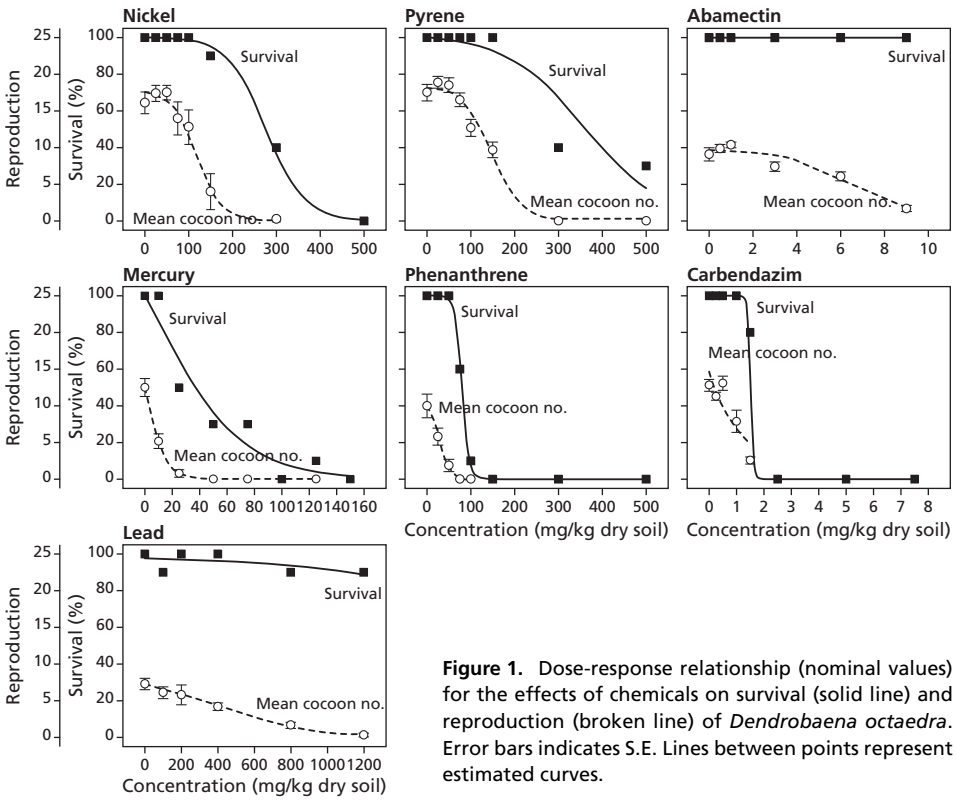

Figure 1. Dose-response relationship (nominal values) for the effects of chemicals on survival (solid line) and reproduction (broken line) of *Dendrobaena octaedra*. Error bars indicates S.E. Lines between points represent estimated curves.

Freeze survival

The freeze treatment in general caused 33 - 53% mortality in controls (Fig 2). The dose response curves differed significantly between the two temperature treatments showing that these two metals interacted with temperature. For nickel, x_0 of the two curves were statistically different (p = 0.02) with estimated LC50s of 261 and 207 mg/kg at 2 °C and –6 °C, respectively. Thus, a significant synergistic interaction between temperature and nickel was observed. For mercury, the two dose-response curves were also different. Here, b was significantly higher at –6 °C (p = 0.0008,) than at 2 °C. Further, LC50 at –6 °C was evidently much lower than at 2 °C (Table 1) showing that mercury drastically reduced freeze-tolerance (or vice-versa) with 100% mortality even at the lowest tested concentration of mercury (Fig 2). Lead did not have any effect on freeze survival (Fig 2).

A significant antagonistic interaction was found between phenanthrene and freezing, where x_0 of the two dose-response curves were significantly different (p = 0.04). The lethality of worms in the control series (2 °C) increased strongly at concentrations above 150 mg/kg dw, whereas the lethality of those worms that were simultaneously exposed to freezing was not affected within the tested range of concentrations (Fig. 2). No significant interaction was found between pyrene and freezing, although a tendency similar to that of phenanthrene exposure was observed (Fig. 2).

No interaction was observed between freezing and exposure to the two pesticides, abamectin and carbendazim. However, exposure at the two lowest concentrations of carbendazim seemed to increase freeze survival considerably (Fig. 2).

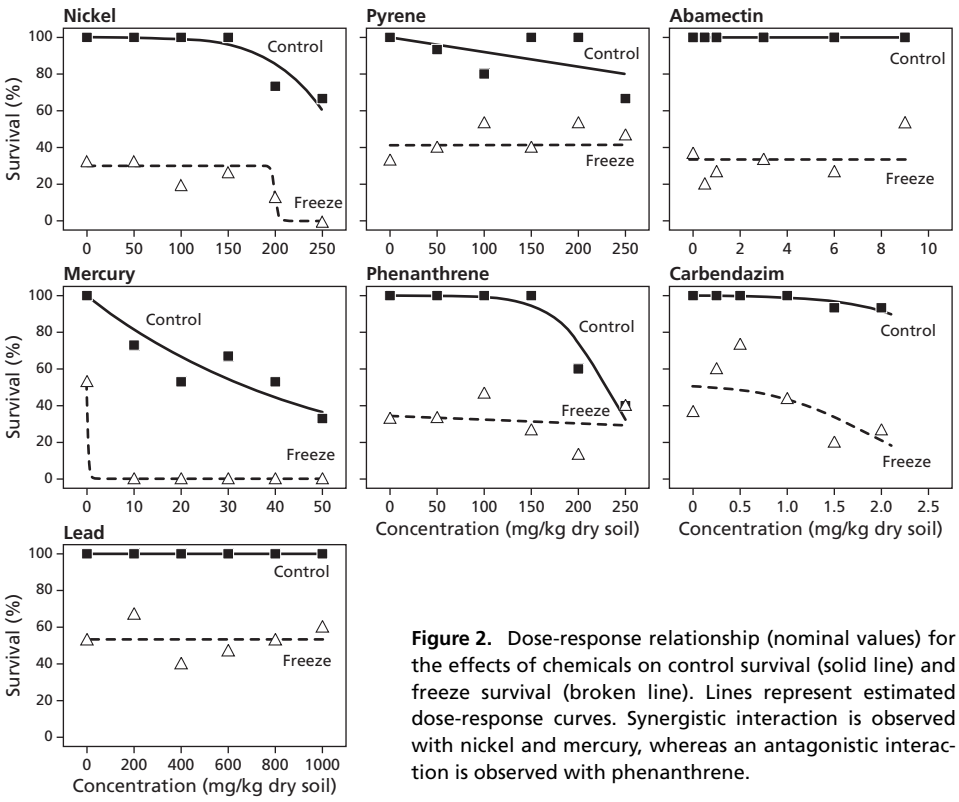

Figure 2. Dose-response relationship (nominal values) for the effects of chemicals on control survival (solid line) and freeze survival (broken line). Lines represent estimated dose-response curves. Synergistic interaction is observed with nickel and mercury, whereas an antagonistic interaction is observed with phenanthrene.

Discussion

Toxicity at 15 °C

Toxicity data for *D. octaedra* are very scarce, so comparisons are, of necessity, made with data from other earthworm species. In general, *D. octaedra* seemed to be more sensitive to metals than other earthworm species. For nickel the LC50 (297 mg/kg dw) and EC50 (121 mg/kg dw) in this study was somewhat lower than values reported by Lock and Janssen [30] in *Eisenia fetida* (LC50 > 1000 mg/kg dw, EC50 = 362 mg/kg dw) or by Scott-Fordsmand et al [31] in *E. veneta* (LC50 = 684 mg/kg dw, EC50 = 300 mg/kg dw). With respect to mercury, both the LC50 (38 mg/kg dw) and the EC50 (7.7 mg/kg dw) from the present study were lower than reported by Lock and Janssen [32], who did not report mortality in *E. fetida*, even after exposure to the highest tested concentration of 100 mg/kg dw, and who found an EC50 (reproduction) of 9.2 mg/kg dw after three weeks of exposure.

In a study by Langdon et al [33] three different species of earthworms were exposed to lead, up to 10,000 mg/kg dw. Only *Aporrectodea caliginosa* showed a minor decrease in survival of 2.5 % at 1000 mg/kg dw, while no mortality was observed in *E. andrei* and *Lumbricus rubellus* at this concentration. Spurgeon et al [34] did not see any significant mortality of lead in *E. fetida* at 2000 mg/kg dw. These previous results are in good agreement with our study, where only a 10 % mortality was observed at 1200 mg/kg dw. Spurgeon et al [34] did not see any decrease in reproduction at 400 mg/kg dw, which indicates that this parameter is more sensitive in our study, with a clear reduction in cocoon production at 400 mg/kg dw and EC50 of 501 mg/kg dw.

In the present study, pyrene caused a comparatively low toxicity and phenanthrene a rather high toxicity compared to other studies. Brown et al [35] recorded a LC50 for pyrene in *L. rubellus* to be 283 mg/kg dw and an EC50 of 90.3 mg/kg dw compared to 368 mg/kg dw and 148 mg/kg dw in our study, respectively. Sverdrup et al [36] recorded a LC50 for pyrene to be 155 mg/kg dw in *E. veneta*. On the contrary, Sverdrup et al [36] recorded a LC50 for phenanthrene to be higher than in the present study (134 mg/kg dw compared to 81 mg/kg dw).

We found no evidence of increased mortality at any of the tested concentrations of abamectin. This is in good agreement with results from a study by Kolar et al [37], who also noted 100% survival in *E. andrei* worms exposed to 9 mg/kg dw. These authors reported a LC50 of 18 mg/kg dw. However, the EC50 (1.03 mg/kg dw) of *E. fetida* was much lower in the study by Jensen et al [38] compared to our study with *D. octaedra* (EC50 = 6.6 mg/kg dw). Contrary to abamectin, carbendazim appeared to be more toxic in the present study compared to other studies: Ellis et al [39] recorded LC50 values of carbendazim to be between 6 and 16 mg/kg dw performed in different test soils.

All these differences in toxicity data can be due to differences in species sensitivity [33] or differences in test soils and thereby a difference in bioavailability of the chemical [40,41,12]. However, the LC50 and EC50 values of the present study are within an order of magnitude of other published results which is within the currently accepted level of variation for inter-laboratory tests [42].

Interaction between chemicals and subzero temperature

In the present study we have examined the effect of seven common compounds, representing at least three different modes of toxicological action, on the freeze tolerance of *D. octaedra*. These compounds include PAHs (pyrene and phenanthrene), pesticides (abamectin and carbendazim) and heavy metals (nickel, mercury and lead). The results suggest that

only two of the seven tested chemicals have an impact on the freeze tolerance of *D. octaedra.* Both of these chemicals (nickel and mercury) are heavy metals. Copper was already known to reduce the freeze tolerance of *D. octaedra* [16,26]. Both of these metals can, as copper, cause lipid peroxidation [21]. Copper, for example, has been shown to alter the membrane phospholipid composition in *D. octaedra* [16]. Polyunsaturated fatty acids that contain two or more double bonds are particularly susceptible to lipid peroxidation [43], and are the same phospholipids that are thought to increase in proportion in membranes during the homeoviscous adaptation that occurs with falling temperatures, an important adaptation to survival of freezing [44,45]. Lead is also known to cause lipid peroxidation [21], and was expected to reduce freeze survival by the same mechanisms as noted for copper. We found no evidence of this, perhaps because the tested concentrations were too low since we did not see any mortality in the controls. We did, however, observe a severe effect of lead on reproduction.

Contrary to our expectations, *D. octaedra* was significantly more susceptible to phenanthrene at the control temperature than at the freezing temperature. This tendency, although not significant, was also observed with pyrene. It is possible that the worms in the control group had higher internal PAH concentrations at the end of the experiment than those exposed to –6 °C, where no further accumulation would be expected because the water of the soil was frozen reducing diffusive uptake. However, no increased survival was observed in the control group during the freezing period, and we therefore reject this as the reason for increased mortality at control conditions. PAHs are likely to accumulate in membranes because of their lipophilic characteristics and structure with no functional groups [22], and might actually increase fluidity [23]. One can speculate that an increase in fluidity may be an advantage during freezing, thereby compensating for the increasing mortality with increasing exposure concentrations, as observed at control conditions.

The two pesticides tested in the present study did not impact freeze tolerance. This is probably due to their quite specific toxic mode of action that does not negatively interfere with physiological adaptations involved in freeze tolerance. Abamectin is a neurotoxin and inhibits the gamma-aminobutyric acid induced neurotransmission causing paralysis in parasites [46,47]. Carbendazim works by inhibiting the fungal development, probably by interfering with spindle formation during mitosis. These modes of action are presumably the same in *D. octaedra*, and both chemicals were inherently toxic with effects on reproduction, but had no effect on freeze tolerance. On the contrary, low concentrations of carbendazim actually increased freeze tolerance from 37 % in controls at –6 °C to 60 and 73 % at 0.25 and 0.5 mg carbendazim/kg dry soil, respectively. Carbendazim has been shown to alleviate effects of water stress on chickpea seedlings [48]. Here, carbendazim acted as a stress protectant by enhancing the accumulation of osmolytes such as proline, sucrose and glucose in the seedlings. Furthermore, increased membrane stability was observed in those chickpea seedlings exposed to increased water stress in combination with carbendazim, compared to those exposed to water stress alone. The increased membrane stability may be due to the observed decrease in lipid peroxidation measured as a lower concentration of malondialdehyde (MDA) in the seedlings exposed to low concentrations of carbendazim. It is well documented that glucose acts as a cryoprotectant in *D. octaedra* [49,7] and that the phospholipid fatty acid composition of cell membranes has an influence on freeze tolerance [8]. It could be speculated that the ability of these chemicals to induce both glucose accumulation and reduce lipid peroxidation in chickpea seedlings might also be the case in *D. octaedra*, which would thus provide some explanation for the increased freeze survival after exposure to low concentrations of carbendazim, but further studies are needed to substantiate this suggestion.

Comparisons between toxicity tests

For most of the tested chemicals, reproduction was affected at lower concentrations than necessary to reduce freeze tolerance. The only two exceptions were mercury and copper, which were particularly potent in their effects on freeze tolerance. At the lowest tested mercury concentration of 10 mg/kg dw, a very strong synergistic interaction was seen with 100% mortality in frozen worms, indicating that this effect may occur at even lower concentrations. Both copper and mercury, and in particular mercury, are known to block aquaporins [50,51], which are integral proteins channelling trans-membranous transport of water and osmolytes such as glycerol [52]. For freeze tolerant organisms it has been proposed that blocking of aquaporins may increase the risk of intracellular freezing which is lethal [53,54]. Nickel did reduce freeze survival at concentrations below those reducing survival at 15 °C; reproduction was, however, even more sensitive. Lead did reduce reproduction at much lower concentrations than observed for survival at both exposure temperatures, but no effect on freeze tolerance was observed within the tested concentrations.

The metal concentrations used in the present study were within the range that can be found at many metal contaminated field sites where *D. octaedra* is often found [55,56] suggesting that simultaneous exposure to freezing temperatures could have an ecologically significant impact on these earthworms in copper and mercury contaminated sites. Our results therefore suggest that this knowledge could be valuable for the development of ecological risk assessment of metals and possibly other chemicals.

Coniferous forests are significant sinks for atmospheric PAHs and have been shown to sequester almost four times more pyrene than a nearby grassland site [57]. This is due to adsorption of PAHs to the lipophilic needle cuticle and subsequent incorporation into soil organic horizon. *D. octaedra*, which is typically found in coniferous forests, is therefore likely to be exposed to high concentrations of PAHs. The present study shows that *D. octaedra* is susceptible to PAH pollution, especially phenanthrene, but that the sensitivity does not increase in areas where freezing impose a further stress. On the contrary, it seems that *D. octaedra* is less sensitive to PAHs at low temperatures. Our study suggests that the safe environmental concentration of PAHs calculated from standard reproduction tests seems protective for effects on freeze survival, and thereby risk assessment in cold areas.

In conclusion, three out of four tested heavy metals significantly reduced the freeze tolerance of *D. octaedra*. This indicates that the assessment of toxicity of metals by traditional laboratory studies, where test organisms are exposed to only one stress factor and otherwise optimal conditions, might underestimate the effect of the metals on survival of field populations living in cold areas. We therefore suggest that science-based extrapolation from laboratory toxicity tests to the field population would benefit from studies including natural stress factors such as freezing. On the other hand, this does not seem to be of equal importance in the case of the tested PAHs and pesticides.

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References

1. Heugens EHW, Hendriks AJ, Dekker T, van Straalen NM, Admiraal W. 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. *Crit Rev Toxicol* 31: 247-284.
2. Friis K, Damgaard CF, Holmstrup M. 2004. Sublethal soil copper concentrations increase mortality in the earthworm *Aporrectodea calignosa* during drought. *Ecotox Environ Safe* 57: 65-73.
3. Stöp-Bowitz C. 1969. A contribution to our knowledge of the systematics and zoogeography of Norwegian earthworms (Annelida Oligochaeta: Lumbricidae). *Nytt Magasin for Zoologi* 17: 169-280.
4. Hendrix PF. 1995. *Earthworm Ecology and Biography in North America*. Lewis Publisher. USA.
5. Dymond P, Scheu S, Parkinson D. 1997. Density and distribution of *Dendrobaena octaedra* (Lumbricidae) in a pine forests in the Canadian Rocky Mountains (Alberta). *Soil Biol Biochem* 29: 265-273.
6. Berman DI, Meshcheryakova EN, Alfimov AV, Leirikh, AN. 2001. Spread of the earthworm *Dendrobaena octaedra* (Lumbricidae: Oligochaeta) from Europe to Northern Asia is restricted by its insufficient freeze tolerance. *Dokl Biol Sci* 377: 145-148.
7. Overgaard J, Slotsbo S, Holmstrup M, Bayley M. 2007. Determining factors for cryoprotectant accumulation in the freeze-tolerant earthworm, *Dendrobaena octaedra. J Exp Zool Part A* 307: 578-589.
8. Holmstrup M, Sørensen LI, Bindesbøl A, Hedlund K. 2007. Cold acclimation and lipid composition in the earthworm *Dendrobaena octaedra*. *Comp Biochem Physiol A*. 147: 911-919.
9. Edwards CA, Bohlen PJ. 1996. *Biology and Ecology of Earthworms*. Chapman & Hall, London, UK.
10. OECD. Organisation for Economic Co-operation and Development, 1984. Test 207: Erathworm acute toxicity test. In: Organization for Economic Co-operation and Development (Eds.), OECD Guideline for Testing of Chemicals.
11. Edwards CA, Bohlen PJ. 1992. The effects of toxic chemicals on earthworms. *Rev Environ Contam Toxicol* 125: 23-99.
12. Van Gestel CAM. 1992. The influence of soil characteristics on the toxicity of chemicals for earthworm: a review. In: Greigh-Smith PW, Becker H, Edwards PJ, Heimbach F (Eds.), *Ecotoxicology of Earthworms*. Intercept Ltd., Hant, UK, pp. 44-54.
13. Sjursen H, Sverdrup LE, Krogh PH. 2001. Effects of polycyclic aromatic compounds on the drought tolerance of *Folsomia fimeteria* (Collembola, Isotomidae). *Environ toxicol chem* 20: 2899-2902.
14. Sørensen TS, Holmstrup M. 2005. A comparative analysis of the toxicity of eight common soil contaminants and their effects on drought tolerance in the collembolan *Folsomia candida*. *Ecotox Environ Saf* 60: 132-139.
15. Skovlund G, Damggard C, Bayley M, Holmstrup M. 2006. Does lipophilicity of toxic compounds determine effects on drought tolerance of the soil collembolan *Folsomia candida*? *Environ Pollut* 144: 808-815.
16. Bindesbøl A, Bayley M, Damgaard C, Hedlund K, Holmstrup M. 2008. Changes in membrane phospholipids as a mechanistic explanation for decreased freeze tolerance in earthworms exposed to sub-lethal copper concentrations. Submitted to ES&T.

17. Aarset AV, Zachariassen KE. 1983. Synergistic effects of an oil dispersant and low temperature on the freezing tolerance and solute concentrations of the blue mussel (*Mytilus edulis L.*). *Polar Res* 1: 223-229.

18. Jensen D, Bayley M, Holmstrup M. 2009. Synergistic interactions between 4-nonylphenol and high but not low temperatures in *Dendrobaena octaedra*. *Ecotox Environ Saf*. 72: 10-16.

19. Deplegde MH. 1987. Enhanced copper toxicity resulting from environmental stress factor synergies. *Comp Biochem Physiol* 87C: 15-19.

20. Holmstrup M, Bayley M, Sjursen H, Højer R, Bossen S, Friis K. 2000. Interactions between environmental pollution and cold tolerance of soil invertebrates: a neglected field of research. *Cryoletters* 21: 309-314.

21. Stohs SJ, Bagchi D. 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radical Bio Med* 18: 321-336.

22. Moriarty,F. 1983. *Ecotoxicology: The study of pollutants in ecosystems*. Academic Press, London.

23. Chaisuksant Y, Yu Q, Connel DW. 1999. The internal critical level concept of nonspecific toxicity. *Rev Environ Contam T* 162: 1-41.

24. Ring A, Danks HV. 1994. Desiccation and cryoprotection: overlapping adaptations. *Cryoletters* 15: 181-190.

25. Bayley M, Petersen SO, Knigge T, Köhler HR, Holmstrup M. 2001. Drought acclimation confers cold tolerance in the soil collembolan *Folsomia candida*. *J Insect Physiol* 47: 1197-1204.

26. Bindesbøl A, Holmstrup M, Damgaard CF, Bayley M. 2005. Stress synergy between environmentally realistic levels of copper and frost in the earthworm *Dendrobaena octaedra*. *Environ Toxicol Chem* 24: 1462-1467.

27. Damgaard CF, Højer R, Bayley M, Scott-Fordsmand JJ, Holmstrup M. 2002. Dose-response curve modelling of excess mortality caused by two forms of stress. *Environ Ecol Stat* 9: 195-200.

28. Greco W, Unkelbach H-D, Pöch G, Sühnel J, Kundi M, Bödeker W. 1992. Concensus on concepts and terminology for combined-action assessment: The Saariselkä agreement. *ACES* 4: 65-69.

29. Carlin L, Louis TA. 1996. *Bayes and Empirical Bayes Methods for Data Analysis*. Chapman and Hall, London, UK.

30. Lock K, Janssen CR. 2002. Ecotoxicity of nickel to *Eisenia fetida*, *Enchytraeus albidus* and *folsomia candida*. *Chemosphere* 46: 197-200.

31. Scott-Fordsmand JJ, Weeks JM, Hopkin P. 1998. Toxicity of nickel to the earthworm and the applicability of the neutral red retention assay. *Ecotoxicology* 7: 291-295.

32. Lock K, Janssen CR. 2001. Ecotoxicity of mercury to *Eisenia fetida*, *Enchytraeus albidus* and *folsomia candida*. *Biol Fertil Soils* 34: 219-221.

33. Langdon CJ, Hodson ME, Arnold RE, Black S. 2005. Survival, Pb-uptake and behaviour of three species of earthworm in Pb treated soils determined using an OECD-style toxicity test and a soil avoidance test. *Environ Pollut* 138: 368-375.

34. Spurgeon DJ, Hopkin SP, Jones DT. 1994. Effects of cadmium, copper, lead and zinc on growth, reproduction and survival of the earthworm *Eisenia fetida* (Savigny): assessing the environmental impact of point-source metal contamination in terrestrial ecosystems. *Environ Pollut* 84: 123-130.

35. Brown PJ, Long SM, Spurgeon DJ, Svendsen C, Hankard PK. 2004. Toxicological and biochemical responses of the earthworm *Lumbricus rubellus* to pyrene, a non-carcinogenic polycyclic aromatic hydrocarbon. *Chemosphere* 57: 1675-1681.

36. Sverdrup LE, Krogh, PH, Nielsen T, Stenersen J. 2002. Relative sensitivity of three terrestrial invertebrate tests to polycyclic aromatic compounds. *Environ Toxicol Chem* 21: 1927-1933.

37. Kolar L, Erzen NK, Hogerwerf L, van Gestel CAM. 2008. Toxicity of abamectin and doramectin to soil invertebrates. *Environ Pollut* 151: 182-189.

38. Jensen J, Diao X, Scott-fordsmans JJ. 2007. Sub-lethal toxicity of the antaparasitc abamectin on earthworm and application of neutral red retention time as a biomarker. *Chemosphere* 68: 744-750.

39. Ellis SR, Hodson ME, Wege P. 2007. The influence of different artificial soil types on the acute toxicity of carbendazim to the earthworm *Eisenia fetida* in laboratory toxicity tests. *Eur J Soil Biol* 43: S239-S245.

40. Chung N, Alexander M. 2002. Effect of soil properties on bioavailability and extractability of phenanthrene and atrazine sequestered in soil. *Chemosphere* 48: 109-115.

41. Spurgeon DJ, Hopkin SP. 1996. Effects of variations of the organic matter content and pH of soils on the availability and toxicity of zinc to the earthworm *Eisenia fetida*. *Pedobiologia* 40: 80-96.

42. Edwards CA. Report of the second stage in development of a standardized laboratory method for assessing the toxicity of chemical substances to earthworms. Report of the Commissions of the European Communities EUR 9360 EN.

43. Valko M, Morris H, Cronin MTD. 2005. Metals, toxicity and oxidative stress. *Cur Med Chem* 12: 1161-1208.

44. Sinensky M. 1974. Homeoviscous adaptation – a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proc Natl Acad Sci U. S. A.* 71: 522-525.

45. Hazel JR. 1995. Thermal adaptation in biological membranes: Is homeoviscous adaptations the explanation? *Annu Rev Physiol* 57: 19-42.

46. Campell WC, Fisher MH, Stapley EO, Albers-Schönberg G, Jacob TA. 1983. Ivermectin: a potent new parasitic agent. *Science* 221: 823-828.

47. Shoop WL, Morzik H, Fisher MH. 1995. Structure and activity of avermectina and milbemycins in animal health. *Vet Parasitol* 59: 139-156.

48. Smita, Nayyar H. 2005. Carbendazim alleviates effects of water stress on chickpea seedling. *Biol Plantarum* 49: 289-291.

49. Rasmussen LM, Holmstrup M. 2002. Geographic variation of freeze-tolerance in the earthworm *Dendrobaena octaedra*. *J Comp Physiol B* 172: 691-698.

50. Zelenia M, Tritto S, Bondar AA, Zeleni S, Aperia A. 2004. Copper inhibits the water and glycerol permeability of aquaporin-3. *J Biol Chem* 279: 51939-51943.

51. Savage DF, Stroud RM. 2007. Structural basis of aquaporin inhibition by mercury. *J Mol Biol* 368: 607-617.

52. Borgnia M, Nielsen S, Engel A, Agre P. 1999. Cellular and molecular biology of the aquaporin water channels. *Annu Rev Biochem* 68: 425-458.

53. Philip BN, Yi S, Elnitsky MA, Lee RE. 2008. Aquaporins play a role in desiccation and freeze tolerance in larvae of the goldenrod gall fly, *Eurosta solidaginis*. *J Exp Biol* 211: 1114-1119.

54. Izumi Y, Sonoda S, Yoshida H, Danks HV, Tsumuki H. 2006. Role of membrane transport of water and glycerol in the freeze tolerance of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyraliae). *J Insect Physiol* 52: 215-220.

55. Lukkari T, Taavitsainen M, Väisänen A, Haimi J. 2004. Effects of heavy metals on earthworms along a contamination gradient in organic rich soils. *Ecotox Environ Saf* 59: 340-348.

56. Kozlow MV, Zvereva EL. 2007. Industrial barrens: extreme habitats created by non-ferrous metallurgy. *Rev Environ Sci Biotechnol* 6: 231-259.

57. Horstmann M, McLachlan MS. 1998. Atmospheric deposition of semi volatile organic compounds to two forest canopies. *Atmos Environ* 32: 1799-1809.

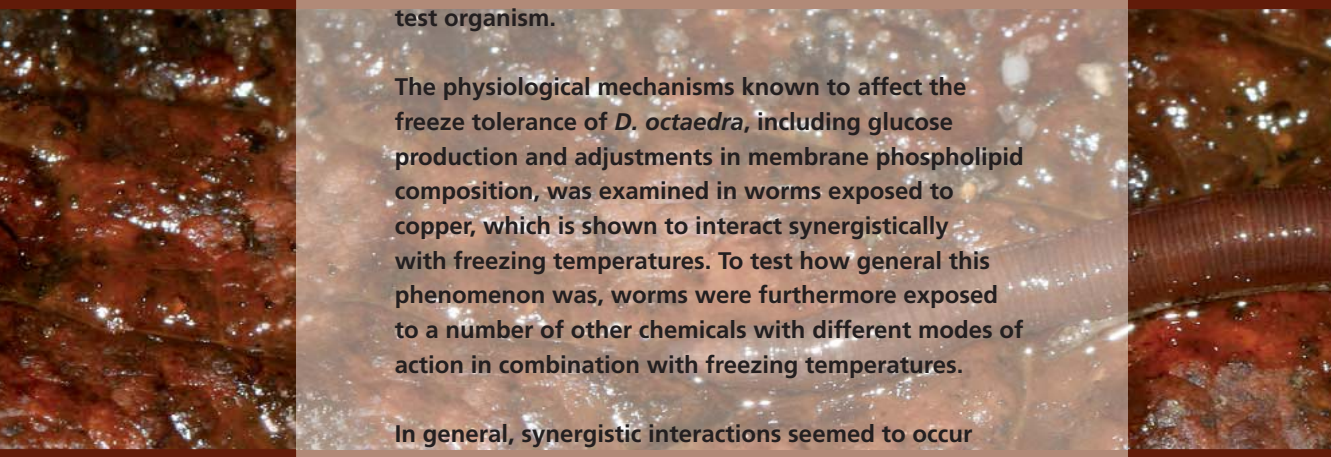
58. Bindesbøl A, Bayley M, Damgaard C, Holmstrup M. 2007. Life-history traits and population growth rate in the laboratory of the earthworm *Dendrobaena octaedra* cultured in copper-contaminated soil. *Appl Soil Ecol* 35: 46-56.

Interactions between climatic and toxic stress

Studies with the freeze tolerant earthworm *Dendrobaena octaedra*

In traditional ecotoxicological studies, test organisms are usually exposed to a single chemical at increasing concentrations, performed under otherwise optimal conditions. However, in natural environments it is very likely that these organisms will also be exposed to other stressful factors, including climatic stressors as frost and drought. This means that traditional laboratory tests may underestimate the toxic effects of chemicals in natural environments.

This PhD thesis examined the interactions between climatic and toxic stress, using the globally distributed freeze tolerant earthworm *Dendrobaena octaedra* as test organism.



The physiological mechanisms known to affect the freeze tolerance of *D. octaedra*, including glucose production and adjustments in membrane phospholipid composition, was examined in worms exposed to copper, which is shown to interact synergistically with freezing temperatures. To test how general this phenomenon was, worms were furthermore exposed to a number of other chemicals with different modes of action in combination with freezing temperatures.

In general, synergistic interactions seemed to occur mostly at high levels of climatic stress in combination with high concentrations of the chemical. It is suggested that it is important to include natural stressors as frost and drought in risk assessment, especially taken into consideration the predictions of future climate change.

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