

Enchytraeidae (Oligochaeta) in a changing climate

Ecology and ecophysiology of enchytraeids exposed to climate changes

PhD Thesis, 2009

Kristine Maraldo



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Preface

This PhD thesis is submitted to the Faculty of Science, University of Copenhagen, Denmark. All of the work has been conducted at the Department of Terrestrial Ecology at National Environmental Research Institute (NERI), Aarhus University, in Silkeborg, Denmark, during the past four years. The PhD is part of the Danish climate change experiment “CLIMATE”, which was initiated in 2005.

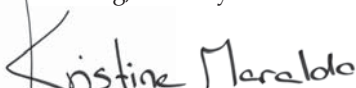
The objective of this PhD was to examine the effect of climate change on enchytraeids both in the field, but also in the laboratory. The introduction of the thesis has the structure of a review paper on the effect of climatic changes on terrestrial enchytraeids. Following the introduction are six papers, all concerning the effects of increased climatic stress, with a special focus on drought stress. Four of the papers have been published, one has been submitted, and one is ready for submission. This thesis was financed by the project CLIMATE (CLIMATE change effects on biological processes In Terrestrial Ecosystems; www.climaite.dk) funded by the The Villum Kann Rasmussen Foundation.

I would like to thank several people, who have all contributed to making this PhD possible. First of all, I would like to thank my supervisor Martin Holmstrup at NERI, who has been of great support, inspiration and always has taken the time to talk to me. My internal supervisor Søren Christensen from University of Copenhagen, for always helping when needed. To the people behind the CLIMATE project, which made it all possible and for some inspiring and pleasant meetings – I am going to miss them! Thank you for introducing me to terrestrial ecology and special thanks goes to the CLIMATE PhD group, which has been of great help and a lot of fun. I also owe a large thank you to a range of enchytraeid people, a special thanks goes to Bent Christensen and his wife, who introduced me to the taxonomy of the enchytraeids, to Dr. Valerie Standen, University of Durham, UK for helping collect soil at Moor House, to Dr. Maria Briones and Noela, University of Vigo – thanks to all of you for your help, inspiration and hospitality. Finally, I would like to acknowledge Philip Vernon from the University of Rennes in France for his help during my stay in France and Rudiger Schmelz and Louise Illum Sørensen for collecting soil in Spain and Finland.

Thank you, to all my colleagues at NERI in Silkeborg. In particular, I wish to thank Paul Henning Krogh, Zdenek Gavor, Elin Jørgensen, Mette Thomson, John Rytter, Lene Birksø, Charlotte Elisabeth Kler, Ninna Skafsgaard, Frankie Henriksen, Lars Henrik Heckman, Helle Weber Ravn and several others for technical assistance, sparring and a lot of fun. A very special thank goes to my fellow PhD students, Anne Mette Bindsbøl, Stine Slotsbo and Dorthe Jensen for sparring, critical review of this thesis, and lots of good times. Tinna Christensen has been very helpful with graphical assistance, Rebekka Andreassen has been very kind to help with the Danish abstract and Lisbeth Jeppesen are thanked for her correction of the language.

Without help and support from friends and family, this thesis would have been an up-hill battle. Finally, I am deeply indebted to Jens, Emma and Anders for always being there and for taking your time to hear about the life of “white earthworms”.

Silkeborg, February 2009


Kristine Maraldo



Abstract

The climate is undergoing rapid changes with rising atmospheric CO₂ concentration, increasing temperature and changes in the hydrological regimes resulting in more frequent and intense drought periods. These three climate change factors will, separately and in combination, affect the biotic and abiotic components of the soil ecosystems. Enchytraeids are an important component in soil ecosystems and affect the decomposition processes and the nutrient mineralisation both directly and indirectly by their activity.

The background for this thesis was to investigate the effect of climate change on field populations of enchytraeids dominated by the species *Cognettia sphagnetorum*. Although enchytraeids are extremely sensitive to long-term desiccation stress, they also have a high degree of resilience when optimal conditions return. Field populations of enchytraeids were exposed in a full factorial in-situ experiment to increased CO₂, temperature and prolonged drought manipulation for three years. In the short term, enchytraeids appear to be unaffected by climate change when all factors were combined. The negative impact of drought was counteracted when CO₂ was present, as drought and CO₂ in combination acted additively during summertime. However, in a long-term drought experiment in which *C. sphagnetorum* was exposed repeatedly to drought during a six year period, the species was, evidently, negatively affected. Even though the enchytraeids had been exposed for a long period, there was no sign of increased drought resistance.

Climate change will, beyond doubt, have an impact on the enchytraeids, however, in a non predictable way. But factors such as extreme events, seasonal and interannual variations are all important factors as well, which can strongly influence the response of enchytraeids in a future climate.



Photo: Frank Vincentz/Wikimedia commons

Danish abstract

Klimaet ændrer sig hastigt. Øgede koncentrationer af CO₂ i atmosfæren, stigende temperaturer og ændringer i mønstret af nedbør påvirker miljøet og fører til hyppigere og mere intense tørkeperioder. Disse tre klima-faktorer påvirker, hver for sig og i samspil, både biotiske og abiotiske dele af jordbundsøkosystemer. Enchytræer, hvide regnorme, udgør en vigtig komponent i jordbundsøkosystemer, hvor de både har direkte og indirekte betydning for nedbrydningsprocesserne og næringsstofkredsløbet i jorden. Deres betydning er særlig vigtig i næringsfattige områder som bl.a. hede og nordlige skovområder.

Baggrunden for denne afhandling var at undersøge effekten af klimaforandringerne på naturlige populationer af enchytræer. Til trods for, at enchytræer er ekstremt følsomme overfor længerevarende tørkeperioder, har de også en høj grad af modstandsdygtighed. I et feltforsøg, der har strakt sig over tre år, blev naturlige enchytræ-populationer udsat for både øget CO₂, forhøjede temperaturer og endelig en langvarig tørkemanipulation. Faktorerne var påført hver for sig, men også i kombinationer. På kort sigt var enchytræerne upåvirkede af manipulationerne, når alle tre faktorer forekom samtidig. Om sommeren blev den negative påvirkning af tørke udlignet af den positive effekt af CO₂ når begge faktorer var til stede. I et andet feltforsøg blev naturlige enchytræ-populationer igennem seks år udsat gentagende gange for længerevarende perioder af tørke, hvilket påvirkede dem negativt. Skønt enchytræerne havde været udsat for øget tørkestress igennem denne længere periode, var der ingen tegn på en genetisk tilpasning til tørkestress.

Klimaændringer vil uden tvivl have betydning for enchytræernes aktivitet og trivsel men det er svært at forudsige præcist hvordan. Omstændigheder som ekstreme hændelser, sæsonvariationer og års variationer er alle vigtige faktorer, som kan have stor indflydelse på enchytræer i et fremtidigt klima.





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Review

Abstract

The potential impacts of multiple climate change factors in the soil ecosystem have received little attention. The climate is undergoing rapid changes with rising atmospheric CO₂ concentration, increasing temperatures and changes in the hydrological regimes resulting in more frequent and intense drought periods. These three climate change factors will, separately and in combination, affect the biotic and abiotic components of the soil ecosystems. This paper reviews the impact of climate change factors on field populations of enchytraeids dominated by the keystone species *Cognettia sphagnetorum*. *C. sphagnetorum* prefers cold and wet environments and, clearly, temperature and moisture regimes play a significant role for its distribution, activity and production. The existing studies on the effect of climate change show that no general pattern has yet emerged even within the same species. Based on two climate change field experiments combining more than one factor, it appears that *C. sphagnetorum* was unaffected by short-term climate change exposure, as no effect was observed. A positive effect of CO₂ was counteracted by the reducing effect of increased drought stress in this species. However, these results are based on short-term climate change experiments and, hence, these results have to be extrapolated with caution. The intensity of the impact from the three factors will vary depending on the local climate, habitat, soil type and vegetation.

The question regarding the adaptation potential in *C. sphagnetorum* was also addressed. According to existing data, it is suggested that *C. sphagnetorum* is not capable of adapting to a drier climate, thus, a reduction of this species can become a reality. A reduction may result in serious disruption in the functioning of the decomposer community due to the key role of *C. sphagnetorum*.

Climatic phenomena, ecosystem processes and human activities are interactive and interdependent, making long-term predictions extremely difficult. Thus, more and longer studies involving all three factors are needed to fully unravel their effects on the enchytraeids, but the effect of the enchytraeids themselves in a future climate also needs to be studied. Climate changes will, beyond doubt, have an impact on the enchytraeids, however, in a non-predictable way. But factors such as extreme events, seasonal and interannual variations are all important factors as well, which in a future climate may strongly influence the response and feedback of enchytraeids in the field.

Introduction

The climate is undergoing changes with rising atmospheric CO₂ concentration, increasing temperatures and changes in the hydrological regimes (IPCC, 2008). Climate change scenarios predict that the CO₂ concentration will increase from 335 ppm to 550 ppm in 2070, temperatures will increase 0.2°C per decade and more intense drought spells will occur in a future northern European climate (IPCC, 2008). Paradoxical as it might seem, an overall warming of the northern regions may result in occasional, but increased, soil freezing, since the snow cover becomes thinner and, thus, has a less insulating impact (Monson et al., 2006; Isard et al., 2007). Although the amount of precipitation is expected to increase, more frequent and intense drought periods are predicted by the climate change models (Arnell, 1999; IPCC, 2008). These climate change factors will, separately and in combination, affect the functioning of soil ecosystems (Swift et al., 1998).



Photo: J.C. Schou/Biopix

The soil ecosystem contains a diverse range of soil organisms from protozoa, nematodes, microarthropods and oligochaetes, which will be directly, but also indirectly, affected by the predicted climate changes. Their presence is important as they, by their activities, accelerate the decomposition and nutrient recycling processes (Seastedt, 1984; Taylor et al., 2004; Huhta, 2006). One group of the Oligochaeta is the Enchytraeidae, also known as pot worms and/or white earthworms due to their pale colour and small size. The Enchytraeidae family contains 27 genera and about 500 species, however, new species are still being described each year (Chalupsky, 1991). Most species are hermaphrodites, but also parthenogenetic spe-

cies and a few species reproducing by fragmentation (fissiparous) such as *Cognettia sphagnetorum* and *Enchytraeus bigeminus*, exist (Christensen, 1964). Enchytraeids are widely distributed from the Arctic to tropical areas (Nurminen, 1965; Petersen and Luxton, 1982; Standen, 1988), but our knowledge of enchytraeids' distribution, productivity and activity is mainly limited to European species, as most work has been performed there.

Soils consist of heterogeneous compartments, which provide a range of different microhabitats. These habitats vary both spatially and temporally and can host different enchytraeid species at different times. Different species also have specific preferences of habitat, and enchytraeids can be divided into euedaphic (soil-living species) like *Achaeta* sp. and *Cernosvitoviella* sp., which are, generally, found deeper in the soil compared to epedaphic species like *Mesenchytraeus glandulosus* and *Cognettia sphagnetorum*, which live in the top soil but can also, occasionally, be found above the soil (Abrahamsen, 1972; Dozsa-Farkas, 1992). Enchytraeids feed on various items but are, generally, said to be 80% microbivorous and 20% saprovorous (Didden, 1993). *C. sphagnetorum* has been found to feed indiscriminately on all available food resources in Scots pine forests (Ponge, 1991), but the feeding preference of *C. sphagnetorum* appears to depend on the locality (Briones and Ineson, 2002).

Enchytraeids are involved both directly and indirectly in the decomposition processes and the nutrient mineralisation (Williams and Griffiths, 1989). Directly, by consuming large amounts of organic matter (Standen, 1978; Abrahamsen, 1990; Setälä and Huhta, 1991; Laakso and Setälä, 1999; Cole et al., 2000), and, indirectly, by creating soil structure and by their feeding activity, which affects the activity and functioning of the microbial community (van Vliet et al., 1993; Cole et al., 2000; Bardgett, 2005). The influences from enchytraeids are especially important in acidic and nutrient-poor ecosystems, e.g. temperate heathland and boreal forests, where they are the dominating group of soil fauna in terms of biomass (Cragg, 1961; Abrahamsen, 1972; Lundkvist, 1982; Setälä and Huhta, 1991; Swift et al., 1998). The dominant enchytraeid species in these types of ecosystems is *C. sphagnetorum* (Cragg, 1961; Lundkvist, 1982; Laakso and Setälä, 1999). This species has been found to have a significant influence on the decomposition process and nutrients cycling and is, therefore, recognised as a keystone species in these ecosystems (Laakso and Setälä, 1999; Setälä, 2002). *C. sphagnetorum* is probably adapted to a temperate oceanic cli-

mate (O'Connor, 1957; Briones et al., 2007) and to predictable environments, such as wet moors and coniferous forests (Lundkvist, 1982). Since climate change scenarios suggest that the climate will become more unpredictable and extreme (IPCC, 2008), this may have severe consequences for the existence of *C. sphagnetorum*, but also for the functioning of the ecosystems, due to its role as keystone organism. Climate change may alter the dynamics of enchytraeids in time and space as well as their spatial patterns and geographical distribution. It seems, therefore, that knowledge of the physiological limits of these organisms is the key to understanding the functional response of organisms to climate change, as there is often a close and predictable link between the climate and the distribution of a given organism (Hodkinson, 1999).

The objective of this paper is to review the existing literature of the effects of climate changes on enchytraeids. The focus will be on the impact of the three most important environmental factors: rising atmospheric CO₂ concentrations, temperatures (both high and sub-zero temperatures) and increased drought stress, but with special emphasis on the effects of drought on the species *C. sphagnetorum*. A more complete description of the responses of enchytraeids to global change would require further research in a wider range of habitats, climates and soil types and is therefore, out of scope of this review. This review is structured by first presenting an overview of the impacts of increased drought stress, temperature and raised CO₂ on enchytraeids. The impact of the different climate stressors will be addressed separately at the ecological and physiological levels. Following this, relevant studies addressing the effects of climate change interactions on enchytraeids and their potential to adapt to these new conditions will be discussed. However, due to the limited number of studies available, these parts will mainly be theoretical and comparative. Finally, other issues of climate change will be addressed.

Enchytraeids and drought stress

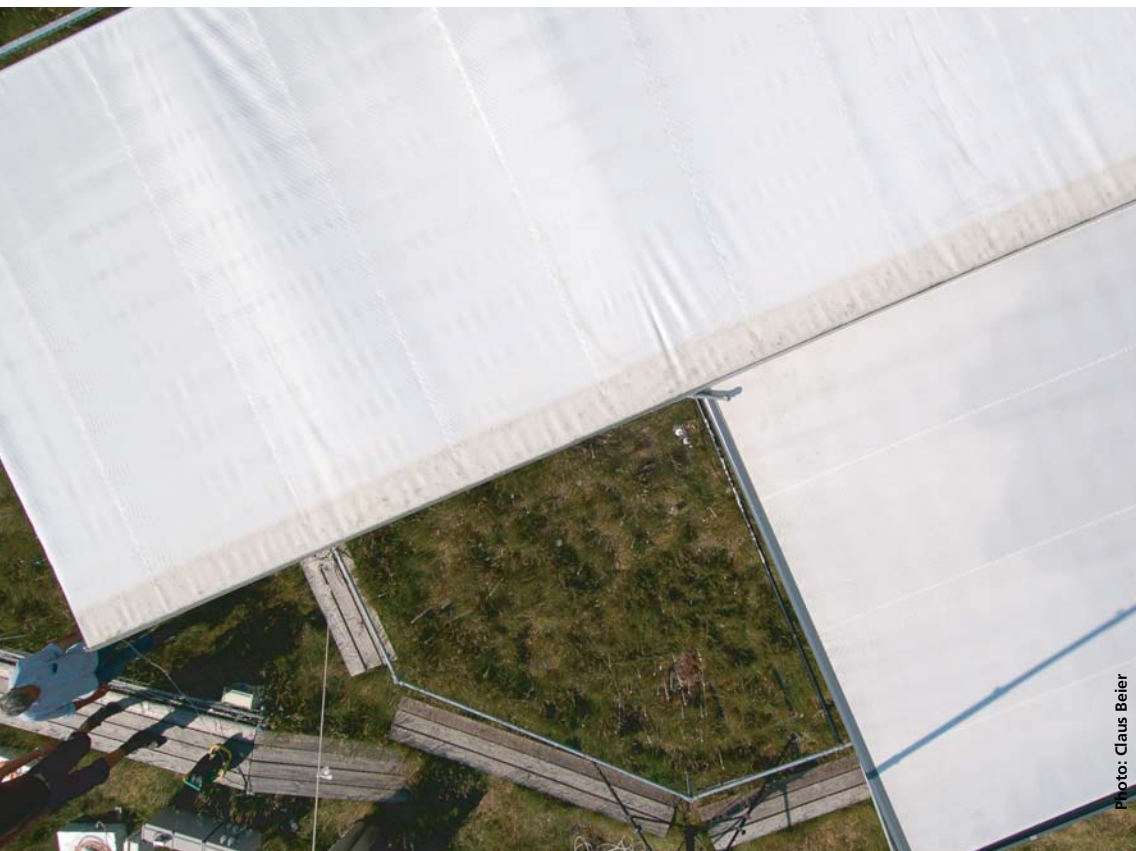
The distribution and performance of enchytraeids is highly dependent on the availability of water (Abrahamsen, 1971; Gröngröft and Miehlich, 1983; Sulkava et al., 1996), and several field studies have reported that enchytraeids are extremely vulnerable to drought stress (Nielsen, 1955b; O'Connor, 1957; Abrahamsen, 1972; Lundkvist, 1982). Both short-term and long-term drought can result in population reductions through mortality and impeded growth and reproduction (Nielsen, 1955a; Springett et al., 1970; Abrahamsen, 1971; Standen, 1980). Climate change models predict that the number and intensity of drought spells will increase. Despite this, only two studies of long-term effects of repeated drought exist (Lindberg et al., 2002; Maraldo et al., 2008). These authors reported a 90% to 65% reduction in enchytraeid populations after eight years of repeated drought in a Swedish coniferous forest (Lindberg et al., 2002) and six years of repeated drought in a Danish heathland (Maraldo et al., 2008), respectively.

Besides the direct effect of drought, the enchytraeid community will also be indirectly affected by the more frequent spells of drought. It has also been proposed that drought can lead to an increase in the microbial C/N ratio, suggesting a change towards a more fungi-dominated microbial community and a decomposition of more complex substrates (Jensen et al., 2003). This could favour more fungivorous and desiccation-tolerant enchytraeid species or other members of soil fauna at the expense of *C. sphagnetorum* (Sulkava et al., 1996; Huhta et al., 1998). Sulkava et al. (1996), found that the presence of microarthropods suppressed the population of *C. sphagnetorum* in dry and medium-moist soil, and that this was

reflected in a decrease of N mineralisation. However, at high moisture the microarthropods failed to influence the enchytraeids, and the N mineralisation remained high. Increased drought stress can, thus, affect the competitive interactions between species and, thereby, cause implications in the mineralisation process in the soil.

The effect of increased desiccation stress

So far two drought survival strategies have been described for enchytraeids. In the first strategy, enchytraeids avoid dry conditions by migrating to deeper and moister microhabitats, but this strategy can only be effective in the short term, unless the food quantity and quality in the deeper soil layers can maintain or increase the population size (Springett et al., 1970; Erman, 1973; Uhia and Briones, 2002). The second strategy leaves the population to survive in the more desiccation tolerant cocoon stage (Nielsen, 1955b; Nielsen, 1955a; Christensen, 1956; Lagerlöf and Strandh, 1997). Some enchytraeid species are even found to cover their egg cocoons with sand and debris, which, can perhaps delay the desiccation of the cocoons (Christensen, 1956). An alternative third strategy, which is connected with the migration strategy, is increased aggregation of worms in moist microhabitat during dry periods, thus reducing the risk of desiccation (Standen and Latter, 1977). However, species like *C. sphagnetorum*, which are known to reproduce asexually by fragmentation and subsequent regeneration, probably lack the second strategy. Consequently, periods with of extended drought spells might have severe consequences for *C. sphagnetorum*, which could, therefore, be more liable to extinction than other enchytraeids (Springett, 1970). However, the potential of *C. sphagnetorum* to survive harsh conditions by cocoon production is still uncertain (Springett et al., 1970; Standen and Latter, 1977). The general opinion is that *C. sphagnetorum* only reproduces asexually by fragmentation, as it allows a



quick recolonisation of the environment (Lundkvist, 1982). However, there are contradictory views, and a study from German spruce forests reports “mature” and newly hatched *C. sphagnetorum* during or after dry periods (Schlaghamersky, 2002). Lundkvist (1983) also reports occurrences of mature individuals of *C. sphagnetorum* after clear-cutting of trees in Swedish coniferous forests, and she suggests that they may be capable of reproducing by cocoons as a response to increased desiccation. *C. sphagnetorum* has also been shown to recover from exposure to severe long-term drought stress (Maraldo and Holmstrup, 2009). Thus, I suggest that *C. sphagnetorum* must have a desiccation tolerant stage, probably cocoons, as seen in other enchytraeid species, as it is rather unlikely that the specimen could have survived soil water potential below -15 bar for periods of weeks (Maraldo and Holmstrup, 2009). However, due to the highly heterogeneous structure of soil, it cannot be rejected that even in very dry conditions moister microhabitats may be present.

Physiological responses to desiccation stress

The majority of enchytraeids live in the top soil and are, therefore, at risk of desiccation, due to their highly permeable surface. Dehydration is harmful as it can cause cellular shrinkage, cellular proteins can become irreversibly denatured, and the cellular membranes can lose their normal conformation (Crowe et al., 1992). Soil water potential (SWP) below pF 4 (corresponding to -9.8 bar) has been shown to be lethal for *C. sphagnetorum*, whereas the optimal SWP has been reported to be between pF 0.2 (-0.0015 bar) and pF 2.2 (-0.16 bar) (Abrahamsen, 1971). But the degree of drought sensitivity is species dependent, and more drought resistant species are also found. For example, *Fridericia galba* has been found to survive in soil with a water-holding capacity (WHC) below 20 % (corresponding to values below -9.8 bar) for more than 49 days (Dozsa-Farkas, 1977). *Enchytraeus albidus*, abundant in rotting seaweeds on sandy beaches, was reported to survive SWP down to -20 bar for a short period of time (days), probably as an adaptation to a strongly fluctuating environment (Maraldo et al., 2009b). *E. albidus* was capable of keeping its water content stable during moist and relatively dry conditions due to an unusually high osmotic pressure of body fluids (ca. 500 mOsm). The body fluid osmolality of most terrestrial earthworms varies in osmolality between 200 and 300 mOsm (Oglesby 1969; Pedersen and Holmstrup 2003). Moreover, during desiccation stress, *E. albidus* accumulated the two osmolytes, glucose and alanine, the concentrations were, however, modest (Maraldo et al., 2009b). The low concentrations of osmolytes mobilisation suggest that the colligative effects are minor (help the cell to maintain its volume and reduce the water loss during desiccation), but the two osmolytes may protect membranes and protein against deleterious effects of desiccation (Crowe et al., 1992). Unfortunately, our knowledge of drought tolerance or drought tolerance strategies in *C. sphagnetorum* is limited, as no laboratory studies have so far examined physiological responses of desiccation in *C. sphagnetorum*.

Enchytraeids and the effects of warming and subzero temperatures

Temperatures, high as well as low, have an influence on the activity, productivity and reproduction of enchytraeids (Abrahamsen, 1972; Standen, 1980; Lundkvist, 1982; Briones et al., 1997; Cole et al., 2002b). In temperate areas, low temperatures are typically the limiting factor during winter, whereas low soil moisture content is often the limiting factor during summer (Lundkvist, 1982). The combination of low soil moisture and high tem-

peratures can increase the desiccation stress for soil organisms, as the evaporation from the soil increases (Abrahamsen, 1971; Lundkvist, 1982; Hodgkinson et al., 1998). This is supported by the observation of Edwards and Lofty (1971), who observed that the density of enchytraeids initially increased in heated plots, but decreased later in the spring, due to decreasing soil moisture caused by the heating. The climate change models predict that the atmospheric warming will be most pronounced at high latitudes, which indicates that enchytraeids in these locations will be particularly influenced by the increased warming. At the same time, the enchytraeids in these areas may also be exposed to more frequent freeze-thaw cycles due to thinner snow cover caused by the enhanced temperatures. This can, especially, have consequences for enchytraeid populations inhabiting sub-arctic and arctic areas (Briones et al., 2007; IPCC, 2008).

Temperature also affects other biological and physical components of the soil ecosystems and may, thus, indirectly impact the enchytraeids. Temperature manipulation experiments have demonstrated negative effects on root productivity, microbial biomass and activity due to warming (Cole et al., 2002b). But increased temperatures have also been shown to affect the structure of the food-web by changing the number of predators (Dolery et al., 2006) and influencing the species diversity (Harte et al., 1996).

The effect of temperature stress

In response to warming and/or sub-zero temperatures, enchytraeids can use the same strategies as pointed out with drought stress. Springett et al. (1970) observed that *C. sphagnetorum* was able to migrate vertically to escape high temperature and dry surface layers, thus affecting the vertical distribution over a short period of time. In cold soils like tundra and alpine soils, cocoon production as protection against ice formation and low soil water availability has been suggested as a way of surviving sub-zero temperatures (Birkemoe et al., 2000; Bauer, 2002).

In general, field studies with temperature increases of 0.5–2°C above ambient temperature have not observed any temperature effects on *C. sphagnetorum* (Haimi et al., 2005b; Maraldo et al., 2008; Maraldo et al., 2009a). This is in contrast to short-term laboratory (Briones et al., 2004) and transplant (Briones et al., 1997) studies, exposing *C. sphagnetorum* to temperatures 2.5–5°C above ambient temperature, where stimulatory effects were observed. These differences in response to warming may be related to the different temperature regimes used, but also different soil moisture content could be involved. All three field studies included annual variations in soil moisture content, whereas the laboratory studies and the transplant study were performed with high and constant soil moisture content. Thus, the enchytraeids in the field could have been suppressed by the low soil moisture content and perhaps, therefore, not benefit from the effect of increased temperatures. Another explanation could be the sampling methodology, as field studies have often been restricted to autumn samplings and, thereby, missed the possibility of seeing a potential positive effect of warming in winter and spring.

Consequently, a future northern European climate with increased minimum temperatures of 2°C to 4°C, but with no changes in soil moisture levels, may not have a detrimental effect on *C. sphagnetorum*. On the contrary, it could have a positive direct effect as growth and activity of enchytraeids will start earlier in the season due to increased winter and spring temperatures (Cole et al., 2002a). A higher enchytraeid growth in spring and autumn could have an impact on the decomposition of soil organic matter (Cole et al., 2002a; Briones et al., 2004). *C. sphagnetorum* has a wide distribution; from the arctic areas (Christensen and Dozsa-Farkas, 2006) to northern Spain (Uhia and Briones, 2002), which are all areas with relatively

low maximum temperatures. The species might, therefore, be adapted to relatively low temperatures, which is supported by studies reporting an optimum temperature of 10°C (Standen, 1973) and an upper threshold temperature of 16°C (Briones et al., 2007).

Enchytraeids living in temperate and arctic areas may experience extended periods of temperatures below the melting point of their body fluids. Studies by Birkemoe et al. (2000) and Bauer (2002) suggest that species in tundra and alpine soils can survive the winter mainly as cocoons, but field surveys indicate that some species are able to hibernate in their adult stage (Birkemoe et al., 2000; Coulson and Birkemoe, 2000; Pedersen and Holmstrup, 2003). Specimens of the enchytraeid *Stercutus niveus* survived a short exposure in frozen litter at -4°C to -5°C (Dózsa-Farkas, 1973; Bauer et al., 1998). Although this and other studies suggest that many species can survive in frozen soil and litter, only a few species have been explicitly shown to survive by freeze tolerance (Pedersen and Holmstrup, 2003; Hartzell et al., 2005; Slotsbo et al., 2008). *C. sphagnetorum* appears to be rather sensitive to freezing temperatures. More than a 70% reduction of the density and biomass was observed in Danish winter-acclimated *C. sphagnetorum* exposed to almost -2°C for two days (Fig. 1). In a Finnish laboratory experiment, no *C. sphagnetorum* survived hard frost (-16°C) treatment, but when the temperature was increased to above zero, a small population of enchytraeids was re-established in samples exposed to hard frost (-16°C) (Sulkava and Huhta, 2003). The authors suggested that a few enchytraeids must have survived the hard frost (-16°C). An alternative explanation could be that the species had survived the harsh temperature in a cold tolerant cocoon stage, as also suggested during severe droughts.



Photo: Murali Narayanan/Wikimedia commons

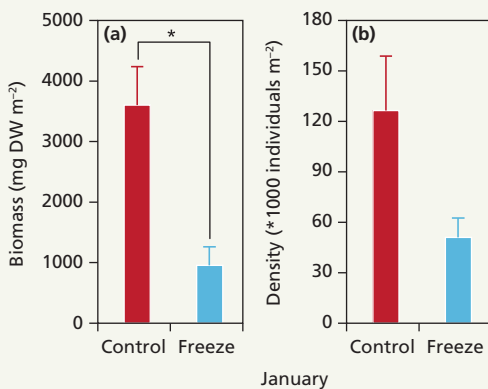


Figure 1. Winter acclimated *C. sphagnetorum* were collected in January 2008 in a Danish heathland (Brandbjerg) using soil cores down to 3 cm depth. The enchytraeids were exposed to a control temperature (field temperature – no freezing; $n=5$) or to freezing temperature of -1.3°C for two days ($n=5$). To ensure that soils froze, an ice crystal was added after 24 h at -1.3°C . After freezing, samples were thawed at 2°C , and enchytraeids extracted to determine the effect of freezing by measuring (a) the biomass (mg DW pr m^2) and (b) density (*1000 individuals per m^2). Significant differences between control and freezing are marked with an asterisk.

Physiological responses to increased temperatures

The physiological responses of enchytraeids exposed to warming have received little investigation. I am aware of only one study, which reports that *C. sphagnetorum* did not tolerate high temperatures, and a short-term exposure to 36 °C for two hours seems to be the limit (Springett, 1967). A well known response to short-term high-temperature stress is the production of Heat Shock Proteins (HSP) (Sørensen et al., 2003). HSPs compose a range of stress proteins, where a variety of members function as molecular chaperones, which prevent the irreversible denaturation of cell proteins (Feder and Hofmann, 1999). HSPs are widespread in living organisms, and, undoubtedly, also in enchytraeids, as they are observed in the closely related earthworms (Homa et al., 2005). However, HSPs have not yet been investigated in enchytraeids.

Physiological responses to sub-zero temperatures

Three different physiological strategies exist whereby enchytraeids can cope with sub-zero temperatures. One strategy, freeze tolerance, is to establish controlled, protective freezing of the extracellular body fluids at high sub-zero temperatures, as observed in *E. albidus* (Slotsbo et al., 2008) and *Fridericia ratzeli* (Pedersen and Holmstrup, 2003). These species accumulate cryoprotectants, especially glucose, in physiologically high concentrations

upon freezing. The second strategy, freeze avoidance, is based on the ability to stay in a supercooled state even at temperatures much below the melting point of body fluids. Because of the intimate contact of enchytraeids with soil water and ice, it seems unlikely that enchytraeids can survive prolonged periods of frost by supercooling. The third option is “cryoprotective dehydration”. Through dehydration and accumulation of osmolytes, soil organisms rapidly equilibrate their melting point to the surrounding temperatures in a frozen environment (Holmstrup et al., 2002). Since enchytraeids are small and their cuticle is very permeable for water, it is possible for them to use cryoprotective dehydration as a survival strategy. A study by (Sømme and Birkemoe, 1997) reports that both freeze tolerance and cryoprotective dehydration seem to exist in enchytraeids, and that the strategy depends on the surrounding thermal and hygric conditions. This

ability to choose between freezing and dehydration, depending on the surrounding conditions, is also known in *F. ratzeli* (Pedersen and Holmstrup, 2003). The physiological responses of *C. sphagnetorum* to increased temperatures or sub-zero temperatures are, despite the species’ ecological importance, not examined.



Photo: J.C. Schou/Biopix

Responses of enchytraeids to elevated CO₂ concentration

Elevated atmospheric CO₂ concentrations may only have negligible direct effects on enchytraeids, since they are adapted to high CO₂ concentrations in soils (van Veen et al., 1991). On the other hand, indirect effects, such as changes in the quantity and quality of litter the rate of root turnover and changes in soil water content, are expected to affect the survival and activity of enchytraeids (Coûteaux and Bolger, 2000). It has been suggested that increased CO₂ can have a positive effect on the soil fauna, as the primary production (Amthor, 2001), root production and biomass (Arnone et al., 2000; Pregitzer et al., 2008), and soil moisture content (Niklaus et al., 2003; Heath et al., 2005) increase due to elevated CO₂.

Enchytraeids have been reported to respond in different ways to increased atmospheric CO₂ concentration (Markkola et al., 1996; Yeates et al., 1997; Yeates et al., 2003; Haimi et al., 2005b). A positive impact of elevated CO₂ has been observed on enchytraeids densities in grassland (Yeates et al., 1997) and in dry heathland dominated by *C. sphagnetorum* (Maraldo et al., 2009a). However, no changes in field populations of *C. sphagnetorum* were observed in Finnish conifer forests after six years of exposure of CO₂ (Haimi et al., 2005b). A negative effect of increased CO₂ on *C. sphagnetorum* has also been reported (Markkola et al., 1996) as well as seasonal variation in CO₂ response (Markkola et al., 1996; Maraldo et al., 2009a). Markkola et al. (1996) observed that summer acclimated enchytraeids responded positively to elevated CO₂, but when the enchytraeids were acclimated to winter conditions, they responded negatively. A similar observation was reported by Maraldo et al. (2009), with positive responses in summer, but no effects of elevated CO₂ in the autumn. In the latter study, no species identification was performed and, thus, it cannot be established whether *C. sphagnetorum* was involved in the reported changes, although, it was the dominant species at the location. It has been suggested that increased CO₂ could affect the reproduction of *C. sphagnetorum* or maybe increase the predation by other members of the soil fauna (Markkola et al., 1996). The observed seasonal variation could also be due to changes in food availability and the number of predators. Mites have been found to increase their grazing of fungi when exposed to elevated CO₂ (Allen et al., 2005). This could also be the case for enchytraeids grazing on fungi or mites feeding on enchytraeids, thereby causing seasonal responses. Moreover, Klironomos et al. (1996) detected that increased CO₂ altered the balance between mycorrhizal and non-mycorrhizal fungi and bacteria, which could have consequences for food availability. An alternative explanation could be that elevated CO₂ enhances the water-use efficiency, which alleviates the water limitation during dry periods in the top soil (Heath et al., 2005). Arnone et al., (2000) suggested that the positive response of soil moisture caused by elevated CO₂ might enhance the soil nutrient availability in seasonally dry grassland. Several authors suggest that the effects of increased CO₂ cascades through multiple components of the soil food web and, so far, no generalised pattern of responses has been identified (Coûteaux and Bolger, 2000; Yeates et al., 2003; Lorange et al., 2004; Allen et al., 2005). An increased allocation of C due to increased root production together with an associated increase in rhizodeposition will typically stimulate the activity of soil organisms (Allen et al., 2005). Elevated CO₂ has also been associated with an upward shift in root length density in the upper soil layer (Arnone et al., 2000; Norby and Jackson, 2000). Root-associated organisms, such as enchytraeids, are suspected to be affected by these changes, but the direction will depend on the plant species, due to the variety of ways in which plants can respond to elevated CO₂ (Wardle et al., 2004). Over time, a reduction in leaf litter nitrogen content will, probably limiting the production of enchytraeids before the potential positive effect of increased soil moisture in nutrient poor environments can be seen (Allen et al., 2005). *C. sphagnetorum* prefers feeding on litter aged

between five and ten years (Standen and Latter, 1977; Briones and Ineson, 2002). Hence, this supports the assumption that the dilution effect will probably not be evident till after a certain period of time has passed.

From the few studies performed, it appears reasonable to conclude that the impact is geographical and species-dependent, but is also affected by the season, soil type, vegetation, and the nutrients status of the soil (Coûteaux and Bolger, 2000).

Enchytraeids and climate change interactions

Increases in atmospheric CO₂ concentrations, higher average temperatures and changes in the hydrological patterns resulting in more frequent and intense drought spells will have direct effects and indirect effects on enchytraeid populations, as presented above. However, one of the major challenges in soil science today is to unravel the impact of all three environmental factors in combination and to understand their feedback mechanisms in the soil ecosystems. The climate change factors interfere with each other, e.g. the combination of increased temperature and low soil water levels could cause an even stronger desiccation stress for the soil organisms, as increased temperatures enhance the evaporation from the soil (Hodkinson et al., 1998). It could also be argued that increased temperatures would have no effect, as low soil moisture acts as a threshold for the activity of the enchytraeids (Sala et al., 2000).

The direction and magnitude of the interactions of the climate change factors depend on a range of biotic and abiotic factors, such as local climate, geographic location and habitat type and their feedback mechanisms (Ives and Carpenter, 2007; Tylianakis et al., 2008). The impact of the interactions can either result in responses that are smaller than expected (antagonistic), additive or greater than expected (synergistic), as has been observed for microbial decomposition, where elevated CO₂ and temperatures interacted synergistically (Fenner et al., 2007). Observed responses like this can create unpredictable feedbacks and support the hypothesis that responses are most likely to be interactive rather than direct or unidirectional (Swift et al., 1998; Sala et al., 2000). Thus, the predictions of the direction and magnitude of these interactions are important in the modelling of the potential impact of climate changes on the soil ecosystem.

Enchytraeids may respond in an unpredictable way to the climate change factors and, consequently, it can be difficult to extrapolate results from single exposure experiments to include all three factors. Despite this, most field studies have focused on one or two factors, but not the combination of them (Table 1). One of the major reasons for this is probably the relatively large financial support these types of studies require. Only two studies have, to my knowledge, focused on the effect of the interactions between two to three climate change factors on natural enchytraeids populations dominated by *C. sphagnetorum* (Haimi et al., 2005b; Maraldo et al., 2009a). In Finnish coniferous forests, field enchytraeid populations were exposed to increased temperatures, CO₂ concentration and the combination of these two for five years. *C. sphagnetorum* did not show any effects of applied treatments, and the same picture emerged in other groups of the soil mesofauna (Haimi et al., 2005b). In the second study, a full factorial experiment with elevated CO₂, increased temperatures and prolonged drought period manipulations was performed in a dry Danish heathland also dominated by *C. sphagnetorum* (Maraldo et al., 2009a). The authors found that all three climate factors in combination did not affect the field population of enchytraeids, even though the increased CO₂ and drought treatments separately affected the populations. No

Table 1. List of field studies of enchytraeid communities dominated by *C. sphagnetorum*, in which either a single or a combination of factors have been applied. The overall effects of treatments are listed; ↑ indicates a significant positive effect, and ↓ indicates a significant negative effect.

Treatment	Length of exposure	Location	Dominating species	Effect	References
Single factor studies					
Prolonged drought and irrigation	8 years	Swedish boreal forest	<i>C. sphagnetorum</i>	Drought ↓ Irrigation ↑	(Lindberg et al., 2002)
Temperature and prolonged drought	6 years	Danish dry heathland	<i>C. sphagnetorum</i>	Drought ↓ Temperature	(Maraldo et al., 2008)
Prolonged drought and irrigation	1 season	Danish dry heathland	<i>C. sphagnetorum</i>	Drought ↓ Irrigation ↑	(Maraldo & Holmstrup, 2009)
Interaction studies					
CO ₂ and temperature	5 years	Finnish boreal forest	<i>C. sphagnetorum</i>	No effects	(Haimi et al., 2005b)
CO ₂ , temperature and prolonged drought	3 years	Danish dry heathland	<i>C. sphagnetorum</i>	Drought ↓ CO ₂ ↑	(Maraldo et al., 2009a)

impact of increased temperature was observed, which can be due to a relatively low degree of warming. Drought was the main limiting factor, causing a reduction in the enchytraeid biomass and density acting independently of the interannual variation in precipitation. But the reducing impact of drought was diminished when CO₂ was present, as drought and CO₂ in combination acted in additive direction during summertime. The authors, therefore, concluded that the combined treatments interacted additively. The enchytraeid populations in both studies were exposed for a relatively short period, thus the full influence of the indirect effects may not have emerged yet. Another implication with both studies is the lack of sampling in other seasons than autumn. Temperature effects may only be visible in winter or spring time where it is the limiting factor (Lundkvist, 1982). This could result in a higher enchytraeid production and activity during these periods.

An alternative approach is to perform a meta-analysis based on existing enchytraeid data, as presented by Briones et al. (2007). In this study, enchytraeid distribution data from more than 44 studies was used and related to a predicted global temperature change scenario. The authors identified a maximum mean annual temperature threshold of 16°C, which could be a critical limit for the present distribution of *C. sphagnetorum*. The authors suggest that above this temperature limit the presence of the species would decline markedly and may be totally lost in some regions (Briones et al., 2007).

For the time being, it is only a matter of speculation how enchytraeids and, especially, *C. sphagnetorum* will be affected by climate changes in the long term. *C. sphagnetorum* has a wide distribution, which complicates the prediction of a general direction in changes. Long-term studies are needed, as the effects of the climate change factors are highly complex, and the effect of e.g. dilution of the nitrogen content of litter cannot be observed till several years after treatments have been applied. In addition to these speculations, one must be aware of a general methodological problem. The manipulations in most climate change experiments are not gradually developing as would be the case in nature. The responses of the applied manipulations could, therefore, result in misleading results, as a gradual shift would, perhaps, give the enchytraeids and other organisms time to adapt to

the new conditions. Moreover, climatic averages, as also applied in most studies, may be of little consequence to enchytraeids, as they experience the full spectrum of climate events and not only average conditions. Infrequent climatic extreme events, such as drought episodes (Nielsen, 1955a) and heat waves (Bragazza, 2008), may have a disproportionate effect on the survival of soil animals (Hodkinson et al., 1998; Scheffer et al., 2001; Piessens et al., 2009). Consequently, these extreme events may expose the soil organisms to conditions below their threshold of existence and, thereby, create serious distributional changes in the soil ecosystems.

Can enchytraeids adapt to climate changes?

Knowledge of environmental adaptation is important for the prediction of how and which populations will be affected by climate changes. Despite this, only few studies have been published on the adaptation of soil invertebrates to climatic stress. Two approaches have been used to examine the ability of a given species to genetically adapt to new environmental conditions; *selection experiments* or *environmental gradient experiments*. The *selection experiment*-approach has been used in a number of ecotoxicological studies involving soil invertebrates, using populations from metal contaminated areas. Various species of soil invertebrates are able to increase their metal tolerance through genetic adaptation, e.g. enchytraeids (Salminen and Haimi, 2001; Haimi et al., 2005a) and collembolans (Posthuma, 1990; Tranvik et al., 1993). The *environmental gradient experimental*-approach has been used in several studies to investigate the ability of soil invertebrates to adapt to local climatic conditions by comparing resistance patterns along geographic gradients; soil arthropods (Bahrndorff et al., 2006; Bokhorst et al., 2008), earthworms (Holmstrup et al., 2007b) and enchytraeids (Slotsbo et al., 2008). For both approaches is it necessary to separate the effects of the environment (phenotypic plasticity) from those of genetics; it must therefore be the off-spring of the field collected organisms, reared under similar conditions in the laboratory, that is used for experiments.

Selection experiments

The principle behind the *selection experiment* approach is to test if a population may have genetically adapted to a specific stressor. By using populations from similar habitats, but in soils with and without a specific stressor, e.g. different metal concentrations, is it possible to test for genetic adaptation to the specific stressor.

Concerning enchytraeids, only one study addresses genetic adaptation to climate change. The study was conducted by using *C. sphagnetorum* populations from a manipulated field experiment where repeated drought stress had been applied for six consecutive years (Maraldo et al., 2008). The authors had expected to see an increased tolerance of drought as a result of directional selection in the populations of *C. sphagnetorum*. However, nothing pointed in that direction as the off-spring from the drought exposed populations had the same high sensitivity to small reductions in soil water potential as those from control plots (Fig. 2). It seems therefore that several years of repeated increased drought stress in the field did not result in selective changes in this particular trait, perhaps because genetic variation in this trait was not present, or due to low clonal diversity, and/or because more time is needed before selection becomes detectable. The authors concluded that *C. sphagnetorum* populations from drought treated plots must have gone through several

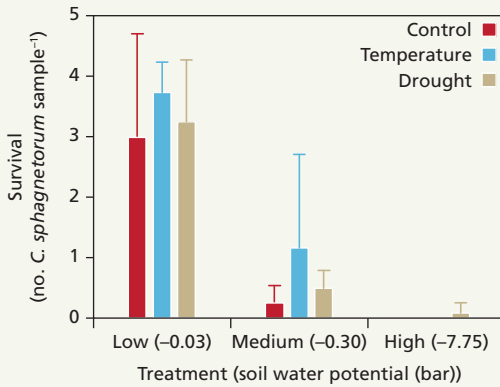


Figure 2. Survival of *C. sphagnetorum* (n=4) exposed to low, medium or high drought intensity. Four *C. sphagnetorum* were added to each test container and exposed to low (-0.03 bar), medium (-0.3 bar) or high drought (-7.75 bar). The enchytraeids used were progeny (F1–F3 generation) of specimens collected in field plots that had received no treatment (control), or had been treated with increased temperature or increased drought for six years. The enchytraeids had been reared under similar conditions in the laboratory. For further information see Maraldo et al. (2008).

genetic bottlenecks, due to the negative effect of drought on enchytraeids, but apparently without any detectable genetic adaptation to drought (Maraldo et al., 2008). This could have consequences for *C. sphagnetorum* populations if drought becomes more frequent and severe in the future.

Environmental gradients

Climate strongly varies with latitudes and altitudes and variations in traits related to the fitness of an organism can be the result of adaptive evolution to climatic conditions. Such variations would suggest a contribution of directional selection causing differentiation among populations. Latitudinal gradients are of particular interest since climate strongly varies with this variable, and this approach can be used as a “space for time substitution” to investigate how populations will be affected by climate changes over time. For example, a study of Collembola collected along a 2000 km latitudinal gradient ranging from Denmark to southern Italy, showed a positive relationship between cold shock resistance and lowest environmental temperature recorded at the site where the populations originated. This was in accordance with a study showing that the cold tolerance of *E. albidus* was related to thermal conditions in the habitat (Slotsbo et al., 2008). Two populations from Greenland were shown to be much more freeze tolerant than a German population. This is consistent with the fact that *E. albidus* from Greenland has to cope with longer winters and lower temperatures, and therefore require a better freeze tolerance than the German worms (Slotsbo et al., 2008).

A similar study was performed with *C. sphagnetorum* collected along a latitudinal gradient spanning more than 3000 km from Spain to Finland (Table 2 and Fig. 3). Offspring from each population were exposed to drought stress for seven days. It was expected that populations from more wet areas (receiving more precipitation) would be more drought sensitive than populations from more dry areas. However, no correlation was detected between drought sensitivity of the respective populations and annual precipitation. Thus, these results suggest that genetic differences in drought resistance among the populations were not present (Table 2 and Fig. 4) as also suggested by Maraldo et al. (2008). Another reason for the result could be that it is difficult to establish the exact microenvironment from where the enchytraeids were collected and several environmental factors may also have affected the selected traits and thereby confound the results (Hoffmann et al., 2003).

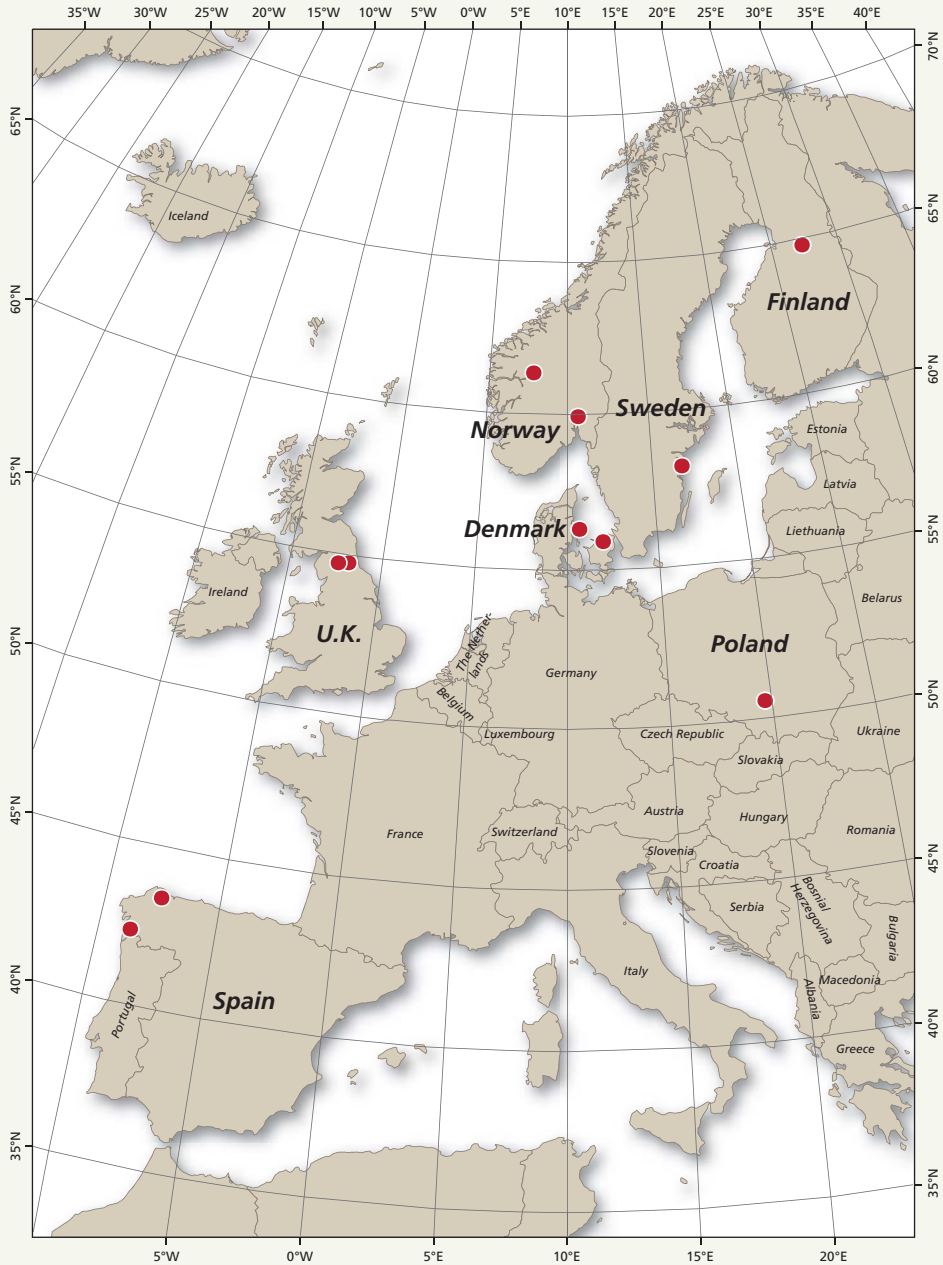


Figure 3. Map showing the positions of sampling sites of *C. sphagnetorum* used in the environmental gradient study shown in Fig 4. Top soil including litter was collected from each location and placed at 5°C until extraction. Individual specimens of *C. sphagnetorum* were after extraction placed in wet soil at 15°C for ten months and reared as described in Maraldo et al. (2008).

Table 2. Selected climatic characteristics and geographic coordinates of the locations from where *C. sphagnetorum* was collected for the comparative drought tolerance experiment presented in Fig. 3. Sources of climatic information are listed below.

Location		Position	Precipitation (mm pr year)	Temperature (24-hr Average°C)
Denmark (DK)	Mols	56°23'N, 10°57'E	720	9.4
	Brandbjerg	55°53'N, 11°58'E	613	8.0
England (UK)	Moor House	54°42'N, 02°23'W	2012	6.1
	Tow Low	54°45'N, 01°49'W	607	8.3
Finland (FIN)	Oulu 1	64°47'N, 27°16'E	453	2.1
	Oulu 2	64°47'N, 27°16'E	453	2.1
Norway (N)	Oslo	59°55'N, 10°38'E	655	5.2
	Årdal	61°19'N, 7°49'E	491	5.9
Poland (POL)	Olkusz	50°32'N, 19°39'E	669	7.5
	Olkusz	50°32'N, 19°39'E	669	7.5
Spain (ES)	Xistral	43°26'N, 07°35'W	1263	9.9
	Zapateria	42°12'N, 08°37'W	1008	13.4
Sweden (S)	Gusum	58°11'N, 16°44'E	530	6.5

Denmark: (Mikkelsen et al., 2008; Sowerby et al., 2008)

England: Moor House 1992-2007 from www.ecl.uk.com and Towlow from www.worldclimate.com

Finland: from www.worldclimate.com 1961-1991

Norway: Oslo from www.worldclimate.com and Årdal from <http://retro.met.no/>

Poland: www.worldclimate.com

Spain: Personal communication from Noela Carrera Pérez (2005-2007)

Sweden: www.worldclimate.com

Adaptation or reduction and consequences

It is evident that temperature and moisture regimes play a significant role for the performance of *C. sphagnetorum*. In areas with constant high soil moisture and high levels of precipitation, the influence of increased temperature may have a positive (Cole et al., 2000) but in some cases also negative (Cole et al., 2002b; Briones et al., 2007). According to Briones et al. (2007), a maximum mean annual temperature of 16 °C will be a critical limit in these areas. This is supported by the study of Cole et al. (2002a), who observed a reduction in the density of *C. sphagnetorum* when the species was exposed to 18 °C for a longer period of time (weeks). However, we still need to know more about *C. sphagnetorum* temperature resistance and its adaptation potential to high temperatures to be able to predict the species' response to a future warmer climate.

In regions with large annual variations in soil moisture content and low levels of precipitations the effect of temperatures may be less important for the distribution and performance of *C. sphagnetorum*, as soil moisture here will be the main limiting factor. In line with the arguments presented in the previous section, I propose that *C. sphagnetorum* can not adapt to a drier climate with the result that the density of this species may be reduced in some regions. This is supported by the observations reported by Lindberg et al. (2002),

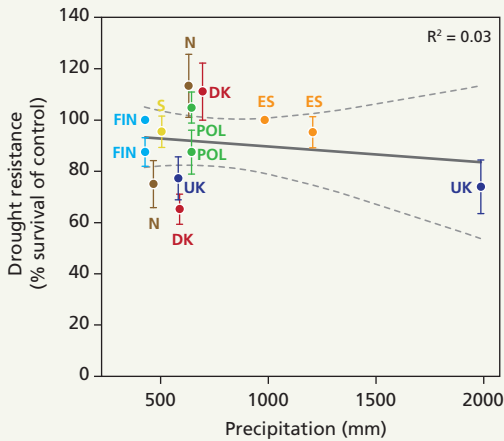


Figure 4. Drought resistance measured as percent survival relative to control survival (mean \pm S.E) plotted against annual precipitation (mm) of the respective location. Drought resistance was determined using a seven-day exposure to high soil moisture and low soil moisture. Four *C. sphagnetorum* were added to vials containing soil with high moisture (-0.01 bar; control) or vials containing dry soil (-0.09 bar; drought). Six vials with four enchytraeids each were used for each location and treatment. The worms used for experiments were progeny (F1–F3 generation) of specimens collected at the different locations (see Table 2 and Fig. 3) and cultured in the laboratory as described by Maraldo et al. (2008). Linear regression is shown as a grey line; grey dotted lines represent the 95% confidence limits of the trend line. No significant correlation ($R^2=0.03$; $p=0.56$) between drought sensitivity and precipitation was observed.

who found a 90% reduction in enchytraeid density (dominated by *C. sphagnetorum*) in Swedish conifer forest after eight years of repeated drought treatments. Other studies have, however, shown that *C. sphagnetorum* does have a potential to recover from long and hard drought stress (Maraldo and Holmstrup, 2009; Maraldo et al., 2009a). However, Nielsen (1955b) also observed that a long and harsh summer drought completely reduced the enchytraeids population and their cocoons, thus leaving the recovery of the population entirely dependent on recolonisation, which is extremely slow in *C. sphagnetorum*, as the species have a low dispersal rate of less than 20 cm per year (Sjögren et al., 1995).

The ability of the ecosystems to withstand (resistance) and recover (resilience) from the disturbance may be dependent on the species' diversity, but also on the presence and importance of keystone species (Sala et al., 2000; Ives and Carpenter, 2007). Changes in enchytraeids' productivity and activity will affect other components of the soil ecosystems, which may lead to serious effects on the soil ecosystem functioning. As suggested by Briones et al. (2007), changes in the density, activity and distribution of *C. sphagnetorum* could contribute to feedbacks between climate and soil C stores in regions at high latitudes due to its key role in decomposition and mineralisation (Briones et al., 2004; Briones et al., 2007). The soil food web is characterised by numerous species, functional groups and trophic levels (Setälä, 2002). This heterogenic structure of the soil food web suggests that the soil ecosystems can contain a high degree of functional redundancy meaning that the functions lost with a loss of a particular species are rapidly replaced by the activities of the remaining species (Setälä, 2002). However, there is an emerging view that below ground processes of decomposition and nutrient mineralisation are influenced mainly by the physiological attributes of the dominant species present (Setälä, 2002). Therefore, one of the challenges in future studies will be to link and unravel the responses of the enchytraeids below ground to the ecosystem functioning above ground. A range of studies have already been performed to estimate the effects of warming on enchytraeids and their functions in moorlands (Briones et al., 1998; Cole et al., 2000), however, no studies have as yet taken all climate changes factors into account. These kinds of studies are needed if we want to improve the understanding the expected effects and feedback mechanisms in the soil ecosystems.

Perspective

The environmental climate change factors which have been discussed in this review are part of the Global Change concept, which beside of the environmental climate change factors, also include a range of anthropogenic factors such as contamination with toxic compounds, nitrogen deposition, increased concentration of O₃ in atmosphere, change of land-use and biotic invasions (Sala et al., 2000; Carpenter et al., 2006; Tylianakis et al., 2008). These additional factors will likely interfere with climate changes as well as with biotic and abiotic elements of the soil ecosystems. The combination effect of climate change factors and environmental contaminations is sparsely investigated in enchytraeids (Puurtilinen and Martikainen, 1997; Maraldo et al., 2006; Kools et al., 2008). In other soil animals such as Collembola (e.g. Højer et al., 2001; Holmstrup et al., 2007a) and earthworms (Friis et al., 2004; Bindesbøl et al., 2005; Jensen et al., 2009) synergistic responses have been reported. The direction of the responses were found to depend on the type of pollutant and the climatic factor involved, however this research is still in its beginning. A similar conclusion has been put forward by Loranger et al. (2004) which examined the effect of increased O₃ and CO₂ in Collembola. The impact of increased nitrogen deposition due to anthropogenic activities has also shown contrasting results on enchytraeid density (Thompson, 2005; Prendergast-Miller et al., 2008) and no clear conclusion can be drawn at this stage. Also aspects like change of land-use or biotic invasion of new species might have consequences for the activity and distribution of enchytraeids, and little is known about possible interactions with climatic changes.

In conclusion, climatic phenomena, ecosystem processes and human activities are interactive and interdependent, making long-term predictions extremely difficult. Climate change will beyond doubt have an impact on the enchytraeids; however, we are just beginning to understand how and to which extent such impacts will occur.



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Paper 1

**Responses to acute and chronic desiccation stress in
Enchytraeus (Oligochaeta: Enchytraeidae)**

Responses to acute and chronic desiccation stress in *Enchytraeus* (Oligochaeta: Enchytraeidae)

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Abstract Enchytraeids are small soil living oligochaete worms with high sensitivity to low soil moisture. The effects of acute and chronic desiccation on survival and reproduction were determined in *Enchytraeus albidus* and *Enchytraeus crypticus*. Further, effects of acute drought stress on the water balance physiology and accumulation of osmolytes were investigated in *E. albidus*. Survival of *E. crypticus* and *E. albidus* was significantly influenced by exposure time. Reproduction was much more sensitive to desiccation than survival and was significantly reduced from -0.06 bar, which was surprising because no dehydration or change in the body fluid osmolality of *E. albidus* occurred until much harsher drought regimes occurred. The body fluid osmolality of *E. albidus* was relatively high, about 500 mOsm. Congruent with this no water loss or changes in osmotic pressure occurred until equivalent or higher water potential values of the environment were reached. Two osmolytes, glucose and alanine, were up-regulated in drought exposed *E. albidus*. Even though enchytraeids display moderate physiological protection to rapid changes in soil moisture (by having a high osmotic pressure) in the short term, populations subjected to long-term drought stress can be severely reduced even under moderate drought levels.

Keywords Water balance physiology · Osmolytes · Body fluid osmolality · *Enchytraeus albidus* · *Enchytraeus crypticus*

Introduction

Enchytraeids are small oligochaete earthworms, which are widespread in arctic to tropical regions (Petersen and Luxton 1982; Römbke 1992). Enchytraeids occur mainly in the organic top layer of soils (Nielsen 1955a; Springett et al. 1970; Dozsa-Farkas 1992), where they take part in the decomposition process both directly, through their own consumption, metabolism and excretion of nutrient-rich faeces, and especially indirectly by stimulating microbial activity through grazing (Didden 1993; Laakso and Setälä 1999; Cole et al. 2000).

The distribution and performance of enchytraeids is highly dependent on the availability of water (e.g. Abrahamsen 1971; Gröngroft and Miehlich 1983; Sulkava et al. 1996). A range of field studies have shown that enchytraeids are extremely vulnerable to drought stress (e.g. Nielsen 1955b; Abrahamsen 1972; Lindberg et al. 2002; Maraldo et al. 2008). Soil water potential below pF 4 (corresponding to -9.8 bar) has been shown to be lethal for *Cognettia sphagnetorum* (Abrahamsen 1971), but the degree of drought sensitivity is species dependent and more drought-resistant species can also be found. For example, *Fridericia galba* has been found to survive in soil with water holding capacity (WHC) above 20% (corresponding to values below -9.8 bar) for more than 49 days (Dozsa-Farkas 1977).

So far two drought survival strategies have been described for enchytraeids. In the first strategy, enchytraeids avoid dry conditions by migrating to deeper and

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moister microhabitats, but this strategy can only be effective in the short term unless the food quantity and quality in the deeper soil layers can maintain or increase the population size (Springett et al. 1970; Uhia and Briones 2002). The second strategy allows the population to survive in the more desiccation tolerant cocoon stage (Nielsen 1955b; Lagerlöf and Strandh 1997). Some enchytraeid species are even found to cover their egg cocoons with sand and debris, which perhaps can delay the desiccation of the cocoons (Christensen 1956).

Despite their ecological importance and well-documented sensitivity to drought, the knowledge of enchytraeids' physiological adaptations to desiccation is limited. The majority of enchytraeids live in the top soil and are therefore at risk of desiccation during drought periods due to their highly permeable surface. Dehydration is harmful as it can cause cellular shrinkage, cellular proteins can become irreversibly denatured and the cellular membranes can lose their normal conformation (Crowe et al. 1992). Certain plants and animal species are known to accumulate low molecular weight metabolites, so-called compatible osmolytes, which may help the cell to maintain its volume and reduce the water loss during desiccation (Yancey 2005). Compatible osmolytes belong to diverse groups of sugars, amino acids, polyols and proteins and are characterised by being non-toxic to cellular processes even in high concentrations (Yancey 2005). However, a range of these compounds also have a protective function besides acting like osmoprotectants, where they prevent protein aggregation (Chakrabortee et al. 2007), stabilize cellular membranes (Crowe 2002), or may have antioxidative properties (Krishnan et al. 2008).

Enchytraeids are semi-aquatic animals and closely related to earthworms. Earthworms has been shown to cope with drought stress by tolerating dehydration or by entering diapause (Holmstrup and Westh 1994; Holmstrup 2001; Friis et al. 2004). Studies have shown that earthworm cocoons dehydrate until they are in equilibrium with the surrounding water potential (Holmstrup and Westh 1994; Petersen et al. 2008) and that the embryo of the cocoons accumulates osmolytes as a response to dehydration. Sorbitol and glucose are reported to be the most abundant osmolytes in earthworm cocoons (Storey and Storey 1996; Petersen et al. 2008). Accumulation of glucose has also been observed in cold exposed *Fridericia ratzelei* (Pedersen and Holmstrup 2003). Thus, we suspect that enchytraeids may respond in a similar way due to their close evolutionary relation with earthworms.

In the field, soil organisms will experience a gradually increasing exposure to desiccation as the soil acts like a buffer. It is therefore more ecologically relevant to use gradual exposure regimes than acute regimes or at least to simulate naturally occurring drought exposures. Earlier

studies report increased desiccation tolerance after a pre-acclimation to relatively mild drought stress (Sjursen et al. 2001; Hayward et al. 2007).

The objective of this study was to investigate the effects of acute and chronic exposures to low soil water contents on survival and reproduction in the two enchytraeids species, *Enchytraeus albidus* and *Enchytraeus crypticus*. In addition, we examined the survival and water balance physiology of adult *E. albidus*, when exposed to realistic desiccation regimes. This was achieved by determining the water content, body fluid osmolality and identification of osmolytes during exposure to increasing desiccation. Finally, it was examined if a pre-acclimation to mild drought could increase the survival of acute drought stress in *E. albidus* by using a gradually increasing drought regime.

Materials and methods

Animals

Information on biogeography distribution of *E. crypticus* is limited; but the species has been found in compost soil in Germany (Westheide and Graefe 1992). *E. albidus* is widely distributed from the high Arctic to temperate Western Europe and can be found in organically rich environments such as decaying seaweed, compost and sewage beds (Christensen and Dozsa-Farkas 2006). Both species reproduce via cocoons and the main morphological difference between the two species is their size. *E. crypticus* is small with an average length of 7 mm and an adult dry weight of 50 µg (Westheide and Graefe 1992). *E. albidus* is larger and adults have an average length of 30 mm and dry weight of 1 mg. Only *E. albidus* was used for the physiological measurements, as the small size of *E. crypticus* precluded such measurements. A German *E. albidus* strain (Tierfischfutter, Jena, Germany) was kept in moist soil at $15 \pm 1^\circ\text{C}$ and weekly fed oatmeal ad libitum. *E. crypticus* was cultured in agar at $20 \pm 1^\circ\text{C}$ and weekly fed oatmeal ad libitum. Only adults of both species with visible clitellum were used for the experiments.

Acute drought exposure

The acute drought experiment followed a method previously described by Holmstrup and Westh (1995). Fully hydrated worms were gently blotted with filter paper to remove surface water and placed in open-top plastic sample vials (3 cm high, 1.6 cm diameter) in the centre of a 160-ml plastic cup (4.2 cm high, 7 cm diameter) containing 25 ml aqueous NaCl solution, sealed with a tightly fitting plastic lid and parafilm. The inner vial was covered with 100 µm net to prevent animals escaping from the vial. The air in this small

closed system quickly equilibrated with the salt solution (following Raoult's law). By using a range of increasing NaCl-solutions a decreasing relative humidity (RH) was created. The different species of enchytraeids were exposed to the same range of decreasing RH, including 99.8% RH (−2.7 bar), 99.6% RH (−5.5 bar), 99.4% RH (−8.3 bar), 99.2% RH (−11.0 bar), 99.0% RH (−13.8 bar), 98.8% RH (−16.6 bar), 98.6% RH (−19.3 bar) and 98.4% RH (−22.1 bar). The control humidity was set to 99.8% RH, due to preliminary results with *E. crypticus*, where 100% RH resulted in moderate mortality. For the experiment with *E. crypticus*, eight worms were added to each test vessel, while only one or two *E. albidus* was added to each vessel. The number of replicates was eight for *E. crypticus* and 18 for *E. albidus*. Vials were placed at $15 \pm 0.1^\circ\text{C}$ for 7 days, followed by 2 days where the NaCl solution was changed to demineralised water to allow the worms to rehydrate. The worms were scored as survived, if they moved after a gentle tactile stimulus.

Chronic drought exposure

Enchytraeus crypticus and *E. albidus* were exposed in soil of varying moisture content for 4 and 6 weeks, respectively. Ten different soil water potential treatments ranging from -0.036 ± 0.00 to 2.444 ± 0.46 bar (mean \pm 95% CI) were set up (Table 1). The different water potential levels were created by mixing soil with different amounts of demineralised water the day before applying the animals. Each treatment was replicated four times. A measure of 10 g wet weight soil was added to 12.5 ml vials with perforated lids together with 33 mg of rolled oat well mixed into the soil. To each test container either eight *E. crypticus* or four *E. albidus* were added. All added enchytraeids were sexually mature adults with visible clitella. Finally, the vials were weighed and placed in a box and covered with damp cloth to reduce temperature fluctuations and to reduce evaporation from the vials. The test containers were

incubated at $15 \pm 0.1^\circ\text{C}$ for 4 weeks (*E. crypticus*) or 6 weeks (*E. albidus*). Every second week each test container was weighed to assure that water had not been lost by evaporation and 17 mg fed dried rolled oat was added. At the end of the experiment the surviving adults and juveniles were extracted. To extract the enchytraeids, the soil of each test container was gently spread into four 200 ml plastic beakers (diameter 7 cm), which were filled with demineralised water, gently shaken, and then left for 24 h at 5°C for sedimentation. Adult and juvenile enchytraeids would move onto the surface of the sediment and were counted within 48 h using a dissection microscope.

The soil consisted of 25% peat and 75% of an agricultural loamy sand soil consisting of 35% coarse sand, 45% fine sand, 9.4% silt, 8.9% clay and 1.7% organic matter. In order to exclude soil animals already present, the soil was dried at 80°C for 24 h and thereafter sifted through a 2-mm mesh to remove larger particles before use. Soil moisture contents and soil water potential was determined as described in Holmstrup (2001) (Table 1).

Water content and osmotic pressure of body fluids

The same experimental produce as described in the acute drought exposure was used. However, the duration of exposure was reduced to four days and there was no rehydration. The control was set to 100% RH (0 bar) as *E. albidus* showed 100% survival at this humidity. Furthermore, an extra treatment was applied to the experiment (98.2% RH, −24.9 bar). Each treatment was replicated seven times and two worms were applied to each vessel. The water content (WC) of surviving worms was calculated gravimetrically from determinations of fresh weight of two worms at the time of sampling and after they have been dried for 24 h at 60°C . The weighing was carried out using a Sartorius Micro SC 2 balance (Sartorius AG, Gottingen, Germany).

Osmotic pressure of body fluids was determined for worms exposed to control 100% RH (0 bar), 99.6% RH

Table 1 Wetness and matric soil water potential of the test soils

	Soil (ml water per g soil)	Nominal wetness (% of dry mass)	Determined wetness (% of dry mass)	Soil water potential (bar)
	0.8 (control)	80	89.89 ± 1.58	-0.036 ± 0.00
	0.70	70	74.81 ± 1.55	-0.039 ± 0.00
	0.65	65	67.67 ± 5.46	-0.049 ± 0.00
	0.60	60	64.41 ± 2.15	-0.053 ± 0.00
	0.55	55	61.05 ± 4.07	-0.078 ± 0.00
	0.50	50	54.71 ± 0.28	-0.098 ± 0.00
	0.45	45	48.42 ± 0.34	-0.127 ± 0.011
	0.40	40	42.46 ± 0.61	-0.756 ± 0.17
	0.35	35	37.21 ± 1.08	-1.422 ± 0.83
	0.30	30	32.48 ± 0.65	-2.444 ± 0.46

The nominal wetness and the determined wetness are expressed as % of the dry mass (mean \pm 95% CI, $n = 3$) and the soil water potential are expressed in bar (mean \pm 95% CI, $n = 3$)

(−5.5 bar), 99.2% RH (−11.0 bar), 98.6% RH (−19.3 bar) and 98.4% RH (−22.9 bar) for 4 days without any rehydration, as described above. Each treatment was replicated five times. Osmotic pressure (expressed in bar) of single individuals was measured by quickly placing the enchytraeids in the sample holder of a Wescor C-52 sample chamber connected to Wescor HR 33T Dew Point Microvolumeter (Wescor 1986) operated in the dew point mode (Holmstrup and Westh 1994; Holmstrup and Sømme 1998). Only surviving enchytraeids were used.

Identification and quantification of responsive osmolytes

A preliminary screening of osmolytes was performed for *E. albidus* exposed to 0 and −11.0 bar, both replicated 20 times. Two worms were added to each vessel and the vessels were placed at $15 \pm 0.1^\circ\text{C}$ for 4 days. Surviving worms were hereafter sampled and freeze dried for 24 h after which the dry weight was determined. Dried worms were crushed and extracted in 0.25 ml 70% ethanol using a rotating glass in a 0.5 ml Eppendorf tube. Samples were centrifuged at 20,000g for 5 min at 4°C and the supernatant saved. The extraction procedure was repeated three times and the combined supernatants were hereafter dried at 60°C for 24 h. The dry sample was stored at 80°C until further analysis. Nuclear Magnetic Resonance spectroscopy (NMR) was then applied to identify those osmolytes that differed significantly between controls and desiccated worms as previously described (Petersen et al. 2008).

Glucose, alanine, glutamine and proline were identified from the NMR spectra as possible responsive osmolyte candidates. An experiment with increasing acute drought stress was set up, following the same procedure as described above. The concentrations of alanine and glucose were determined from two separate experiments. Surviving *E. albidus* from each replicate were gently collected in Eppendorf tubes after drought exposure. Six worms (5–7 mg DW) were pooled in each Eppendorf tube and three to four replicates were made for each treatment. The samples were thereafter freeze dried, weighed and placed at -80°C until further analysis.

Glucose determination was performed using a spectrophotometrically based enzyme assay as previously described (Overgaard et al. 2007). Alanine was quantified by thin layer chromatography (TLC) using a slight modification of the method described by Hjorth et al. (2006). Only the concentration of alanine could be determined, since preliminary results had shown that concentrations of glutamine and proline were below the present TLC-method's detection limit of 0.05 and 0.10 mM, respectively. Each sample was extracted in 1.4 ml 10% ethanol and then homogenised on ice using an ultrasonic homogenizer (Ultrasonic

Homogenizer, Cole-Parmer Instruments, IL, USA). The homogenate was left on ice for 10 min, spun for 5 min at 20,000g and the supernatant was stored in a 5 ml glass vial. This procedure was repeated three times and the pooled supernatants were freeze dried for 24 h and stored at -20°C . The samples were protected against light during the extraction. The freeze dried extracts were dissolved in 300 μl 10% ethanol and insoluble residuals were removed by ultrasonic homogenization for 15 min followed by filtration through a polypropylene 0.45 μm filter (Whatman[®], Clifton, New Jersey, USA). Ten μl samples of the extract were applied to a HPTLC-plates with a 0.1 mm layer of cellulose (Merck 16092) in 12 mm bands, 15 mm from the lower edge of the plate using an Automatic TLC sampler 4 (CAMAG, Muttenz, Switzerland). The plates were developed in eluent with the composition, butanol:formic acid:deionised water (40:10:10) (see Hjorth et al. 2006 for further details).

Survival after gradual and acute exposure

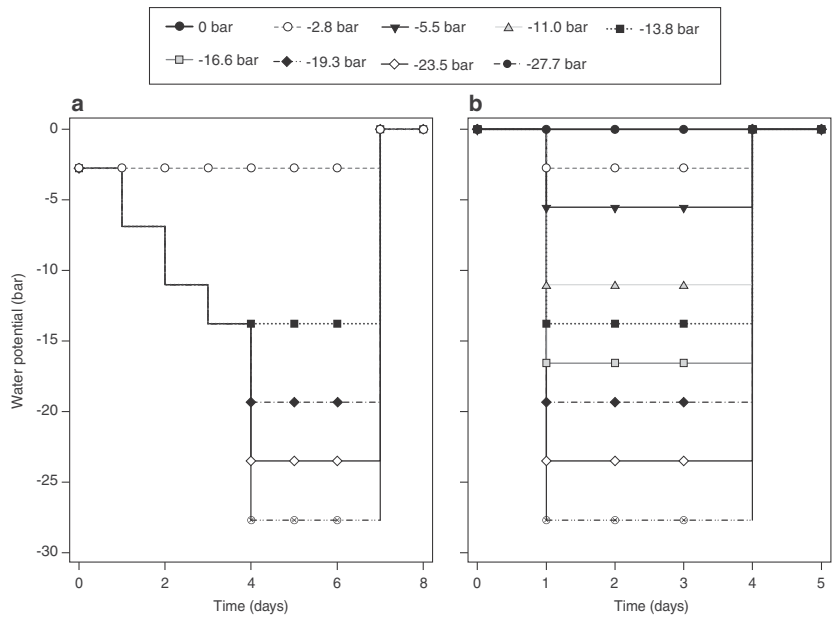
Two experiments were designed to compare the effects of gradually increasing desiccating conditions with the effects of acute exposure to harsh desiccation. Both experiments were performed as described in previous sections. The first experiment compared the survival of gradually and acutely exposed *E. albidus* with gradually desiccated worms exposed to a series of decreasing humidities by replacing the salt solutions of the desiccation chambers daily over a 3-day period (Fig. 1). After this period the worms were left at −13.8 bar (99.0% RH), −19.3 bar (98.6% RH), −23.5 bar (98.0% RH) or −27.7 bar (98.0% RH) for 3 days (Fig. 1). Each treatment was replicated ten times with two worms in each sample. Acutely exposed worms were exposed directly to the same range of decreasing soil water potential as the gradual (Fig. 1). The worms were hereafter rehydrated for 2 days, by replacing the NaCl-solution with demineralised water. The second gradual exposure experiment was performed to verify the trends observed in experiment one, however, only using one final drought level (−23.5 bar).

Data analysis and statistical methods

Normally distributed datasets were subjected to one-way ANOVA followed by the post-hoc Tukey-test to test for differences between mean values. If data did not fulfil the homogeneity of variance log or square root transformation was applied prior to statistic analyses or otherwise an appropriate non-parametric test was performed. Binomial data was analysed by Pearson Chi-Square test for significant differences.

Estimation of the drought stress causing 50% mortality (LD50) and 50% effect on the reproduction (ED50),

Fig. 1 Experimental protocol for gradual (a) and acute (b) drought exposure of *E. albidus*



respectively, was performed with the SAS/STAT procedure NLMIXED. Nonlinear modelling was used to estimate drought-response relationships by fitting the binomially distributed mortality data to the mortality rate (m) formula: $m = c(1 - c)\Phi(a + bd)$, where c is control mortality rate, Φ is the cumulative normal probability function, a slides the curve along the x axis, b determines the slope, and d is the drought in $-bar$. The LD50 for acutely exposed *E. albidus* was estimated using the formula, $m = c \exp(ad)$. Nonlinear modelling was also used to estimate drought-response relationships by fitting the normally distributed reproduction data to the reproduction rate (r) formula: $r = c \exp(d - 0.03583)(b)$. LD50 or ED50 values were compared by calculating contrasts between pairwise incubation periods or species under the hypothesis that there was no difference. PROC NLMIXED then constructs approximate F tests using the delta method (SAS Institute Inc. 2004).

All tests were performed using Enterprise Guide® Version 4 (SAS Institute Inc. 2004), applying, a significance level of 5%. All results are presented as mean \pm SE if nothing else is mentioned.

Results

Effect of acute and chronic drought exposure

The duration of exposures had a negative influence on the survival on both species. The LD50 values of chronic exposures were more than five times lower than LD50

values of the acute exposures (Table 2). In the acute test, the survival of both *E. crypticus* (Mann–Whitney-test; $P = 0.044$) and *E. albidus* (Pearson χ^2 -test; $P = 0.0469$) were significantly different from control at -11 bar (Fig. 2a, c). The two species were almost equal in their drought sensitivity as there was no significant difference between the LD50 values.

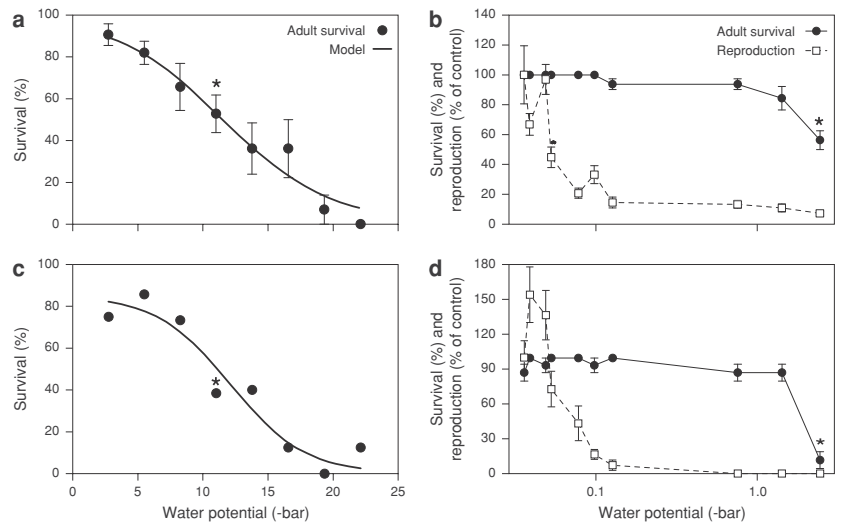
Survival and reproduction in both species were affected by the chronic drought stress, even at very moderate soil water potentials (Fig. 2b, d). The smaller species, *E. crypticus*, showed a significantly (NLMIXED; $P < 0.005$) better survival than the larger species, *E. albidus* (Table 2). However, the LD50 values of *E. crypticus* and *E. albidus* were in the same range, -2.5 to -2.0 bar, respectively. The reproduction of both species was also significantly reduced by the treatment even at high soil moisture levels and there

Table 2 The 50% lethality drought level (LD50, $-bar$) (mean \pm 95% CI) and the drought level causing a 50% reduction of reproduction (ED50, $-bar$) (mean \pm 95% CI) for *E. crypticus* and *E. albidus* in acute and chronic drought exposure studies

Exposure type	<i>E. crypticus</i>	<i>E. albidus</i>
Acute exposure (LD50)	12.3 ^a (10.5–14.2)	13.9 ^a (11.3–16.6)
Chronic (LD50)	2.5 ^a (2.2–2.8)	2.0 ^b (1.7–2.3)
Chronic (ED50)	0.07 ^a (0.05–0.08)	0.06 ^a (0.05–0.08)

The chronic exposures were 28 and 54 days, respectively, for *E. crypticus* and *E. albidus*. Different superscript letters indicate a significant difference between species

Fig. 2 The percent survival (mean \pm SE) of acute drought exposure (a) in *E. crypticus* ($n = 8$) and (c) *E. albidus* ($n = 18$). The survival and reproduction of chronic drought exposure in (b) *E. crypticus* (mean \pm SE; $n = 4$) and (d) *E. albidus* (mean \pm SE; $n = 4$) exposed for 4 or 6 weeks, respectively. Asterisks indicate significant differences from the control treatment. Note that the X axis is log metric in the chronic exposure experiment



was no significant difference in the sensitivity of the two species (Fig. 2b, d).

Water content and osmotic pressure of body fluids

The water content of *E. albidus* remained constant even at water potentials down to -15 bar. However, water content was significantly (ANOVA, Tukey; $P < 0.005$) reduced at -19.3 bar. The survival of *E. albidus* started to decrease when specimens had lost 22.5% of the initial water content (Fig. 3a). The dehydration continued to increase at even lower water potentials and at -24.9 bar *E. albidus* specimens had lost 39.0% of the initial water content.

The osmotic pressure of body fluids was also significantly affected by the treatments (ANOVA; $P < 0.0001$) and was significantly higher than control values when animals were exposed to a water potential of -19.3 bar (Fig. 3b; Tukey; $P < 0.05$). The osmotic pressure of body fluids was slightly higher than the water potential of the environment but largely unchanged until the water potential of the environment exceeded -19.3 bar.

Accumulation of osmolytes

The NMR spectroscopy analysis revealed an accumulation of glucose and the amino acids alanine, glutamine and proline, in response to the drought treatment (data not shown). Nevertheless, the concentration of proline and glutamine was below the detection limit of the TLC-method. Neither glucose nor alanine were upregulated until the water potential of the environment had reached -19.3 bar. The concentration of glucose was significantly

(ANOVA, $P = 0.009$) affected by the drought treatment (Fig. 4a) and was significantly (Tukey, $P < 0.05$) upregulated from $1.5 \pm 0.5 \mu\text{g mg}^{-1}$ DW in controls to $8.4 \pm 1.9 \mu\text{g mg}^{-1}$ DW at -19.3 bar.

Alanine production showed a different pattern with a significant (Tukey, $P < 0.05$) reduction of alanine at -5 bar and a significant (Tukey, $P < 0.05$) increase at -19 bar (Fig. 4b). Alanine accumulated from $1.2 \pm 0.4 \mu\text{g mg}^{-1}$ DW in the control to $3.0 \pm 0.5 \mu\text{g mg}^{-1}$ DW at -19.3 bar.

Contribution of water loss to increases in osmotic pressure

The osmotic contribution of the water lost was estimated by using the results from the water content and body fluid osmotic pressure determinations. The water content can pragmatically be divided into two fractions: osmotically inactive water (OIW) and osmotically active water (OAW) (Holmstrup and Westh 1994). OAW is readily removed by mild desiccation, whereas the OIW (typically 15–25% of the total water content) is bound by membranes and protein, and is only removed during severe dehydration or freeze-induced dehydration at low temperature (Holmstrup and Westh 1994). Block and Bauer (2000) reported that the amount of OAW in *E. albidus* is 62.2% of the total water content, equivalent to 2.3 mg mg^{-1} DW in fully hydrated animals (Fig. 3a). Under the assumption that OIW is constant in the various desiccation treatments it was estimated that water loss accounted for 61% of the observed increased osmotic pressure at -19.3 bar (Fig. 3b). The osmotic contribution from dehydration decreased to about 47% of the observed increase in osmotic pressure at -22.1 bar.

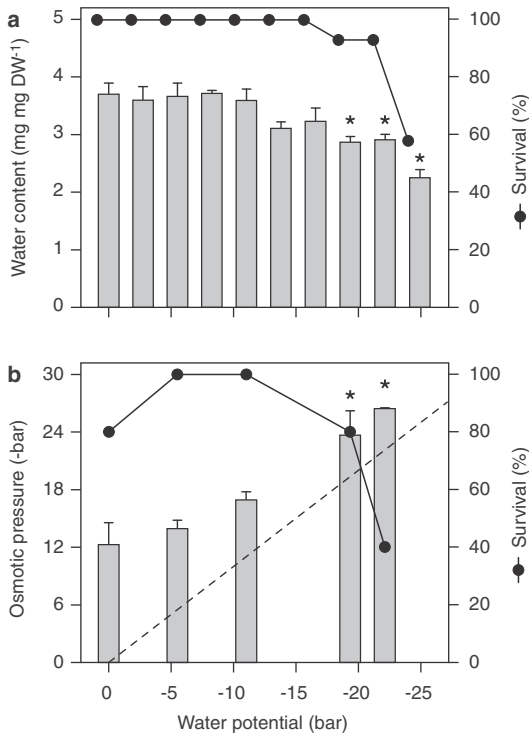


Fig. 3 **a** The water content (mg water mg⁻¹ DW) (mean \pm 95% SE; $n = 7$) and survival (%) of *E. albidus*. **b** Body fluid osmolality (bar) (mean \pm 95% SE; $n = 5$) and survival (%) of *E. albidus*. The grey dotted line illustrates equality between osmotic pressure and water potential of the environment. Asterisks indicate significant differences from the control treatment

Effect of gradually increasing drought exposures

The gradual drought treatment did not significantly increase (Pearson χ^2 -test; $P > 0.05$) the survival of *E. albidus* at any drought level (Fig. 5a). However, a tendency to improved survival due to gradual desiccation could be observed in the first experiment. But the results from the second experiment with gradual desiccation did not support this trend, as no significant difference could be observed (Pearson χ^2 -test; $P > 0.05$) (Fig 5b).

Discussion

The present study is one of the first to investigate enchytraeids' water relations and osmotic pressure when subjected to realistic drought conditions. A priori, we expected that *E. albidus* would dehydrate and reach equilibrium with the water potential of the surrounding environment rather quickly due to its highly permeable surface. However, water

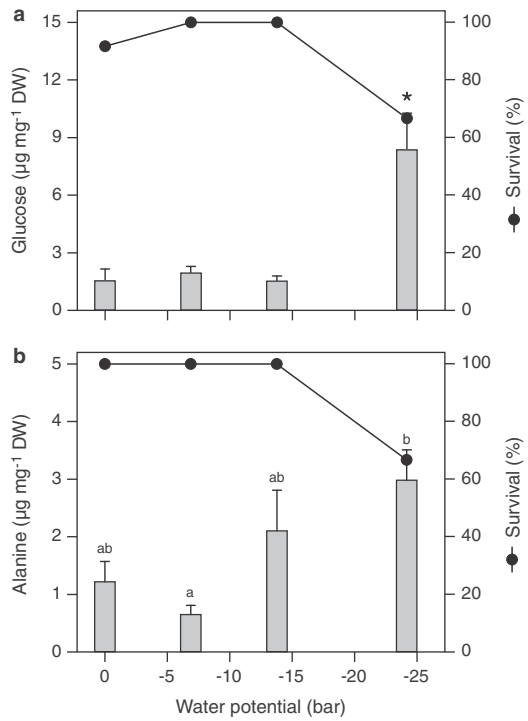
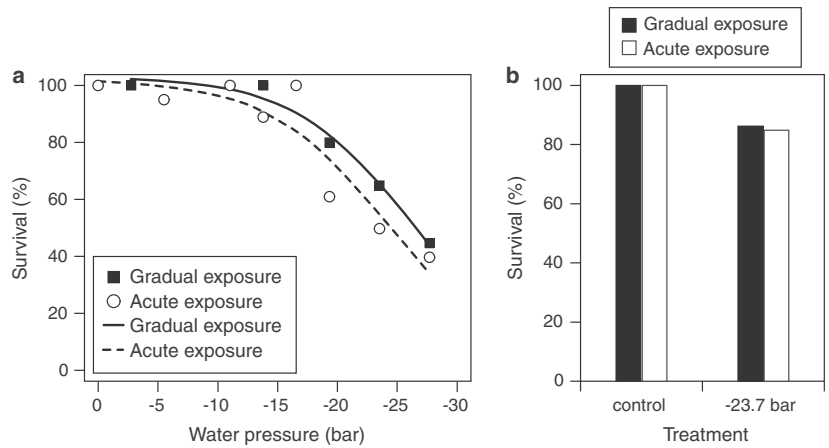


Fig. 4 **a** Concentration of accumulated glucose ($\mu\text{g mg}^{-1}$ DW, mean \pm SE; $n = 4$) and **b** concentration of alanine ($\mu\text{g mg}^{-1}$ DW, mean \pm SE; $n = 3$) in *E. albidus* exposed for four days at increasing drought stress. Different letters signify significant differences between mean values and asterisks indicate significant differences from the control treatment. The black line shows the % survival of *E. albidus*

content of *E. albidus* remained stable even under relatively dry conditions. The water content did not decrease before -19.3 bar (-15 bar is equivalent to the wilting point of plants) due to an unusually high osmotic pressure of body fluids even under moist conditions (control). It seems, therefore, that *E. albidus* is adapted to live in environments with strong fluctuations within a certain limit of humidity such as in decaying seaweed in the littoral zone. By having a relatively high body fluid osmolality (ca. 500 mOsm), we suggest it is adapted to survive acute and rapid changes in soil moisture. The body fluid osmolality of most terrestrial earthworms varies in osmolality between 200 and 300 mOsm (Oglesby 1969; Holmstrup et al. 2007; Pedersen and Holmstrup 2003), thus suggesting that *E. albidus* differs from these species and in fact displays typical osmoconformity as do many marine annelids (Schmidt-Nielsen 1990). Enchytraeids are recognised as being semi-aquatic animals (O'Connor 1967) and especially *E. albidus* which can be found in the coastal zone (Christensen and Dozsa-Farkas 2006).

Fig. 5 **a** Survival of *E. albidus* exposed either gradually (filled square) or acutely (filled circle) to increasing drought stress for 3 days. Each point represents the mean of two worms and 10 replicates. **b** Survival of *E. albidus* exposed to drought either gradually or acutely



The ability to tolerate excessive water loss may be an adaptation to drought, however, no large water loss was observed in *E. albidus* subjected to quite severe drought. On the contrary, the survival of *E. albidus* was reduced when the dehydration exceeded 23% of the initial water content. It may, therefore, be proposed that this species has a high content of OIW, which is also supported by the study of Block and Bauer (2000) reporting that 37.8% of the total water content in *E. albidus* was OIW. Thus, even small water losses may be detrimental in species with high proportions of OIW because OAW may be depleted rapidly.

Acute and chronic drought exposure

During acute exposure more than 50% of *E. crypticus* and *E. albidus* were able to survive water potentials below -11.0 bar, but chronic exposure increased their sensitivity considerably. *E. crypticus* was slightly, but significantly, more desiccation resistant than *E. albidus* when exposed to chronic drought stress. The size of worms could probably influence the ability to tolerate desiccation because the dehydration process will take longer in larger individuals before the equilibrium water content is reached. Thus, large organisms are favoured because a longer time is available for distribution of osmoprotectants and a slower rate of tissue dehydration will take place. However, our results suggest, in contrast, that *E. crypticus* even though it has a much lower fresh weight may be the most desiccation tolerant. Unfortunately, water balance physiology of *E. crypticus* is difficult to undertake because of its small size. Block and Bauer (2000) reported that the closely related and smaller species, *E. buchholzi*, had OIW of $83 \pm 2.9\%$ (mean \pm SD). Although this latter result seems unrealistic it suggests that, even within the same genus, a large variation in water relations can be found. Both

E. crypticus and *E. albidus* were extremely drought sensitive during chronic exposures as even modest soil water potentials affected the reproduction and survival. It is unclear why both enchytraeid species died at water potentials where their body fluid osmotic pressure (-12.3 bar) was lower than the treatments (-2.2 bar) which should ensure that body water was not lost. There is no reason to believe that running the experiments at 15°C had any negative influence on drought tolerance since both species reproduced and seemed to thrive at this temperature. Factors like time span and accumulation of toxic excretory production could all influence the survival; however, more experiments are needed to clarify this. The degree of sensitivity is probably species dependent as *Cognettia sphagnetorum* has been found to survive in soil with a soil water potential of -10 bar for more than 8 months (Abrahamsen 1971). Dozsa-Farkas 1977 also observed that *Fridericia galba* could survive in soil with soil moisture around -10 bar for more than 49 days. These results are congruent with O'Connor's (1967) ranking of enchytraeid genera in their degree of adaptation to a terrestrial environment, where *Lumbricillus* < *Enchytraeus* < *Henlea* < *Mesenchytraeus* < *Fridericia*, although this was based on field observations and not experiments. He proposed that well adapted species tended to have a thicker integument supposedly diminishing water loss rates. These findings were supported by a study of Lagerlöf and Strandh (1997) who found that the cocoons of *Enchytraeus*, *Henlea* and *Fridericia* were the most common genera after drought exposure. The reason for the presence of *Enchytraeus* was explained by the observation of Christensen (1956), who found that *Enchytraeus* cover their egg cocoons with sand and debris and therefore perhaps reduce the evaporation from the cocoons. This suggests to us that populations of *E. albidus* may survive chronic drought stress by producing drought-resistant egg cocoons. Other members of soil fauna

have been found to use various strategies to survive chronic drought exposure. Drought avoiding earthworms can besides migration, also enter diapause (Friis et al. 2004). Drought tolerant soil organisms such as nematodes have been found to survive extreme dehydration in an anhydrobiotic state (Crowe et al. 1992). Anhydrobiotic soil organisms can survive the loss of practically all water including OIW. A third strategy found in Collembola is passive absorption of water vapour (Bayley and Holmstrup 1999). However, none of these adaptations were observed in *E. albidus* and this support the suggestion that *E. albidus* is surviving chronic drought stress by producing cocoons that are more drought tolerant than hatched individuals.

The reproduction of both species was even more sensitive and was significantly reduced from -0.06 bar, which was surprising because no dehydration or change in the body fluid osmotic pressure of *E. albidus* are expected at these soil water potentials. These results suggest that the hatching of the cocoons is only successful during optimal soil moisture conditions.

Production of protective osmolytes

Both glucose and alanine were upregulated in *E. albidus* during desiccation, however, only in modest concentrations. Glucose has been found to accumulate during cold and drought exposures in in collembola (Holmstrup et al. 2001), earthworms (Holmstrup et al. 2007) and enchytraeids (Holmstrup and Sjørnsen 2001; Pedersen and Holmstrup 2003). The concentration of accumulated glucose was in the same range ($8 \mu\text{g mg}^{-1}$ DW) as determined in drought-exposed *F. ratzelei* ($13.5 \pm 7.7 \mu\text{g}^{-1}$ DW; mean \pm SE) (Pedersen and Holmstrup 2003) but much lower than in frozen *E. albidus* (Slotsbo et al. 2008) and in the freeze tolerant earthworm, *Dendrobaena octeaedra*, where concentrations up to $140 \mu\text{g mg}^{-1}$ DW has been measured (Holmstrup et al. 2007). In the present case glucose mobilisation has a negligible effect on the osmotic pressure of body fluids in drought exposed *E. albidus*. However, glucose and other sugars are known to protect membranes and protein against deleterious effects of freezing and desiccation (Crowe et al. 1992; Storey and Storey 1996). Thus, it seems more likely that glucose accumulation had such non-colligative effects even though firm conclusions cannot be made.

Accumulation of alanine has been observed in a number of insects (Fields et al. 1998; Michaud and Denlinger. 2007), earthworms (Bundy et al. 2003) and earthworm cocoons (Petersen et al. 2008). The low concentration of alanine mobilisation suggests that the colligative effect of alanine is minor. Other studies have proposed that the accumulation of alanine is a consequence of anaerobic or partly anaerobic conditions when the water content

becomes low (Bundy et al. 2003; Petersen et al. 2008). Yet, due to the highly permeable surface of the enchytraeids we do not believe that the alanine production was a result of anaerobic conditions. Alanine is synthesised from pyruvate and an explanation can be that alanine is accumulated as a non-toxic excretory product under conditions where normal ammonotelic excretion is hampered by desiccation.

The free amino acids, glutamine and proline, were also upregulated during drought as revealed by NMR spectroscopy. Nevertheless, the concentrations of both amino acids were below the detection limit of the applied TLC method and their colligative contribution to osmotic pressure of body fluids can therefore be neglected. However, they may be involved in other protective responses in cells during dehydration. Glutamine has been found to have a positive effect on low temperature survival in mammalian cells at concentration as low as 2 mM (Kruuv et al. 1988) and to accumulate during rapid cold-hardening in crickets (Tomeba et al. 1988). Proline has been found to stabilize membranes (Storey and Storey 1996) and protein (Maheshwari and Dubey 2007) during cell shrinkage. Proline production has also been observed in diptera larvae exposed to freezing (Shimada and Riihimaa 1990). However, at present we can only speculate about putative physiological functions of these two amino acids in *E. albidus*.

Effect of gradual and acute desiccation on survival

Soil invertebrates which inhabit the upper layer of the soil must be able to survive both short- and long-term periods of drought. A range of studies, e.g. on earthworm cocoons (Petersen et al. 2008), the Antarctic midge *Belgica antarctica* (Hayward et al. 2007) and the collembolan *Folsomia candida* (Sjørnsen et al. 2001), have observed that a pre-acclimation can increase the survival of subsequent severe drought exposure. Thus, we therefore expected that a gradual desiccation process would increase the drought tolerance of *E. albidus*. However, this was not the case and the explanation could be that this species has adapted to an environment with strong and rapid fluctuations. Thus, slow rates of desiccation do not induce any additional stress tolerance.

Conclusion

Chronic exposure to even relatively mild drought had large negative effects on reproduction and survival in both *E. albidus* and *E. crypticus*, but the two species did, however, survive the acute drought stress better. *E. albidus* is adapted to live in environments with strong fluctuations in humidity. By having a relatively high osmotic pressure

of its body fluids, it can endure rapid changes in soil moisture without drastic water loss.

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Paper 2

Recovery of enchytraeid populations after severe drought events

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Recovery of enchytraeid populations after severe drought events

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Abstract

The potential impact of changes in precipitation patterns associated with climate changes was investigated in Enchytraeidae (Oligochaeta) in a Danish heathland. The amount of precipitation was manipulated during spring and summer in an experimental field site in order to reveal effects of three different drought regimes: weak drought (WD), medium drought (MD) and high drought (HD). Enchytraeids were sampled every six to eight weeks (0–9 cm depth) and soil water potential (SWP) and soil water content (SWC) was measured on a regular basis.

The enchytraeid populations were generally reduced due to natural drought. The HD treatment significantly reduced the moisture level of the soil further with SWP below –15 bar (5 and 10 cm depth) and SWC around 5% (vol/vol) for more than two months. As a result almost no enchytraeids were found in HD plots after two months with high drought stress. Nevertheless, the HD-treated enchytraeid populations recovered within two months, as there was no significant difference in biomass and density of the different treatments at that time. During periods with extreme low SWP enchytraeids were practically absent in the top soil (0–3 cm), but a few animals were found in 3–6 cm. During this period SWP was around –15 bar even in 20 cm depth, indicating that active stages could not have survived. Thus, we suggest that the species present must be dependent on a drought tolerant stage, as vertical migration could not have supported the observed recovery.

SWP and SWC were both significantly correlated with the total density and biomass of enchytraeids. However, density was better correlated with the SWP and SWC compared to biomass, which can be due to hatching of cocoons and increased fragmentation rate.

Keywords: Dry heathland; *Cognettia sphagnetorum*; desiccation; Soil water potential; soil water content

Introduction

A major part of earth's terrestrial primary production is decomposed in the soil by soil micro-organisms, but soil meso- and macrofauna are by their activities accelerating the decomposition and nutrient recycling processes (Seastedt, 1984; Cole et al., 2000; Cole et al., 2002; Taylor et al., 2004). Enchytraeids, small soil living oligochaete worms, are widely distributed from the Arctic to tropical areas (Petersen and Luxton, 1982), and are typically located in the organic top layer of soils (Springett, 1967; Springett et al., 1970b). Enchytraeids affect both directly and indirectly the decomposition processes and the nutrient mineralisation; directly by consuming large amounts of organic matter (Standen, 1978; Setälä and Huhta, 1991; Laakso and Setälä, 1999; Cole et al., 2000) and indirectly by creating improved soil aggregate structure. Further, by their feeding activity, they affect the activity and function of the microbial community (van Vliet et al., 1993; Cole et al., 2000; Rantalainen et al., 2004; Bardgett, 2005). Especially in nutrient poor habitats, like temperate heathland and coniferous forests, enchytraeids contribute significantly to the decomposition (Laakso and Setälä, 1999). In these ecosystems the species *Cognettia sphagnetorum* dominate and is recognised as a keystone species (Laakso and Setälä, 1999; Cole et al., 2000).

Climate change scenarios for northern Europe predict that the precipitation pattern will change (IPCC, 2008), and it is expected that these changes will result in frequent harsh drought spells even though the annual amount of rainfall will increase (IPCC, 2008). Enchytraeids are extremely vulnerable to drought stress (Nielsen, 1955b; Lindberg et al., 2002; Maraldo et al., 2008) due to their highly permeable surface (Maraldo et al., 2009c). Long-term experimental field studies have shown that more frequent drought spells may reduce the biomass and density of enchytraeids in dry heathland (Maraldo et al., 2008) and coniferous forests (Lindberg et al., 2002).

So far, two drought survival strategies have been suggested to function in enchytraeids; either enchytraeids migrate to moister micro-habitats, which only can be a short-term strategy unless the food quantity and quality in the new habitat can maintain the population size (Springett et al., 1970; Uhi and Briones, 2002), or population resistance is ensured by a desiccation tolerant cocoon stage (Lagerlöf and Strandh, 1997). However, species like *C. sphagnetorum* which are known to reproduce asexually by fragmentation and subsequent regeneration are thought to lack the latter strategy. Thus, periods with extended drought spells may have severe consequences for *C. sphagnetorum* which may be more liable to extinction than other enchytraeid species.

Soil moisture can be expressed as soil water potential (SWP) and/or soil water content (SWC). The only information provided by SWC is the relative amount of water in the soil and not the availability to soil organisms. On the contrary, SWP expresses a measure of the available fraction of water, as it also accounts for the adhesive (i.e. greater surface area means more water is adsorbed via electrostatic forces) and cohesive forces. Thus, magnitude of water in soil is highly dependent on the soil type and texture, which makes it difficult to compare the water availability for the soil fauna in different studies where soil moisture is reported as SWC. Despite this, most studies manipulating soil moisture report only SWC (e.g. Sulkava et al., 1996; Frampton et al., 2000; Huhta and Hanninen, 2001; Tsiafouli et al., 2005). Using SWP as a measure of water availability would facilitate comparisons of drought effects in studies covering various species and soil types.

The objective of this study was to determine the population dynamics of enchytraeids exposed to different drought treatments in a dry Danish heathland dominated by *C. sphagnetorum*. The treatments were applied to reflect drought scenarios in a North European climate of differing intensity and length: dry spring with sporadic precipitation followed

by wet early summer (weak drought; WD), dry spring followed by wet early summer (medium drought; MD), and a final treatment with long-term spring and summer drought (high drought; HD). The different treatments were applied by manipulating the length of reduced precipitation (using transparent coverings) and the amount of irrigation. A second aim of the study was to examine the relationship between soil moisture, determined as SWP and SWC, and the size and dynamics of enchytraeid field populations. We hypothesise that enchytraeids' density and biomass will be better correlated with SWP compared to SWC, since SWP reflects directly the water availability.

2 Material and methods

2.1 The field site

The experimental site was situated at Brandbjerg, Denmark (55°53'N 11°58'E) on a hilly nutrient-poor sandy deposit with a dry heath/grassland ecosystem dominated by a grass (*Deschampsia flexuosa*) and an evergreen dwarf shrub (*Calluna vulgaris*). The yearly mean temperature of this area is 8.0°C and the yearly mean precipitation amounts to 613 mm (Mikkelsen et al., 2008). The enchytraeid community was dominated by *Cognettia sphagnetorum*, but also *Achaeta affinis* and *Enchytronia parva* contributed to the community (Maraldo et al., 2009b).

2.2 Manipulations

The experiment consisted of 15 plots (1.0 by 1.0 m), which were laid out in a randomised block design with three treatments replicated five times. The vegetation of the plots was dominated by *D. flexuosa* and mosses. The treatments, MD and HD, were applied by covering the plots with transparent plastic roofs (approximately 1.5 by 1.5 m) placed one meter above the ground. The WD plots were not covered at any time. In the MD treatment the aim was to reach the permanent wilting percentage in the surface soil layers (-15 bar in 5 cm depth), whereas in the MD and HD the aim was to reach the wilting percentage in the root depths of *D. flexuosa* (-15 bar in 20 cm depth). Precipitation was excluded from 21 April until 21 May, 2008, in the MD treatment, and from 21 April until 16 June 2008, in the HD treatment. After these respective periods the coverings were removed and plots watered with 10 mm or 20 mm tap water in MD and HD, respectively. Due to unusually low precipitation during the spring and early summer (Table 1) the WD and MD plots received 90 mm water from May to July, whereas the drought treated plots received 50 mm from June to July (Table 1). Watering was applied weekly in doses of 10 mm per week immediately after SWP and SWC were measured and soil samples for enchytraeids were collected, with the exception of the beginning of June where WD and MD were watered twice in a week.

Soil temperature (5 cm) was recorded continuously in two WD and two HD plots (at 15-minute intervals) using TinyTag datalogger with external PT100 thermistor-type temperature probes (Orion Components, Chichester, UK). Daily average temperature data and daily average precipitation (mm) from February to November, 2008, was obtained from the field site weather station.

Table 1. Time period for drought manipulation, amount of irrigation added (mm) and the amount of precipitation (mm) received in each treatment during the experimental period. The amount of water received from irrigation and from precipitation was added in total water applied.

Treatment	Weak drought (WD)	Medium drought (MD)	High drought (HD)
	Spring drought with sporadic precipitation followed by irrigation	Spring drought followed by irrigation	Long-term drought for two months
Drought treatment (days)	0	36	62
Applied drought period	21 April – 16 June	21 April – 21 May	
Irrigation (mm)	90	90	50
Irrigation period	21 May – 16 July	21 May – 16 July	16 June – 16 July
Precipitation (mm)	373	357	343
Precipitation period	21 April – 10 November	21 May – 10 November	16 June – 10 November
Total water applied (mm)	463	447	392

2.3 Sampling of enchytraeids

Enchytraeids were collected every six to eight weeks from 14 April to 10 November, 2008. For this purpose a cylindrical soil corer with an inner diameter of 5.5 cm was used in 0–9 cm depth. Immediately after sampling each soil core was divided into layers of 3 cm thickness with a knife. All soil samples were collected beneath *D. flexuosa* and were stored at 5 °C until extraction which was carried out within one week. The extraction was a modified version of O'Connor's wet funnel extraction (O'Connor, 1955), with a stepwise increase in temperature from 25 °C to 50 °C at the sample surface in five hours. The enchytraeids were collected in tap water and stored for 24 h at 5 °C before being counted. The total number of enchytraeids per m² was determined (integrating 0–9 cm depth) and biomass (dry weight; mg DW m⁻²) was determined for all samples. Biomass was determined after drying the worms at 60 °C for 24 h using a Sartorius Micro SC 2 balance with a precision of 0.01 mg (Sartorius AG, Goettingen, Germany).

2.4 Soil water potential and soil water content

Soil water potentials (SWP) were measured weekly in 5, 10 and 20 cm depth in all treatments from late April to 11 August before watering the plots. Representative measurements were made for each plot with permanently installed soil psychrometers connected to a Wescor HR 33T Dew Point Microvoltmeter (Wescor, 1986) operated in the dew point mode. Measurements were performed early in the morning in order to avoid large temperature gradients. Representative soil moisture contents (SWC) (vol % water content) was measured for each plot in 0–5 cm using a Thetaprobe ML2x (Delta-T Devices Ltd, Cambridge, UK). SWP and SWC were measured weekly from 30 April to 11 August. Additionally, soil samples (diameter 6 cm; depth 5 cm) were collected in each plot to determine the soil gravimetric water content in 0–5 cm depth at the time of enchytraeid sampling. These soil samples were also used for extraction of microarthropods (results reported elsewhere)

with the following procedure. The soil cores were transferred directly into plastic tubes and covered with plastic lids during transportation to the laboratory. Samples were weighed and extracted over one week in a high gradient extraction apparatus (MacFadyen type), where the temperature was increased stepwise from 25 °C to 50 °C in seven days. The dry soil samples were weighed and gravimetric soil water content was calculated.

2.5 Data analysis and statistical methods

The results were analysed as a randomized ANOVA block design using the GLM model procedure with type III sum of squares. One-way ANOVA with treatments as fixed factors and blocks as random factors were applied to test for effects of main factors on density and biomass of enchytraeids in 0–9 cm depth and in each of the different soil depths. A pairwise comparison between treatments was performed using the post hoc Tukey test in the PROC GLM procedure (SAS Institute Inc., 2004). In order to examine for correlations between SWP, SWC and the enchytraeid population linear regression analysis was performed. For this purpose the SWP and SWC in 5 cm and 0–5 cm depth, respectively, were correlated with the total biomass and total density of enchytraeids. Pearson Correlation Coefficients was used to test for significant regression and a Wilcoxon Signed Ranks Test was used to test if one or the other of the different soil moisture measures provided better correlation coefficients.

Data was square root transformed to improve normality and homogeneity of variance if needed. Statistics were carried out using SAS Enterprise Guide® 4.1 and for all tests, a significance level of 0.05 was applied.

3 Results

3.1 Soil moisture

The spring 2008 was characterized by having a relatively low amount of precipitation (Fig. 1). The daily averaged soil temperatures (5 cm depth) in plots covered by roofs were one degree lower compared to temperatures in the WD plots during the experimental period.

The WD plots received less than 16 mm of rain from the 21 of April to 21 of May and the SWP in the WD treatment fell below –10 bar for a period of two weeks. It was therefore necessary to irrigate the plots to maintain the difference in SWP and SWC between the treatments,

The SWP was clearly affected by the reduced precipitation in the HD plots, whereas there was no statistically significant difference between WD and MD at any time even though WD plots had received more water in May. Once the roofs were removed the SWP in MD and HD plots rapidly rose to the same level as in the WD plots (Fig. 2). A clear (ANOVA; $p < 0.05$) treatment effect was observed in the top soil of the HD plots from 2 June to 26 June. The SWP was in this period below –35 bar and stayed there for a least three weeks being significantly lower than in WD and MD plots. The same pattern was observed in the 10 cm soil layer (Fig 2). Here the SWP was below –30 bar for more than two weeks from the beginning of June and a significant (ANOVA; $p < 0.05$) treatment effect was observed from 26 May to 26 June. The SWP in 20 cm was generally higher than in 5 and 10 cm, but still around –15 bar for a period of one to two weeks. It should be noted that the psychrometric method is not suitable for measurements higher than –1 bar (Wescor, 1986).

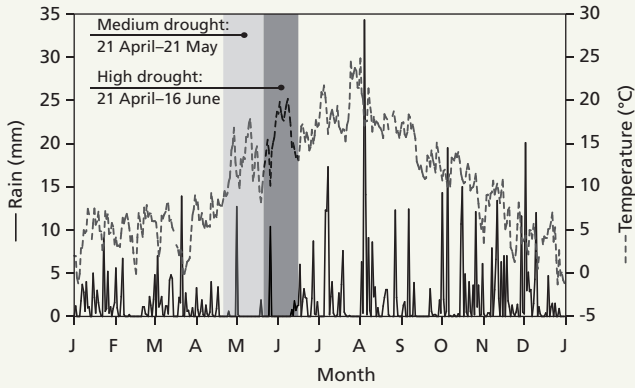


Figure 1. Daily average precipitation (mm) (mean \pm S.D; $n=5$) and daily average temperature ($^{\circ}\text{C}$) in period January 2008 to January 2009. The light grey shading shows the applied drought period for medium drought (MD) treatment and dark grey shading shows the applied drought period for high drought treatment (HD).

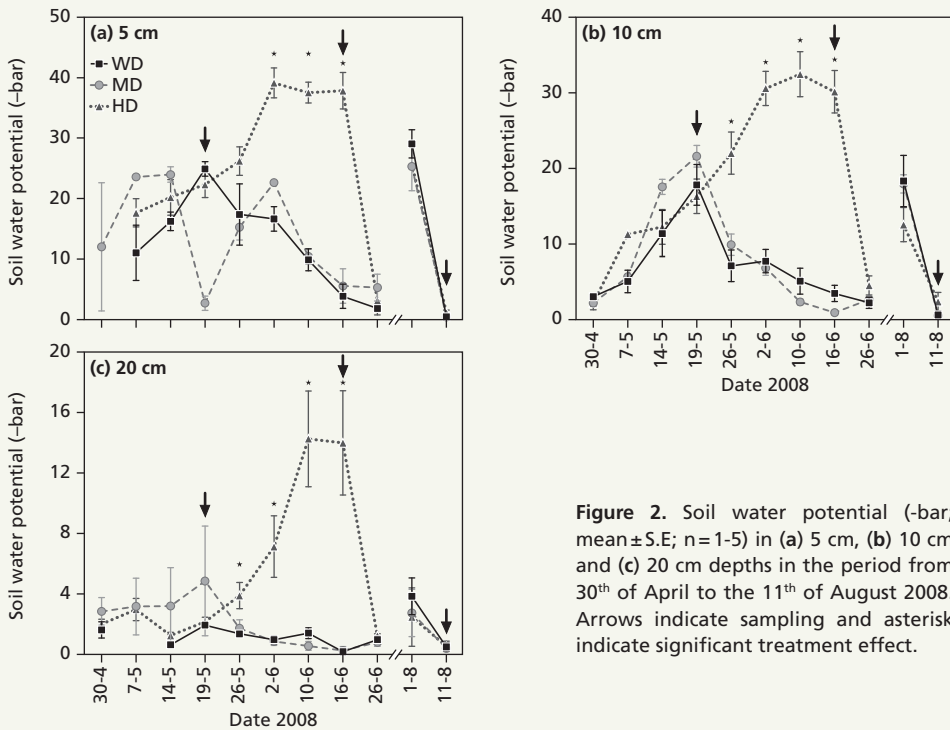


Figure 2. Soil water potential (-bar; mean \pm S.E; $n=1-5$) in (a) 5 cm, (b) 10 cm and (c) 20 cm depths in the period from 30th of April to the 11th of August 2008. Arrows indicate sampling and asterisk indicate significant treatment effect.

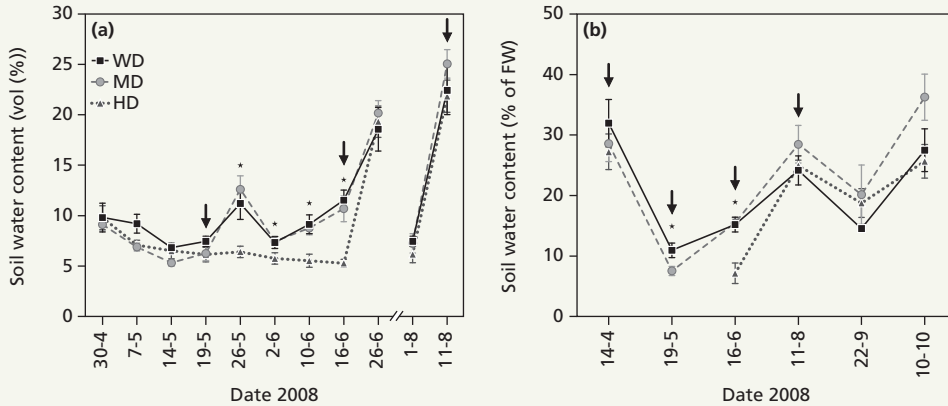


Figure 3. (a) Soil water content (vol %) in 5 cm depth from 30 April to the 11 August 2008. (b) Soil water content (% of FW) in 0–5 cm depth from 14 April to the 11 August 2008. Arrows indicate sampling and asterisk indicate significant treatment effect.

It is therefore likely that soil water potentials shown as being around -1 bar in the present study (Fig. 2) in reality indicate much higher soil moisture levels.

The SWC (gravimetric and volumetric) was negatively influenced by the removal of precipitation showing a comparable pattern as seen for SWP. SWC was around 5% v/v for almost two months, whereas the SWC measured gravimetrically was between 5 and 20% of soil fresh weight in the same period (Fig. 3). Both approaches revealed a significant (ANOVA; $p < 0.05$) reduction of the soil water in the drought treated plots.

3.2 Effect of drought on enchytraeids

A large reduction in total enchytraeid biomass was observed between the April sampling and the May sampling (Fig. 4 and Table 2). In the May sampling almost no enchytraeids were found in any of the treatments and no significant difference was observed in the total biomass (0–9 cm) between the treatments. However, the biomass was significantly (ANOVA; $p = 0.0457$) reduced in the 3–6 cm soil layer in the MD plots compared to WD (Table 2). By June the total biomass was significantly (ANOVA; $p = 0.0371$) influenced by treatment and the total biomass was significantly (Tukey; $p = 0.0087$) reduced in the HD plots compared to MD plots (Fig. 4). The same pattern was observed in the 0–3 cm soil layer (ANOVA: $p = 0.0541$) and in the 3–6 cm soil layer (ANOVA: $p = 0.0096$). From August and forward no significant differences were revealed between the treatments.

The density of the field enchytraeid population was also affected by the treatments and very low numbers were found in May and June. No significant treatment effect was observed in the total density (0–9 cm) in May. But the density in the 3–6 cm soil layer was significantly (ANOVA: $p = 0.0373$) lower in the MD plots compared to WD plots (Table 2). In June the total density was significantly reduced in HD plots compared to MD plots (ANOVA; $p = 0.0105$; Fig. 4), and similar effects were observed in 0–3 cm (ANOVA; $p =$

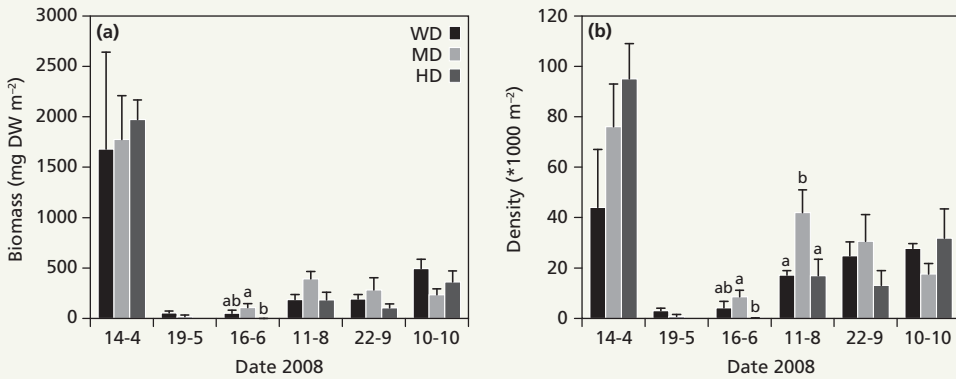


Figure 3. (a) The total biomass (mg dry weight/m²; mean \pm S.E; n = 5) in each treatment from the 14 April to 10 November 2008. (b) The total density (*1000 ind./m²; mean \pm S.E; n = 5) in each treatment from the 14 April to 10 November 2008. Different letters show significant differences between treatments at the respective date.

0.0058), 3–6 cm (ANOVA; $p = 0.0302$) and 6–9 cm (ANOVA; $p = 0.0184$) soil layers (Table 2). In August a significant effect of treatment was still observed on the total enchytraeid density (ANOVA; $p = 0.0202$). This was due to a significantly (ANOVA; $p = 0.0114$) higher density of enchytraeids in the MD plots compared to WD and HD plots (Fig. 4). From September and onward the treatment effect had disappeared (Fig. 4 and Table 2).

The vertical distribution of biomass and density was also significantly influenced by treatment (Table 2). In April, more than 60% of the enchytraeids and their biomass were found in the top soil and between 25% and 37% were found in 3–6 cm soil layer. In May, worms were only observed in 3–6 cm and 6–9 cm soil layers. In June, after one month of irrigation in the WD and MD plots, between 8 and 25% of the biomass and density was again found in the top soil. However, a larger proportion of worms were still present in 3–6 cm soil layer. The vertical distribution was back to April-level in September (Table 2).

3.3 Soil moisture and enchytraeids

When plotting the total number of enchytraeids per m² against SWP, density was clearly positively correlated with SWP (linear regression, $R^2 = 0.33$, $p < 0.001$; Fig. 5a). Similarly, the total biomass was positively correlated with SWP (linear regression, $R^2 = 0.26$, $p = 0.002$; Fig. 5b), meaning that high drought stress results in fewer enchytraeids. SWC was positively correlated with both total density (linear regression, $R^2 = 0.42$, $p < 0.001$; Fig. 5c) and total biomass (linear regression, $R^2 = 0.30$, $p < 0.001$; Fig. 5d). The same pattern emerge with gravimetrically determined SWC data; again both total density (linear regression, $R^2 = 0.18$, $p < 0.001$; Fig. 5e) and total biomass (linear regression, $R^2 = 0.18$, $p < 0.001$; Fig. 5f) was positively correlated with increasing soil water content. However, none of the moisture measures were superior to the others in predicting responses of enchytraeids ($p > 0.05$).

Table 2. Biomass (mg DW pr m²) and density (1000* pr m²) (mean±S.E; n=5) of weak drought (WD), medium drought (MD) and high drought (HD) plots in 0-3 cm, 3-6 cm and 6-9 cm soil layers. Each date was tested for treatment effects in each depth. Significant differences between treatments in the respective depths are marked with different letters.

Date	15 April	21 May	16 June	11 August	21 September	10 November
Biomass						
<i>WD</i>						
0-3 cm	1767 ± 1036	0 ± 0	8 ± 4ab	130 ± 51	121 ± 31	358 ± 106
3-6 cm	256 ± 128	39 ± 13a	20 ± 12ab	56 ± 114	49 ± 31	134 ± 81
6-9 cm	9 ± 2	15 ± 9	24 ± 16ab	1 ± 1	21 ± 8	3 ± 2
<i>MD</i>						
0-3 cm	1811 ± 210	0 ± 0	23 ± 16b	265 ± 55	202 ± 73	224 ± 61
3-6 cm	267 ± 73	6 ± 6b	50 ± 18b	105 ± 40	67 ± 46	6 ± 2
6-9 cm	79 ± 73	12 ± 9	35 ± 17b	23 ± 10	13 ± 10	1 ± 1
<i>HD</i>						
0-3 cm	1288 ± 467		0a	133 ± 74	69 ± 28	318 ± 101
3-6 cm	606 ± 316		0 ± 0a	31 ± 7	26 ± 17	32 ± 10
6-9 cm	58 ± 22		0a	19 ± 6	9 ± 7	10 ± 7
Density						
<i>WD</i>						
0-3 cm	41.5 ± 24.3	0	0.4 ± 0.2ab	10.1 ± 1.1	16.4 ± 4.6	20.5 ± 1.9
3-6 cm	9.2 ± 3.4	2.6 ± 0.9a	2.4 ± 1.5ab	6.6 ± 1.5ab	4.7 ± 2.1	6.6 ± 0.9
6-9 cm	1.8 ± 0.4	0.5 ± 0.3	1.4 ± 0.8ab	0.4 ± 0.3	3.7 ± 2.1	0.7 ± 0.4
<i>MD</i>						
0-3 cm	76.4 ± 5.3	0	1.8 ± 0.9b	26.3 ± 5.6	21.3 ± 7.1	15.0 ± 4.2
3-6 cm	10.3 ± 2.1	0.3 ± 0.3b	4.3 ± 1.8b	13.4 ± 0.4b	7.9 ± 3.9	2.3 ± 0.7
6-9 cm	4.3 ± 1.9	0.5 ± 0.4	2.5 ± 0.8b	2.3 ± 1.5	1.4 ± 0.7	0.3 ± 0.2
<i>HD</i>						
0-3 cm	66.6 ± 22.1		0 a	14.1 ± 6.6	7.2 ± 2.7	23.6 ± 9.2
3-6 cm	24.7 ± 11.2		0.1 ± 0.1a	1.7 ± 0.4a	4.0 ± 2.7	5.8 ± 2.3
6-9 cm	3.7 ± 3.1		0a	1.1 ± 0.4	1.9 ± 1.4	2.4 ± 1.5

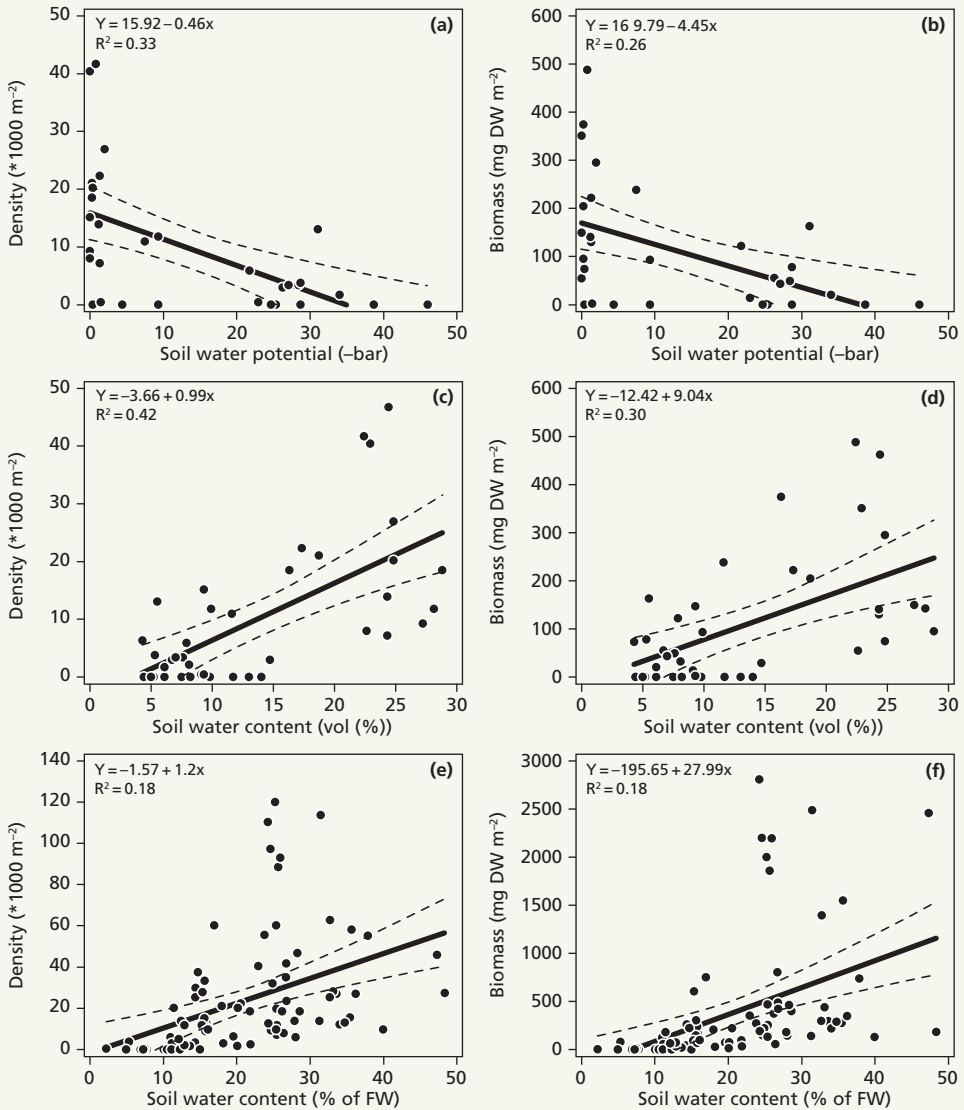


Figure 5. Linear regression between soil water potential (-bar) and (a) total enchytraeid density (*1000 ind. pr m²) and (b) total biomass (mg pr m²). Linear regression between soil water content (vol(%))(c) total density (*1000 ind. pr m²) and (d) total biomass (mg pr m²). Linear regression between soil water content (% of FW) (e) total density (*1000 ind. pr m²) and (f) total biomass (mg pr m²). Note, that the x-axis in (a) and (b) are in -bars, (c) and (d) are in soil water content (vol(%)) and (e) and (f) are in soil water content (% of FW). Linearly regression is shown as the black line, and the grey dotted lines represent the 95% confident limit of the linearly regression.

4 Discussion

In this study we manipulated the amount of precipitation to simulate three different drought scenarios reflecting the expected changes in precipitation in a future climate. The applied drought treatment clearly reduced the enchytraeids for more than two months in the plots. However, even when the enchytraeid population was exposed to long-term hard drought stress, they were still capable of re-populating the plots. None of the enchytraeid populations did, however, return to the same level as before the treatments were applied during our study, meaning that full recovery of the population requires a relatively long period with optimal moisture and temperature conditions. Thus, density and biomass were by the end of the experiment still reduced by 39 % and 70 %, respectively, compared to background sampling in April.

4.1 Drought responses

Several field studies report that the level of soil moisture is clearly important for enchytraeid activity and production in various habitats (Nielsen, 1955a; Abrahamsen, 1972; Lindberg et al., 2002; Maraldo et al., 2008). The severity of drought-induced stress depends on intensity and duration of drought conditions, and both short- and long-term drought exposures can result in population reductions through mortality, and impeded growth and reproduction (Nielsen, 1955b; Abrahamsen, 1972; Lindberg et al., 2002; Maraldo et al., 2008). In this study we compared different lengths of drought spells, as we operated with a long-term high intensity drought exposure (HD) and a short-term and low intensity drought (WD and MD). Unfortunately, all three treatments were influenced by a natural drought spell and high temperature which drastically reduced the number of enchytraeids in the plots in May. Due to this natural drought WD and MD treatments did therefore not differ as much as planned. These unforeseen conditions complicate the interpretation of the results, but our study shows that the enchytraeids were equally capable of rapid re-population after exposure to long-term and high-intensity drought stress or short-term and milder drought stress. However, one or two month of drought exposures as in this study, can probably both be considered as long-term drought and our results also show that there is almost no difference between the population dynamics in the treatments. The soil water potential fluctuates diurnally due to cyclic evapo-transpiration patterns caused by temperature fluctuation and plants removing and delivering water in the root zone (Fiscus and Huck, 1972; Erman, 1973). Thus, enchytraeids in the plots were probably also exposed to short-term (hours or days) low water availability as well as to long-term exposures.

Soil water potential below pF 4 (corresponding to -9.8 bar) has been shown to be lethal for *C. sphagnetorum* whereas the optimal SWP has been reported to be between 0.2 pF (-0.0015 bar) and 2.2 pF (-0.16 bar) (Abrahamsen, 1971). These results are in agreement with our observations, as almost no enchytraeids were observed in periods with SWP below -10 bar. However, the degree of drought tolerance differs between species, as *Enchytraeus albidus* has been found to survive SWP down to -20 bar (Maraldo et al., 2009c). *E. albidus* survived short-term (days) hard drought stress, but when the drought stress was applied for longer periods (weeks) mortality occurred at milder levels of drought (Maraldo et al., 2009c). We suggest that *C. sphagnetorum* may respond in a similar manner, and that it may even be more droughts sensitive since this species seems to be adapted to more stable mesic environments such as wet moors and coniferous forests (O'Connor, 1955).

Even in dry soils moist microhabitats can be present due to the spatial heterogeneity of the soil. Enchytraeids can avoid adverse environmental conditions by migration to these

moister microhabitats, but this movement can only be a short-term strategy unless the food quantity and quality in the new habitat can maintain or increase the population size (Springgett et al., 1970; Erman, 1973; Uhi and Briones, 2002). Springgett et al. (1970) observed that *C. sphagnetorum* was able to migrate vertically to escape dry surface layers and high temperature, thus affecting the distribution over short periods of time. In the present study, a higher proportion of the density and biomass was observed in the 3–6 cm soil layer and in the 6–9 cm soil layer in May and June. This shift in distribution may be due to migration from the top soil to deeper soil layers and/or due to high mortality rates in the top soil. This is consistent with the study of O'Connor (1957), where he concludes that the variation in the vertical distribution was rather due to different mortality rates than to vertical migration, as he both saw a reduction of enchytraeids in the litter layer and the deeper soil layer. The observed partial recovery of the population in this study could therefore originate from enchytraeids migrating back to top soil layers from deeper and less desiccated soil layers. A parallel study of the seasonal phenology of enchytraeids at the site observed that the euedaphic species *Achaeta affinis* was the first species that fully recovered after the very dry period in May and June (Maraldo et al., 2009a). This genus is found to be more drought tolerant due to its relatively thick cuticle and drought tolerant cocoons (O'Connor, 1957). It could be argued that the observed recovery was due to migration from areas outside the plots. However, this seems unlikely in this case since the dispersal rate in soil of *C. sphagnetorum* is known to be limited to less than 20 cm pr year (Sjögren et al., 1995).

C. sphagnetorum was the dominant species at the site and the responses and recovery of this enchytraeid is therefore of particular interest. However, little is known of the degree of drought tolerance or drought tolerance strategies in this species, as no laboratory study so far has examined physiological responses of desiccation in *C. sphagnetorum*. Hard frost, which also result in low soil moisture as the water is bound in ice, has been shown to have a fatal effect on enchytraeids. Thus, no adult *C. sphagnetorum* survived hard frost treatment in a laboratory experiment, but when soil samples were thawed and kept at temperatures above 0°C for a period, a small population of enchytraeids was re-established in the hard frost exposed samples (Sulkava and Huhta, 2003). The authors suggested that a few adult enchytraeids must have survived the hard frost (-16°C), but this seems unlikely as we have observed a more than 70% reduction in density in winter acclimated *C. sphagnetorum* exposed to -2°C for two days (Maraldo et al. unpublished results) suggesting that recovery after hard frost is based on a cold tolerant stage, perhaps cocoons. Most authors believe that *C. sphagnetorum* survive desiccation by migrating to more moist microhabitats (Springgett et al., 1970; Standen and Latter, 1977; Uhi and Briones, 2002). But also increased aggregation in certain suitable microhabitats has been suggested as a response towards increased desiccation (Standen and Latter, 1977). The potential of *C. sphagnetorum* to survive harsh conditions in a cocoon stage ("egg-capsules") is still under discussion; the general opinion is that *C. sphagnetorum* only reproduces asexually by fragmentation, which allows a quick recolonisation of the environment (Lundkvist, 1982; Lundkvist, 1983). However, there are contradictory views and a study from German spruce forest reports of both mature and newly hatched *C. sphagnetorum* during or after dry periods (Schlaghamersky, 2002). The soil moisture level in June was very low down to 20 cm and additional sampling outside the plots confirmed that enchytraeids were practically absent down to 15 cm soil depth at this time. We therefore find it unlikely that upward migration from the deeper soil layers can explain the observed recovery. Furthermore the soil layers below 9 cm depth at the site consist of fine sand and almost no organic matter and poor soil structure providing no attractive habitat for enchytraeids. Thus, we suggest that *C. sphagnetorum* must have a desiccation tolerant stage, probably a cocoon stage, since it is unlikely that the specimens

could have survived a SWP below -12 bar in 20 cm soil depth. However, more studies are needed to confirm this suggestion.

4.2 Enchytraeids and soil moisture

To our knowledge, this is the first study relating drought responses of soil fauna to SWP in a field situation. Although several studies have shown that the numbers of enchytraeids are correlated with soil moisture (Nielsen, 1955b; Abrahamsen, 1972), these studies have considered the effects of SWC which makes comparisons between field studies difficult and does not provide any mechanistic (physiological) explanation of the various responses. Measuring SWP is advantageous since it expresses more directly the water availability to the soil organisms, and it makes inter-study comparisons possible irrespective of chemical-physical properties of the soils in question (Hillel, 1998). We therefore expected that enchytraeid dynamics would be better correlated with SWP than with SWC, but this was not the case. On the other hand, enchytraeid populations are not only influenced by moisture conditions at the time of sampling, but also by moisture conditions to which the population has been exposed to in the past. We therefore tried to include information of the past soil moisture levels in the correlation analysis using the average SWP and SWC of the two weeks before sampling (data not shown). However, for both soil moisture measurements the correlations became weaker. Continuous measurements of SWP and SWC are probably required to describe the enchytraeid dynamic in a Danish dry heathland, as daily variation in SWP, SWC and temperature will influence the growth and reproduction rate. SWC measured gravimetrically seemed to be a weaker predictor than SWC (vol %) and SWP. Furthermore, the density of the enchytraeids was better correlated with SWP and SWC than biomass, which can be due to hatching of cocoons (Lagerlöf and Strandh, 1997) and to an increased fragmentation rate (Lundkvist, 1982).

Lundkvist, (1982) observed in Swedish forest soils such an increased fragmentation rate in *C. sphagnetorum* after dry spells and an increased biomass when moisture conditions had returned to normal levels. We propose that this could also be the case in dry heathland.

5 Conclusion

The enchytraeid population in a Danish heathland was able to recover from more than two months of hard drought. This shows that enchytraeids communities dominated by *C. sphagnetorum* have a high potential to recover from severe drought stress events. Our results strongly suggest that this species must have a desiccation tolerant stage, as known from other enchytraeids species. However, this needs more investigation, as cocoon production by *C. sphagnetorum* has not yet been observed.

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Paper 3

**Counteracting effects of elevated CO₂ and drought episodes:
studies of enchytraeid populations in dry heathland**

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Counteracting effects of elevated CO₂ and drought episodes: studies of enchytraeid populations in dry heathland

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Abstract

The potential impacts of interactions of multiple climate change factors in soil ecosystems have received little attention. Therefore, in this study we investigate the effects of *in situ* exposure to elevated atmospheric CO₂ concentration, increased temperatures and prolonged drought episodes on field populations of Enchytraeidae (Oligochaeta) in a dry heathland (Brandbjerg, Denmark). Increased CO₂ had a positive effect on enchytraeids, whereas drought significantly reduced the population size. Elevated temperature did not result in any detectable effects. No consistent interactions between the three factors were observed. Interestingly, the positive effect of increased CO₂ and the negative effect of drought were cancelled out when applied in combination. Thus, in the combined drought and CO₂ treatment, and when additionally combined with increased temperature, the total biomass of enchytraeids were similar to those in the ambient plots at all three sampling occasions. The positive effect of increased CO₂ was seasonally dependent, as the response was only observed during summer, and not in autumn. The impact of the drought was more consistent, with enchytraeids reduced at all sampling occasions. Moreover, the negative effect of drought seemed to be dependant on the inter-annual variability of precipitation. The year with a dry summer and autumn showed a stronger impact of drought on the enchytraeids, compared to the year with wet summers and autumns. Our study emphasises the importance of multifactorial experimental design as a means to investigate effects of climatic changes.

Keywords: CLIMAITE; interactions, increased temperature, prolonged summer drought, elevated CO₂ level, *Cognettia sphagnetorum*.

Introduction

The climate of northern Europe is undergoing rapid changes and is experiencing rising atmospheric CO₂ concentration, increasing temperature and the emergence of a more extreme pattern of precipitation (IPCC, 2008). These three factors will alone and in combination affect the function of soil ecosystems (Swift *et al.*, 1998; Taylor *et al.*, 2004). Despite the potential for interactions, the majority of climate change studies so far have focussed on one or perhaps two factors, but seldom all three in combination (Mikkelsen *et al.*, 2008). Fenner *et al.*, (2007) observed that the positive effect of elevated temperature and CO₂ on microbial decomposition interacted synergistically; in other words, the combined effect of the two factors in combination was greater than that expected from the isolated effects of each treatment. Alternatively, combining two or more factors can also result in responses that are less than expected (antagonistic) or simply additive responses. However, synergistic responses as observed by Fenner *et al.*, (2007) can create an even stronger positive or negative feedback and support the hypothesis that responses are most likely to be interactive rather than direct or unidirectional (Swift *et al.*, 1998). The main climate change related factors, interfere with several biotic and abiotic factors, as well as with seasonal and inter-annual variations in temperature and precipitation, which complicates the construction of a general model (Tylianakis *et al.*, 2008). Thus, it is necessary to identify the directions of the interactions to predict the responses of climate change in the soil ecosystem (Mikkelsen *et al.*, 2008; Swift *et al.*, 1998; Tylianakis *et al.*, 2008).

A major part of global terrestrial primary production is decomposed in the soil by soil micro-organisms and soil fauna controlling the decomposition and nutrient recycling processes and therefore plays an important role in the terrestrial ecosystem (Cole *et al.*, 2000; Cole *et al.*, 2002b; Seastedt, 1984; Taylor *et al.*, 2004). Enchytraeids, small oligochaete worms, are widely distributed from the Arctic to tropical areas (Petersen & Luxton, 1982), and typically inhabit the organic horizon in soils (Springett, 1967; Springett *et al.*, 1970). Enchytraeids affect the decomposition processes and nutrient mineralisation, both directly and indirectly; directly, by consuming large amounts of organic matter (Cole *et al.*, 2000; Laakso & Setälä, 1999; Setälä & Huhta, 1991; Standen, 1978) and indirectly, by their feeding activity and modifications of soil structure; both of which affect the activity and function of the microbial community (e.g. Bardgett, 2005; Cole *et al.*, 2000). Despite the importance of enchytraeids, only one study has, to our knowledge, focused on the effect of the interactions between two climate change factors on natural enchytraeid populations (Haimi *et al.*, 2005). The presence of enchytraeids is especially important in nutrient poor ecosystems, e.g. temperate heathland and northern coniferous forests, where they dominate the soil fauna (Cragg, 1961; Swift *et al.*, 1998). The dominant enchytraeid species in these types of ecosystems is *Cognettia sphagnetorum* (Cragg, 1961; Lundkvist, 1982; Laakso & Setälä, 1999). This species has a significant influence on the decomposition process and is recognised as a keystone species in such nutrient poor ecosystems (Laakso & Setälä, 1999; Setälä, 2000). *C. sphagnetorum* is adapted to temperate oceanic climate (Briones *et al.*, 2007; O'Connor, 1957) and to mesic environments, such as wet moors and coniferous forests (Lundkvist, 1982). Since climate change scenarios indicate that the climate in these regions will become more variable and extreme (IPCC, 2008), the climate changes may have severe consequences for the existence of *C. sphagnetorum* (Maraldo *et al.*, 2008).

Elevated atmospheric CO₂ concentrations may only have negligible direct effects on enchytraeids as they are adapted to high CO₂ concentrations in soils (Van Veen *et al.*, 1991). The indirect effects, however, such as changes in quantity and quality of plant litter, are expected to affect the survival and activity of enchytraeids (Coûteaux & Bolger, 2000). It

has been shown that increased CO₂ can stimulate primary production (Amthor, 2001), root production and biomass (Arnone *et al.*, 2000; Pregitzer *et al.*, 2008) and increase the level of soil moisture (Heath *et al.*, 2005; Niklaus *et al.*, 2003). Yeates *et al.*, (1997) observed a positive effect of elevated CO₂ on the density of enchytraeids, whereas no changes in field populations of *C. sphagnetorum* was observed in Finnish conifer forest after six years of exposure of CO₂ (Haimi *et al.*, 2005). Negative effects of elevated CO₂ on enchytraeids have also been reported (Markkola *et al.*, 1996; Yeates *et al.*, 2003). The effects of CO₂ may therefore be geographical and species dependent as well as dependent on season, soil type, vegetation or nutrients status of the soil (Coûteaux and Bolger, 2000).

A range of field studies revealing that enchytraeids are extremely vulnerable to drought stress (e.g. (Lindberg *et al.*, 2002; Maraldo *et al.*, 2008; Nielsen, 1955b) and soil water potential below pF 4 (corresponding to -9.8 bar) has been shown to be lethal for *Cognettia sphagnetorum* (Abrahamsen, 1971; Nielsen, 1955b). So far, two strategies for survival of drought stress have been described: migration to moist microhabitats e.g. (Briones *et al.*, 1998; Springett *et al.*, 1970) or survival in the more desiccation tolerant cocoon stage (Lagerlöf & Strandh, 1997). Since *C. sphagnetorum* is known to reproduce asexually by fragmentation and subsequent regeneration, this species could be more liable to extinction than other enchytraeids if more frequent periods with severe drought becomes reality (Springett *et al.*, 1970).

Elevated temperature has been found to stimulate or have no effects on the populations' dynamics of enchytraeids (Briones *et al.*, 1997; Haimi *et al.*, 2005). Effects are, however, species and geographically dependent (Briones *et al.*, 1997; Haimi *et al.*, 2005). *C. sphagnetorum* has a wide distribution; from arctic areas (Christensen & Dozsa-Farkas, 2006) to northern Spain (Uhia & Briones, 2002), which are all areas with rather low maximum temperatures. Considering this, and its optimum temperature of 10°C (Standen, 1973) and a suggested mean annually threshold temperature of 16°C (Briones *et al.*, 2007), the species may therefore be considered to be adapted to lower temperatures. Consequently, increasing the minimum temperatures in a northern European climate by 2°C to 4°C, might be hypothesised to stimulate *C. sphagnetorum*, as their growth and activity would begin earlier in the season (Cole *et al.*, 2002a; Cole *et al.*, 2002b).

The objective of this study was to investigate the short term interactive impacts of increased CO₂, increased temperatures and drought on field populations of enchytraeids dominated by *C. sphagnetorum*. We hypothesise that increasing drought stress will have severe negative consequences for field populations of enchytraeids, but that the response could be counteracted by a potential stimulatory effect of temperature and CO₂.

Material and methods

The field site

The experimental site is situated at Brandbjerg, Denmark (55°53'N 11°58'E) on a hilly nutrient-poor sandy deposit with a dry heath/grassland ecosystem dominated by a grass (*Deschampsia flexuosa*) and an evergreen dwarf shrub (*Calluna vulgaris*). The mean annual temperature is 8.0°C and the mean annual precipitation is 613 mm. The experiment was initiated in October 2005 and includes treatments of elevated CO₂ concentration (CO₂), increased temperature (T), drought in late spring/summer (D), and untreated controls for reference (A). The treatments were applied singly and in all possible combinations (A, T, D, CO₂, TD, TCO₂, DCO₂, TDCO₂). Each experimental treatment is replicated six times,

(total of 48 plots) in a split plot design with six octagons at ambient CO₂ and six receiving elevated CO₂ (Mikkelsen *et al.*, 2008).

The CO₂ was distributed by a Free Air Carbon Enrichment (FACE) system with a target concentration of 510 ppm (Mikkelsen *et al.*, 2008). CO₂ fumigation started 30 min after sunrise and ended 30 min before sunset all year round, except during periods with full snow cover of the vegetation. The temperature enhancement was achieved by “passive nighttime warming”, where a light scaffolding (0.5 m height) carrying a curtain reflects the outgoing infrared radiation (Beier *et al.*, 2004). The curtains were automatically pulled over the vegetation at sunset and retracted at sunrise. In case of rain or heavy winds (> 7 m s⁻¹) during the night the curtains were automatically retreated to avoid hydrological disturbance or damage to the curtains. The drought treatment was applied with water proof curtains automatically pulled over the vegetation during rain events to remove the water from the plots and retracted when the rain stopped. The automatic operation of the curtains according to day and night and climatic conditions were controlled by an astronomic watch (Astro-switching, SC 28/172 4×3, Hugo Müller GmbH & Co. KG, VS-Schwenningen, Germany), a rain sensor (A. Thies GmbH & Co. KG, Goettingen, Germany) and a wind sensor (Windwächter Plus 500, Vestamatic GmbH, Mönchengladbach, Germany). The drought treatment was applied during the growing season in May and continued until soil water content reached 5 vol % water content in the top 20 cm of the soil. Drought treatment was applied twice in 2006; from the 3rd of June to the 20th of June 2006 and again from the 22nd of July to the 4th of August. In 2007 the drought treatment was applied from the 5th of May to the 22nd of June. Temperature was recorded every minute at 0 and 5 cm depth and at 20 cm above the soil surface with cable temperature sensors (TF25/Pt100 Thermokon, Mittenaar, Germany). The soil moisture was measured in 0-20 cm depth by TDR probes (PRENART Equipment ApS, Frederiksberg, Denmark). See (Mikkelsen *et al.*, 2008) for further information of the CLIMAITE field site.

Sampling and species identification

Pre-treatment samples were collected in November 2004, a year before the treatments were applied. Soil cores were collected in 0-6 cm depth and the total number of enchytraeids was determined after extraction. For this purpose a cylindrical soil corer with an inner diameter of 5.5 cm was used. Immediately after sampling each soil core was divided into layers of 3 cm thickness with a knife. The samples were kept at 5°C until extraction which was initiated within one week.

Samples were collected on three occasions during the experimental period: once in 2006 and twice in 2007. To reduce the number of destructive samplings, no samples were collected in 2005, as no effects were expected to be observed only after a few weeks of treatment. One soil core was collected within an area of 1 m² in each plot in 0-9 cm depth for determination of number and biomass of enchytraeids, in late October 2006 and 2007. A third sampling was performed on the 23rd of August 2007, where only enchytraeid biomass was determined. The soil core holes were filled with a new labelled soil core from outside the experimental field site but with similar vegetation. This was done to reduce the gradual destruction of the plots due to sampling. All soil samples were collected beneath *D. flexuosa* and were stored at 5°C until extraction, which was initiated within two weeks.

The extraction procedure was a modified version of O'Connor's wet funnel extraction (O'Connor, 1955), with a stepwise increase in temperature at the sample surface from 25°C to 50°C in five hours. The enchytraeids were collected in tap water and stored for no more than 48 h at 5°C before enumeration. The total number of enchytraeids per m² was deter-

mined integrating 0-9 cm depth. Total biomass (dry weight; mg DW m⁻²) was determined after drying at 60°C for minimum 24 h with a precision of 0.01 mg (Micro SC 2, Sartorius AG, Goettingen, Germany).

The species composition of the enchytraeid community at the field site was determined in October 2006 and 2007 using four and five extra soil samples, respectively, collected outside the experimental plots. Specimens were identified to species level according to (Nielsen & Christensen, 1959, 1961, 1963).

Data analysis and statistical methods

Linear mixed models were applied to analyse the effect of treatment on the biomass and density of enchytraeids in the total soil column, as well as in the different depths and the vertical distribution of biomass and density. Random effect terms were block, octagon and plot, respecting the nested structure of the design. Pre-treatment was added as a random effect, if a significant pre-treatment effect was observed, otherwise, each sampling occasion was treated as independent, as enchytraeids have a highly patchy distribution and large variation can be found over small distances. Main effect terms were the treatment factors: CO₂, temperature (T) and drought (D); in addition, all interaction terms between the factors CO₂, T and D were included. The models were gradually simplified, starting with third order interactions, taking out non-significant interactions until only significant terms were left. Data was transformed ($\log(1+x)$) to improve normality and homogeneity of variance if needed. Statistics were carried out using SAS[®] 9.1 (SAS Inc., 2004) and for all tests, a significance level of 0.05 was applied.

Analysis of soil water contents under the experimental treatments was conducted using linear mixed effects models with a similar random effect error structure of block, octagon and plot with the corresponding nesting, however pre-treatment data was included as a fixed effect in all analyses. The model effects on soil water was determined from daily data from 12:00 by first fitting the fully factorial fixed effect model, followed by model reduction until all effect p-values were less than 0.25. Responses with p-values less than 0.05 are presented as significant. Soil water responses were analysed using R software (R Development Core Team, 2008).

Results

Climatic data

The attainment of the daytime CO₂ target concentration 510 ppm was generally good (mean 480±51 ppm) and the ambient concentration was on average 380 ± 41 ppm. The mean hourly air temperature, measured at 2 m height at the meteorological station mast, was 9.9°C (range -11.4 to 32.3°C) and 10.4°C (range -5.7 to 31.7°C) in 2006 and 2007, respectively. The mean daily temperature increase in the temperature treatments compared to the ambient plots, measured at 5 cm depth ranged from 0.3°C in the winter to 0.7°C in the summer months (Fig.1a and Fig.2a). The maximum mean daily temperature elevation was 1.2, 2.1 and 2.8°C in the 5 cm depth, soil surface and 20 cm height sensors, respectively. The annual mean precipitation was 627 and 852 mm in 2006 and 2007, respectively. Despite 2007 being a wetter year, the drought treatment reduced precipitation by approximately 8% in both years. In 2006, the sum of precipitation from the beginning of the drought to the time of sampling was 274 mm and 222 mm in the ambient and drought

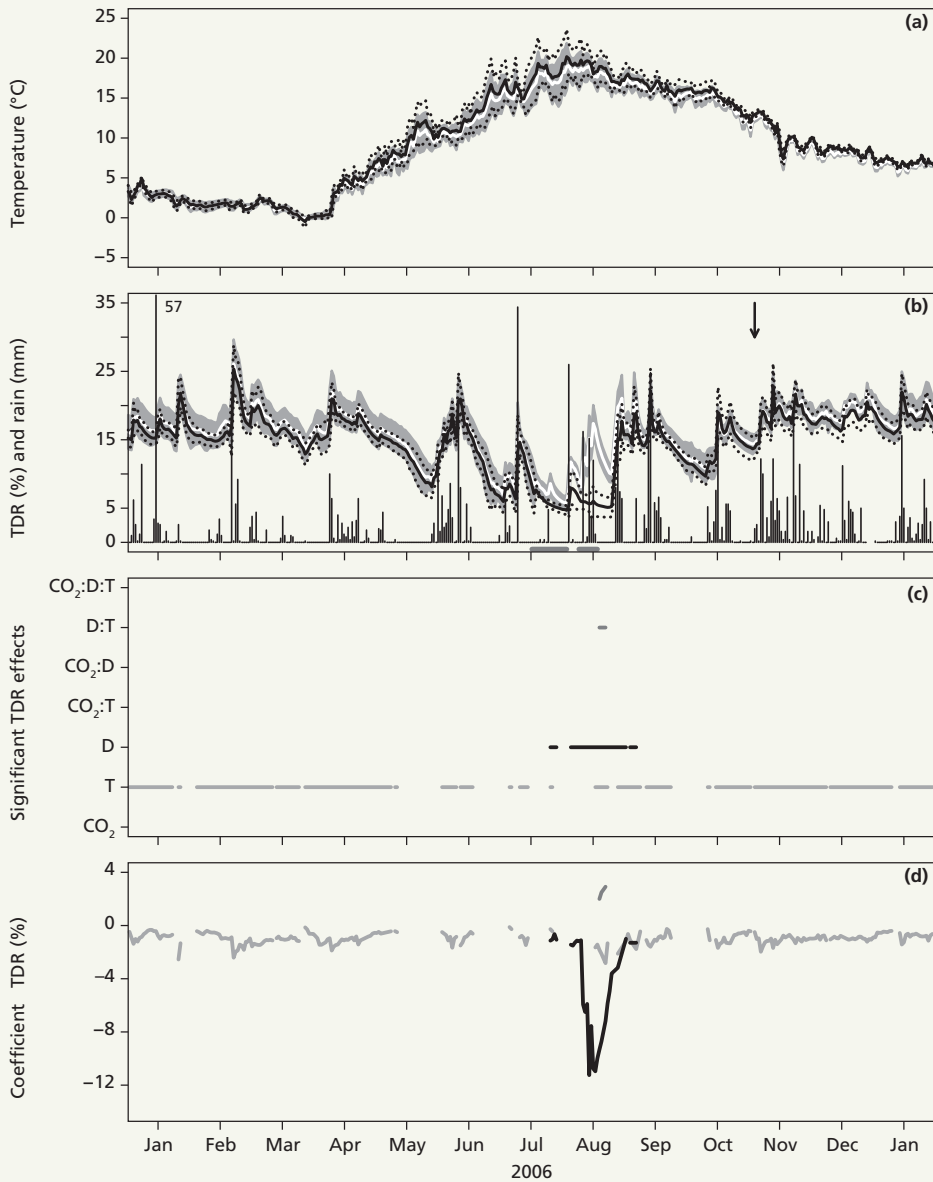


Figure 1. Environmental conditions in 2006. (a) Daily temperature (°C; mean \pm SD) at 5 cm depth in the ambient (white line \pm grey area) and temperature (black line \pm dashed line) treatments. (b) Daily soil water content (TDR %; mean \pm SD) over 0–20 cm depth in the ambient (white line \pm grey area) and drought (black line \pm dashed line) treatments and daily rainfall (vertical bars). The drought period is marked as a dark grey bar and enchytraeid sampling points are marked with arrows. (c) Significance of effects in the linear mixed effects model analysis of TDR. Lines represent periods with significant ($p < 0.05$) effects detected on TDR (%) at 0–20 cm depth. The identity of the effect is marked on the y-axis. (d) Response coefficients of the significant effects shown in Fig. 1c (shades and weights of the lines correspond).

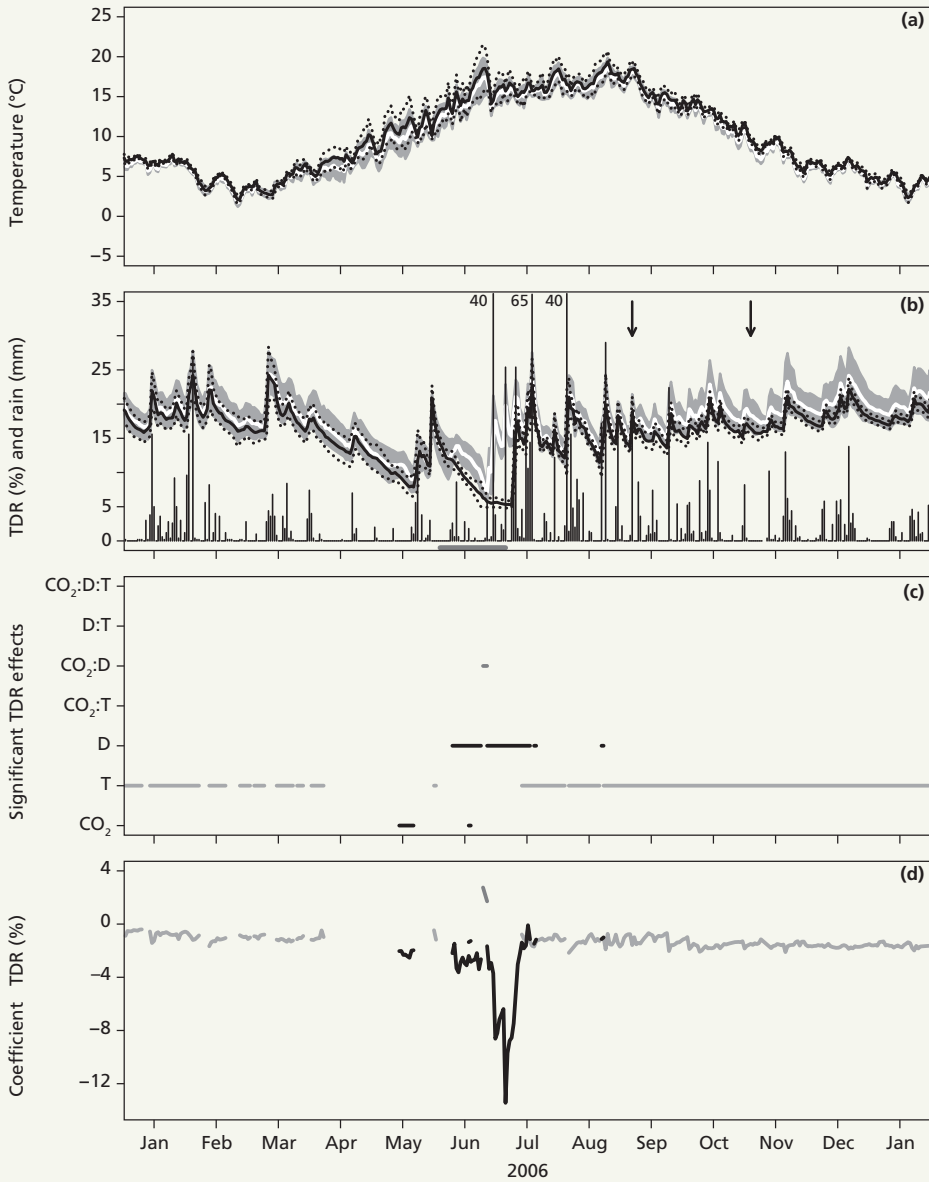


Figure 2. Environmental conditions in 2007. (a) Daily temperature (°C; mean±SD) at 5 cm depth in the ambient (white line±grey area) and temperature (black line±dashed line) treatments. (b) Daily soil water content (TDR %; mean±SD) over 0-20 cm depth in the ambient (white line±grey area) and drought (black line±dashed line) treatments and daily rainfall (vertical bars). The drought period is marked as a dark grey bar and enchytraeid sampling points are marked with arrows. (c) Significance of effects in the linear mixed effects model analysis of TDR. Lines represent periods with significant ($p < 0.05$) effects detected on TDR (%) at 0-20 cm depth. The identity of the effect is marked on the y-axis. (d) Response coefficients of the significant effects shown in Fig. 1c (shades and weights of the lines correspond).

treated plots, respectively (Fig. 1b). In 2007 the sum of precipitation, in the similar period was 565 mm in ambient plots and 495 mm in the drought treated plots (Fig. 2b).

The mean \pm SD hourly soil water content at 0-20cm depth in the ambient treatment was $15.7 \pm 3.9\%$ (range 5.8–26.1) and $17.3 \pm 3.3\%$ (range 7.2–24.4) in 2006 and 2007, respectively. The temperature treatment had a general small negative effect on soil water content in both 2006 and 2007, reducing the soil water content by up to 3% (Fig. 1c and Fig. 2c). The most pronounced effect on soil water was the result of the drought treatments, resulting in peak reductions of 11 and 13% in 2006 and 2007 respectively (Fig. 1d and Fig. 2d). During the 2006 drought there was an amelioration effect (positive) of the CO₂ treatment on soil water content in 0–60 cm depth (data not shown) however a similar effect was not found at 0–20 cm depth (Fig. 1c and d). During the 2007 drought, there was a short period where a DCO₂ interaction with a positive coefficient was detected (Fig. 2c and d), implying that the CO₂ treatment resulted in higher soil moisture in plots with drought applied (Fig. 2c and d).

Enchytraeid community at the field site and pre-treatment sampling

Eight different species of enchytraeids were identified (Table 1). *C. sphagnetorum* was the dominant species at the field site, especially in the top soil (0-3 cm) where they contributed with up to 90% of the total enchytraeid community (Table 1). They contributed less to the community in the 3-6 cm and 6-9 cm soil layers with 27 and 33%, respectively; these layers were dominated by *Achaeta affinis* and *Enchytronia parva* (Table 2).

The pre-treatment sampling revealed a significant block effect in total enchytraeid density ($p = 0.003$) and a similar pattern was observed in the top soil layer (0-3 cm) enchytraeid density ($p = 0.0008$; Fig 3; Table 3).

One year of climate change manipulation (2006)

The drought treatment significantly affected the total enchytraeid biomass ($p = 0.0016$), which was reduced by 65% compared to ambient (Fig. 4a). The same pattern in biomass was observed in the different soil layers; 0-3 cm ($p = 0.046$), 3-6 cm ($p = 0.007$) and 6-9 cm ($p = 0.021$) (Table 2). The total density of enchytraeids was also significantly ($p = 0.0023$) influ-

Table 1. Abundance of enchytraeid species (10^3 individuals m^{-2}) from soil close to the experimental field site in 2006 (mean \pm S.E.; N = 4) and 2007 (mean \pm S.E.; n=5).

Species	Autumn 2006			Autumn 2007		
	0-3 cm	3-6 cm	6-9 cm	0-3 cm	3-6 cm	6-9 cm
<i>C. sphagnetorum</i>	43.0 \pm 4.1	0.1 \pm 0.1	0	82.1 \pm 14	2.4 \pm 1.0	0.3 \pm 0.1
<i>Enchytronia parva</i>	0.2 \pm 0.2	0.3 \pm 0.2	0.1 \pm 0.1	5.8 \pm 3.9	9.3 \pm 5.3	0.8 \pm 0.3
<i>Achaeta</i> sp.	0	0	0	0	0.3 \pm 0.3	0
<i>Achaeta affinis</i>	1.3 \pm 1.3	1.9 \pm 1.4	0.6 \pm 0.5	0.8 \pm 0.6	0.8 \pm 0.3	0.1 \pm 0.1
<i>A. bohémica</i>	0	0	0	0.1 \pm 0.1	0.4 \pm 0.4	0.1 \pm 0.1
<i>Fridericia</i> sp.	0	0	0	0	0.1 \pm 0.1	0
<i>Fridericia bisetosa</i>	0	0	0	1.0 \pm 1.0	0	0
<i>Marionina</i> sp.	0.3 \pm 0.3	0	0.1 \pm 0.1	0	0	0

Table 2. Enchytraeid biomass (mg DW m⁻²; mean ± S.E.; n=6) for each treatment and each depth from autumn 2006 to autumn 2007. No significant effect is marked by N.S. and significance at $p < 0.05$ by an asterisk.

Treatment	Autumn 2006			Summer 2007			Autumn 2007		
	0-3 cm	3-6 cm	6-9 cm	0-3 cm	3-6 cm	6-9 cm	0-3 cm	3-6 cm	6-9 cm
A	803±172	589±333	8±6	531±71	74±37	21±40	1663±199	132±76	31±26
D	434±112*	53±25*	10±7*	257±105*	38±18*	10±13	1056±225*	150±83	85 ±78
T	1149±236	128±54	27±16	513±137	80±44	36±30	1996±690	164±89	8±4
CO ₂	1046±235	312±240	15±10	726±145*	443±337*	136±119	2744±872	53±27	161±154
TD	413±170	52±24	9±9	306±84	111±80	7±5	859±217	96±73	18±8
TCO ₂	735±105	275±199	15±9	1034±296	413±225	76±36	1418±194	387±232	60±54
DCO ₂	715±152	28±16	2±1	514±153	16±13	2±1	1465±245	196±116	159±126
TDCO ₂	787±384	80±34	16±8	404±93	146±65	14±8	1995±684	85±33	4±2
Block	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

enced by the drought treatment and was reduced by 47 % compared to the enchytraeid density in the ambient plots. The 3-6 cm soil layer ($p = 0.0056$) and 6-9 cm layer ($p = 0.0136$) showed also a significant effect of drought (Table 3). No significant effects of warming and elevated CO₂ was observed as well as no significant interaction effects of the experimental treatments in the enchytraeid biomass or density, in the total soil column and the different soil layers (Fig. 4 and 5; Table 2 and 3).

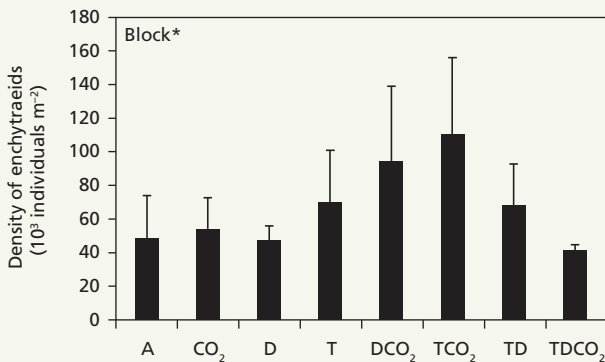


Figure 3. Density of enchytraeids (10³ individuals m⁻²) in pre-treatment samples from the 0-6 cm soil layer (n=6, mean ± S.E.).

Table 3. Enchytraeid density (10^3 individuals m^{-2} ; mean \pm S.E.; $n=6$) for each treatment and each depth from autumn 2004, autumn 2006 and autumn 2007. No significant effect is marked by N.S. and significance at $p < 0.05$ by an asterisk.

Treatment	Autumn 2004			Autumn 2006			Autumn 2007			
	Depth	0-3 cm	3-6 cm	6-9 cm*	0-3 cm	3-6 cm	6-9 cm	0-3 cm	3-6 cm	6-9 cm
A		37 \pm 19	10 \pm 7		32 \pm 6	26 \pm 14	1 \pm 0	70 \pm 5	9 \pm 3	2 \pm 1
D		34 \pm 5	12 \pm 7		24 \pm 8	7 \pm 4*	1 \pm 1*	75 \pm 22	26 \pm 15	14 \pm 13
T		51 \pm 23	17 \pm 9		43 \pm 8	9 \pm 2	3 \pm 1	76 \pm 19	13 \pm 6	1 \pm 0
CO ₂		30 \pm 8	22 \pm 13		50 \pm 11	29 \pm 20	2 \pm 1	110 \pm 38	7 \pm 3	13 \pm 11
TD		59 \pm 21	8 \pm 4		31 \pm 15	5 \pm 2	1 \pm 0	43 \pm 11	8 \pm 4	2 \pm 1
TCO ₂		55 \pm 13	53 \pm 34		38 \pm 5	19 \pm 12	1 \pm 1	72 \pm 10	24 \pm 12	4 \pm 4
DCO ₂		61 \pm 22	32 \pm 54		30 \pm 8	3 \pm 2	0 \pm 0	102 \pm 28	33 \pm 18	23 \pm 18
TDCO ₂		28 \pm 5	12 \pm 3		38 \pm 16	6 \pm 2	1 \pm 1	77 \pm 20	7 \pm 2	1 \pm 0
Block		*	N.S.		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

*The soil depth 6-9 cm was not sampled in 2004

Two years of climate change manipulation (2007)

Summer

CO₂ was found to have a significant ($p = 0.017$) stimulatory effect on the enchytraeid population in the summer 2007. The total enchytraeid biomass in the CO₂ plots was increased by 108% compared to ambient plots (Fig. 4). The drought treatment significantly ($p = 0.0059$) reduced the total enchytraeid biomass by more than 50%. A similar pattern was observed in the 0-3 cm soil layer (CO₂: $p = 0.045$ and D: $p = 0.0134$) and in the deeper soil layer 3-6 cm (CO₂: $p = 0.0237$ and D: $p = 0.0336$) (Table 2). Moreover, in the combined drought and CO₂ treatment (DCO₂) the negative effect of drought was counteracted by the positive effect of elevated CO₂ (Fig. 4c). No significant interaction between CO₂ and drought was detected ($p = 0.759$) indicating a simple additive effect of these treatments on enchytraeid biomass. In the vertical distribution of biomass, however, a significant interaction between CO₂ and D was observed; a significantly ($p = 0.0474$) higher proportion of enchytraeid biomass was found in 0-3 cm soil layer in the combined treatments at the expense of biomass in the 3-6 cm soil layer (Fig. 6b).

Autumn

In autumn 2007, the enchytraeid population was still affected by the drought treatment; the total biomass was significantly ($p = 0.033$) reduced by 29% compared to ambient plots (Fig. 4). This reduction was also observed in the 0-3 cm ($p = 0.0359$) soil layer, but not in the 3-6 cm and 6-9 cm soil layers (Table 2). The enchytraeid density was not significantly affected by treatments in the autumn 2007. No significant interactions were detected in the enchytraeid biomass or density (Fig. 4 and 5; Table 2 and 3).

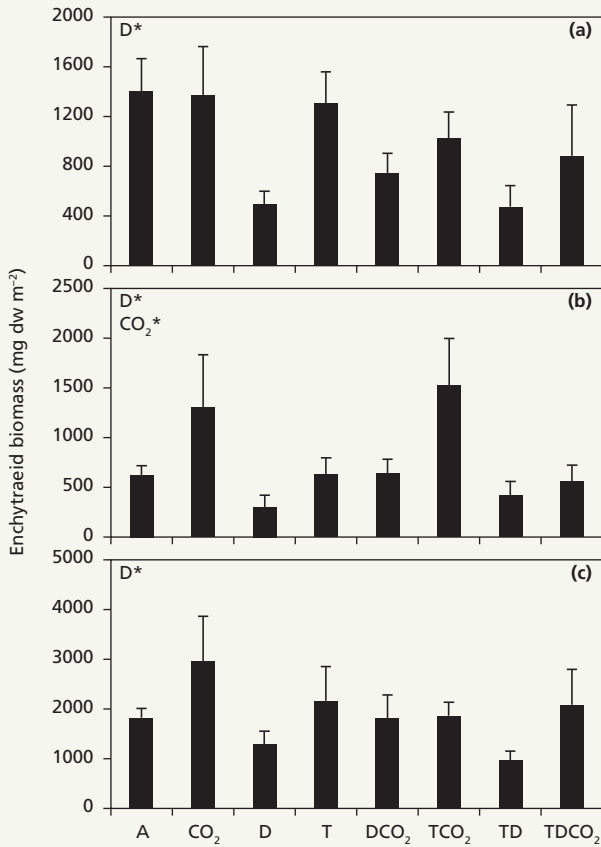


Figure 4. The biomass of enchytraeids (mg DW m⁻²) in samples collected from the 0–9 cm soil layer (n=6, mean±S.E.) in (a) autumn 2006, (b) summer 2007 and in (c) autumn 2007. Annotations show which effects were determined to be significant and the significance level ($p < 0.05$) is marked by an asterisk (*).

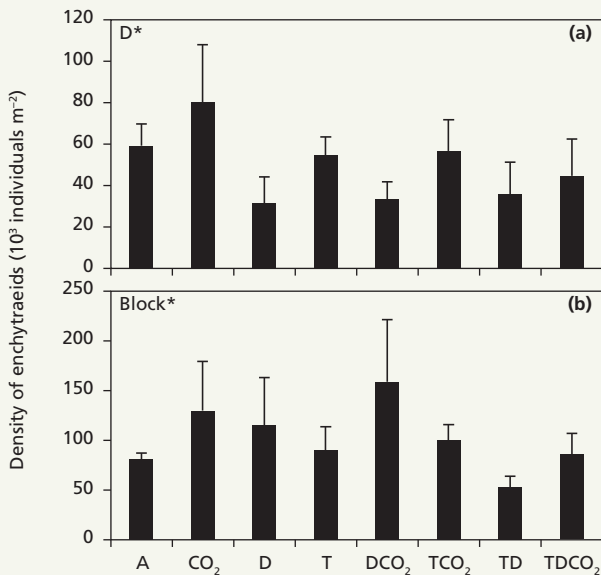


Figure 5. Density of enchytraeids (10³ individuals m⁻²) in samples collected from the 0–9 cm soil layer (n=6, mean±S.E.) in (a) autumn 2006 and in (b) 2007. Annotations show which effects were determined to be significant and the significance level ($p < 0.05$) is marked by an asterisk (*).

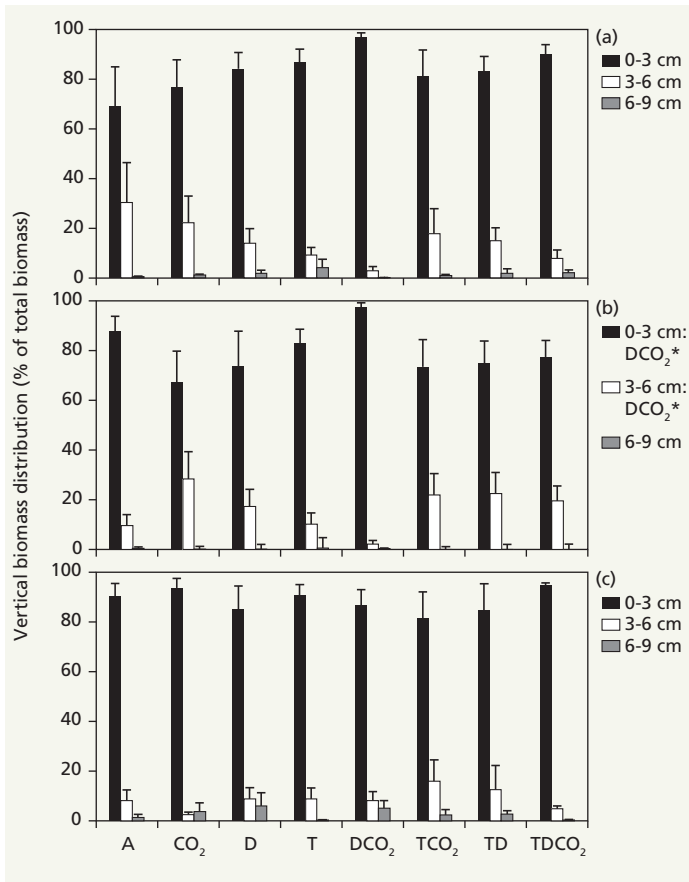


Figure 6. The vertical distribution of enchytraeid biomass as a percentage of the total biomass (n=6, mean±S.E.) in the total soil column in (a) autumn 2006, (b) summer 2007 and in (c) autumn 2007. Annotations show which effects were determined to be significant and the significance level ($p < 0.05$) is marked by an asterisk (*).

Effects of interannual variation of precipitation

The amount of precipitation was higher in 2007 than in 2006, which was a more typical year for the region. Hence, the enchytraeid population in 2007 experienced a longer period of soil water content above 15% compared to 2006, due to the higher amount of precipitation and the earlier application of drought in 2007. The difference in precipitation between the two years was reflected in a generally higher enchytraeid biomass in 2007 than in 2006. In autumn 2007 the total biomass of ambient plots was 31% higher than in 2006 (Fig. 4). In the drought treated plots this difference between years was larger; the biomass was 160% higher in autumn 2007 compared to autumn 2006 (Fig. 4). The same pattern was observed for enchytraeid density (Fig. 5). However, the difference between the two years was even more pronounced in the drought treated plots (Fig. 5).

Discussion

The climate change manipulations at the CLIMAITE field site, although representing a likely scenario for year 2075 (Mikkelsen *et al.*, 2008), were applied acutely and not developing slowly as would occur in reality. Thus, the observed responses should be interpreted with this in mind; a gradual shift in climatic factors could give the enchytraeids and other organisms the possibility to adapt genetically. However, this potential problem is inherent in all climate change field experiments and is near impossible to circumvent.

Enchytraeid responses to elevated CO₂ concentration

The effects of elevated CO₂ were detectable in the summer sample in 2007, but not in autumn of 2006 or 2007. Such seasonality in CO₂ effects was also observed by Markkola *et al.* (1996). Markkola *et al.* (1996) noted that populations of enchytraeids that were acclimated to summer conditions responded positively to elevated CO₂, whereas winter acclimated enchytraeids responded negatively. The authors suggested that increased CO₂ could have affected the reproduction of *C. sphagnetorum* or increased the predation by other members of the soil fauna (Markkola *et al.*, 1996). The grazing activity of mites on fungi has been found to increase when exposed to elevated CO₂ (Allen *et al.*, 2005); this could be the case for enchytraeids, thereby causing positive responses that were only evident in the growing season. Another potential explanation is that elevated CO₂ enhances the water-use efficiency of the vegetation, which alleviates the water limitation in the top soil during drought periods (Heath *et al.*, 2005). However, we did not observe a general increase in soil moisture (0–20 cm) in the CO₂ treated plots and more fine-scaled measurements would be necessary to determine if there are changes in the near surface distributions of soil moisture.

Several authors have suggested that the effects of increased CO₂ seem to originate through multiple components of the soil food web and, so far, no general pattern of response has been identified (Allen *et al.*, 2005; Coûteaux & Bolger, 2000; Loranger *et al.*, 2004). Both the biotic and abiotic environment of the enchytraeids are expected to be altered by exposure to elevated CO₂, due to changes in litter quality and quantity (Coûteaux & Bolger, 2000). Also, an increased allocation of C due to increased root production together with an associated increase in rhizodeposition will typically stimulate the activity of soil organisms and thereby provide increased food availability for enchytraeids (Allen *et al.*, 2005). The reduction in leaf litter nitrogen content will probably become limiting to the production of enchytraeids before the potential positive effect of increased soil moisture in nutrient poor environments such as heathlands, over time (Allen *et al.*, 2005). *C. sphagnetorum* reportedly prefer to feed on litter with an age between five and ten years (Briones & Ineson, 2002; Standen & Latter, 1977) and therefore the dilution effect will probably not be evident before this period of time has passed. It would therefore be interesting to perform a new sampling after five to ten years of treatment to elucidate if the positive effect of CO₂ in summer still persists and to examine if a soil fauna community composition change occurs.

Responses to prolonged drought period

The drought treatment was the only climate change factor revealing a consistent significant effect on the enchytraeid population. This is in accordance with our hypothesis, that drought would be a main limiting factor after a relatively short period of treatments and is in agreement with a range of field studies showing that the level of soil moisture is clearly important for enchytraeid activity and production in various habitats (Abrahamsen, 1972; Nielsen,

1955a). Both short- and long-term drought exposures can result in population reductions through mortality and impeded growth and reproduction (Abrahamsen, 1971; Maraldo *et al.*, 2008; Nielsen, 1955b; Springett *et al.*, 1970; Standen, 1980). So far, only two studies of long-term effects of repeated drought exist reporting 90% and 65% reductions in enchytraeid populations after eight years of repeated drought in a Swedish coniferous forest (Lindberg *et al.*, 2002), and six years of repeated drought in a Danish heathland (Maraldo *et al.*, 2008), respectively. The observed reduction in biomass and density in this study does not necessarily reflect a general decrease throughout the year, as the enchytraeid population could have recovered during the winter and spring following the drought treatment. However, the dominant species, *C. sphagnetorum*, is a slow growing species at low temperature (Springett, 1970; Standen, 1973). It has also been suggested that *C. sphagnetorum* populations in cool temperate regions only peak once a year in the autumn (Lundkvist, 1982) and that the enchytraeid community can take several years to recover following disturbance/drought (Cole *et al.*, 2002a). Thus, the reduction due to drought seen in autumn is probably representative for a large part of the year. Enchytraeids can avoid desiccating conditions by migration to moister soil layers, but this movement can only be a short-term strategy unless the food quantity and quality in the new habitat can maintain or increase the population size (Springett *et al.*, 1970); however, no consistent downward migration was observed. During severe droughts, as used in this study, it is unlikely that enchytraeids can find moist microhabitats, and they will therefore inevitably be exposed to stressful soil water potentials that would probably result in high mortality (Maraldo *et al.*, 2008).

Enchytraeid responses to increased temperature

Effects of warming were not detected in the present study, however, the applied daily warming of between 0.2 and 0.6 °C (25 to 75 percentiles at 5 cm depth) was modest in comparison to predicted climatic changes (IPCC, 2008). Two other long-term field studies, with temperature increased by 0.5–2 °C above ambient temperature, have, in accordance with our study, not observed any temperature effects on *C. sphagnetorum* (Haimi *et al.*, 2005; Maraldo *et al.*, 2008). However, short-term laboratory (Briones *et al.*, 2004; Cole *et al.*, 2002a) and transplant studies, exposing *C. sphagnetorum* to temperatures 2.5–5 °C above ambient temperature, did reveal stimulatory effects (Briones *et al.*, 1997; Briones *et al.*, 2004; Cole *et al.*, 2002b). These differences between field and laboratory experiments may be related to the different temperature regimes used, but different soil moisture contents in these studies may also be responsible for the observations. Both of the long-term studies were performed in the field with annual variations in soil moisture content, whereas the laboratory studies and transplant study were performed with high and constant soil moisture contents. Another explanation could be that stimulatory effects of warming do in fact occur in the field situation, but are overlooked due to timing of the field sampling. Thus, if sampling is performed in autumn, potential positive effects of increased temperature in winter and spring may not be detected. We can therefore not conclude that temperature does not have any effect at all on the enchytraeid population.

Increased temperature can lead to enhanced evaporation from the soil and thereby reduce the soil moisture in soil. Edwards & Lofty (1971), found that the enchytraeid density initially increased in heated plots but decreased later on in spring due to decreasing soil moisture content caused by the heating. In this study we also observed a significant decrease in soil moisture content in the temperature treated plots and we can not reject that this could have had a negative influence on the enchytraeid's response, although the reduction in soil moisture was small.

Inter-annual variation in the enchytraeid population

The negative effect of drought was more pronounced for the enchytraeid population in 2006 with a drier summer and autumn as compared to 2007, which had a wet summer and autumn. In general the enchytraeid population was much higher in 2007 compared to 2006, and especially in the drought treated plots the difference was pronounced. This demonstrates that the enchytraeid population is capable of rapid population growth when optimal conditions are present, as also seen in field experiments manipulating the soil water by regulating the amount of precipitation and providing irrigation (Maraldo & Holmstrup, 2009). Thus, it seems reasonable to suggest that years with high amounts of precipitation can reduce the negative impact of drought from the previous year, despite the effects of drought being evident in the second year of this scenario. Long-term studies are needed to examine the strength of this recovery caused by inter-annual variation in precipitation.

Combination effects

This study did not detect any interaction effects among the three factors when considering the enchytraeid population after either one or two years of manipulation, meaning that effects of one factor did not influence the response of others. An interesting result of this study was that the positive effect of increased CO₂ and the negative effect of drought counteracted each other when applied in combination. Thus, the total density and biomass of enchytraeids in this combination treatment, and when additionally combined with increased temperature, were at the same level as in ambient plots at all three sampling occasions. Nevertheless, when looking at the vertical distribution of enchytraeids, a significant interaction between drought and CO₂ was observed in the summer 2007. Here, the proportion of enchytraeid biomass in the DCO₂ treatment was higher in the 0–3 cm soil layer than in either the D or CO₂ treatments, and correspondingly lower in the 3–6 cm layer. Thus, in this particular case a non-additive (synergistic) interaction seemed to occur, perhaps due to more suitable moisture for the enchytraeids in the top soil in this combination as observed for a short period in 2007 (Fig. 2c).

Conclusion

Drought was clearly the main limiting factor in our experiment, causing a reduction in the enchytraeid biomass and density independent of the year-to-year variation of precipitation. The negative impact of drought was, however, ameliorated by increased CO₂ concentrations, as the effects of drought and CO₂ combined additively to cancel out the individual effects of each factor. Our study therefore emphasises the importance of multifactorial experimental designs as a means to investigate effects of climatic changes. It should be stressed that our results represent the short-term temporal responses, whereas long-term effects may potentially emerge, due to the effect of elevated CO₂ on litter quality and the implications for decomposition processes in the soil; such effects should form the focus of longer term experiments.

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Paper 4

Can field populations of the enchytraeid, *Cognettia sphagnetorum*, adapt to increased drought stress?



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Can field populations of the enchytraeid, *Cognettia sphagnetorum*, adapt to increased drought stress?

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ABSTRACT

The ability to evolve increased drought tolerance in response to climate change was investigated in the enchytraeid, *Cognettia sphagnetorum*. Populations exposed to reduced precipitation or increased night time temperature for more than six years were collected in mixed *Calluna*/grass heathland at the Mols Laboratory, Denmark. The level of prolonged drought and increased temperature corresponded to a predicted climate change scenario and has been applied since 1999. In autumn 2005, enchytraeids were sampled in 3 cm intervals down to 9 cm depth and total number, biomass, diversity and soil organic matter were determined. The drought treatment resulted in a significant reduction of the density and biomass of enchytraeids, as well as changes in the species composition. In total, five different genera were found at the site in all three treatments (control, temperature and drought). *C. sphagnetorum* was the dominant species, especially in the upper 0–3 cm, and was clearly affected by the drought treatment. *C. sphagnetorum* from all plots were cultured in the laboratory to rear second or third generation adults. Results showed that populations of drought treated plots had not developed an increased drought resistance compared to populations of control or warming plots even after several years of a putative severe selection. Lack of adaptive potential in *C. sphagnetorum* suggests that more frequent periods with drought in the future will have a very strong negative influence on enchytraeid density, biomass and diversity.

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1. Introduction

Climate change predictions suggest that years with extreme summer drought and floods are likely to become more frequent in a future northern European climate due to changes in the precipitation pattern. In addition, the minimum temperature is expected to increase all year round together with an increased concentration of CO₂ (Houghton et al., 2001). Each of these factors can directly, indirectly and in combination affect the biodiversity and the function of terrestrial ecosystems (Swift et al., 1998; Taylor et al., 2004). The survival of a population depends on the ability of the individuals to either adapt to or avoid stress (Lopes et al., 2004). A range of studies has been investigating the ability of animals to adapt to local changing climatic conditions by comparing resistance patterns along geographic gradients (Hoffmann et al., 2003; Holmstrup and Loeschke, 2003; Bahrndorff et al., 2006; Holmstrup et al., 2007). Likewise, a number of ecotoxicological studies, with populations from metal contaminated areas, report on

various species of soil invertebrates which are capable of increasing their metal tolerance through genetic adaptation, e.g. enchytraeid worms (Salminen and Haimi, 2001; Haimi et al., 2005a) and collembolans (Posthuma, 1990; Tranvik et al., 1993). Genetic adaptation to desiccation stress may also take place in response to increased occurrence of drought episodes. However, no studies have investigated the ability of soil organisms to adapt to increased drought by using long-term exposed populations from manipulated field experiments.

A major part of earth's terrestrial primary production is decomposed in the soil and soil micro-organisms and soil fauna are by their activities accelerating the decomposition and nutrient recycling processes (Seastedt, 1984; Cole et al., 2000, 2002; Taylor et al., 2004). Enchytraeids, small oligochaete worms living in the organic top layer in soil, are key organisms of temperate heathland ecosystems contributing to the decomposition processes (Standen, 1978; Petersen and Luxton, 1982; Laakso and Setälä, 1999). The enchytraeid species *Cognettia sphagnetorum* is a keystone species in temperate heathland (Laakso and Setälä, 1999), and can be present in high numbers.

The most important environmental factors determining the performance of enchytraeids are the availability of water

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(Abrahamsen, 1971; Gröngröft and Miehl, 1983; Sulkava et al., 1996; Beylich and Achazi, 1999), temperature (Edwards and Lofty, 1971; Standen, 1978; Briones et al., 1997) and pH (Didden, 1993; Graefe and Beylich, 2003; Cole et al., 2006). Thus, in the northern hemisphere a number of species will typically reach their annual minimum in summer due to desiccation stress (Nielsen, 1955b; Springett et al., 1970; Abrahamsen, 1971; Lagerlof and Strandh, 1997). So far, two strategies for survival of drought have been described for enchytraeids: either the worms migrate vertically to more moist microhabitats (e.g. Nielsen, 1955b; Springett et al., 1970; Standen, 1973; Briones et al., 1998; Uhia and Briones, 2002) or survive in the more desiccation tolerant cocoon stage (Lagerlof and Strandh, 1997). *C. sphagnetorum* is known to reproduce asexually by fragmentation and subsequent regeneration. Thus, during periods with severe drought this species could be more liable to extinction than other enchytraeids (Springett et al., 1970).

Increased temperature has been shown to have stimulatory effects or no effects on the populations dynamics of enchytraeids. Effects are, however, species and geographically dependent (Standen, 1973; Sulkava et al., 1996; Briones et al., 1997, 2004; Haimi et al., 2005b).

In the present study we investigated the impacts of reduced precipitation and increased temperature on enchytraeid populations in a long-term field experiment, which had been running for six years (1999–2005). Based on earlier studies, we hypothesize that, (I) increased temperature will lead to increased abundance, biomass and species richness of the enchytraeids. (II) Drought will have a negative effect on the abundance, biomass and species richness in the natural populations of enchytraeids.

We further hypothesize that the applied drought treatment could lead to increased desiccation tolerance in populations of enchytraeids. As a model organism *C. sphagnetorum* was chosen because it fulfils two important criteria. Firstly, it is the dominant enchytraeid species at the field site. Secondly, it has a relatively stationary behaviour; the dispersal distance and rate is estimated to be extremely low (Sjogren et al., 1995) meaning that migration in or out of the experimental plots is negligible. Thus, after exposure to enforced drought for six years, we expect that a directional selection will have occurred in *C. sphagnetorum*, resulting from genetic differences in desiccation tolerance which developed in the differently exposed populations. Alternatively, due to the asexual reproduction of *C. sphagnetorum*, the species might lack necessary genetic variability to adapt to climate changes.

2. Materials and methods

This study consists of a field experiment and a laboratory experiment. Soil samples were taken in field plots which since 1999 have been subjected to experimental manipulations of precipitation and temperature. The number, biomass and species composition of enchytraeids were determined from soil samples. In addition, specimens of the dominating enchytraeid, *C. sphagnetorum*, were isolated from these samples and laboratory cultures were established for further testing of drought tolerance in the laboratory.

2.1. Field experiment

2.1.1. Study site

The experimental site is located at Mols, Denmark (DK) (56°23' N, 10°57' E) and is a part of the European research projects "Climate-driven changes in the functioning of heath and moorland ecosystems" (CLIMOOR) and "Vulnerability assessment of shrubland ecosystems in Europe under climatic changes" (VULCAN) (Beier et al., 2004). The site is a semi-natural ecosystem formerly subjected to low intensity grazing with no management activities

for nearly 10 years prior to the start of the experiment in 1999. The soil at Mols is a sandy podzol with a shallow organic layer, which is dominated by the ericaceous shrub *Calluna vulgaris* and with *Deschampsia flexuosa* as a codominating grass.

2.1.2. Set up of field experiment

The field treatments, temperature, drought and control, were initiated in three replicated 20 m² plots at Mols in 1999. Drought treatment was imposed for a 2-month period in the spring/summer seasons from 1999 to 2005 by covering the vegetation with transparent waterproof covers. In order to reduce unwanted influences by wind, temperature and light conditions, the plots were only covered during rain events and left open during dry conditions. This was done by activating the transparent covers automatically via rain sensors to cover the plots whenever it rained while the covers were removed after the rain stopped. For the part of year without drought treatment, the drought plots were run parallel to the control plots. The temperature treatment was designed to mimic an increased minimum night temperature rather than the general diurnal temperature. The temperature plots were covered by a light scaffolding carrying a curtain made of high-density polyethylene mesh, which reflects the infrared (IR) radiation. The curtains reflected 97% of the direct and 96% of the diffuse radiation and allowed transfer of water vapor. The curtains were automatically drawn over the vegetation to reduce the loss of IR radiation when light intensities fell below 0.4 W m⁻² (sunset). At sunrise the curtains were retracted to leave the plots open during the day. The control plots had the same size as the drought and temperature treated plots, but were left untreated. For further information on the field site and the experimental design (see Beier et al., 2004; Schmidt et al., 2004).

2.1.3. Meteorological data

Meteorological data was obtained from the VULCAN database. Temperature of air and soil were recorded by bihourly measurements with a 110 Thermocouple Reference Thermistor Type, probe 107 (Campbell scientific, Logan, UT, USA). Soil temperature was recorded at 2 and 10 cm depth. The average soil temperature was increased by about 0.8 °C in the warming treated plots (Beier et al., 2004). During some periods the average monthly soil temperature was increased by more than 1 °C and increases up to almost 2 °C were also observed. The largest increases were observed in early spring, autumn and winter. The precipitation was measured monthly by rain gauges inside the plots. The drought treatment was applied for one to two months every year in the growing season and during these periods the precipitation was almost eliminated from the plots (reductions from 83% to 100%). The mean annual precipitation in the drought treated plot was reduced by approximately 25% with some minor variations between years (Table 1). Soil water content was determined by Theta probes (ML-2, Delta-T) in the period 2000–2004. The measurements were made in 5–10 cm depth, and it was found that the drought treatment significantly reduced the soil water content by 33–50% during the drought periods (Sowerby et al., in press).

2.1.4. Sampling and extraction of animals

A preliminary sampling outside the experimental plots was conducted 20th September 2005 to examine the density, vertical distribution and species composition of the enchytraeids in the area. For this purpose a cylindrical soil corer with an inner diameter of 5.5 cm was used. Six samples were taken in the depth 0–12 cm; enchytraeids were extracted, identified to species or genus level and counted. On the 25th October 2005, six soil cores (sub-samples) were taken within an area of 1 m² in each plot in 0–9 cm depth. Immediately after sampling each soil core was divided into layers of 3 cm thickness with a knife. The samples were kept at 5 °C

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Table 1

The total annual precipitation (mm) in the control, temperature and drought treated plots at Mols, Denmark

Year	Drought period	Annual precipitation (mm)		
		Control	Temperature	Drought
1999	18th May–31st July	882	853	624
2000	25th May–30th July	730	730	574
2001	27th May–31st July	639	639	526
2002	21st May–31st July	846	846	607
2003	17th May–6th June	576	576	434
	18th June–31st July			
2004*	16th June–13th August	636	636	566
	25th September–20th October			
2005*	15th June–14th September	607	607	431

*Precipitation was measured at the site for 1999–2003. For 2004 and 2005 precipitation was estimated based on monthly measurements for the region made by the Danish Meteorological Institute. The monthly measurements for Mols and for the region during 1999–2003 showed a good relationship ($Mols(precip) = 1.0555 \cdot Region(precip) - 6.5316$; $R^2 = 0.68$) which was used to estimate the precipitation in experimental field plots during 2004 and 2005 from the regional monthly measurements.

until extraction which was initiated within two weeks. The extraction was a modified version of O'Connor's wet funnel extraction (O'Connor, 1955), with a stepwise increase in temperature from 25 °C to 50 °C in five hours. The enchytraeids were collected in tap water and stored for 24–48 h at 5 °C before being identified and counted.

2.1.5. Abundance and biomass

Total number, biomass and species identification of the enchytraeids were all determined within 48 h after extraction. The total number of enchytraeids per m² was determined based on all sub-samples (integrating 0–9 cm depth), whereas biomass (dry weight; mg DW m⁻²) was determined for three of the six sub-samples. Biomass was determined after drying the worms at 60 °C for 24 h using a Sartorius Micro SC 2 balance with a precision of 0.01 mg (Sartorius AG, Goettingen, Germany). The remaining three sub-samples were used for species identification. The preliminary results suggested that *C. sphagnetorum* was the dominant species at the field site. This species was therefore the only species identified to species level whereas all other enchytraeids found in the final sampling were identified to genus level according to (Nielsen and Christensen, 1959, 1961, 1963).

2.1.6. Soil organic matter content

Soil from each sub-sample was after the wet extraction dried for 24 h at 80 °C and stored at room temperature until soil organic matter (SOM) content was determined by loss on ignition after 3 h at 600 °C in a furnace.

2.2. Laboratory experiment

2.2.1. Enchytraeid populations

The objective of the experiment was to examine for genetic adaptation to desiccation stress in the Mols populations. Therefore, *C. sphagnetorum* individuals used had to be of F1 or later generations, raised under similar conditions in order to separate the effects of the environment from those of genetics. From each experimental field plot, a laboratory population of *C. sphagnetorum* was established, i.e. three replicated populations from each treatment (control, temperature and drought). Between 10 and 50 individuals from each plot were allowed to reproduce in culture for a period of 31 weeks in order to obtain a sufficient number of adult worms (minimum of 35 segments, see Sjøgren et al., 1995). *C. sphagnetorum* were cultivated in moist soil, which consisted of 25% moor soil from the experimental study site mixed with 75% peat.

Prior to use, the soil was dried at 80 °C for 24 h to kill any animals present and then sifted through a 2-mm sieve to remove larger particles. Water was added so that the soil appeared moist. The worms were fed rolled oats and placed at 20 ± 1 °C until further experimentation. Food was given once a week and water lost by evaporation was replaced by adding demineralised water.

2.2.2. Experimental preparation

The soil used was a mixture of 67% peat and 33% soil collected at an agricultural site (Foulum, Denmark). Foulum soil was loamy sand consisting of 35% coarse sand, 45% fine sand, 9.4% silt, 8.9% clay and 1.7% organic matter. Prior to use, the soil was dried at 80 °C for 24 h and sifted through a 2-mm mesh. The levels of soil water potential used in the experiment were chosen based on relevant literature and the results of a preliminary experiment (data not shown).

Three days before the laboratory experiment was started three different soil water potentials were established representing low (-0.03 ± 0.00 bar; mean ± 95% C.I), medium (-3.03 ± 0.36 bar) and high (-7.75 ± 0.82) drought intensities, respectively. The drought treatments were created by adding appropriate amounts of water to dry soil followed by thorough mixing. The water used had been inoculated with natural assembly of micro-organisms from Mols soil. This was done by mixing 0.5 kg FW (fresh weight) top soil collected at the field site with 1 L demineralised water. The slurry was shaken for 1 h, sieved through a 50 µm mesh and diluted 1:7 with demineralised water. Ten g FW soil from each drought treatment (low, medium and high) were put into vials, followed by 15 mg rolled oats that was mixed into the soil. For each drought treatment, 36 vials were prepared. All vials were closed with perforated lids to allow air exchange, placed in a box and covered with damp cloth. The vials were kept at 15 ± 1 °C until addition of enchytraeids.

2.2.3. Laboratory experiment

At day 0 of the experiment, four *C. sphagnetorum* individuals of similar size originating from the same cultured enchytraeid population were added randomly to each of the 12 vials (low, medium and high drought treatment). Per laboratory drought treatment this resulted in 12 replicates for each enchytraeid population formerly exposed to one field climate treatment (drought, warming or control). All the vials were weighed and kept at 15 ± 1 °C. Every two to three weeks were the samples weighed to ensure that soil water content remained constant. After 13 weeks the experiment was stopped and surviving worms were counted as described above. To document the initial and the final soil water potential and soil moisture, five replicates of each soil treatment without worms were included in the experiment. The soil water potential of moist soil was determined by use of a soil moisture 2710 tensiometer (Gravquick, Herlev, Denmark) covering the range 0 to -1 bar (accuracy ± 0.02 bar). For medium and dry soils the soil water potential was determined by a psychrometric vapour pressure depression technique using Wescor C-52 sample chambers connected to a Wescor HR 33 T Dew Point Microvoltmeter operated in the dew point mode (Wescor, Logan, UT, USA). An overview of the soil moisture and soil water potential used in the experiment is presented in Table 2.

2.3. Statistics

2.3.1. Field experiment

The results were analysed as a randomized ANOVA block design using the GLM model procedure with type III sum of squares. One-way ANOVA with treatments as fixed factors and blocks as random factors were applied to test the effects of the main factors on the density and biomass of enchytraeids. A pairwise comparison

Table 2
Moisture and soil water potential of the test soils used for laboratory drought tolerance experiment ($n = 3-5$, mean \pm 95% C.I.)

	Intensity of drought		
	Low	Medium	High
Nominal moisture (% of dry mass)	166.00	66.00	50.00
Initial moisture (% of dry mass)	188.5 \pm 1.5	80.1 \pm 1.7	50.5 \pm 0.2
Finally determined moisture (% of dry mass)	180.8 \pm 7.17	74.1 \pm 0.9	57.1 \pm 1.4
Soil without addition of water (% of dry mass)	7.9 \pm 0.2	7.9 \pm 0.2	7.9 \pm 0.2
Initial soil water potential (bar)	-0.03 \pm 0.00	-3.03 \pm 0.36	-7.75 \pm 0.82
Finally soil water potential (bar)	-0.03 \pm 0.00	-2.47 \pm 0.32	-3.79 \pm 0.64

between treatments was performed using the post hoc Tukey test in the PROC GLM procedure.

The composition of the enchytraeid community was analysed by PRIMER version 5.0 (Plymouth Routines In Multivariate Ecological Research, PRIMER-E Ltd. Plymouth). All species abundance data were square root transformed in order to reduce the influence of very abundant species and consequently increase the contributions of rare species. A Bray-Curtis similarity matrix was subsequently calculated from the transformed species abundance (Clarke and Green, 1988; Clarke, 1999). To test for effects of warming or drought an Analysis of Similarities (ANOSIM) was performed. Finally, a SIMPER-procedure, which quantifies the contribution of the species to any observed changes, was conducted (Clarke and Green, 1988; Clarke, 1999).

2.3.2. Laboratory experiment

A two-way ANOVA, with drought level and population as fixed factors, were applied to test the effect of drought and field treatment (the genetic component) on mean survival.

For both field and laboratory studies, data was transformed (either by log or square root transformation) to improve normality and homogeneity of variance if needed. Statistics were carried out using SAS Enterprise Guide 3.0 (SAS, 2007) and for all tests, a significance level of 0.05 was applied.

3. Results

3.1. Field experiment

3.1.1. Abundance and biomass of enchytraeids

The total number of enchytraeid worms (ANOVA; $P < 0.0001$) and the number of worms in the upper 0–3 cm were significantly influenced by the treatments (ANOVA; $P < 0.0001$). This was due to a significant reduction in the drought treated plots (Table 3). Warming had no significant effect on the number of worms at any soil depth. *C. sphagnetorum* was the most abundant species in the 0–3 cm layer and contributed more than 90% of the total enchytraeid population. However, below 3 cm, the number and percent dominance of this species rapidly declined (Figs. 1 and 2). The

Table 3

Mean numbers per m^2 of enchytraeids in different depths and total number of enchytraeid genera at the Mols site ($n = 3$, mean \pm C.I. 95%)

Soil layer	Number of enchytraeids ($\times 10^3 m^{-2}$)		
	Control	Temperature	Drought
0–3 cm	30.4 \pm 17.0 ^a	39.7 \pm 26.7 ^a	6.5 \pm 0.6 ^b
3–6 cm	4.7 \pm 5.9	5.4 \pm 2.5	2.9 \pm 3.7
6–9 cm	0.5 \pm 0.7	1.6 \pm 0.6	0.9 \pm 1.2
0–9 cm	35.6 \pm 17.8 ^a	46.7 \pm 27.4 ^a	10.4 \pm 5.7 ^b
Number of genera	1.9 \pm 0.6	2.9 \pm 0.6	1.9 \pm 0.6

Different letters signify significant differences between mean values of the different treatments (ANOVA, Tukey; $P < 0.05$).

genus *Enchytronia* was dominant in the 3–6 cm layer. Nevertheless, there was no significant difference in the relative vertical distribution of *C. sphagnetorum* between the treatments (Fig. 1). The abundance of *C. sphagnetorum* in the upper 0–3 cm and of *Enchytronia* spp. in 3–6 cm depth were both negatively affected by drought (Fig. 2).

The drought treatment significantly reduced the biomass of enchytraeids (Table 4), both in the total soil column (ANOVA, Tukey; $P = 0.017$) and in 0–3 cm depth (ANOVA, Tukey; $P < 0.0001$). The biomass of the enchytraeids was not influenced by temperature neither in the total soil column nor in the different depths.

The soil organic matter (SOM) content was not significantly influenced by treatment (ANOVA; $P = 0.16$) (Table 5). However, the number of enchytraeids was positively correlated with SOM content (linear regression, control: $R^2 = 0.395$; $P < 0.0001$, temperature: $R^2 = 0.295$; $P < 0.0001$ and drought: $R^2 = 0.1217$; $P < 0.0097$).

3.1.2. Effects on the enchytraeid community

In total, five genera were found at the site. In addition to *C. sphagnetorum* and *Enchytronia* spp. also *Achaeta* spp., *Buchholzi* spp. and *Fridericia* spp. were found, although in low numbers. All five genera were found in the three treatments. The community structure in the three treatments was significantly different as illustrated by a MDS ordination plot (Fig. 3). The ANOSIM analysis (global $R = 0.35$, $P = 0.001$) showed that the control and warming treated plots ($R = 0.108$, $P = 0.09$) were not statistically separable. However, the drought treated plots and the control plots ($R = 0.408$, $P = 0.0001$) as well as the drought treated plots and warming treated plots ($R = 0.496$, $P = 0.0002$) were clearly separated. The SIMPER analysis revealed that these differences were due to a reduced density of *C. sphagnetorum* and *Enchytronia* spp., which explained more than 80% of the dissimilarity between the plots.

3.2. Laboratory experiment of drought tolerance

C. sphagnetorum was highly sensitive to even small decreases in soil water potential (Fig. 4).

The numbers surviving at both medium and high drought stress levels were significantly ($P < 0.0001$) different from those at low drought (Fig. 4). Even the mildest drought level (-0.03 bar) affected population as no fragmentation was observed. No significant population effect ($P = 0.324$) was observed showing that tolerance of drought was not significantly different in *C. sphagnetorum* originating from the three field treatments.

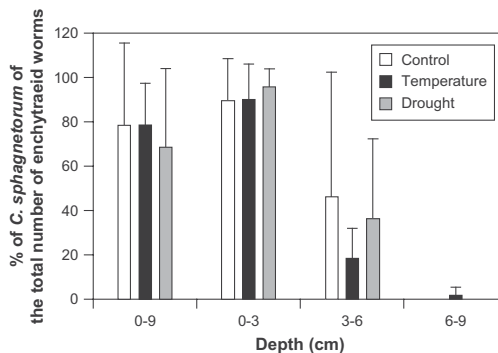


Fig. 1. The relative distribution of *C. sphagnetorum* ($\times 10^3 m^{-2}$) ($n = 3$, mean \pm C.I. 95%) from the control, temperature and drought treated plots. Samples were taken in the depth of 0–3, 3–6 and 6–9 cm are the sum of all three depths. Different letters signify significant differences between mean values (ANOVA, Tukey, $P < 0.05$).

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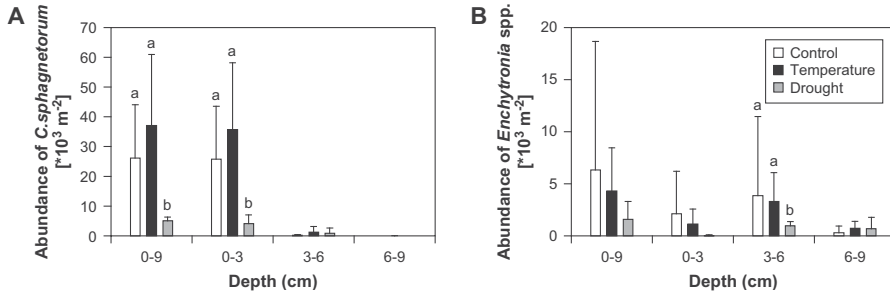


Fig. 2. The vertical distribution of *C. sphagnetorum* (A) and *Enchytronia* spp. (B) ($\times 10^3 \text{ m}^{-2}$) ($n = 3$, mean \pm C.I. 95%) from the control, temperature and drought treated plots. Samples were taken in the depth of 0–3, 3–6 and 6–9 and 0–9 cm are the sum of all three depths. Different letters signify significant differences between mean values (ANOVA, Tukey, $P < 0.05$).

4. Discussion

4.1. Has adaptation to drought occurred?

With this study we have tried to address the important question of whether a population is able to adapt to changing climatic conditions in its habitat, or whether populations will suffer from stress and decline in numbers over a longer period. *C. sphagnetorum* used in this experiment had been exposed to enforced drought stress in the field for several generations. As an estimate, we propose that more than 12 generations have elapsed during the six years of the experiment taking into account that the life cycle time in *C. sphagnetorum* may be variable depending on temperature and moisture conditions (Springett, 1967; Standen, 1973; Briones et al., 1997). Thus, we expected to see an increased tolerance of drought as a result of directional selection, in the populations of *C. sphagnetorum* exposed to repeated drought. However, nothing in our results points in this direction. On the contrary, populations from drought plots had the same high sensitivity to small reductions in soil water potential as those from control plots. This suggests that enforced drought had not resulted in selection, perhaps because genetic variation in this trait is not present. Even though *C. sphagnetorum* is reproducing asexually it could have a high level of clonal diversity as seen in the parthenogenetic earthworm, *Den-drobaena octaedra* (Hansen et al., 2006), but this need further investigation. It is also possible that *C. sphagnetorum* need more than six to 12 generations to adapt. Studies of *Drosophila* have shown that many species can rapidly adapt to increased drought whereas some species have no potential for adaptation to desiccation stress (Hoffmann et al., 2003).

However, our results are consistent with field observations where the abundance and biomass of enchytraeids, and *C. sphagnetorum* in particular, was much lower in the drought treated plots (Lindberg et al., 2002). Thus, it becomes evident that *C. sphagnetorum* populations of the drought treated plots went through

bottlenecks, but apparently without any detectable genetic adaptation to increased drought tolerance. It is therefore possible that this species could become extinct at Mols if summer droughts in a future climate become harsher and more frequent.

4.2. Effects of drought on field populations

Soil moisture is clearly an important factor for enchytraeid populations in various habitats (Nielsen, 1955a; Abrahamsen, 1972). Both short- and long-term drought can result in population reductions through mortality and impeded growth and reproduction (Nielsen, 1955b; Springett et al., 1970; Abrahamsen, 1971; Standen, 1980). So far, only one study of long-term effects of repeated drought exists (Lindberg et al., 2002). These authors reported a 90% reduction in enchytraeid populations after eight years of repeated drought in a Swedish coniferous forest. The severe reduction in number and biomass observed in this study due to drought does not necessarily reflect a general decrease throughout the years, as the enchytraeids could have recovered during the winter and spring following the drought treatment. However, the dominant species *C. sphagnetorum* is a slow growing species at low temperature (Standen, 1973) suggesting that the drought treatment probably affected the population over a large part of the years. It should also be noted that the severity of the drought treatments depended on the year to year variation in temperature and precipitation.

Enchytraeids can avoid adverse environmental conditions by migration to moister habitats, but this movement can only be a short-term strategy unless the food quantity and quality in the new habitat can maintain or increase the population size (Springett et al., 1970; Uhia and Briones, 2002). *C. sphagnetorum* prefers soil with high levels of SOM (Standen and Latter, 1977). At the experimental field, SOM was 80% lower in the 3–6 cm layer compared to the 0–3 cm layer. We therefore assume that the food quantity and quality was not sufficient to support the population of *C. sphagnetorum* in the deeper soil layers. Soil can act as a thermal and hydric buffer and soil biotic communities may be less sensitive to changes in air temperature and dry periods than surface communities

Table 4

Mean biomass of enchytraeids per m^2 in different depths at the Mols site ($n = 3$, mean \pm C.I. 95%)

Soil layer	Biomass of enchytraeids (mg DW m^{-2})		
	Control	Temperature	Drought
0–3 cm	601.6 \pm 353.8 ^a	547.2 \pm 440.5 ^a	192.1 \pm 95.0 ^b
3–6 cm	24.3 \pm 18.8	40.2 \pm 38.6	26.1 \pm 43.7
6–9 cm	2.9 \pm 3.7	8.39 \pm 1.7	7.1 \pm 10.3
0–9 cm	630.6 \pm 361.1 ^a	642.6 \pm 378.6 ^a	225.3 \pm 148.8 ^b

Different letters signify significant differences between mean values of the different treatments (ANOVA, Tukey; $P < 0.05$). Where no letters are shown no significant differences were found.

Table 5

Soil organic matter content (SOM) ($n = 3$, mean \pm 95% C.I.) of control, temperature and drought plots at Mols, Denmark after six years of treatment

Soil layer	Average SOM (% of dry weight)		
	Control	Temperature	Drought
0–3 cm	35.4 \pm 12.5	21.8 \pm 3.4	24.0 \pm 15.1
3–6 cm	6.7 \pm 1.8	4.6 \pm 0.4	5.8 \pm 1.5
6–9 cm	2.9 \pm 0.5	2.2 \pm 0.1	2.7 \pm 0.5

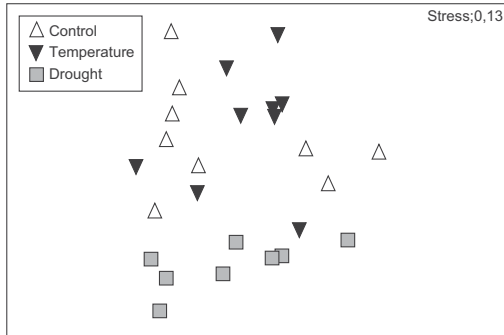


Fig. 3. Ordination plots of the enchytraeid community at the Mols. Samples from the control plots (Δ), temperature treated plots (\blacktriangledown) and drought treated plots (\blacksquare). The symbols represent the total community in the soil column. The distance between the points corresponds to the enchytraeid species composition: the shorter the distance, the higher similarity between treatments.

(Whitford et al., 1981). However, during severe droughts, as used in this study, enchytraeids may experience great difficulties in finding moist microhabitats, and will therefore inevitably be exposed to decreasing and stressful soil water potentials that would result in high mortality.

Many enchytraeid species show strong seasonal fluctuations, whereas it has been observed that the number of *C. sphagnetorum* often remains relatively constant during the year (Springett et al., 1970; Standen, 1973). The observed seasonal fluctuation in numbers of other species is probably connected to different drought tolerance strategies. Some enchytraeid species exposed to increased drought produce desiccation tolerant cocoons while adult worms die (Nielsen, 1955b; Springett et al., 1970). None of the *C. sphagnetorum* from Mols were observed with a clitellum and we conclude, that *C. sphagnetorum* lacks this strategy.

4.3. Effects of temperature on field populations

In a long-term study (six years) in a northern coniferous forest no effects of warming were found on the number of *C. sphagnetorum* (Haimi et al., 2005b). This is consistent with our results even though the mean annual temperature was increased by 2 °C in their study compared with 0.8 °C in our study. However, another short-

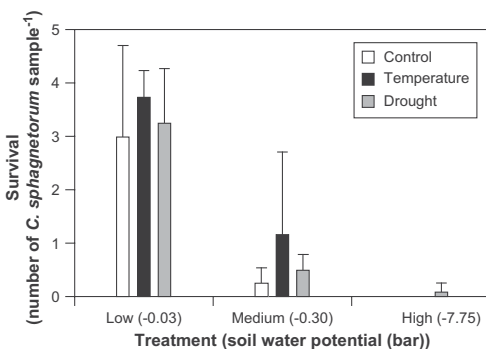


Fig. 4. Survival of *C. sphagnetorum* ($n = 4$) exposed to low, medium or high drought intensity. Four *C. sphagnetorum* were added to each test container. They were progeny (F1–F3 generation) of specimens collected in control, temperature treated and drought treated plots from the field site and cultured in the laboratory.

term study (one year) using a mean annual temperature increase of 2.5 °C, showed that increased temperature can have stimulatory effect on enchytraeids (Briones et al., 1997). These differences in response to warming may be related to genetic variation in *C. sphagnetorum* and to other climatic conditions at the studied sites. Increased temperature can increase the evaporation from the soil and thereby decrease the soil moisture. But the low temperature increase observed at Mols did not affect the enchytraeid population.

4.4. Indirect effects of temperature and drought (trophic relationships)

The community structure of the enchytraeids was affected by the enforced drought treatment, and this was due to a reduction of *C. sphagnetorum* and *Enchytronia* spp. and not the loss of a particular species/genus. However, this result could be biased because only *C. sphagnetorum* was identified to species, whereas the other specimens were identified only to genus level. We may therefore have lost valuable information of more drought and warming sensitive or tolerant species. Lindberg et al. (2002) did not observe any changes in the species composition after eight years of drought treatment similar to the one used in the present study. However, only two species dominated the habitat in the study of Lindberg et al. (2002). In a comparative ecological analysis of Norwegian coniferous forest soils a higher density of *Achaeta* sp., *Enchytronia parva* and *Enchytraeus norvegicus* was observed in dry compared to wetter soils (Abrahamsen, 1972). This suggests that other factors are interacting with the treatment effects and that the same species in different habitats can respond differently to a treatment (Gröngröft and Miehlén, 1983). Increased temperature and prolonged drought period affects also other components of the terrestrial ecosystem e.g. plants, macro-fauna and micro-organisms, which the enchytraeids interact with or feed upon. It has been proposed that drought can lead to an increase in the microbial C/N ratio, suggesting a change towards a more fungi-dominated microbial community and decomposition of more complex substrates (Jensen et al., 2003). This could lead to fungivorous enchytraeid species being favoured at the expense of other species. Thus, the enchytraeid community could be both directly but also indirectly affected by more frequent drought episodes due to change in food quantity and quality.

Important parameters like SOM and vegetation (personal obs., I.K. Schmidt) were all influenced by the applied treatments in the field experiment, and changes in these factors would probably also affect the composition of the enchytraeid communities.

4.5. Conclusion and perspectives

We had expected that enchytraeid populations exposed to increased drought stress for six years would have increased their tolerance to these adverse conditions by adaptation, however, this had apparently not occurred in the dominant species, *C. sphagnetorum*. The effect of enforced summer drought had a strong negative influence on enchytraeids, whereas the effect of moderate warming was negligible in the present study. Drought also affected species composition with a decreased abundance of the two dominant species. Our results corroborate the predictions of Briones et al. (2007) suggesting that the functionally important enchytraeid species in heathland and coniferous forest ecosystems, *C. sphagnetorum*, could be at risk if the prevailing predictions of future climate holds true. This could have implications for the decomposition processes in the soil of these terrestrial ecosystems on the long term. Climate change models predict that warming will occur in combination with increased occurrence of drought and increased atmospheric CO₂ levels. In future studies it will therefore

be important to evaluate how these factors in combination will affect the populations of soil organisms.

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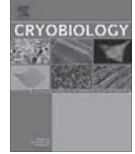
Paper 5

**Freeze tolerance and accumulation of cryoprotectants
in the enchytraeid *Enchytraeus albidus* (Oligochaeta)
from Greenland and Europe**



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Freeze tolerance and accumulation of cryoprotectants in the enchytraeid *Enchytraeus albidus* (Oligochaeta) from Greenland and Europe [☆]

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ABSTRACT

The freeze tolerance and accumulation of cryoprotectants was investigated in three geographically different populations of the enchytraeid *Enchytraeus albidus* (Oligochaeta). *E. albidus* is widely distributed from the high Arctic to temperate Western Europe. Our results show that *E. albidus* is freeze tolerant, with freeze tolerance varying extensively between Greenlandic and European populations. Two populations from sub Arctic (Nuuk) and high Arctic Greenland (Zackenber) survived freezing at $-15\text{ }^{\circ}\text{C}$, whereas only 30% of a German population survived this temperature. When frozen, *E. albidus* responded by catabolising glycogen to glucose, which likely acted as a cryoprotectant. The average glucose concentrations were similar in the three populations when worms were frozen at $-2\text{ }^{\circ}\text{C}$, approximately $50\text{ }\mu\text{g glucose mg}^{-1}$ tissue dry weight (DW). At $-14\text{ }^{\circ}\text{C}$ the glucose concentrations increased to between 110 and $170\text{ }\mu\text{g mg}^{-1}$ DW in worms from Greenland. The average glycogen content of worms from Zackenberg and Nuuk were about $300\text{ }\mu\text{g mg}^{-1}$ DW, but only $230\text{ }\mu\text{g mg}^{-1}$ DW in worms from Germany showing that not all glycogen was catabolised during the experiment. Nuclear magnetic resonance spectrometry (NMR) was used to screen for other putative cryoprotectants. Proline, glutamine and alanine were up regulated in frozen worms at $-2\text{ }^{\circ}\text{C}$ but only in relatively small concentrations suggesting that they were of little significance for freeze survival. The present study confirms earlier reports that freeze tolerant enchytraeids, like other freeze tolerant oligochaete earthworms, accumulate high concentrations of glucose as a primary cryoprotectant.

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Introduction

Enchytraeids are small oligochaete earthworms mainly living in the top layer of the soil. Here they play an important role in decomposition of dead plant material. The species *Enchytraeus albidus* is widely distributed from the high Arctic to temperate Western Europe and can be found in organically rich environments such as decaying seaweed, compost and sewage beds [4,19].

Ectothermic invertebrates living in temperate and Arctic areas may experience extended periods of temperatures below the melting point of their body fluids. Three different strategies exist whereby ectothermic invertebrates can cope with subzero temperatures. One strategy, freeze tolerance, is to establish controlled, protective freezing of the extracellular body fluids at high subzero temperatures. Another strategy, freeze avoidance, is based on the ability to stay in a supercooled state even at temperatures much below the melting point of body fluids. For small permeable soil

invertebrates there is a third option, cryoprotective dehydration. Through dehydration such organisms rapidly equilibrate their melting point to the surrounding temperature in a frozen environment [12,23,32].

Despite enchytraeids' importance for decomposition in areas where the soils are frozen for long periods during winter, the physiological and biochemical adaptations to subzero temperatures have only been studied in a few species. Specimens of the enchytraeid, *Stercutus niveus*, survived a short exposure in frozen litter at -4 to $-5\text{ }^{\circ}\text{C}$ [8]. Although this and other studies suggest that many species can survive in frozen soil and litter, only a few species have been explicitly shown to survive by freeze tolerance [16,22,27]. Because enchytraeids are small and their cuticle is very permeable for water, they may use cryoprotective dehydration as a survival strategy. A study by Sømme and Birkemoe [27] reports that both freeze tolerance and cryoprotective dehydration seem to exist in enchytraeids and that the strategy depends on the surrounding thermal and hygric conditions [27]. This ability to choose between freezing or dehydration, depending on the surrounding conditions, is also known from the nematode, *Panagrolaimus davidi* [29] and the enchytraeid, *Fridericia ratzeli* [22]. However, it seems unlikely that enchytraeids can survive prolonged periods of frost

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in their natural environment by supercooling because of the intimate contact with soil ice which inevitably will result in inoculative freezing.

Freeze tolerant animals undergoing freezing of their body fluids and animals using cryoprotective dehydration face the problems of dehydration of cells; therefore they require physiological and biochemical mechanisms that protect them against dehydration injuries [12]. A well known protection mechanism is the accumulation of cryoprotectants such as sugars and polyols, but also amino acids may have cryoprotective abilities [30,32]. Glucose is a widely occurring cryoprotectant in oligochaetes probably because it is the primary blood sugar of these animals [14]. Accordingly, glucose has been shown to accumulate in frozen individuals of the enchytraeid, *F. ratzelei* [16].

The aim of the present study was to investigate if *E. albidus* is a freeze tolerant species, and to explore which types of low molecular weight cryoprotectants are produced by the species, if any. An earlier work by Kähler [18] reported that *E. albidus* from the coast of the Wadden Sea (Northern Germany) were able to survive for a few hours in sea water frozen to -13°C . However, the study did not reveal whether freeze tolerance was the basis for winter survival during ecologically realistic periods of time. We therefore examined survival of *E. albidus* subjected to natural cooling rates in frozen soil at various subzero temperatures. Nuclear magnetic resonance spectrometry (NMR) was used to screen for putative cryoprotectants of individuals subjected to -2°C in soil as a basis for further chemical analysis of selected metabolites. Further, the supercooling ability, water content and melting point of cold acclimated worms was investigated.

Material and methods

Test animals

The two populations from Greenland (Zackenberglund and Nuuk) were collected in 2004 and 2007, respectively. Both populations were found in decaying seaweed and transported back to the laboratory in the seaweed. The specimens of both populations were identified as *E. albidus* by an expert in enchytraeid taxonomy (Prof. Bent Christensen, University of Copenhagen, Denmark). A German population also used for experiments was a laboratory culture, which was obtained in 2005 from a fish food company (Tierfischfutter, Jena, Germany). The three populations were kept in moist soil at $15 \pm 1^{\circ}\text{C}$ and weekly fed oatmeal ad libitum. The soil consisted of a mixture of 75% loamy sand and 25% peat. The loamy sand consisting of 35% coarse sand, 45% fine sand, 9.4% silt, 8.9% clay and 1.7% organic matter. Prior to use, the soil was dried at 80°C for 24 h and sieved through a 2 mm mesh. Appropriate soil moisture was achieved by adding 0.8 ml demineralised water per g dry soil. All three populations were cold acclimated at 2°C for 36 days prior to the experiment. Pilot experiments had shown that this cold acclimation protocol induces freeze tolerance in *E. albidus*.

Osmolyte identification

NMR was used to screen for osmolytes up regulated in frozen worms. From the German population, five cold acclimated worms (2°C) and five worms frozen at -2°C for 5 days were sampled for identification of osmolytes.

Enchytraeids from each treatment were pooled in a 1.5 ml Eppendorf tube and freeze dried for 24 h after which the dry weight was determined. Dried worms were crushed and extracted in 0.25 ml 70% ethanol. Samples were centrifuged at 20,000g for 5 min at 4°C and the supernatant saved for measurements. The pellet was re-suspended and the procedure was repeated three

times after which the pooled supernatants were dried at 60°C for 24 h and stored at -80°C until further analysis.

The samples were re-suspended in 650 μl of 50 mM phosphate buffer made up in D_2O (pH 7.4). The samples were vortexed and 600 μl were transferred to a 5 mm NMR tube. The buffer contained 50 mg l^{-1} of the chemical shift reference 3-(trimethylsilyl)-propionic acid-D4, sodium salt (TSP). NMR measurements were performed at 25°C on a Bruker Avance-2 700 spectrometer, operating at a ^1H frequency of 700.09 MHz, and equipped with a 5 mm HCN triple resonance probe. ^1H NMR spectra were acquired using a single 90° -pulse experiment with a Carr-Purcell-Meiboom-Gill (CPMG) delay added in order to attenuate broad signals from high molecular weight components. The total CPMG delay was 40 ms and the spin-echo delay was 200 μs . Water was suppressed by pre-saturation during the relaxation delay of 1.5 s. A total of 256 transients of 16 K data points spanning a spectral width of 24 ppm were collected, corresponding to a total experiment time of 10 min. For assignment purposes a 2-dimensional ^1H - ^1H TOCSY spectrum with 80 ms mixing and ^1H - ^1H NOESY with 1 s mixing were acquired. The spectra were processed using INMR (www.inmr.net). An exponential line-broadening of 0.5 Hz was applied to the free-induction decay prior to Fourier transformation. All spectra were referenced to the TSP signal at -0.017 ppm and baseline corrected.

Experimental protocol

Five worms were placed in small vials containing 5 g of the moist soil used for cultures and 20 mg oatmeal. The vials were, with exception of the controls ($+2^{\circ}\text{C}$), transferred to a walk-in freezer at a temperature of $-1.3 \pm 0.2^{\circ}\text{C}$. Five replicates of five enchytraeids were used for each treatment. To ensure that all worms froze at the same temperature, an ice crystal was added after 24 h at -1.3°C , which has been shown to ensure inoculative freezing in other worms, irrespectively of their supercooling point being lower than this temperature [2]. Subsequently the vials were transferred to four custom made programmable cooling cabinets preset at -2°C and accurate to $\pm 0.2^{\circ}\text{C}$. In one cabinet the temperature was kept at -2°C , in the other three the temperature was gradually lowered ($-0.042^{\circ}\text{C h}^{-1}$) until temperature had reached -8 , -14 and -15°C , respectively. They were kept at their target temperature until six days after the coldest cabinet had reached its final temperature (-15°C). In that way each group remained at subzero temperatures for equal time, namely 25 days. Afterwards, all vials were placed at $+2^{\circ}\text{C}$ to thaw and after 24 h the survival was determined. The worms that reacted normally to tactile stimuli and showed no visible freezing damage were scored as survivors.

In order to investigate accumulation of cryoprotectants, representative worms were sampled during the experiment. The sampled worms were quickly cleansed of excess soil, frozen in liquid nitrogen and stored at -80°C until analysis. The concentrations of glucose, glycogen and the amino acid alanine were determined for controls (2°C) and frozen worms (-2°C) of all three populations. In addition, glucose was measured for the Nuuk and Zackenberg populations frozen at -14°C . Glucose was not measured for the German worms frozen at -14°C where high mortality was observed. Since it was impossible to distinguish between live and dead animals immediately upon thawing such measurement seemed inappropriate.

Quantification of cryoprotectants and glycogen

Glucose and glycogen analysis was carried out as described by Overgaard et al. [21] using spectrophotometrically based enzymatic test kits.

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Alanine, glutamine, proline, an un-identified compound which was probably an alanine-bound residue (X-alanine), and another unidentified metabolite (Y) were identified as possible osmolyte candidates from the NMR spectra. Only alanine was quantified, however, because our preliminary studies suggested that concentrations of glutamine and proline were below the detection limit of the applied TLC method and possibly too low to have relevance as a cryoprotectant.

Alanine was quantified by Thin Layer Chromatography (TLC) using a slight modification of the method described by Hjorth et al. [10]. Each sample (approximately 5 mg DW) was extracted in 1.4 ml 10% ethanol and then homogenised on ice using an ultrasonic homogenizer (Ultrasonic Homogenizer, Cole-Parmer Instruments, Illinois, USA). The homogenate was left on ice for 10 min, spun for 5 min at 20,000g and the supernatant stored in a 5 ml glass vial. This procedure was repeated three times and the pooled supernatants were freeze dried for 24 h and stored at -20°C . The samples were protected from light during the extraction.

The freeze dried extracts were dissolved in 400 μl 10% ethanol and insoluble residuals were removed by ultrasonic homogenisation for 15 min followed by filtration through a polypropylene 0.45 μm filter (Whatman). Ten microlitres samples of the extract were applied to HPTLC-plates with a 0.1 mm layer of cellulose (Merck 16092) in 12 mm bands, 15 mm from the lower edge of the plate using an Automatic TLC sampler 4 (CAMAG, Muttenz, Switzerland). The extracts from the German worms were applied undiluted whereas the extracts from Nuuk and Zackenberg worms were diluted (dilution factor 5–11). The plates with German and Nuuk extracts were developed in eluent with the following composition 1-butanol: formic acid: deionised water (40:10:10). For the Zackenberg extract it was necessary to use a different eluent with following composition: 1-propanol: 0.6 mol l^{-1} NaCO_3 : deionised water (31:2:12) to separate alanine from an un-identified amino acid. Alanine was identified using co-dimensional chromatography as described previously [10].

Water content, melting point and supercooling point

Dry weight, water content, melting point and supercooling point were determined for cold acclimated (2°C) worms. Water content (WC) of individual enchytraeids was calculated from measurements of fresh weight and dry weight after drying for 24 h at 60°C . Twelve individuals from each population were used. The weighing was carried out using a Sartorius Micro SC 2 balance accurate to $\pm 1 \mu\text{g}$ (Sartorius AG, Goettingen, Germany).

The body fluid melting point of single individuals were measured by quickly placing an enchytraeid in the sample holder of a Wescor C-52 sample chamber connected to a Wescor HR 33 T Dew Point Microvoltmeter (Wescor, Logan, Utah) operated in the dew point mode as previously described [17]. Melting point of six individuals from each population was determined.

The supercooling points were measured using copper-constantan thermocouples as described by Pedersen and Holmstrup [22]. The enchytraeids were surface dried with filter paper and attached to the thermocouple by means of adhesive tape. The cooling rate was approximately $1^{\circ}\text{C min}^{-1}$. Supercooling points of seven individuals from each population was determined.

Calculations

The effect of glucose accumulation on melting point depression and thereby the ice content at different freezing temperatures was estimated using the assumption that 64% of the worm's water content could be regarded as osmotically active water [3]. Another assumption was that all glucose was osmotically active resulting in 1 M of glucose being equivalent to approximately 1 Osmol kg^{-1} .

The melting point depression was then calculated using the osmolar melting point depression constant ($-1.86^{\circ}\text{C Osmol}^{-1} \text{kg water}$). The ice fraction at a given temperature, F , was calculated according to the formula: $F = 1 - (\text{MP}/T)$, where MP is the melting point and T is ambient temperature.

Statistical methods

Two-way ANOVA was used to test for treatment and population effects, and interaction of population and treatment on glycogen, glucose and alanine concentrations. Differences in water content, melting point and supercooling point between populations were tested by one-way ANOVA. To show significant differences in pair-wise comparisons a Tukey post hoc test was used. Some data were transformed prior to statistical analysis to improve normality. All statistical analyses were performed using Sigmasat for Windows Version 2.03 (SPSS Inc., Chicago, IL). All results are presented as mean \pm SE.

Results

Freeze survival

Immediately after removing the vials from freezing cabinets specimens were without any signs of activity but seemed to have normal water content. The populations from Nuuk and Zackenberg had 100% survival at all temperatures tested. The population from Germany was less freeze tolerant, it had 80% survival at -2°C , but the survival rate fell with lower temperatures (Fig. 1).

Cryoprotectants and glycogen

The NMR analysis of German *E. albidus* suggested that glucose, alanine, glutamine, proline, an unidentified alanine-bound metabolite (X-alanine), and another unidentified metabolite (Y) became up regulated as a response to freezing of body fluids (Fig. 2). The unidentified metabolites (X-alanine and Y) could both be part of a larger molecule as indicated by broader signals than for regular alanine, but firm conclusions could not be made from the NMR spectra. However, the NMR spectra did indicate that no other common cryoprotectants (e.g. glycerol, trehalose or sorbitol) were up regulated.

The average glucose concentrations of cold acclimated but unfrozen worms were in the range $1\text{--}2 \mu\text{g mg}^{-1}$ DW in all three

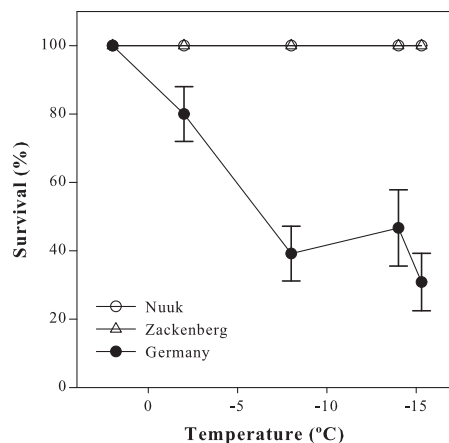


Fig. 1. Survival of *Enchytraeus albidus* (mean \pm SE, $n = 5$) from Nuuk, Zackenberg and Germany as a function of decreasing temperature.

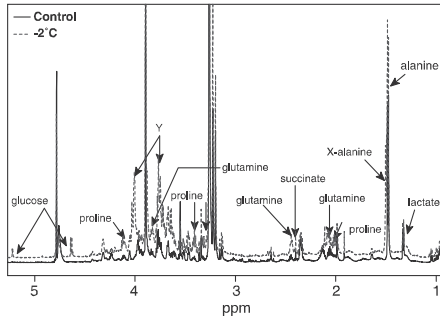


Fig. 2. NMR spectra of cold acclimated (2 °C; black line) and frozen *Enchytraeus albidus* (−2 °C; dashed line) from Germany. Metabolites that showed large increases or were suspected to do so, and are clearly visible are labelled. “Y” is an unknown metabolite that was up regulated at −2 °C.

populations (Fig. 3A). The glucose concentration increased significantly (ANOVA; $P < 0.001$) in response to freezing of body fluids and the average glucose concentration rose at −2 °C to 47.4 ± 2.5 , 37.8 ± 2.0 and $39.9 \pm 4.7 \mu\text{g mg}^{-1}$ DW for Nuuk, Zackenberg and Germany populations, respectively. At this temperature there were no significant differences in glucose between the populations (ANOVA; $P = 0.599$). The average glucose concentration in the worms at −14 °C, was significantly higher than the concentrations observed at −2 °C (ANOVA; $P < 0.001$). Furthermore the glucose concentration at −14 °C in the Zackenberg worms ($167.6 \pm 10.8 \mu\text{g mg}^{-1}$ DW) were significantly (ANOVA; $P = 0.007$) higher than in the Nuuk worms ($103.6 \pm 8.4 \mu\text{g mg}^{-1}$ DW).

The average glycogen concentration for the cold acclimated but unfrozen worms were 269.9 ± 9.7 , 320.3 ± 17.7 and $231.1 \pm 22.9 \mu\text{g mg}^{-1}$ DW for Nuuk, Zackenberg and Germany populations, respectively (Fig. 3B). Only Zackenberg and Germany differed significantly in concentration at 2 °C (ANOVA, Tukey; $P < 0.001$). In response to freezing, the glycogen concentration fell to levels significantly lower than those found before frost (ANOVA; $P < 0.001$) in all the populations. At −2 °C, the average concentrations were 190.7 ± 6.8 , 256.8 ± 11.3 and $89.4 \pm 15.4 \mu\text{g mg}^{-1}$ DW for Nuuk, Zackenberg and Germany populations, respectively, and all were significantly different from each other (ANOVA; $P < 0.001$).

Freezing at −2 °C caused a significant increase in the concentration of alanine (ANOVA, $P = 0.004$) in all three populations (Fig. 4) to about $7\text{--}10 \mu\text{g mg}^{-1}$ DW. There was no significant difference between the populations (ANOVA, $P = 0.114$).

Water content, melting point and supercooling point

The enchytraeids from Zackenberg had both the lowest dry weight and the lowest water content whereas the enchytraeids from Germany had the highest dry weight and the highest water content (Table 1). There was no significant difference in the melting point of body fluids at 2 °C (ANOVA; $P = 0.185$) or the supercooling point (ANOVA; $P = 0.665$) between the populations (Table 1).

Discussion

Freeze tolerance

The present study shows that *E. albidus*, when subjected to ecologically relevant cooling regimes, possesses a considerable tolerance to low temperatures over extended periods of time which has not been demonstrated before in any enchytraeid species. Kähler [18] exposed

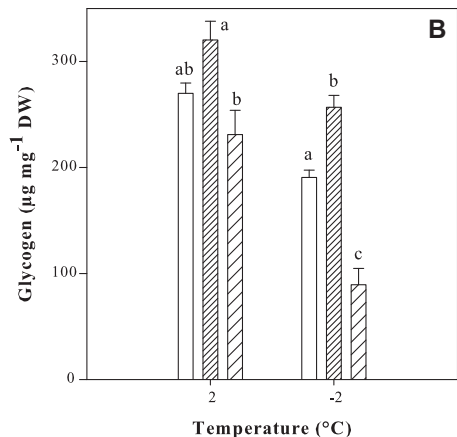
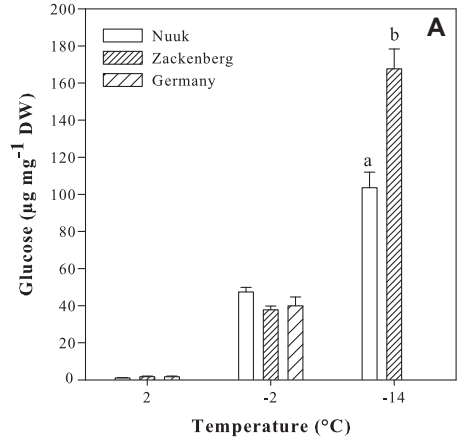


Fig. 3. Glucose (A) and glycogen (B) concentration (mean \pm SE, $n = 5$) of *Enchytraeus albidus* from Nuuk, Zackenberg and Germany, respectively, at different temperatures. Different letters indicate significant differences between populations within a given temperature. Glucose was not determined for the German population at −14 °C.

E. albidus to rapid cooling to −13.5 °C in sea water and observed 50% mortality already after 10 h but no information was given as to when the sea water froze or whether the exposed enchytraeids were in fact frozen. Bauer et al. [1] cooled *E. albidus* rapidly to −10 or −20 °C (2.5 °C min^{-1}) and observed no survival under these conditions. Until now, studies on cold tolerance of *E. albidus* have not revealed which cold strategy exists in this species. In the present study all specimens from the two Greenland populations survived temperature down to −15 °C and held at subzero temperatures for 25 days. The supercooling point of *E. albidus* was around −7 °C, and because of the inoculative freezing due to close contact with ice in the vials, no individuals could have survived by supercooling in this experiment. Moreover, since individuals immediately upon thawing seemed fully hydrated it is certain that all individuals had internal ice at the lower temperatures tested. We therefore conclude that *E. albidus* can survive winter frost using a freeze tolerance strategy.

Cryoprotective dehydration, a cold tolerance strategy known from other enchytraeid species [22], was not observed in the present study as the characteristic severe dehydration of speci-

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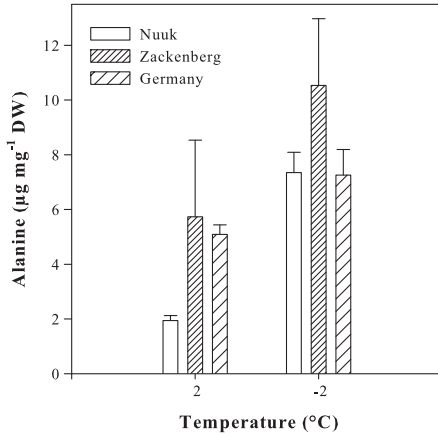


Fig. 4. Alanine concentration (mean \pm SE; $n = 5$) of *Enchytraeus albidus* from Nuuk, Zackenberg and Germany at 2 and -2 °C.

Table 1

The dry weight (DW), water content, melting point of body fluids and supercooling point of *Enchytraeus albidus* from Nuuk, Zackenberg and Germany

Location	DW (mg)	Water content (mg mg ⁻¹ DW)	Melting point (°C)	Supercooling Point (°C)
Nuuk	2.04 \pm 0.15 ^a	2.92 \pm 0.09 ^a	-0.95 \pm 0.07 ^a	-7.14 \pm 0.20 ^a
Zackenberg	0.94 \pm 0.08 ^b	2.58 \pm 0.08 ^b	-1.00 \pm 0.07 ^a	-7.30 \pm 0.14 ^a
Germany	1.38 \pm 0.18 ^c	3.48 \pm 0.11 ^c	-0.82 \pm 0.06 ^a	-7.04 \pm 0.26 ^a

Values are mean \pm SE. Different superscript letters indicate significant differences between populations when reading vertically.

mens was not observed. However, it can not be ruled out that *E. albidus* also has the ability to use cryoprotective dehydration as a survival strategy, but further experiments are needed to clarify this.

Geographic difference in freeze tolerance

Our results clearly demonstrate variations in freeze tolerance according to the geographic region of *E. albidus*. Specimens from Greenland were more freeze tolerant than German specimens. This is consistent with the fact that *E. albidus* from Greenland must cope with longer winters and lower temperatures, and therefore need a better freeze tolerance than the German worms. These results resemble the geographic variation seen in freeze tolerance of the earthworm *Dendrobaena octaedra* [15,24]. The test animals were kept for more than one generation under the same constant laboratory conditions, therefore the reason for the difference in freeze tolerance must be due to genetic adaption to the different environments, from where they were collected.

Enchytraeids from Zackenberg and Nuuk were much more freeze tolerant and had significantly lower water content than worms from Germany. Difference in water content could influence freeze tolerance because lower water content would give a higher concentration of glucose and hereby perhaps give a better protection against freezing.

Cryoprotectants

E. albidus accumulated glucose as the dominating cryoprotectant when frozen at subzero temperatures. At -2 °C, the average

glucose level increased to around 40 $\mu\text{g mg}^{-1}$ DW. At the same time glycogen levels fell by 70–142 $\mu\text{g mg}^{-1}$ DW in the three populations tested suggesting that glucose was produced by the rapid catabolism of glycogen. Indeed, glycogen is the principal source for mobilisation of carbohydrate cryoprotectants in many other cold hardy animals including freeze tolerant insects, earthworms and frogs [9,15,21,25,28].

In freeze tolerant earthworms there exist a correlation between the size of the glycogen storage and freeze tolerance [14,15]. A large amount of glycogen may be an advantage in relation to freeze tolerance, because it may maximise cryoprotectant production and possibly can be used as a source of energy during prolonged freezing of the body fluids. It seems reasonable that the same could be the case in *E. albidus*. Thus, Zackenberg worms had significantly higher glycogen storage than the less freeze tolerant German population. On the other hand, the greater drop in glycogen of German worms should be expected to result in a larger accumulation of glucose than observed at -2 °C. However, no obvious explanation for this discrepancy can be given.

At high subzero temperatures (-2 °C) *E. albidus* produced less glucose than the freeze tolerant earthworm *D. octaedra* which under the same circumstances may reach concentrations of 70–140 $\mu\text{g mg}^{-1}$ DW [15]. *D. octaedra* seems to accumulate glucose rapidly in response to subzero temperatures, and reach a maximum within one day at -2 °C. In this species the glucose concentration does not increase when temperature is further lowered [21,24]. In contrast we found that *E. albidus* continues to accumulate considerable amounts of glucose since at -14 °C the average glucose concentrations resemble the concentrations found in *D. octaedra* at -2 °C [15,21]. At -14 °C the average glucose concentration was significantly higher in the Zackenberg population than in the Nuuk population, however, this was not manifested in a differing freeze tolerance. Further studies using lower exposure temperatures are needed to determine if differences exist in freeze survival between the two Greenland populations.

In this study we found that *E. albidus* produces glucose as the primary cryoprotectant thus resembling most other freeze tolerant earthworms [13,14,21] and frogs [28]. Glucose acts as an osmolyte and thereby decreases the speed of ice formation and lower the ice fraction at a given temperature. Furthermore it contributes to the dilution of potentially toxic solutes by colligative means [20]. Glucose also has the ability to stabilize membranes and proteins during freeze-induced dehydration of cells [6,7].

In addition to glucose there was an up regulation of the amino acid alanine at -2 °C. The role of alanine is more ambiguous. Alanine is a compatible compound, in the meaning that it is not toxic even in high concentrations [30]. In its presence alanine contributes to increase the osmolality, and thereby lower the melting point, but compared to glucose, in this case, it plays a minor role. In other cases the accumulation of free amino acids such as proline may contribute to stabilize proteins and membranes [26] and alanine could have a similar function. Another possibility is that the up regulation in alanine is an effect of anoxia during freezing of the body fluids since alanine is a well known end-product of anaerobic metabolism [11]. However, it is uncertain whether enchytraeids experience anoxia in a frozen state at -2 °C, because of their small size and highly permeable skin.

The effect of glucose on the ice fraction

Since it has been shown for other animals that glucose accumulation enhances freeze tolerance [5] there can be no doubt that the high concentrations of glucose in *E. albidus* must play an important role in their ability to tolerate freezing of their body fluids. The increase in osmolality that follows the glucose

mobilisation lowers the melting point of the worms' body fluids, which consequently will decrease the amount of ice formed at equilibrium [31]. The melting point of *E. albidus* without glucose accumulation is approximately $-1\text{ }^{\circ}\text{C}$ (Table 1). Considering the average glucose levels in frozen worms, this melting point will by the glucose concentration at $-2\text{ }^{\circ}\text{C}$, in theory, be depressed by around $0.18\text{--}0.26\text{ }^{\circ}\text{C}$ depending on the population. Although this is only a minor depression of the melting point it will have considerable effect on the ice content. Based on the assumptions described earlier it can be estimated, that glucose accumulation at $-2\text{ }^{\circ}\text{C}$ will reduce the ice content by 25%, 23% and 16% in the Nuuk, Zackenberg and German worms, respectively. Reduced ice content helps to ensure controlled tissue dehydration due to extracellular freezing and ensure transport of glucose between tissues. Although concentrations were higher at $-14\text{ }^{\circ}\text{C}$, glucose would only reduce the ice content by 4% and 8% (Nuuk and Zackenberg worms, respectively), at this temperature. Nevertheless, this could still be important for survival. In addition to the depression of the melting point, increased glucose levels may also stabilise membrane and protein structures during the period where the body fluids are frozen [6,26]. To this end it should be noted that the concentration of glucose in the unfrozen part of the body fluids may reach Molar concentrations since only water contributes to the ice growth.

In conclusion, this study shows that *E. albidus* from Greenland was highly freeze tolerant; the German population also displayed freeze tolerance, but to a lesser extent. The extent of freeze tolerance could be related to the ability to synthesise glucose in high concentrations. Further, a difference in water content could also have influenced the glucose concentration. In future studies it would be interesting to investigate if *E. albidus* may utilise cryoprotective dehydration as a cold hardiness strategy and further explore the role of the unusually high glycogen contents both as a cryoprotectant and as a fuel for metabolism during the long Arctic winter.

Acknowledgments

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Photo: Kristine Maraldo

Paper 6

Effects of copper on enchytraeids in the field
under differing soil moisture regimes



EFFECTS OF COPPER ON ENCHYTRAEIDS IN THE FIELD UNDER DIFFERING SOIL MOISTURE REGIMES

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Abstract—The aims of this study were to investigate the combined effects of drought stress and copper pollution on enchytraeids under natural conditions in the field and to compare the results of laboratory toxicity tests with results of the field study. Such studies were conducted to increase the understanding of interactions between chemicals and natural stressors and assess the predictive value of standardized laboratory tests with enchytraeids. The combined effect of copper and summer drought on enchytraeids was investigated in an old copper-contaminated field site at Hygum, Denmark, in three areas with different copper burdens. Each area consisted of five plots, which were divided into two subplots: one control and one drought subplot in which precipitation was excluded for a 45-d period during summer. Enchytraeids were sampled in spring (before the enforced drought began) and in autumn (after recovery from drought). Clear effects of copper were evident in both the field and the laboratory experiment. The field population density and species composition was highly affected by copper at concentrations in the range 300 to 500 mg Cu/kg dry soil and higher. In particular, a greatly impoverished species diversity was found in the copper-polluted areas. The effects of copper in the field compared reasonably well with the results of the laboratory tests. Surprisingly, possible effects of summer drought in the field were not detected in the autumn sampling, perhaps because of rapid recovery of the enchytraeid populations in both unpolluted and copper-polluted areas.

Keywords—Drought Enchytraeid communities *Enchytraeus crypticus* Heavy metal Risk assessment

INTRODUCTION

Risk assessment of heavy metals and other soil pollutants is often based on toxicity data derived from simple single-species tests. These systems are controlled systems with chemical and physical factors (e.g., moisture and temperature) held constant typically at optimum conditions. However, a number of environmental conditions, such as aging, pH, organic matter (OM) content, species interactions and climatic variations, can differ between laboratory and field [1–3]. These variations could influence the bioavailability and hence the ecotoxicity of heavy metals or could alter the physiology and behavior of species and so interact with uptake and excretion rates of, for example, zinc and copper [4,5]. Because of these variations in conditions between field and laboratory, single-species test systems might not always give a reliable estimate of heavy metal effects on soil organisms.

Enchytraeids are widespread in temperate and cold soils. They are important for the decomposition of dead plant material and recycling of plant nutrients in soil [6,7]. Because of their ecological importance, a number of field studies have addressed heavy metal pollution effects on enchytraeids [8–11]. However, none have concerned copper alone, perhaps because metal-polluted areas are often contaminated with mixtures of heavy metals. Moreover, most of these studies report effects on a single species, *Cognettia sphagnetorum*, which is dominant in coniferous forest soil. Other soil ecosystems such as grasslands often harbor a large variety of enchytraeid species. Thus, there is a need to broaden the range of enchytraeid species in ecotoxicological studies.

One of the most important environmental factors deter-

mining the performance of soil fauna is the availability of water [12]. Enchytraeids are probably among the soil organisms most sensitive to desiccation [6], and during summer droughts, populations will typically reach their annual minimum as a result of desiccation stress [13]. A number of laboratory studies have shown that drought in combination with a toxic substance can lead to potentiation of effects; that is, the combination of the two stressors are larger than if the effects were additive [5,14,15]. No previous studies have investigated whether the effects of summer drought on enchytraeids are larger in areas with an existing pollution burden compared with unpolluted areas.

The objective of this study was to investigate the combined effect of copper and summer drought on enchytraeids in an abandoned arable field. Copper is known as an essential metal but can be toxic at increased soil concentrations if biologically available [16]. Three areas with background levels, medium copper contamination, and a high copper contamination were chosen for experimentation in an old copper-contaminated field site. In these areas, the effects of drought on the enchytraeid community were assessed. The field results were compared with results obtained in standardized laboratory tests, and the effects of copper on survival and reproduction in the enchytraeid *Enchytraeus crypticus* were evaluated to examine the predictive value of such tests commonly used in the risk assessment of toxic chemicals.

MATERIALS AND METHODS

The study consisted of a field study and a laboratory study with field-collected copper-contaminated soil and uncontaminated field soil spiked with copper. All experiments were conducted with soil from the Hygum site, Denmark. The Hygum site occupies a total of 7,200 m² and is situated on flat land

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at Hygum, Jutland, Denmark. At this site, timber preservation with CuSO_4 (no other toxic substances) took place from 1911 to 1924, resulting in a soil copper concentration gradient ranging from background levels up to about 3,400 mg Cu/kg soil. From 1924 to 1993, the field was plowed and harrowed every year, and the copper present in the soil was therefore homogeneously mixed in the upper approximately 25 cm of the soil. The soil type at Hygum (0–20 cm depth) is a sandy loam having 26% coarse sand, 23% fine sand, 33% silt, 14% clay, and 4% OM. For further information about the Hygum site, see references [2,17,18].

Site and soil factors

At the Hygum site, three areas were chosen in spring 2004 (unpolluted and medium and high copper contamination), each consisting of five plots. Each plot was further divided into two subplots (control and drought) measuring 0.5 by 0.5 m and separated by a 1-m buffer zone. The unpolluted area had copper concentrations between 20 and 42 mg Cu/kg soil, the medium contamination area had copper concentrations between 277 and 501 mg Cu/kg soil, and the high contamination area had copper concentrations ranging from 655 to 1,143 mg Cu/kg soil. Along the border of each subplot, a 20-cm plastic barrier was inserted into the ground to a depth of approximately 5 cm to prevent or diminish migration in and out of the subplots. The drought subplots were covered with a transparent plastic roof (1×1 m) placed 1 m above the ground in the period June 19 to August 25, eliminating most precipitation during this period.

The plant community of the unpolluted area was dominated by mosses such as *Pohlia nutans* and the grasses *Lolium perenne* and *Agrostis stolonifera*. In the medium and high contamination areas *A. stolonifera* was highly dominant. The total grass biomass in October 2004, estimated according to Strandberg et al. [18], was largely equal in the unpolluted and highly polluted areas: 457 ± 46 g dry weight (dry wt)/m² and 473 ± 20 g dry wt/m², respectively. In the medium-polluted area, the grass biomass was somewhat smaller, 329 ± 46 g dry wt/m².

Soil water potential was measured at weekly intervals in a subset of control and drought plots with Wescor soil psychrometers connected to a Wescor HR33T Microvoltmeter operated in the dew point mode (Wescor, Logan, UT, USA). The output from the soil psychrometers was transformed to soil water potential (bar) with a calibration curve derived from known NaCl solutions. The soil water potential in six control and six drought subplots (evenly distributed across the three areas with different copper levels) was measured at 3 and 10 cm depth, and a mean value for control and drought plots was calculated. Soil temperature of two drought subplots and two control subplots (at 2 cm depth) was recorded at 15-min intervals with Tinytalk dataloggers connected to PT100 thermistors (Gemini Data Loggers, Chichester, UK).

Sampling of enchytraeids

Enchytraeids were sampled on two occasions. On June 19, 2004, two soil cores (diameter 5.5 cm; height 3 cm) were sampled from topsoil of each subplot. The soil appeared dry at this time because of low precipitation during May and June. A preliminary extraction of a few additional samples showed that enchytraeid numbers were extremely low. To get an estimate of the "potential" enchytraeid population size and species composition, 15 ml of water was added to each soil sample, and they were incubated at 20°C for 13 d to allow hatching

of cocoons [19]. After this time, the samples were extracted by wet funnel extraction [20], in which the temperature increased stepwise from 25 to 50°C in 5 h. The enchytraeids were collected in water and stored at 5°C until counting (total enchytraeids), which was done within 48 h after extraction. Additional soil cores were sampled from three drought subplots and three control subplots of each of the three contamination levels for identification and enumeration at the species level according to Nielsen and Christensen [21]. On October 19, a similar set of soil cores was sampled. These samples were extracted immediately because soil moisture was optimal for enchytraeids at this time.

Laboratory experiment

The experimental design was a modified version of the International Standard Organization 16387 enchytraeid reproduction test (ERT) with *E. crypticus* [22]. Two experiments were performed: *E. crypticus* exposed to increasing concentrations of copper in contaminated field soil and *E. crypticus* exposed to newly copper-spiked field soil from the unpolluted area. The soil was sampled from the 0- to 20-cm layer at Hygum in six areas along the copper gradient from uncontaminated (21 mg Cu/kg soil) to highly contaminated soil (1,601 mg Cu/kg soil). To exclude soil animals already present, the soil was dried at 80°C for 24 h and thereafter sifted through a 2-mm mesh to remove larger particles. The enchytraeids were obtained from a permanent laboratory culture of *E. crypticus* held at 20°C on agar and fed dried and rolled oats. The test soil (20 ± 0.02 g dry soil per test container) was either spiked with five ml of aqueous CuSO_4 (Merck, Darmstadt, Germany) solution or 5 ml of demineralized water the day before addition of animals. In the experiment with spiked soil, the following concentrations were used: control, 80, 160, 320, 640, and 960 mg Cu/kg soil. In the experiment with contaminated field soil, the following concentrations were used: control, 213, 462, 615, 878, 1,202, and 1,601 mg Cu/kg soil. Four replicate test containers of each treatment were made. To each test container, 10 sexually mature adult *E. crypticus* with visible clitellum were added. The enchytraeids were fed 33 mg of cooked rolled oat flakes subsequently dried at 105°C for 24 h and finely ground. The test containers were incubated in a climate room at $20 \pm 1^\circ\text{C}$ for two weeks. After the first week, 17 mg of cooked oat flakes was added to each test container. After two weeks, the surviving adults and juveniles were extracted. To extract the enchytraeids, the soil of each test container was gently spread into four 200-ml plastic beakers (diameter 7 cm), which were filled with demineralized water, gently shaken, and then left for 24 h at 5°C for sedimentation. Adult and juvenile enchytraeids moved onto the surface of the sediment and were counted within 48 h with a dissection microscope.

Copper concentration, pH, and OM content

The total copper concentration, pH, and OM content of soil was measured both in field and laboratory studies. For pH and OM measurement of field soil, one soil core (diameter 5.5 cm; height 6 cm) from each subplot was used. For both field and laboratory experiment, the total copper concentration was determined for three soil cores of each subplot. The pH was measured by adding 20 ml of demineralized water to 10 g of soil (dry wt) in flasks shaken for 2 h on a shaking table. After a short sedimentation period, the samples were analyzed with a pH meter (LAB pHM220®, Radiometer Analytical, Lyon, France). Organic matter was measured as the loss on ignition

Table 1. Total soil copper concentration, soil pH, and organic matter (OM) content in control and drought plots of the three areas used in the field experiment. Total copper concentration is calculated from the average of three subsamples per subplot ($n = 5$). Soil pH and OM are calculated from the average of one sample per subplot ($n = 5$). Values are given as means (minimum–maximum). Different letters signify significant differences between mean values ($p < 0.05$)

Treatment	Unpolluted		Medium		High	
	Control	Drought	Control	Drought	Control	Drought
Cu (mg/kg)	32 A (23–38)	33 A (20–42)	395 B (277–501)	417 B (351–496)	890 C (655–1,069)	944 C (819–1,143)
pH ^a	6.5 AB (6.2–7.3)	6.3 AB (6.2–6.4)	6.5 A (6.4–7.0)	6.6 A (6.5–6.7)	6.0 B (5.7–6.6)	6.1 B (5.9–6.5)
OM (%)	6.6 A (6.2–6.9)	6.4 A (5.0–7.2)	9.3 B (7.6–11.2)	10.2 B (6.7–13.5)	10.5 B (8.0–11.6)	10.3 B (9.3–11.6)

^a Soil (10 g dry wt) in 20 ml of demineralized water, shaken for 2 h, sedimented, then analyzed.

after 3 h at 600°C in a furnace. The total amount of copper in the soils was determined with atomic absorption spectrometry (Perkin-Elmer 4100, Ueberlingen, Germany). To extract the total copper concentration, 0.3 g of dried soil was sifted through a 2-mm mesh and crushed in a mortar. Two milliliters of 7 N HNO₃ pro analysi (Merck) was added, heated to 80°C for 17 h, and finally heated to 110°C until dry. Another 1 ml of 7 N HNO₃ was added to each sample, and the procedure was repeated. The samples were dissolved in 5 ml of 0.1 M HCl and then analyzed by atomic absorption spectrometry. Only total copper was determined in this study. However, previous studies have shown that water, CaCl₂, and diethylenetriaminepentaacetic acid–extractable copper fractions are correlated linearly to the total copper concentration in Hygum soil [2,23].

Statistics

For analysis of field results, a multilocation model was employed. The copper contamination area was the location with a known level of copper that was intentionally selected to study the interaction between Cu contamination and drought. Five blocks were nested within each area, and each block included two treatment plots: control and drought. In accordance with mixed model terminology, the area and drought treatments were fixed factors, and the blocks nested within area were random factors [24]. Appropriate *F* tests were standard output from the PROC MIXED procedure of SAS [25]. Because plots were subject to subsampling, these were included as repeated measures. Comparisons of means were made by specifying contrasts of the treatment pairs with the Proc Mixed procedure [25]. For all tests, a significance level of 0.05 was applied. The community composition was analyzed by Primer Version 5.0 (Plymouth Routines In Multivariate Ecological Research, PRIMER-E, Plymouth, UK). The species data were fourth root–transformed before the cluster analysis to reduce the influence of abundant species. A non-parametric multidimensional scaling, based upon the Bray–Curtis similarity matrix, and ordination of the enchytraeid community matrix was performed [26]. The BIOENV-procedure was used to examine the relationship between species composition and environmental factors (total copper, pH, OM). The BIOENV procedure ranks correlations according to a similarity matrix on the basis of the environmental variables and the community similarity matrix, thereby identifying the parameters that best explain the variation in enchytraeid species composition. The environmental factors were all log-transformed before analysis. Finally, to test for significant differ-

ences between species compositions of the three areas, an analysis of similarities was performed [26].

The no observed effect concentration (the highest tested concentration of copper with no statistically significant effect) and lowest observed effect concentration (the lowest tested concentration showing a statistically significant effect) of the ERT were determined by comparing the control with each of the concentrations by a Dunnett's test in an analysis of variance (ANOVA) procedure. Estimation of the concentration causing 10 and 50% mortality (LC10 and LC50, respectively) was by probit analysis, and the estimation of the concentration causing 10 and 50% effect on reproduction (EC10 and EC50, respectively) was by the inhibition concentration (ICp) approach [27].

RESULTS

Soil chemistry, temperature, and moisture in the field study

Total extractable copper concentration was significantly different among the three study areas (Table 1; $F = 1.096$, $p < 0.01$), but the copper concentrations in drought and control subplots were not different within each of the study areas (Table 1; $F = 0.46$, $p = 0.0063$). Soil pH varied only a little among the three study areas, ranging from 6.0 to 6.6. However, pH was significantly lower in the highly contaminated than in the medium-contaminated area (Table 1; $p < 0.05$). Soil pH in the unpolluted area was not significantly different from any of the contaminated areas. The OM content in the unpolluted area was significantly lower than in the medium and high contamination areas ($p < 0.05$).

The plastic roofs used to manipulate soil moisture had only a slight effect on soil temperatures. Because of malfunctioning thermosensors in the control plots, comparison of temperatures in control and drought plots could only be made during the first two weeks of the drought treatment. During this period, the mean temperature at 2 cm depth differed by less than 1°C between treatments.

In covered subplots, soil water potential was clearly lower than in corresponding control plots, both at 3 cm (Fig. 1; one-way ANOVA, $F = 27.9$; $p < 0.00001$) and 10 cm depth (Fig. 1; one-way ANOVA, $F = 28.6$; $p < 0.00001$). When soil moisture was at its lowest (August 11), the wilting point of plants (approximately –15 bars; mean values) was reached at 3 cm depth in drought subplots. Because of low precipitation and high temperatures, the soil water potential was at this time also quite low in the corresponding control plots, approximately –7 bars (mean values). It should be noted that the

Effects of copper on enchytraeids in the field

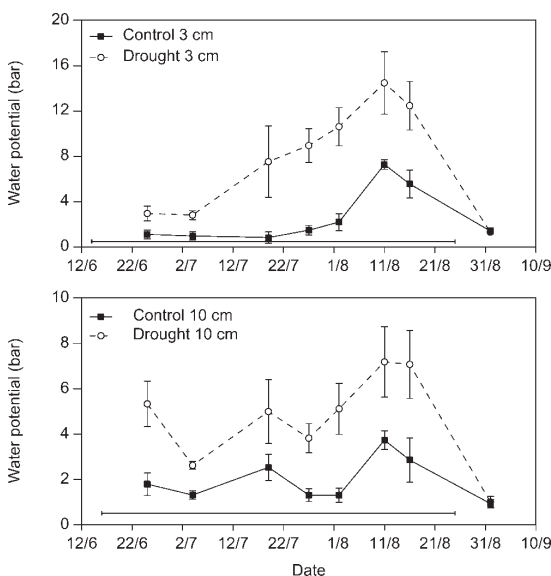


Fig. 1. Soil water potential in control and drought subplots at 3 (A) and 10 cm depth (B). Values are shown as means \pm standard error ($n = 6$). Horizontal lines indicate the duration of the drought treatment. Note that high positive water potential corresponds to low water content.

psychrometric method is not suitable for measurements higher than -1 bar [28]. It is therefore likely that soil water potentials shown as being around -1 bar in this study (Fig. 1) in reality indicate much higher soil moisture levels, which is consistent with our visual observations in the field. Once the roofs were removed on August 25, the soil water potential quickly became equal in control and drought subplots, with a high soil moisture content (Fig. 1). Precipitation was high in the period up to the autumn sampling on October 19, and drought conditions did not occur during this period (data not shown).

Effects of drought treatment on enchytraeids

Data from the spring sampling was analyzed to test whether the total number of enchytraeids of subplots destined for control or drought treatment, respectively, differed. Overall, the control and drought subplots differed significantly ($F = 10.2$, $p < 0.0024$). However, pairwise comparisons showed that this was only the case in the area with background copper concentrations ($p < 0.05$), whereas the two areas with high copper content did not differ (Table 2). The results of the autumn sampling revealed an overall significant effect of drought ($F = 4.9$, $p < 0.034$), but again, the significant difference was seen only in the area with background copper levels (Table 2). Drought and copper ($F = 1.29$, $p = 0.29$) had no significant interaction. Likewise, the species composition was not affected by drought (Table 3).

Effects of copper on enchytraeids and species number

The total enchytraeid number was significantly influenced by copper concentration in the spring sampling ($F = 40.1$, $p < 0.0001$). The unpolluted area with background copper levels had a significantly higher number of enchytraeids than the areas with medium and high copper pollution (Table 2; $p < 0.05$). In the autumn sampling, the unpolluted area seemed to

have the highest number of enchytraeids, but this was not supported by the overall ANOVA ($F = 1.8$, $p = 0.2$). However, for control subplots, the number of enchytraeids was significantly higher in the unpolluted area than in the area with highest copper concentration ($p < 0.05$). There was no interaction between copper and drought in the autumn sampling ($F = 1.29$, $p = 0.29$). When plotting the number of enchytraeids per sample against copper concentration of the respective subplot, density was clearly negatively correlated with copper concentration in the spring sampling (linear regression, $R^2 = -0.34$, $p < 0.003$; Fig. 2A). Similarly, the autumn enchytraeid density of control subplots was negatively correlated with copper concentration ($R^2 = -0.41$, $p < 0.02$; Fig. 3A), whereas this was not the case for subplots previously exposed to drought ($R^2 = -0.31$, $p = 0.1$; Fig. 3A). However, when considering the pooled control and drought subplots in the autumn sampling, a significant negative correlation was seen between enchytraeid density and copper concentration ($R^2 = -0.37$, $p < 0.004$; Fig. 3A).

In the spring sampling, nine species were detected in the unpolluted plots (Table 3; Fig. 2B). This was in clear contrast to only three species found in the copper-polluted areas. In the autumn sampling, seven species were found in the unpolluted plots, largely representing the same species as found in spring in this area, whereas only three species were found in the copper-polluted areas (Table 3; Fig. 3B). In the unpolluted area, *Fridericia connata* was the dominant species, but *Achaeta bohemica* and *Enchytronia parva* also were abundant. In the copper-polluted areas *Fridericia ratzeli* and *Enchytraeus cf. buchholzi* were largely the only species found both in spring and autumn samplings.

Enchytraeid community structure

On the basis of the BIOENV procedure, it was found that copper concentration (Spearman rank correlation coefficient of 0.730, $p = 0.001$) best explained the distribution of enchytraeids collected in the autumn. The differences in species composition between the three areas are illustrated by a multidimensional scaling ordination plot (Fig. 4). The analysis of similarities analysis for the autumn sampling (global $R = 0.66$, $p = 0.001$) showed that the unpolluted and the high-copper areas ($R = 0.99$, $p = 0.002$) and the unpolluted and the medium-copper areas ($R = 0.84$, $p = 0.002$) were clearly separated, whereas the medium- and high-copper areas ($R = 0.23$, $p = 0.052$) were not statistically separable. This was in agreement with the result of the spring sampling (global $R = 0.416$, $p = 0.001$). Here, the unpolluted and the high-copper areas were well separated ($R = 0.79$, $p = 0.002$), the unpolluted and the medium-copper areas were overlapping but clearly different ($R = 0.52$, $p = 0.002$), and the medium-copper and high-copper areas were clearly not separable ($R = 0.05$, $p = 0.26$).

Laboratory study

Copper toxicity was clearly different in spiked soil and contaminated field soil. Copper-induced mortality of adult *E. crypticus* was much higher in the spiked soil than in contaminated field soil (Figs. 5A and 6A and Table 4). No mortality was seen in contaminated field soil up to 1,601 mg Cu/kg soil, whereas mortality in spiked soil began to increase above 600 mg Cu/kg dry soil, and no survival at all was observed at 960 mg Cu/kg soil. Reproduction in control soils was largely equal in the two tests, but copper effects were different (Figs. 5B

Table 2. Total number of enchytraeids per sample (mean \pm standard deviation) in control and drought subplots of three areas with differing soil copper concentrations. Different letters within rows (A, B, C) and within columns (X, Y) signify significant differences between mean values ($p < 0.05$)

	No. of enchytraeids (sample ⁻¹)		
	Unpolluted	Medium	High
Spring sampling			
Control ($n = 5$)	39.9 \pm 20.6 AX	40.0 \pm 2.4 BX	17.7 \pm 13.8 CX
Drought ^a ($n = 5$)	27.4 \pm 11.3 AY	3.0 \pm 5.6 BX	12.0 \pm 3.2 AX
Autumn sampling			
Control ($n = 5$)	41.2 \pm 18.6 AX	25.9 \pm 19.3 ABX	17.5 \pm 7.6 BX
Drought ($n = 5$)	25.8 \pm 9.5 AY	20.1 \pm 19.2 AX	17.4 \pm 8.1 AX

^a Plots destined for drought treatment but sampled before the treatment was initiated.

and 6B and Table 4). For example, at a concentration of approximately 600 mg Cu/kg soil, reproduction in spiked soil was about three juveniles per surviving adult but nearly seven juveniles per surviving adult in contaminated field soil. It is remarkable that reproduction was taking place even at 1,601 mg Cu/kg of contaminated soil.

DISCUSSION

Effects of drought

Several studies have shown that soil moisture is an important factor for enchytraeid populations in various habitats. Thus, both short- and long-term drought can result in population reductions through mortality or impeded growth and reproduction [13,29,30]. The drought treatment applied in this study resulted in desiccation levels in the upper soil layers that were expected to have detrimental effects on enchytraeids [29,31]. When soil moisture was at its lowest, the soil water potential was on average about -15 bar, equivalent to the wilting point of plants. However, in deeper soil layers, desiccation stress was lower, meaning that enchytraeids could have avoided the most intense desiccation stress by migration to moister soil layers [6]. Even so, it is likely that moisture conditions in the drought treatment subplots were much more stressful than in control subplots during the period when drought was applied. We did not observe any increased difference in enchytraeid numbers between control and drought treatment at the autumn sampling, probably because of rapid recovery of the drought-exposed enchytraeid populations. During the two-month recovery period, precipitation was plentiful, creating optimal soil moisture conditions for population growth by hatching of drought-resistant cocoons (as seen in the spring sampling), recolonization of the upper soil layers by upward migration from deeper soil layers, or both [13,19]. It is important to stress that the drought levels applied in this study were environmentally realistic [32] but did not result in any long-term effects on enchytraeids either in unpolluted soil or in soil with high copper content when populations were allowed to recover from the drought stress.

Effects of copper in the field

We observed large differences in enchytraeid abundance, species richness, and species composition between the unpolluted area and the copper-polluted areas. Besides different concentrations of copper, these areas also differed slightly in soil pH (6.0–6.5). These differences in pH seem to be too small to have any influence on enchytraeid abundance or species composition when compared with previous field studies [6].

Soil OM in the copper-polluted areas, however, was significantly higher than in the unpolluted area, perhaps having an influence on enchytraeid community structure. Enchytraeids are often considered as being 50% saproverous, 25% bacterivorous, and 25% fungivorous [6]. It could therefore be speculated that a higher OM content would be stimulating, or at least would not have a negative influence on the enchytraeid populations studied here. It is therefore reasonable to conclude that the differences in abundance, species richness, and species composition between the three study areas must be because of the variations in copper concentration of the studied areas. This is in agreement with the result of the BIOENV procedure, which indicates copper as the main determining environmental factor. Furthermore, the results of the ERT with contaminated field soil also support this conclusion because effects on reproduction were evident at the copper levels found in the copper-polluted areas (Fig. 6B).

Striking differences were found in the species richness between the unpolluted and copper-polluted areas. A total of 10 enchytraeid species were found at the Hygum site. The polluted areas were dominated almost exclusively by *F. ratzei* and *E. cf. buchholzi*, whereas nine different species were found in the unpolluted area dominated by *F. connata*, *A. bohemica*, and *E. parva*. It is interesting to note that the species found in the copper-polluted areas are known as "stress-tolerant" species when considering other natural stress factors such as drought and cold [13,31]. Apparently this also holds true when it comes to stress from copper pollution. Defense mechanisms against copper toxicity by metallothionein-like sequestration and detoxification have been shown in *E. buchholzi* [33], whereas little is known of copper toxicity in *F. ratzei*.

When inspecting the data, it appears that species richness was considerably more responsive to copper than total abundance (Figs. 2 and 3) as predicted from general hypotheses [34]. One should be cautious in describing dose-response relationships of field data because more than one factor might vary between treatments. However, a large decrease in species number seemed to occur between 40 and 280 mg Cu/kg soil, suggesting a threshold level in between these concentrations. In fact, this is consistent with the threshold level at 100 mg Cu/kg soil derived by Bengtsson and Tranvik [35] for earthworms and enchytraeids. A range of other studies have found results similar to ours in this study. For example, Bengtsson and Rundgren [8] found a total of eight enchytraeid species in Swedish unpolluted spruce forest (other species than found in our study). However, in areas close to a brass mill, where soil and litter was polluted mainly with copper and zinc in

Table 3. The species composition (mean ± standard deviation; n = 2–3) in control and drought plots of the three areas with differing soil copper concentrations

Species	No. (sample ⁻¹)											
	Spring sampling						Autumn sampling					
	Unpolluted		Medium		High		Unpolluted		Medium		High	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
<i>Fridericia connata</i>	28.3 ± 40.6	7.7 ± 6.7	—	—	—	—	22.7 ± 21.2	13.3 ± 4.0	—	—	—	—
<i>F. ratzeili</i>	2.3 ± 1.2	1.7 ± 1.2	2.3 ± 2.3	2.3 ± 3.2	22.5 ± 21.9	9.3 ± 7.2	—	0.3 ± 0.6	1.0 ± 1.7	2.7 ± 3.8	16.3 ± 17.9	3.3 ± 4.2
<i>Achaeta bohemica</i>	5.7 ± 1.5	2.3 ± 2.3	—	—	—	—	15.7 ± 13.0	6.0 ± 6.1	—	—	—	—
<i>Encyrtoria parva</i>	6.0 ± 5.6	3.7 ± 4.0	—	—	—	—	12.7 ± 11.9	19.0 ± 24	—	—	—	—
<i>Henleia perpusilla</i>	2.0 ± 3.5	0.3 ± 0.6	—	—	—	—	2.3 ± 2.5	0.3 ± 0.6	—	—	—	0.3 ± 0.6
<i>Marionina communis</i>	3.3 ± 5.8	1.0 ± 1.7	—	—	—	—	—	—	—	—	—	—
<i>Enchytraeus cf. Buccholzi</i> ^a	3.3 ± 2.6	1.7 ± 2.9	1 ± 1.0	—	1 ± 1.0	9.3 ± 14.5	8.3 ± 8.7	0.3 ± 0.6	16.0 ± 7.8	13.0 ± 11.8	4.3 ± 1.5	3.3 ± 3.2
<i>F. balboides</i>	0.7 ± 1.2	—	—	—	—	—	—	—	—	—	—	—
<i>F. maculata</i>	—	—	—	—	—	2 ± 3.5	—	—	—	—	—	—
<i>F. galba</i>	—	—	—	—	—	—	5.7 ± 9.8	—	—	—	—	—

^a *Enchytraeus cf. buchholzi* refers to a complex of closely related species only identifiable when sexually mature. In this study, *E. buchholzi* sensu stricto was strongly dominant, but two additional species not yet identified were found in low numbers.

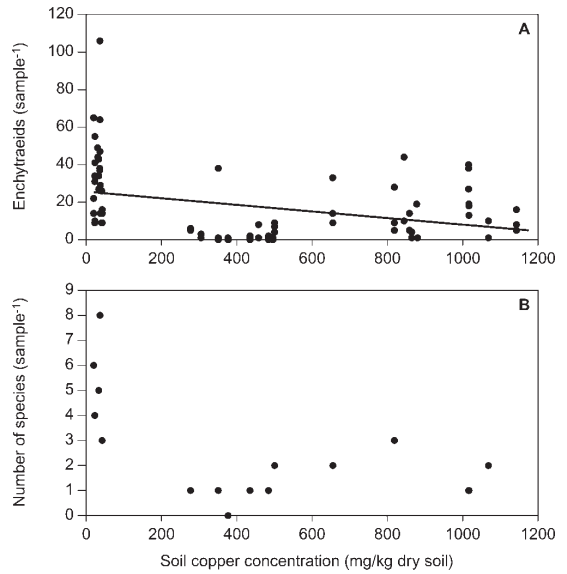


Fig. 2. Total number of enchytraeids (A) and total number of enchytraeid species (B) per soil sample in the 2004 spring sampling plotted against the soil copper concentration in the respective subplot. Soil samples cover an area of 23.8 cm² and a depth of 3 cm. Note that soil samples were wetted and incubated at 20°C for 13 d before extraction. A linear regression line is indicated (pooled data).

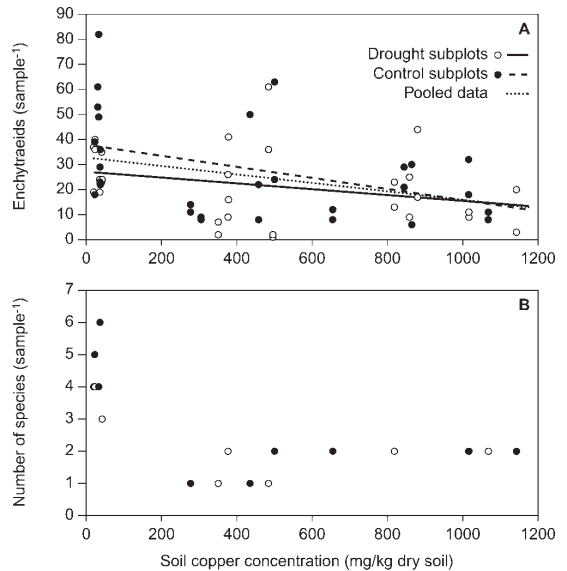


Fig. 3. Total number of enchytraeids (A) and total number of enchytraeid species (B) per soil sample in the 2004 autumn sampling plotted against the soil copper concentration in the respective subplot. Soil samples cover an area of 23.8 cm² and a depth of 3 cm. Soil samples from the drought-exposed subplots are marked as open circles and soil samples from the control subplots are marked as closed circles. Linear regression lines are shown as well.

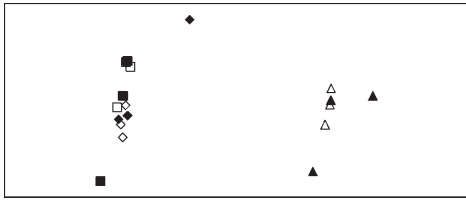


Fig. 4. Ordination plots of the enchytraeid community at the Hygum (Denmark) site (autumn sample). Samples are from the unpolluted area (Δ), medium contamination area (\square), and high contamination area (\diamond). The control subplots are marked as open points, whereas the drought-exposed subplots are marked as filled points. The distance between the points corresponds to enchytraeid species composition: the shorter the distance, the higher similarity between treatments.

concentrations up to 1,600 and 2,200 mg/kg soil, only two species were found, and total abundance was drastically reduced. Yeates et al. [10] found reduced abundance of enchytraeids in grassland contaminated with copper, chromium, and arsenic. In their study, copper was the contaminant with greatest effect, apparently beginning at concentrations of around 425 mg Cu/kg soil. Comparable results were also found when studying the enchytraeid community in the area of a former Dutch zinc smelter [36]. In a Polish forest contaminated with cadmium, lead, zinc, and copper, Kapusta et al. [37] demonstrated that enchytraeid communities were little influenced by relatively low levels of heavy metals, even though metal concentrations in enchytraeid tissues were clearly elevated [38].

Comparison of field and laboratory study

Because of the rather unusual contamination at Hygum (only copper), it is possible to compare results of the ERT with enchytraeid community responses in the field and to evaluate the performance of such standard tests in predicting the

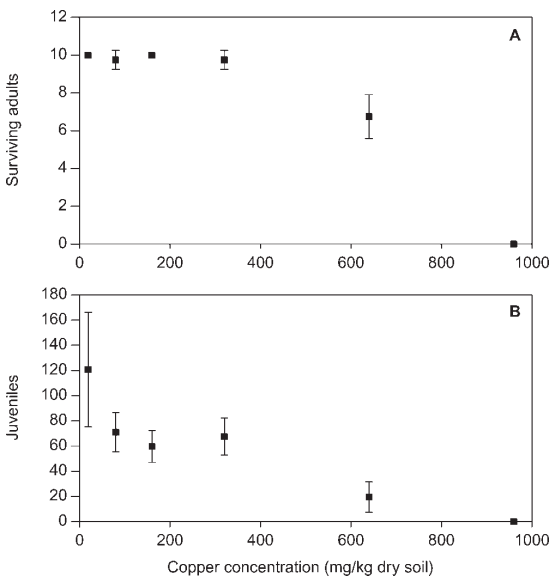


Fig. 5. Effects of copper added as CuSO_4 to unpolluted soil on adult survival (A) and juvenile production (B) in a laboratory test with *Enchytraeus crypticus*. Values are shown as means \pm standard error.

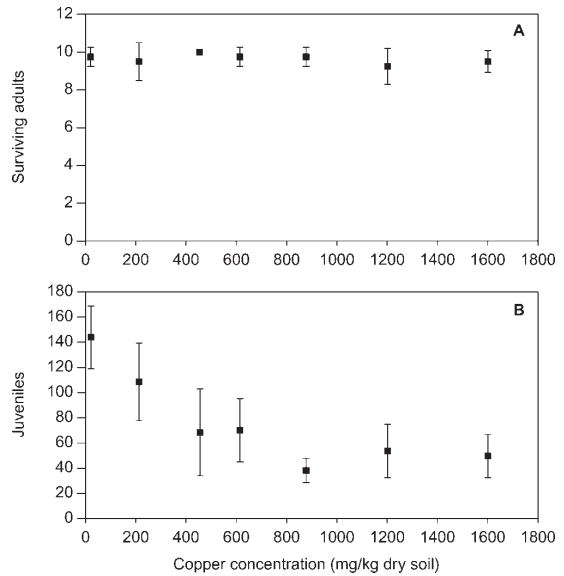


Fig. 6. Effects of copper in contaminated field soil on adult survival (A) and juvenile production (B) in a laboratory test with *Enchytraeus crypticus*. Values are shown as means \pm standard error.

effects in a field situation. The typical way to run the ERT is to mix the pure chemical in question into a test soil and establish the dose-response relationship for both adult survival and reproduction [39,40]. However, this often overestimates direct toxicity effects, primarily because aging processes are not taken into account. Several examples show that aging of metal contamination will decrease toxicity [2,41,42]. In this study, we also observed a clearly higher toxicity when soil was spiked with CuSO_4 than when contaminated field soil was tested. In fact, if the spiked soil test was used as a guiding toxicity value, this would have predicted that enchytraeids could not persist at concentrations above approximately 800 mg Cu/kg soil. Contrary to this, it was observed that enchytraeids were indeed existing in quite high population densities in the field even at 1,000 mg Cu/kg soil, although species

Table 4. Effects of copper on *Enchytraeus crypticus* in reproduction tests with spiked soil from the unpolluted area at Hygum (Denmark) or field-collected copper-contaminated soil. Brackets indicate \pm standard deviation

Effect	Maturity	Copper concentration (mg/kg dry soil)	
		Spiked soil	Field soil
NOEC ^a	Adult	640	>1,601
	Juvenile	320	213
LOEC	Adult	960	>1,601
	Juvenile	640	455
LC10	Adult	522 (± 84)	>1,601
LC50	Adult	775 (± 10)	>1,601
EC10	Juvenile	35 (± 10)	99 (± 56)
EC50	Juvenile	341 (± 146)	439 (± 130)

^a NOEC = highest concentration with no observed effect; LOEC = lowest concentration with an observed effect; LC10, LC50 = concentration causing 10 and 50% mortality, respectively; EC10, EC50 = concentration causing 10 and 50% effect on reproduction, respectively.

richness was reduced. *Enchytraeus crypticus*, a widely used test species, is undoubtedly closely related to *E. buchholzi*. As such, it is probable that *E. crypticus* also is relatively tolerant to various stressors. It could therefore be argued that this species is not representative of the enchytraeid community as a whole and that other less stress tolerant species could make a better choice as test species.

Nevertheless, it seems that a standard laboratory ERT of spiked soil can be used as a rather conservative indicator of copper toxicity to enchytraeids in the field, even accounting for additional stress from a relatively long drought period. Previous laboratory studies have suggested that drought stress can potentiate the toxic effects of copper or other contaminants (or vice versa) in earthworms and Collembola [5,14,43]. It should be noted that the drought stress applied in these laboratory studies was more severe than in our field study. Only one study has so far investigated the combined effects of drought and soil contamination in enchytraeids [44]. It would therefore be interesting to continue this line of research to get a better understanding of cumulative stressors in the environment and how they should be integrated into risk assessment of soil contaminants.

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The climate is undergoing rapid changes with rising atmospheric CO₂ concentration, increasing temperature and changes in the hydrological regimes resulting in more frequent and intense drought periods. These three climate change factors will, separately and in combination, affect the biotic and abiotic components of the soil ecosystems. Enchytraeids are an important component in soil ecosystems and affect the decomposition processes and the nutrient mineralisation both directly and indirectly by their activity.

The background for this thesis was to investigate the effect of climate change on field populations of enchytraeids dominated by the species *Cognettia sphagnetorum*. Field populations of enchytraeids were exposed in a full factorial in-situ experiment to increased CO₂, temperature and prolonged drought manipulation for three years. In the short term, enchytraeids appear to be unaffected by climate change when all factors are combined. The negative impact of drought was counteracted when CO₂ was present, as drought and CO₂ in combination acted additively during summertime. However, in a long-term drought experiment in which *C. sphagnetorum* was exposed repeatedly to drought during a six year period, the species was, evidently, negatively affected. Even though the enchytraeids had been exposed for a long period, there was no sign of increased drought resistance.

Climate change will, beyond doubt, have an impact on the enchytraeids, however, in a non predictable way. But factors such as extreme events, seasonal and interannually variations are all important factors as well, which can strongly influence the response of enchytraeids in a future climate.

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